Production of oligosaccharides from lignocellulosic biomass

Nanthakumar Arumugam

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biotechnology

Department of Biotechnology and Food Technology, Faculty of Applied Sciences,

Durban University of Technology, Durban, South Africa

2020

Supervisor: Dr. Santhosh Kumar Kuttan Pillai

Co-supervisor: Prof. Suren Singh

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the award of the Doctor of Philosophy in Biotechnology, to the Durban University of Technology, Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.

Student Number: 21452678

Date: .

Student: Mr. Nanthakumar Arumugam

Date:

Supervisor: Dr. Santhosh Kumar Kuttan Pillai

Date: _

Co-Supervisor: Prof. Suren Singh

DEDICATION

To My Beloved Parents

and Family

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……I

LIST OF ACRONYMS AND ABBREVIATIONS ……..III

LIST OF FIGURES……...……...VII

LIST OF TABLES……...…XIV

PUBLICATIONS AND CONFERENCE PRESENTATIONS...…...…….…….....XV

ABSTRACT……..…XVII

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW 1

1.1 INTRODUCTION……...1

1.2 LIGNOCELLULOSE……....4

1.2.1 Cellulose…….....5

1.2.2 Hemicellulose…….6

1.2.2.1 Xylans…….7

1.2.2.2 Xyloglucans……9

1.2.2.3 Mannans……9

1.2.2.4 Arabinans…….....10

1.2.3 Lignin…….....10

1.2.4 Importance of lignocellulosic biomass……11

1.2.5 Challenges in the processing of lignocelluloses……..12

1.3 BIOREFINERY APPROACH……....13

1.4 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS……...14

1.4.1 Physical pretreatments…….....15

1.4.1.1 Comminution……..15
1.4.1.2 Ultrasonic pretreatment……..16
1.4.2 Chemical pretreatments……...16
1.4.2.1 Acid hydrolysis……16
1.4.2.2 Alkaline hydrolysis……..17
1.4.2.3 Organosolv……...17
1.4.2.4 Oxidative delignification……18
1.4.2.5 Ionic liquid pretreatment……19
1.4.3 Physico-chemical pretreatments…….....19
1.4.3.1 Liquid hot water…….....19
1.4.3.2 Steam explosion……...20
1.4.3.3 Ammonia fibre explosion (AFEX) and ammonia recycle percolation (ARP)……20
1.4.3.4 CO_2-explosion……21
1.4.3.5 Microwave-assisted pretreatment……...22
1.4.4 Biological pretreatments……..22
1.5 LIGNOCELLULOSE DEGRADING ENZYMES…….....23
1.5.1 Hemicellulases……24
1.5.2 Xylanases……..24
1.5.2.1 Sources of xylanases……26
1.5.2.2 Xylanase production……29
1.5.2.3 Xylanases from various glycoside hydrolase families……30
1.5.2.4 GH10 xylanase- mode of action……..31
1.5.2.5 GH11 xylanase- mode of action……..32
1.5.2.6 GH30 xylanase- mode of action……..32
1.5.3 Accessory enzymes for xylan degradation……..34
1.5.3.1 Xylosidase……34
1.5.3.2 Arabinofuranosidase……...34
1.5.3.3 Glucuronidase……35

1.5.4 Application of xylanases……..36
1.6 VALUE-ADDED PRODUCTS FROM LIGNOCELLULOSE…….…….....38
1.6.1 Bioethanol……39
1.6.2 1,2 and 1,3-Propandiol……40
1.6.3 Biohydrogen……40
1.6.4 Lactic acid……41
1.6.5 Succinic acid……42
1.6.6 Furfural……43
1.6.7 Oligosaccharides……..44
1.6.7.1 Oligosaccharides from lignocellulose……44
1.6.7.2 Xylooligosaccharides……...45
1.6.7.3 Benefits of oligosaccharides as a prebiotic…….....48
1.7 AIM OF THE STUDY……49
1.7.1 Objectives……50

CHAPTER TWO

EXTRACTION OF XYLAN FROM BIOMASS AND THEIR CHARACTERIZATION 51

2.1 Introduction……51

2.2 Materials and methods……53

2.2.1 Selection of raw materials ……...……53

2.2.2 Extraction of xylan from biomass……...53

2.2.3 Quantification of total sugars……...……...54

2.2.4 Fourier transform infrared spectroscopy (FTIR)...……54

2.2.5 Monosaccharide analysis……55

2.2.6 Determination of uronic acids in the extracted polysaccharides……..55

2.2.7 Enzymatic removal of contaminating polysaccharides……..56

2.2.8 Thin layer chromatography (TLC) analysis of the hydrolysed products

……..56

2.2.9 MALDI-TOF mass spectroscopy……57

2.2.10 NMR spectrometry……57

2.3 Results and discussion……..57

2.3.1 Delignification of biomass by NaClO and NaOH extraction……57

2.3.2 Acidic delignification of biomass by $NaClO_2$ and NaOH…..……59

2.3.3 Fourier transform infrared spectroscopy analysis of extracted xylans…..60

2.3.4 Monosaccharide analysis of isolated xylans and starch removal……...64

2.3.5 NMR analysis of isolated xylans……..69

2.4 Conclusion……74

CHAPTER THREE

PRODUCTION AND CHARACTERIZATION OF XYLOOLIGOSACCHARIDES, AND THE INTERACTION STUDIES OF XYLANASE-XYLAN COMPLEX 75

3.1 Introduction……75

3.2 Materials and methods……77

3.2.1 *Thermomyces lanuginosus* SSBP……..……77

3.2.2 Production of xylanase……77

3.2.3 Other enzymes used in the study……78

3.2.4 Protein estimation and enzyme assay……78

3.2.5 Partial purification of xylanase……..…78

3.2.6 Xylooligosaccharide production by enzymatic hydrolysis……79

3.2.7 Sequence retrieval and analysis……...……80

3.2.8 Phylogenetic analysis……...80

3.2.9 Protein 3D structure modelling and validation……..80

3.2.10 Docking studies……81

3.3 Results and discussion..……81

3.3.1 Production and purification of GH11 xylanase from *T. lanuginosus* SSBP..……81

3.3.2 Optimization of xylan concentration and enzyme dosage for XOS production……82

3.3.3 Hydrolysis of peanut shell, bambara, cowpea and sorghum xylan by GH10, GH11 and GH30 xylanases……..84

3.3.4 MALDI-TOF MS analysis of XOS from different xylans……...87

3.3.5 Sequential analysis of xylanases for structural elucidation……93

3.3.6 Phylogenetic analysis……...96

3.3.7 Docking studies on xylan and xylanase……101

3.4 Conclusion……110

CHAPTER FOUR

APPLICATION OF OLIGOSACCHARIDES AS A PREBIOTIC IN FOOD PRODUCTS 111

4.1 Introduction……...111

4.2 Materials and methods……...113

4.2.1 Isolation of probiotic bacteria……...113

4.2.2 Identification and phylogenetic analysis of the isolated bacteria……114

4.2.3 Measurement of bacterial growth on XOS…….....115

4.2.4 Development of prebiotic functional foods…….....115

4.2.4.1 Preparation of amadumbe and bambara groundnut flour…..115

4.2.4.2 Development of amadumbe-bambara spread……..116

4.2.4.3 Texture analysis of spread samples……...116

4.2.4.4 Accelerated stability tests……...116

4.2.4.5 Nutritional analysis of the spread samples……...117

4.2.4.6 Sensory analysis of the spread samples……117

4.2.4.7 Preparation of XOS enriched yogurt……117

4.2.4.8 Physicochemical analysis of yogurt samples……118

4.2.4.9 Microbial analysis of yogurt samples……118

4.2.4.10 Nutritional and sensory analysis of yogurt samples……..…..118

4.2.5 Statistical analysis……..118

4.3 Results and discussion……118

4.3.1 Isolation and identification of probiotic bacteria……..118

4.3.2 Phylogenetic analysis of probiotic microorganisms……119

4.3.3 Measurement of bacterial growth on XOS derived from lignocellulosic materials……..122

4.3.4 Development of prebiotic functional foods……..125

4.3.4.1 Textural properties of the formulated spread……..125

4.3.4.2 Accelerated phase separation of plain and XOS enriched spreads……126

4.3.4.3 Physiochemical properties of plain and prebiotic yogurts…...127

4.3.4.4 Viability test of yogurt samples……130

4.3.4.5 The nutritional profile of XOS enriched spread and yogurt...131

4.3.5 Sensory evaluation……133

4.4 Conclusion……135

5 CONCLUDING REMARKS 137

5.1 Future perspectives……141

REFERENCES 143

ACKNOWLEDGEMENTS

First and foremost, I would like to express my heartfelt gratitude and regards to Dr. Santhosh Kumar Kuttan Pillai for giving me the opportunity to do research under his guidance. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly. Without his patient guidance, support, insight and encouragement, this thesis work would have been impossible. I am really fortunate to have met and worked with him.

My genuine gratitude to my co-supervisor, Prof Suren Singh for his continuous support and guidance. I am gratefully indebted for the opportunity he provided me to work in Dr. Peter Biely's Lab at Slovak Academy of Sciences during my pursuit of the PhD.

My special thanks go to Dr. Peter Biely for allowing me to do part of PhD work under his supervision. His limitless help and fruitful discussions aided me overcome every obstacle I encountered during the project. I have also been constantly inspired by his enduring dedication to science and enthusiasm to push forward the frontier of human knowledge. I also want to thank Dr. Vladimír Puchart for training me on every research technique required to thrive in the lab when I knew nearly nothing about instrumentational biochemistry and for sharing with me his brilliant ideas regarding work, and making the lab such a pleasant place to work in.

My heartfelt thanks must also go to the post-doctoral fellows, Dr. Prashant Kishor Bhagwat and Dr. Koel De Mukherjee for their intellect, and timely inputs at crucial junctures right from the commencement of the thesis till the last-minute revisions.

I would like to extend my special thanks to all staff and post-graduate students in the Department of Biotechnology and Food Technology for their help and assistance throughout the course of this study.

My thanks to my lab mates and friends: Dr. Bibhuti Ranjan, Ms. Deepti Yadav and Dr. Jetendra Kumar Roy for their much-appreciated support in the research environment. I had the privilege of knowing people from diverse cultures and the fun times spent with my lab mates.

My thanks to my friends from other institutions; Dr. B. Ravindran, Dr. A. Vasanthakumar and Dr. M. Arul, Dr. Deepak Gusain and Ms. Sabana Ara Begum for their support and assistance.

My sincere thanks to Dr. M. K. Rajesh for cultivating research enthusiasm in me during the beginning of my research career.

My thanks to my best friends Dr. Shafeeq Rahman, Dr. P. Anburajan, Dr. S. Poorna Chandrika and Mr. P. Selvan, for their moral support.

I would like to extend my gratitude to Dr. Sheena Kumari, Nandana and Nanditha for their kind support and being my family during my stay in Durban.

My gratitude to National Research Foundation (NRF) for the NRF Innovation Doctoral grant (Grant No: 101418 & 109712), ARC-DUT-UFS consortium and DUT Department of Research and Postgraduate Support, for their financial support.

My heartfelt thanks to my Parents M. Arumugam and A. Poovathal for all the sacrifices made and being the guiding light in all my pursuits. I would like to thank and acknowledge the encouragement provided by my family members: my Brother A. Mohan raj and sister-in-law M. Sathya Priya; my Sister T. Tamil Selvi; my niece T. Madhumathi and Nephew T. Arun for their full and enduring support in all my educational endeavours.

LIST OF ACRONYMS AND ABBREVIATIONS

%LA- Percentage of lactic acid

°C- Degree Celsius

µg- microgram

µl- microlitre

^{1}H-NMR spectroscopy- Proton Nuclear Magnetic Resonance spectroscopy

3D- Three dimensional

Å- Angstrom

ADT- AutoDockTools

AFEX- Ammonium Fibre Explosion

Ala- Alanine

AOAC- Association of Official Analytical Chemists

AOS- Arabinooligosaccharides

Ara- Arabinose

Ara*f*- Arabinofuranose

ARC- Agricultural Research Council

Arg- Arginine

ARP- Ammonium Recycled Percolation

Asn- Asparagine

Asp- Aspartic acid

BamX- Bambara xylan

BSA- Bovine serum albumin

BX- Beechwood xylan

C- Control

CAZy- Database of Carbohydrate-Active Enzymes

C-C- Alkene group

CFU- Colony Forming Units

CH_2- methylene group

Cl_2- Chlorine

cm- centimetre

C-O- Carbonyl group

CO_2- Carbon dioxide

C-OH- Aldehyde group

COO^-- Carboxyl group

cP- centipoise

CPX- Cowpea xylan

D_2O- Deuterium oxide

Da- Dalton

DHB- 2,5-Dihydroxybenzoic acid

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

DNS- 3,5-Dinitrosalicylic acid

DOPE- Discrete Optimized Protein Energy

DP- Degree of Polymerization

DTGS- deuterated triglycine sulfate

F- Glc-Xyl-Gal-Fuc

$FeCl_3.6H_2O$- Ferric chloride hexahydrate

FOS- Fructooligosaccharides

FTIR- Fourier Transform Infrared spectroscopy

Fuc- Fucose

g- gram

Gal- Galactose

GH- Glycoside Hydrolase

GLC- Gas-liquid chromatography

Glc- Glucose

Glc_2- Maltose

Glc_3- Maltotriose

Glc_4- Maltotetraose

GlcA- Glucuronic acid

Glc*p*- Glucopyranose

Gln- Glutamine

GOS- Galactooligosaccharides

GOS- Galactooligosaccharides

G_x- ions of cellooligosaccharides

h- hours

H_2CO_3- Carbonic acid

H_2O_2- Hydrogen peroxide

H_2SO_4- Sulfuric acid

HCl- Hydrochloric acid

HMF- 5-Hydroxymethyl furfural

Ile- Isoleucine

KBr- Potassium bromide

kcal- kilocalories

kDa- Kilo Dalton

KH_2PO_4- Potassium dihydrogen phosphate

kJ- Kilojoules

K_m- Michaelis constant

KOH- Potassium hydroxide

kV- kilovolt

L- Glc-Xyl-Gal

Leu- Leucine

LHW- Liquid hot water process

M- molar

m/z- Mass-to-charge ratio

MALDI-TOF MS- Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

MeGlcA- 4-O-methyl-D-glucuronic acid

$MeGlcA^2Xyl_2$- Aldotriouronic acid

$MeGlcA^3Xyl_3$- Aldotetraouronic acid

$MeGlcA^3Xyl_4$- Aldopentaouronic acid

$MeGlcA_{H\text{-}1}$- $MeGlcA_{H\text{-}5}$- H-1 signals of MeGlcA

$MeGlcA_{H\text{-}5}$- H-5 signals of MeGlcA

MeGlcAXyl- Aldobiouronic acid

$MeGlcAXyl_5$- Aldohexaouronic acid

$MeGlcAXyl_6$- Aldoheptaouronic acid

$MeGlcAXyl_7$- Aldooctaouronic acid

mg- milligram

min- minutes

ml- Millilitre

mm- millimetre

mM- millimolar

MOS- Mannooligosaccharides

MPa- Mega pascal

MRS agar- De Man, Rogosa and Sharpe agar

MW- Molecular weight

MWCO- Molecular weight cut-off

N- Newton (Unit of force)

$NaBH_4$- Sodium borohydride

NaClO- Sodium hypochlorite

$NaClO_2$- Sodium chlorite

NaOH- Sodium hydroxide

NCBI- National Center for Biotechnology Information

NDOs- Non-digestible oligosaccharides

NH_4OH- Ammonium hydroxide

nm- Nanometre

O_2- Oxygen

O_3- Ozone/trioxygen

OCH_3- methyl ether group

OD- Optical density

O-H- Hydroxyl group

PDA- Potato Dextrose Agar

PDB- Protein Data Base

Pen- Pentose

pH- Power of hydrogen ion concentration

ppm- Parts per million

Pro- Proline

ProSA- Protein Structure Analysis

PSX- Peanut shell xylan

PTT- Pretreatment

r.f.- radio frequency

RMSD- Root-mean-square deviation

rpm- Revolutions per minute

s- seconds

S- Standard

SCFA- Short chain fatty acid

SD- Standard deviation

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Ser- Serine

SSX- Sorghum xylan

T- temperature

TCA cycle- Tricarboxylic acid cycle

TFA- Trifluoroacetic acid

Thr- Threonine

TLC- Thin layer chromatography

Trp- Tryptophan

Tyr- Tyrosine

U- International unit of enzyme activity

UKZN- University of KwaZulu Natal

v/v- Volume by volume

V_{max}- Maximum rate of reaction

w/v- Weight by volume

w/w- Weight by weight

wt%- Weight percentage

x g- Gravitational force

X- Glc-Xyl

XOS- Xylooligosaccharides

Xyl- Xylose

Xyl_2- Xylobiose

Xyl_3- Xylotriose

Xyl_4- Xylotetraose

Xyl_5- Xylopentaose

Xyl_6- Xylohexaose

Xyl_7- Xyloheptaose

Xyl_{int}- H-1 signal of internal xylose

Xyl*p*- Xylopyranose

$Y_{XOS/xylan}$- Yield (amount of XOS produced per amount of xylan)

$\alpha\text{-}Glc_{H\text{-}1}$- H-1 signal of α-1,3-linked glucose

α-L-Ara- H-1 signal of α-1,3-linked arabinose

µmol- micromole

LIST OF FIGURES

Figure 1.1	Illustration of the main components of lignocellulose (adapted from Isikgor and Becer 2015). The basic components of cellulose, hemicellulose and lignin were explained with their biochemical structures	5
Figure 1.2	The basic biochemical structure of cellulose containing glucose chain linked with β-linkages (adapted from George and Sabapathi, 2015)	6
Figure 1.3	Heterogeneous chemical structure of hemicellulose contains xylose backbone substituted with the common substitutions such as arabinose, glucuronic acid, acetyl and feruloyl groups (adapted from A. E. da Silva *et al.*, 2012)	7
Figure 1.4	Chemical structure of lignin with the basic components called coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol subunits (adapted from Prieur *et al.*, 2017)	11
Figure 1.5	Diagrammatic representation of the strategy for a biorefinery approach (adapted from Kurian *et al.*, 2013). General sources of biomass were processed through biorefinery system and converted into value-added products	14
Figure 1.6	Mode of action of GH10 and GH11 xylanases on alkali extracted glucuronoxylan (adapted from Biely *et al.*, 2016). The smallest XOS released by GH10 xylanase is $MeGlcAXyl_3$, whereas $MeGlcAXyl_4$ is the product released by GH11 xylanase which further converted into single units by the action of glucuronidase and β-xylosidase	31
Figure 1.7	Mode of action of GH30 xylanase on alkali extracted glucuronoxylan (adapted from Biely *et al.*, 2016). The basic XOS produced by GH30 xylanase depends on the MeGlcA substitution on xylan main chain	33
Figure 2.1	FTIR spectra of beechwood xylan (control)	61

Figure 2.2 FTIR spectra of peanut shell xylan 61

Figure 2.3 FTIR spectra of bambara xylan 62

Figure 2.4 FTIR spectrum of cowpea xylan 63

Figure 2.5 FTIR spectrum of sorghum xylan 63

Figure 2.6 TLC analysis of the soluble fractions of peanut shall xylan, xylan I (NaClO and NaOH extraction, A) and xylan II (acidic delignification and NaOH extraction, B) treated with α-amylase (1), xyloglucanase (2), β-1,3(4)-glucanase (3) and GH11 xylanase (GH11). S, standards of xylose and maltooligosaccharides; C, control (xylan incubated without enzyme); Xyl, xylose; Glc_1, glucose; Glc_2, maltose; Glc_3, maltotriose; Glc_4, maltotetraose 66

Figure 2.7 MALDI-TOF MS spectrum of xyloglucan oligosaccharides released from peanut shell xylan (NaClO and NaOH extraction) by xyloglucanase. Abbreviations: G_x, ions of neutral cellooligosaccharides; Explanation for the coding: X, Glc-Xyl; L, Glc-Xyl-Gal; F, Glc-Xyl-Gal-Fuc; A, B, D, Disubstituted backbone Glc by two pentoses or side chain Xyl substituted with another pentose 67

Figure 2.8 TLC analysis of products after α-amylase treatment of xylans from bambara, cowpea and sorghum biomass; S_n, standards (S_1-Glucose and maltooligosaccharides, maltose to maltotetraose; S_2- Xyl, xylose); 1, control bambara xylan; 2, products released by α-amylase from bambara xylan; 3, control cowpea xylan; 4, products released by α-amylase from cowpea xylan; 5, products released by α-amylase from commercial starch 6, products released by α-amylase from sorghum xylan 69

Figure 2.9 ^{1}H-NMR spectrum of birchwood xylan. Description of signals: OCH_3, methyl ether group; $MeGlcA_{H-1}$ and $MeGlcA_{H-5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose 70

Figure 2.10 ^{1}H-NMR spectra of peanut shell xylan isolated by (A) one-step NaClO and NaOH extraction procedure and, (B) NaOH extraction after $NaClO_2$-delignification and NH_4OH pretreatment. Description of signals: OCH_3, methyl ether group; $MeGlcA_{H-1}$ and $MeGlcA_{H-5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; α-L-Ara, H-1 signal of α-1,3-linked arabinose; α-Glc_{H-1}, H-1 signal of α-1,3-linked glucose 71

Figure 2.11 ^{1}H-NMR spectrum of xylan isolated by NaOH extraction of $NaClO_2$-delignified and NH_4OH pretreated bambara biomass. Description of signals: OCH_3, methyl ether group; $MeGlcA_{H-1}$ and $MeGlcA_{H-5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; α-Glc_{H-1}, 3-linked- H-1 signal of α-1,3-linked glucose 72

Figure 2.12 ^{1}H-NMR spectra of xylan isolated by NaOH extraction of $NaClO_2$-delignified and NH_4OH pretreated cowpea (A) and sorghum biomass (B). Description of signals: OCH_3, methyl ether group; $MeGlcA_{H-1}$ and $MeGlcA_{H-5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; Ara, 3-linked, H-1 signal of α-1,3-linked arabinose 73

Figure 3.1 SDS-PAGE profile of GH11 xylanase produced from *T. lanuginosus* SSBP. Lane 1, Protein marker; lane 2, purified xylanase 82

Figure 3.2 Optimization of xylan concentration for XOS production. Each point represents the mean (n=3) ± SD 83

Figure 3.3 Optimization of enzyme dosage for XOS production. Each point represents the mean (n=3) ± SD 84

Figure 3.4 TLC analysis of XOS produced from peanut shell xylan and beechwood xylan by GH10, GH11 and GH30 xylanases; C, control (xylan incubated without enzyme); S, standards; Xyl, Xylose; Xyl_2, Xylobiose; Xyl_3, Xylotriose; Xyl_4, Xylotetraose; $MeGlcA^2Xyl_2$, aldotriouronic acid; 85

MeGlcA^{3}Xyl$_{3}$, aldotetraouronic acid; MeGlcA^{3}Xyl$_{4}$, aldopentaouronic acid; MeGlcAXyl$_{5}$, aldohexaouronic acid; MeGlcAXyl$_{6}$, aldoheptaouronic acid

Figure 3.5 TLC analysis of XOS produced from bambara (BamX), cowpea (CPX), sorghum (SSX) and beechwood xylans (BX) by GH10, GH11 and GH30 xylanases; C_1, C_2 and C_3, Controls (xylan incubated without enzyme); S, oligosaccharide standards; Xyl, xylose; Xyl$_{2}$, xylobiose; Xyl$_{3}$, xylotriose; Xyl$_{4}$, xylotetraose; MeGlcA^{2}Xyl$_{2}$, Aldotriouronic acid; MeGlcA^{3}Xyl$_{3}$, Aldotetraouronic acid; MeGlcA^{3}Xyl$_{4}$, Aldopentaouronic acid; MeGlcAXyl$_{5}$, Aldohexaouronic acid; MeGlcAXyl$_{6}$, Aldoheptaouronic acid 86

Figure 3.6 MALDI-TOF MS spectra of hydrolysates of peanut shell xylan by GH10, GH11 and GH30 xylanases. The numbers are m/z values of Xyl$_{n}$, ions of neutral xylooligosaccharides; MeGlcA^{i}Xyl$_{n}$, ions of acidic oligosaccharides; +22, Disodium salts of acidic oligosaccharides 88

Figure 3.7 MALDI-TOF MS spectrum of xylooligosaccharides produced from bambara xylan by GH10, GH11 and GH30 xylanases. Abbreviations: Xyl$_{x}$, ions of neutral xylooligosaccharides; MeGlcAXyl$_{x}$, ions of acidic oligosaccharides; MeGlcAXyl$_{x}$ +22, ions of sodium salts of acidic oligosaccharides 90

Figure 3.8 MALDI-TOF MS spectrum of xylooligosaccharides produced from cowpea xylan by GH10, GH11 and GH30 xylanases. Abbreviations: Xyl$_{x}$, ions of neutral xylooligosaccharides; MeGlcAXyl$_{x}$, ions of acidic oligosaccharides; MeGlcAXyl$_{x}$ +22, ions of sodium salts of acidic oligosaccharides 91

Figure 3.9 MALDI-TOF MS spectrum of xylooligosaccharides produced from sorghum xylan by GH10, GH11 and GH30 xylanases. Abbreviations: Pen$_{x}$, ions of neutral and/or arabinosylated xylooligosaccharides; 92

MeGlcAXyl$_x$, ions of acidic oligosaccharides; MeGlcAXyl$_x$ +22, ions of sodium salts of acidic oligosaccharides

Figure 3.10 Schematic diagrams from the PDBsum for xylanase of *C. mixtus*. (A) The wiring diagram shows the protein's secondary structure (α-helices and β-sheets) together with various structural motifs such as β and γ turns. (B) Topology diagram of the structural domain illustrating the β-strands (large arrows) and, locations of the α-helices (red cylinders). The small arrows indicate the direction of the protein chain, from N-terminus to C- terminus. (C) Insight of *Cm*GH10 xylanase domains identified using the InterProScan server 94

Figure 3.11 Schematic diagrams from the PDBsum for xylanase of *T. lanuginosus*. (A) The wiring diagram shows the protein's secondary structure (α-helices and β-sheets) together with various structural motifs such as β and γ turns, and β hairpins. The yellow linking bars labelled H1 represent disulphide bond. (B) Topology diagram of the structural domain illustrating the linkages of the β-strands (large arrows) the locations of the α-helices (red cylinder). The small arrows indicate the directionality of the protein chain, from the N- terminus to the C-terminus. (C) Insight of *Tl*GH11 xylanase domains identified using the InterProScan server 95

Figure 3.12 Schematic diagrams from the PDBsum for xylanase of *E. chrysanthemi*. (A) The wiring diagram shows the protein's secondary structure (α-helices and β-sheets) together with various structural motifs such as β and γ turns, and β hairpins. (B) Topology diagram of the structural domain illustrating the linkages of the β-strands (large arrows), the location of the α-helices (red cylinders). The small arrows indicate the direction of the protein chain, from the N- terminus to the C- terminus. 96

(C) Insight of *Ec*GH30 xylanase domains identified using the InterProScan server

Figure 3.13 Phylogenetic analysis of the amino acid sequences of xylanases from family GH10. *C. mixtus* is highlighted in bold letters 97

Figure 3.14 Phylogenetic analysis of the amino acid sequences of xylanases from family GH11. *T. lanuginosus* was highlighted with bold letters 99

Figure 3.15 Phylogenetic analysis of the amino acid sequences xylanases from GH30 family. *E. chrysanthemi* is represented as *Dickeya chrysanthemi* in bold letters 101

Figure 3.16 Stereo view of the modelled structure of xylanase from (A) *C. mixtus*, (B) *T. lanuginosus* and (C) *E. chrysanthemi*. Cyan and grey colour represent the modelled and template structures 103

Figure 3.17 Ramachandran plot of xylanase generated from Procheck server (A) *C. mixtus,* (B) *T. lanuginosus* and (C) *E. chrysanthemi.* Percentage of residues falling in the favoured region for three organisms is 90.02%, 91.9% and 92% respectively 104

Figure 3.18 Schematic illustration of the binding of *Tl*GH11 xylanase (*T. lanuginosus*) and aldopentaouronic acid MeGlcA^{3}Xyl$_{4}$. (A) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (B) Cartoon view of the complex; (C) Three letter code of the residues of the enzyme interacting with the ligand (D) Inter-molecular H-bonds are represented by green bonds. In (B) and (D) the ligand is shown in blue sticks and the enzyme in pink colour 105

Figure 3.19 Schematic illustration of complex structure of *Cm*GH10 xylanase (*C. mixtus*) and aldopentaouronic acid. (A) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (B) Cartoon view of xylanase (grey) 108

and aldopentaouronic acid (purple) (C) Three letter code of the residues of the enzyme interacting with the ligand

Figure 3.20 Schematic illustration of complex structure of *Ec*GH30 xylanase (*E. chrysanthemi*) and aldopentaouronic acid. (A) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (B) Cartoon view of xylanase (blue-grey) and aldopentaouronic acid (red) (C) Three letter code of the residues of the enzyme interacting with the ligand 109

Figure 4.1 Phylogenetic tree of *L. plantarum* NS6 and other closely related *Lactobacillus* species based on 16S rRNA sequences. The tree was generated using the neighbour-joining method 121

Figure 4.2 Phylogenetic tree of *L. lactis* NS9 and other closely related *Lactococcus* species based on 16S rRNA sequences. The tree was generated using the neighbour-joining method 121

Figure 4.3 Phylogenetic tree of *B. animalis* NS11 and other closely related *Bifidobacterium* species based on 16S rRNA sequences. The tree was generated using the neighbour-joining method 122

Figure 4.4 Accelerated phase separation after centrifugation of plain and XOS enriched spread samples 127

Figure 4.5 Viability of bacteria in plain and XOS enriched yogurt 131

Figure 4.6 Radar plot for consumer acceptability of plain spread and XOS enriched spread 134

Figure 4.7 Radar plot for consumer acceptability of plain yogurt and XOS enriched yogurt 135

LIST OF TABLES

Table 1.1	World production of agricultural crops	2
Table 1.2	Leading manufacturers of xylanases in the world	25
Table 1.3	Recent reports on the production and properties of xylanases from fungi and bacteria	27–28
Table 1.4	Production of XOS from different agricultural residues via biological conversion	46–47
Table 2.1	Recovery of xylan by one-step and two-step alkali extraction methods from agricultural biomass	60
Table 2.2	Monosaccharide analysis of extracted xylans (sugars are estimated as molar ratios in comparison to xylose)	65
Table 3.1	Sequence and structural information of xylanase from different organisms. DOPE score for all five models are provided along with the lowest score in bold letters using Modeller 9.20. Verification results of the models with the lowest DOPE scores were presented as Verfy3D and Z score	102
Table 3.2	Description of Docking input and output results. Pose and scoring values are for the best docking regions (active sites) which are highlighted in Bold letters	106
Table 4.1	Growth characteristics of *L. plantarum* NS6, *L. lactis* NS9 and *B. animalis* NS11 on XOS from different sources and glucose	124
Table 4.2	Texture analysis of plain spread and XOS enriched spread	126
Table 4.3	The physicochemical properties of plain and XOS enriched yogurt	129
Table 4.4	Nutritional profile of plain spread and XOS enriched spread	132
Table 4.5	Nutritional profiles of freeze-dried plain and prebiotic yogurt	133

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications in international journals

- **Arumugam N**, Biely P, Puchart V, Gerrano A S, De Mukherjee K, Singh S and Kumar S. 2019. Xylan from bambara and cowpea biomass and their structural elucidation. *International Journal of Biological Macromolecules*, **132**: 987–993. **(Impact factor: 4.784)**

- **Arumugam N**, Biely P, Puchart V, Singh S and Pillai S. 2018. Structure of peanut shell xylan and its conversion to oligosaccharides. *Process Biochemistry*, **72**: 124–129. **(Impact factor: 2.883)**

Book chapters

- Kumar S, **Arumugam N,** Permaul K and Singh S. 2016. Thermostable enzymes and their industrial applications. *Microbial Biotechnology: An interdisciplinary approach*, (Eds). Shukla, P, CRC press, Taylor and Francis Group, Pp.115–162. ISBN: 978-1-4987-5677-8.

- Roy J K, **Arumugam N**, Ranjan B, Puri A K, Pillai S, Singh S and Mukherjee A K. 2019. An overview of raw starch digesting enzymes and their applications in biofuel development. *Microbial fermentation and enzyme technology*. (Eds). Thatoi H N, Das Mohapatra P K, Mondal K C and Mohapatra S, CRC press, Taylor and Francis Group, (In press).

Publication under preparation

- **Arumugam N**, Bhagwat P K, Amonsou E O, Singh S and Pillai S, (2019). Development of a novel amadumbe-bambara spread and probiotic yogurt enriched with prebiotic XOS and its analysis.

International conferences

- Pillai S, **Arumugam N**, Puri A K and Singh S. 2019. Production of xylan and xylooligosaccharide from indigenous African agricultural crops. New Horizons in Biotechnology (NHBT-2019), 20-24 November 2019, Trivandrum, India.

- Pillai S, **Arumugam N**, Okuofu S and Singh S. 2019. Untapped agricultural biomass into a value added product- an enzymatic approach. 14th World Bioenergy Congress and Expo, 06–07 June 2019, London, UK.

- **Arumugam N**, Biely P, Puchart V, Singh S and Kumar S. 2017. Structural elucidation of xylan and xylooligosaccharides from selected underutilised African crops. Biotech France 2017 conference, 28–30 June 2017, Paris, France.

- Kumar S, **Arumugam N**, Gerrano A and Singh S. 2016. Xylooligosaccharides: An emerging dietary fibre for food based Applications. 18th World Congress of Food Science and Technology (IUFoST–2016), Royal Dublin Society, Ballsbridge, 21–25 August 2016, Dublin, Ireland.

National conferences

- **Arumugam N**, Biely P, Puchart V, Singh S and Pillai S. 2018. Valorization of peanut shells for the production of xylooligosaccharides by *Thermomyces lanuginosus* (GH11) xylanase. SASM conference 2018, 4–7 April 2018, Johannesburg, South Africa.

- **Arumugam N**, Roy J K, Kumar S and Singh S. 2016. Exploring lignocellulosic biomass of South African crops for xylooligosaccharide production. Abstract proceedings of The South African Society for Microbiology's 19th Biennial Congress (SASM 2016), 17-20 January 2016, Umhlanga, Durban, South Africa.

ABSTRACT

Lignocellulosic biomass is the most abundant plant material present on earth which is primarily composed of cellulose, hemicellulose and lignin. The composition of lignocellulosic biomass varies depending on the type of plant material and the conditions at which the plants grow. Exploration of lignocellulose for the production of value-added compounds including all types of platform chemicals, biofuels and bioactive compounds is gaining momentum. However, extensive research needs to be carried out to minimize the cost of production to make the processing of this biomass more viable. In the last two decades, several agricultural biomass types have been studied to facilitate the production of biochemicals and biofuels at a low cost. Biomass such as peanut shells, bambara, cowpea and sorghum are some of the indigenous crops of South Africa that are yet to be explored for value addition. Therefore, this study was designed to characterize the underutilized agro-residues such as peanut shell, bambara, cowpea and sorghum biomass for the enzymatic production of prebiotic xylooligosaccharides (XOS) and their application.

This work is the first report on the isolation and structural elucidation of xylan from bambara and cowpea biomass. Many physical and chemical pretreatments were tested to improve the quality and yield of xylan. Among those, acid delignification and alkali extraction processes yielded the xylan with good quality. A two-step extraction of sodium chlorite-delignified material yielded purer xylan from peanut shell (15.5% yield), bambara (12.3% yield), cowpea (13.6% yield) and sorghum (32.2% yield). FTIR spectra confirmed the functional groups of the extracted xylans. Further analysis using ^{1}H NMR together with monosaccharide analysis revealed that all the xylans were glucuronoxylan and shared similar functional groups as that of hardwood glucuronoxylan. Enzymatic hydrolysis of xylan extracted from peanut shell, bambara, cowpea and sorghum resulted in the maximum yield of XOS, at optimized parameters of 1% substrate concentration and 10 U enzyme dosage. TLC analysis of the hydrolysates of the GH11 xylanase from *Thermomyces lanuginosus* SSBP was compared against GH10 and GH30 xylanases regarding their modes of action. The GH11 xylanase was very efficient on all the

xylans evaluated without xylose release. XOS released by GH11 xylanase had a higher DP than GH10 xylanase, but with similar aldouronic acids to GH30 xylanase. Several oligosaccharides were observed in the hydrolysates *viz.*, Xyl_2, Xyl_3, $MeGlcA^3Xyl_3$, $MeGlcAXyl_4$ and $MeGlcAXyl_5$. MALDI-TOF MS characterization corroborated with the TLC results and showed signals for both neutral and acidic oligosaccharides.

A series of computational approaches were also used to study the structure of GH10, GH11 and GH30 xylanases and their interactions with xylan. The phylogenetic analysis of xylanase sequences revealed the closest species from their respective genus with maximum similarity. Secondary structures, topology and catalytic domains were confirmed using PDBsum server. 3D modelling of the three xylanases were done using the structural data and their stability was verified. The binding pattern of the xylanases was identified by docking them with a template $MeGlcAXyl_4$ which showed that GH11 xylanase having good binding ability and strong bonding with the substrate. GH11 xylanase-substrate complex was found as the best-suited docking model for polymeric xylan with a binding affinity of −10.24 kcal/mol. The *in silico* investigation suggested that *Tl*GH11 was found to be more suitable for coupling with xylan over GH10 and GH30 xylanases.

The prebiotic efficacy of XOS was also evaluated using *in vitro* fermentation of XOS with probiotic bacteria. This revealed that, *Lactobacillus plantarum* NS6, *Lactococcus lactis* NS9 and *Bifidobacterium animalis* NS11 had a high growth rate on XOS from the four biomass types. Further, XOS from bambara biomass was used as a functional food supplement in a novel amadumbe-bambara spread and prebiotic yoghurt formulations. The XOS enriched spread was comparable to the commercial spreads with respect to nutritional and sensory characteristics. The prebiotic yoghurt also retained significant probiotic counts during storage. The physicochemical and sensory evaluation of both amadumbe-bambara spread and yoghurt confirmed that both products have improved traits with higher consumer acceptance. Results from our study have shown the prebiotic potential of XOS during *in vitro* fermentation, however, further studies are recommended exploring the animal model and human clinical trials which would form a basis for their commercialization.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The current world population is around 7.7 billion which is estimated to reach 9.7 billion by 2050 (United Nations, 2019). Considering the steady growth, the human race not only has a huge challenge of feeding the large population but also supposed to secure the natural resources for upcoming generations. The agriculture industry is the main food sector that supplies sufficient food to the growing population. This makes the agriculture industry to improve its production with innovative farming techniques. High production rates have been observed in recent years' scaling up the yields. According to the report of Food and Agricultural Organisation of the United Nations (FAO), there is inclined production of crops which is in proportion to the ever-increasing global population (Table 1.1).

Large production of agricultural goods generates large quantities of waste, referred to as 'biomass'. Five billion metric tons of crop residues were generated in 2013, which is increasing with enhanced crop production (Cherubin *et al.*, 2018). The large biomass or crop residues accumulated due to farming activities should be effectively utilized, however, most of the time, this biomass remains unutilized. Burning of crop residues for the preparation of the subsequent production cycle is a very simple and pragmatically followed practice worldwide. This is a very detrimental practice that subsequently affects the soil microbiota, soil fertility, along with the generation of greenhouse gases. These crop residues have essential biomolecules which can be effectively utilized, if treated using biotechnology.

The crop residues which are left over after harvest and processing of the plant materials/agricultural crops result in the creation of a large quantity of solid wastes on earth, constituting an environmental problem. Majority of the agricultural residues thus accumulated

are either burnt or dumped for waste disposal and limited portion are used as fodder or fuel, or for mulching purposes (Menon and Rao 2012; Naidu *et al.*, 2018; Kumari and Singh 2018). However, some portion of the agricultural wastes also contributes (energy crops, residues and by-products) to energy production (Carvalho *et al.*, 2013).

Table 1.1 World production of agricultural crops

	World		Africa	
	2007	2017	2007	2017
Bambara	0.10	0.18	0.10	0.18
Barley	131.1	147.4	4.41	6.61
Cassava	227	292	117.5	178
Cowpea	5.33	7.41	5.01	7.11
Maize	792	1134	48.4	84.1
Millet	33.7	28.4	17.3	12.8
Oats	24.9	25.9	0.18	0.21
Olives	16.9	20.8	2.55	3.73
Peanut	375	471	8.68	12.3
Rapeseed	50.6	76.2	0.10	0.17
Rice	656	769	21.0	36.5
Rye	15.1	13.7	0.08	0.10
Sorghum	62.7	57.6	25.8	27.2
Soybeans	220	352	1.41	3.12
Sugar beet	247	301	7.94	15.9
Sugarcane	1605	1841	91.3	92.1
Sunflower	26.5	47.8	0.93	2.37
Wheat	607	772	18.8	27.1

All values in million tons. Source: FAOSTAT (http://www.fao.org/faostat/en/#data/QC. accessed on 02/10/2019).

The use of the terminology 'waste' is incongruous for organic matter, which may have enormous applications. Bio-based products are better than petroleum refineries which have a great advantage that they can be produced from a variety of feedstocks at a larger scale using various technologies (Arevalo-Gallegos *et al.*, 2017). Recycling of renewable biomass is a feasible to solve the environmental impact of pollutants (Chapla *et al.*, 2010; Krüger *et al.*, 2018). The physico-chemical and biological characteristics of lignocellulosic biomass make them a potential substrate for a broad range of value-added products. Therefore, research over the last two decades has shifted towards alternate strategies for using agricultural waste as feedstock for the production of fuels, solvents, chemicals and polymers (Diaz *et al.*, 2018). Valorization of excessive biomass to produce chemicals or energy has acquired substantial attention due to the unsustainability of traditional energy resources as well as the demand for more conscious management of agricultural wastes (Celinska and Grajek, 2009; Krüger *et al.*, 2018).

Lignocellulosic biomass is comprised of cellulose, hemicellulose and lignin as the basic components hence it serves as the biggest reservoir of hexoses and pentoses. Those sugars can be utilized as a potential raw material to produce biofuels, chemicals and other economic by-products (Kaparaju *et al.*, 2009; Hassan *et al.*, 2018). The exploitation of lignocellulose for producing value-added products primarily requires rigorous pretreatment and saccharification to acquire fermentable sugars (Jung *et al.*, 2014). The classical methodology used for the conversion of lignocelluloses into simple sugars is accompanied by the formation of constituents that are toxic to the environment (Teixeira *et al.*, 2014). This warrants the need to explore biological processes, such as the use of microorganisms and/or their enzymes in lignocellulose hydrolysis. These processes have high specificity, mild reaction conditions, less undesirable product generation, negligible substrate loss, and are environmentally friendly (Zabed *et al.*, 2019). Simultaneous conversion of pretreated lignocellulose into value-added products along with the production of cellulases and hemicellulases, which performs the enzymatic hydrolysis for producing fermentable sugars, plays a major contribution for the reduction of total production cost of the end product (Haldar and Purkait, 2020). Hence, the

use of biological treatments can overcome the limitation of chemical treatments in lignocellulosic biomass utilization.

1.2 LIGNOCELLULOSE

Lignocellulose is the principal biomass produced from the agricultural industry which is a predominant renewable biopolymer in the world (Sreedevi *et al.*, 2013). The lignocellulosic biomass consists of cellulose (40–50%), hemicelluloses (25–30%), lignin (15–20%) and some other basic components (Kumari and Singh 2018). The major portion of the biomass is occupied by cellulose and hemicellulose, which are the rich sources of fermentable carbohydrates (Bhalla *et al.*, 2013). The lignocellulosic biomass can be present in most of the waste materials such as wood residues (wood chips, paper mill discards and sawdust), waste paper, grasses, agricultural residues (peelings, cobs, nutshells, straw, stover, bagasse, stalks), domestic wastes (sewage and plant based garbage), municipal solid wastes, food industry wastes etc. (Menon and Rao, 2012; Singh *et al.*, 2014; Isikgor and Becer, 2015; Mazlan *et al.*, 2019).

The structure of lignocellulosic materials is very complex where cellulose fibres are embedded in a lignin-hemicellulose matrix (Hasunuma *et al.*, 2013). The cellulose polymers are stuffed into microfibrils where the hydrogen bonds make them stable. Hemicelluloses and pectin bind through the microfibrils and the whole structure are covered by lignin (Figure 1.1). The middle lamella, which connects the neighbouring cells, is almost completely made of lignin (Pandey, 2009). To access carbohydrates in this framework, pretreatments should be used to breakdown the lignin sheath before applying the enzymes for saccharification (Haldar and Purkait, 2020).

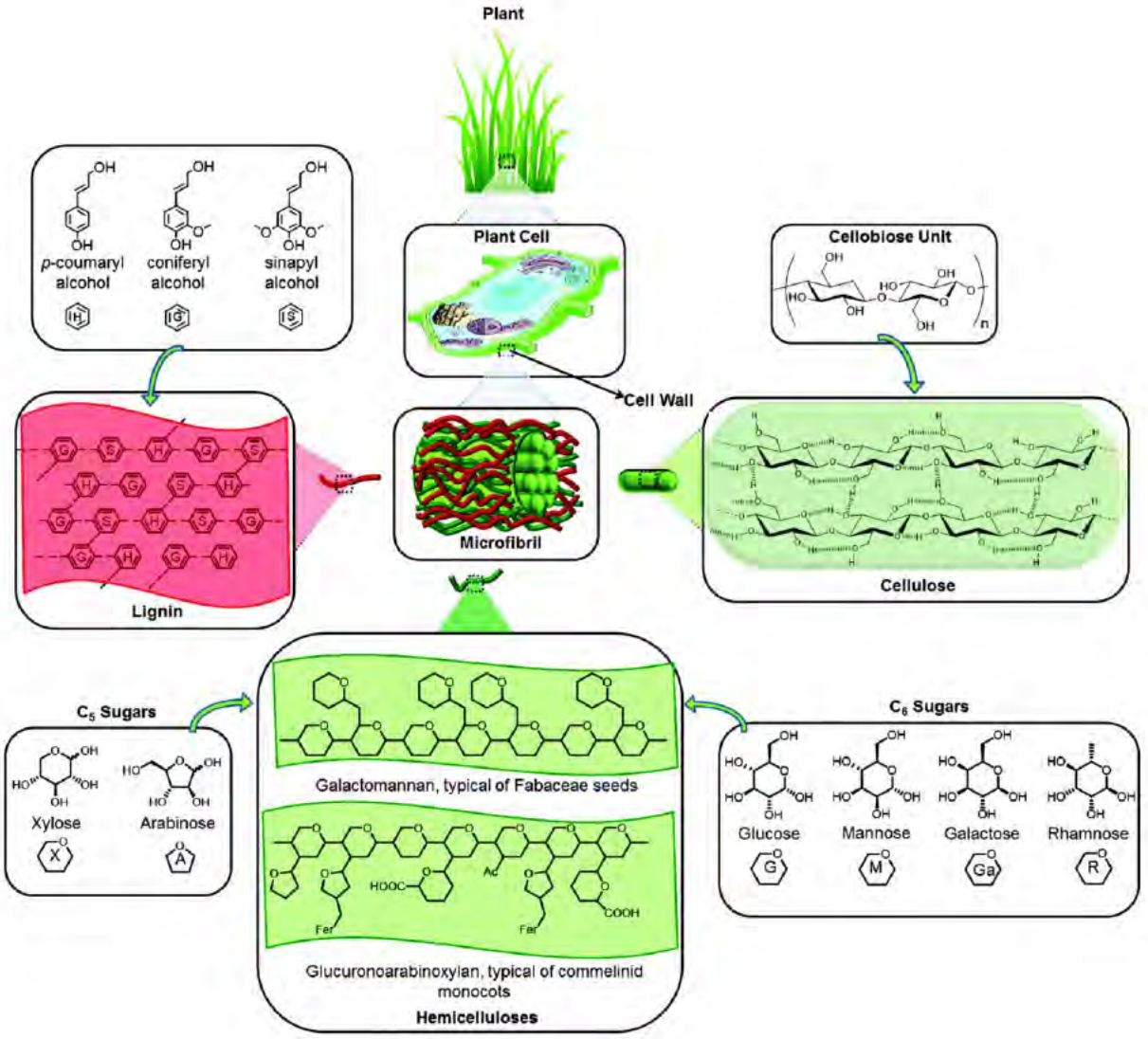

Figure 1.1 Illustration of the components present in lignocellulose (adapted from Isikgor and Becer, 2015). The basic components of cellulose, hemicellulose and lignin are explained with their biochemical structures.

1.2.1 Cellulose

Cellulose is a homogeneous linear polymer of 1,4 linked β-D-glucose units (Figure 1.2), with a degree of polymerization (DP) ranging from 10,000 to 15,000 units (Walton 2009; Menon and Rao 2012). Cellulose comprises huge number of hydrogen bonds between glucose molecules that provide a crystalline and unbreakable matrix framework (Limayem and Ricke, 2012). The crystallinity of cellulose makes them poorly reactive to hydrolysis (Nguyen *et al.*, 2014a). The

β-(1,4) glycosidic linkages in the cellulose can be broken down by cellulases, which concert the complex cellulose into simple glucose molecules (Kazemi *et al.*, 2014). Several microorganisms are active in the degradation of cellulose which includes fungi such as *Trichoderma, Aspergillus, Rhizopus* and *Penicillium* species (Chandel *et al.*, 2013), bacteria such as *Clostridium thermocellum*, *Bacillus, Pseudomonas* species etc. (Peng *et al.*, 2011; Sreedevi *et al.*, 2013; Kazemi *et al.*, 2014). However, only a few of these microorganisms produce large amount of enzymes that are effective in the complete breakdown of cellulose (Khokhar *et al.*, 2012).

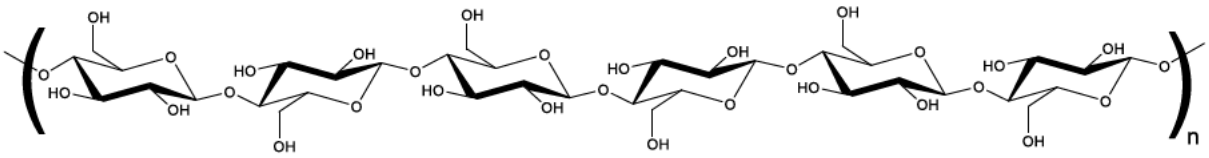

Figure 1.2 The basic biochemical structure of cellulose containing glucose chain linked with β-linkages (adapted from George and Sabapathi, 2015).

1.2.2 Hemicellulose

Hemicellulose, which is the most prevalent component in woods and agricultural biomass is an amorphous and irregular structure formed by a heterogeneous class of polysaccharides consisting of pentoses as well as hexoses (Menon and Rao 2012; Gao *et al.*, 2014). Hemicellulose (Figure 1.3) possesses a hetero-polysaccharide structure with xylan as the main backbone, usually made of xylose units (Biely *et al.*, 2016). A range of branching frequencies for xylan was observed depends upon the nature, composition and resource of raw materials used, however, the individual classification of hemicellulose is based upon the dominant monomeric sugar present. There are four types of hemicelluloses, classified based on the sugar backbone, *viz.*, xylans, mannans, xyloglucans and β-glucans with mixed linkages (Samanta *et al.*, 2015). The common hemicelluloses present in the softwood are glucomannans and arabinoxylans, whereas, hardwood hemicellulose comprised most frequently xylans (Limayem and Ricke, 2012).

Although the proportion and structure of hemicelluloses in plant cell walls are well established, their eco-physiological relevance was not well known (Hoch, 2007). Hemicelluloses are more soluble in water than other components of lignocellulose due to their lower degree of polymerization and the presence of branched side groups (Walton, 2009; Xue *et al.*, 2016). It is also confirmed that hemicelluloses are partially susceptible to mobilization, especially when the hemicellulose portion is loosely attached to cellulose fibrils (Schädel *et al.*, 2009). The variable binding strength of hemicellulose with cellulose defines the various intracellular functions of hemicelluloses in the cell wall (Sun *et al.*, 2004).

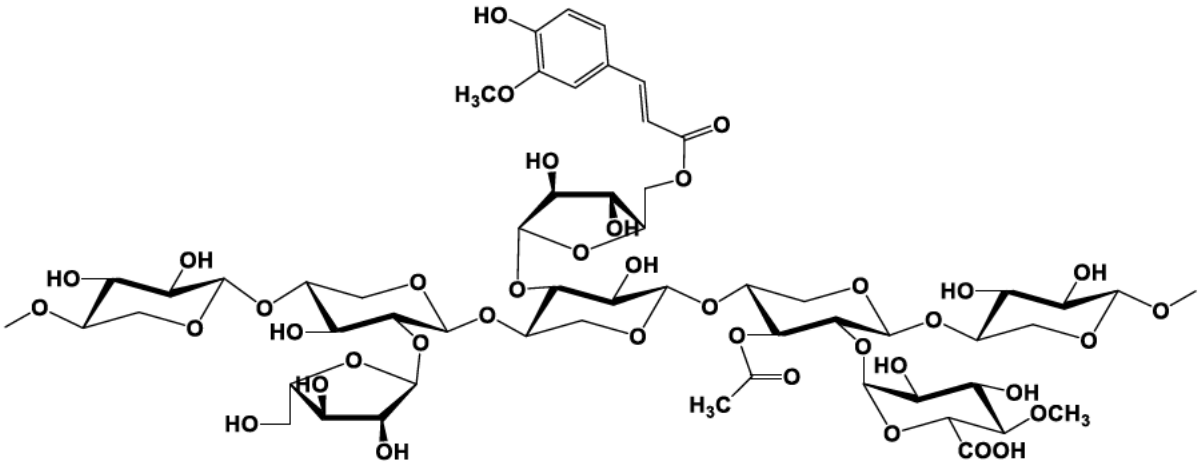

Figure 1.3 Heterogeneous chemical structure of hemicellulose contains xylose backbone substituted with the common substitutions such as arabinose, glucuronic acid, acetyl and feruloyl groups (adapted from da Silva *et al.*, 2012).

1.2.2.1 Xylans

Xylans are non-crystalline complex heteropolysaccharides that are composed of (1,4) linked β-D-xylose units in the main chain of the backbone (Álvarez *et al.*, 2017). Apart from xylose, xylans may comprise glucuronic acid (also have 4-*O*-methyl ether), arabinose, acetic and ferulic acids as substitutions (Cantu-Jungles *et al.*, 2017). Depending on the plant source and the extraction method, the structure and composition of xylan may vary (Singh *et al.*, 2015). In general, most of the xylan comprises substituents in their main chain, however, some of the

biomass including esparto grass, tobacco stalks and guar seed husk were found with unsubstituted linear xylans (Saha, 2003). Therefore, xylans can be classified as linear homoxylan, glucuronoxylan, arabinoxylan and glucuronoarabinoxylan (da Silva *et al.*, 2012). The arabinoxylans consist of α-L-arabinofuranose residues as substituents attached to (1,2)- and/or (1,3) positions of β-(1,4)-linked D-xylopyranose units in the main xylan backbone (Heikkinen, 2016). Generally, the polymeric structure of arabinoxylan comprises a range between 1500 and 5000 residues of xylose/arabinose. The arabinose substitution may also be found on other groups in the xylan backbone such as glucuronic acids, acetyl groups and ferulic acid crosslinks.

Glucuronoxylan is another type of xylan which contains 4-*O*-methyl-D-glucuronic acid (MeGlcA) side residue attached to every 10^{th} xylose residue of the xylan main chain (Biely *et al.*, 2016). In nature, glucuronoxylan is partially acetylated and also networked with lignin through ester bonds between the carboxyl group of MeGlcA and lignin alcohols. If xylan is treated with any alkali, it causes complete deacetylation and apparently leads to the interruption of ester linkages with lignin (Lagaron *et al.*, 2016). However, the bonding between xylose and MeGlcA is not getting affected. There is evidence that indicate a mixture of heterogeneous xylan molecules varying in the number of MeGlcA substitution can be acquired during alkaline extraction. Acetylated glucuronoxylan extracted by DMSO from delignified hardwood pulp contains significantly lower MeGlcA content than the alkali extracted polysaccharide thus the average ratio Xyl:MeGlcA is 10:1 (Naran *et al.*, 2009). The distribution of MeGlcA residues of xylan obtained from Hinoki cypress and softwood Japanese cedar was observed with a strange arrangement (Ishii *et al.*, 2010) that provides the evidence for heterogeneity and complexity of xylan. Hardwood xylan is more water-soluble and has greater MeGlcA content (e.g., from beechwood) which is responsible for its heterogeneous nature (Biely *et al.*, 2015). Therefore, the physicochemical properties such as solubility was determined by the MeGlcA content. The chances of xylan association with cellulose via H-bond present in the unsubstituted regions will increase when they comprise the lower frequency of side residues (Biely *et al.*, 2016).

1.2.2.2 Xyloglucans

Xyloglucan is a type of hemicellulose present mainly in the primary cell walls of dicots and some monocots (Madeira *et al.*, 2017). In dicots, especially in the primary cell wall of growing plants, xyloglucan comprises 20–25% (w/w), whereas in monocots, its content is much lower (2-10% (w/w)) (Pauly and Keegstra, 2016). Xyloglucan comprises a β-(1,4)-glucan backbone which has α-(1,6)-xylose substitution, as well as occasional fucosyl or galactosyl residues (Eckardt, 2008). *O*-6 α-D-xylose substitution covers up to 75% of the glucose residues and a portion of the xylose residues consists further β-D-galactose substitution. The backbone of xyloglucan comprises a basic repetition of four oligosaccharides which vary in number and galactose substitution. The xyloglucan chains form highly viscous solutions when they are self-aggregated in water (Kochumalayil *et al.*, 2013). The oligosaccharides produced from the hydrolysis of xyloglucan has been coded with single-letter codes to simplify the identification of xyloglucan oligosaccharides (Tuomivaara *et al.*, 2015).

1.2.2.3 Mannans

Mannan polymers are mainly classified as glucomannans and galactomannans which are present in the secondary cell walls of softwoods and leguminous plants (Schädel *et al.*, 2009). Glucomannans are also present in the seeds of certain annual plants, for example, some lilies and irises as storage polysaccharides. Furthermore, bulbs, tubers and roots of many plants are also containing glucomannans (Singh *et al.*, 2018a). Many of these glucomannans are also water-soluble. Glucomannans are composed of mannose and glucose in the ratio 1/1 to 2/1 (Hagglund, 2002). Glucomannan present in hardwoods has a linear chain of β-(1,4)-linked mannose and glucose residues. Another type of mannan termed as galactomannan is stored in the seed endosperm of leguminous plants. Unlike linear mannans, the galactomannans are soluble in water and can assimilate water, which aids in the water holding capacity of the seeds. A glucomannan main chain composed of galactosyl substitutions at α-(1,6) position is called galactoglucomannan.

Some of the sources of mannans include soybeans, coffee beans, ivory nuts, alfalfa seeds, sugar beets, palm kernel, coconut kernel, tubers of orchids, konjac tubers, copra meal and cell walls of some fungi, yeast and bacteria (van Zyl *et al.*, 2010; Singh *et al.*, 2018a). In many cases, mannans are highly insoluble in water and very thick. Mannan has been suggested as the reason for hardness of palm kernels, such as the ivory nut which is the basic characteristic of kernels (Singh *et al.*, 2018b).

1.2.2.4 Arabinans

Arabinans are pectic polysaccharides usually consist of a backbone of (1,5)-linked-α-L-arabinofuranosyl units, and could have a linear or branched structure (Cordeiro *et al.*, 2012). They widely occur in the primary cell wall of various plant parts such as fruits, root, stem and seeds, which are also considered as dietary fibres (Zhu *et al.*, 2018). Arabinans are generally considered to be associated with rhamnogalacturonan-I in the pectic network as the side chains at the *O*-4 position of some rhamnosyl residues (Ding *et al.*, 2015; Colodel *et al.*, 2018). Few palm species from *Arecaceae* family such as *Phoenix canariensis* and *Rhopalostylis sapida* contains mostly of arabinan in their primary cell wall It is reported that arabinan and arabinan-rich pectin retains a wide range of biological activities including gastro-protective, immunological and anticoagulant activities (Cordeiro *et al.*, 2012; Fernández *et al.*, 2013; Mandal *et al.*, 2013; Xia *et al.*, 2015).

1.2.3 Lignin

Lignin is an aromatic biopolymer covalently linked to hemicellulose which confers a high level of compactness and rigidity to the lignocellulose (Figure 1.4). It is found in most terrestrial plants typically with 15–30% occupancy (Lupoi *et al.*, 2015; Gillet *et al.*, 2017). It is mainly composed of coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (Wang *et al.*, 2019). Softwoods generally contain more lignin than hardwoods, whereas the lowest level of lignin was found in grasses and softwoods (Ponnusamy *et al.*, 2019). Any biomass that has high lignin

content is not suitable to be used as a substrate in fermentation, as lignin makes the biomass resistant to chemical and biological degradation (Nguyen *et al.*, 2014a).

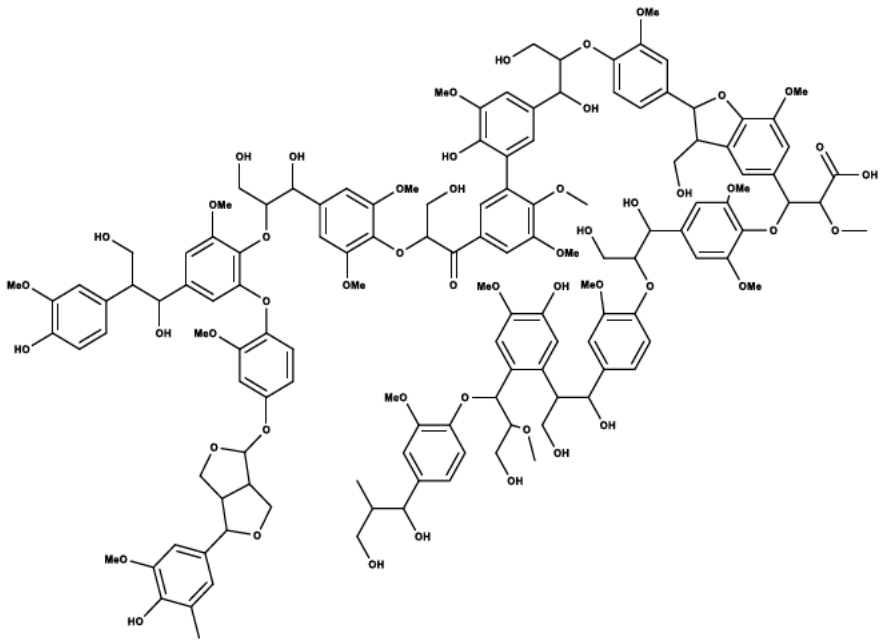

Figure 1.4 Chemical structure of lignin with the basic components such as coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol subunits (adapted from Prieur *et al.*, 2017).

In contrast to most natural polymers, lignin molecules have an extremely complicated structure due to their natural variability. Thus, only minimal studies were focused on isolation of lignin and its hydrolysis was carried out in the past. Subsequently, lignin depolymerization and conversion has gained interest and has potential as a sustainable biopolymer to produce fuels and bulk chemicals, and is an alternative to the petrochemical industry (Gillet *et al.*, 2017).

1.2.4 Importance of lignocellulosic biomass

The depletion of fossil fuels and their deleterious effect on global warming also urge to move on for alternative sources. The current demand in the oil market and the rise in fuel prices have established a global challenge for biofuel production from lignocellulosic materials (Johnravindar *et al.*, 2017; Rodionova *et al.*, 2017). Traditionally biofuels and biochemicals are derived mainly from food crops which are termed as first-generation biofuels. This practice

creates many problems such as net energy losses, greenhouse gas emissions, competition for primary food crops (Kumari and Singh, 2018). To reduce the clash between the usage of food crops for food consumption, and biochemicals and biofuels production, the biomass should be focused as the substrate for second generation biofuel production (Rodionova *et al.*, 2017; De Bhowmick *et al.*, 2018). Evolution in the alternative energy will surely unwind the food and biofuel production conflict along with reduced CO_2 emissions and also ensure a promising source for sustainable bioenergy (Kaparaju *et al.*, 2009; Kumari and Singh, 2018). Ease of availability, high carbohydrate content and low cost make the biomass wastes as suitable substrates for value-addition (Bao *et al.*, 2014).

With the development of new technologies; enormous biomass waste can be converted to different valuable biomaterials representing a significant prospect to fully exploit our resources (FitzPatrick *et al.*, 2010; De Bhowmick *et al.*, 2018). Being less energy-intensive and having the perspective of producing value-added products during the manufacturing process, bioprocess technologies have some significant advantages in contrast with conventional chemical methods (Wong *et al.*, 2012; Diaz *et al.*, 2018). Utilization of these materials not only solves the proper disposal of the wastes but also provides an attractive opportunity for more sustainable processing of agricultural resources (Chu *et al.*, 2014).

1.2.5 Challenges in the processing of lignocelluloses

The conversion of lignocellulosic biomass into simple molecules is a tedious and harsh process. The hydrolysis of cellulose is difficult due to its large molecular structure, which discloses the crystalline nature and poor solubility of cellulose (Brodeur *et al.*, 2011). Lignin surrounds the cellulose which prevents direct interaction between cellulose and hydrolysing materials such as chemicals and catalysts. (Zhao *et al.*, 2009). To achieve the enzymatic hydrolysis of biomass, crystalline cellulose disruption, increasing the surface area, hemicellulose and lignin removal should be performed before applying the enzymes (Brodeur *et al.*, 2011). The exploitation of the biomass for value addition typically requires extensive pretreatment. However, the

recalcitrant nature of lignocellulosic biomass leads to a complicated bioconversion process than that of starchy and sugary materials. In general, prior to microbial fermentation the substrate must be converted to fermentable sugars (Mussatto *et al.*, 2010).

Microorganisms and their enzymes used in biorefinery applications should be thermotolerant and pH stable to handle the extreme environmental conditions during the bioprocessing. This is significant in some cases where the products released acts as inhibitors – for examples organic acids and biofuels (Buschke *et al.*, 2013). In addition, the contaminants present in the raw materials could interfere the fermentation and also may release toxic products into the media (Muthaiyan *et al.*, 2011). A complete utilization of sugars in lignocellulosic materials could results in the maximum yield of desired products (Harmsen *et al.*, 2013).

1.3 BIOREFINERY APPROACH

Biorefinery is defined as "the sustainable processing of biomass into a spectrum of marketable products and energy" (Chandel *et al.*, 2018). This concept incorporates the processes for biomass conversion and equipment to generate chemicals, fuels and power from biomass (Figure 1.5), in preference to traditional oil refinery processes (Kurian *et al.*, 2013). Rich lignocellulose sources such as green biomass, feedstocks and wood industrial waste streams are predominantly alluring for sustainable biorefinery concepts since they are easily available, low cost and eco-friendly (Buschke *et al.*, 2013). An array of technologies and unit operations exists or under development which includes hydrothermal liquefaction, hydrogenation, hydrothermolysis, oxidation, gasification, pyrolysis, hydrodeoxygenation and fermentation (Kajaste, 2014; De Bhowmick *et al.*, 2018). To launch the cost-effective fermentation of lignocellulosic biomass, a combined saccharification and fermentation strategy should be implemented, however, the microorganisms that can hydrolyze both the cellulose and hemicellulose, and accomplish product biosynthesis is necessary (Hasunuma *et al.*, 2013).

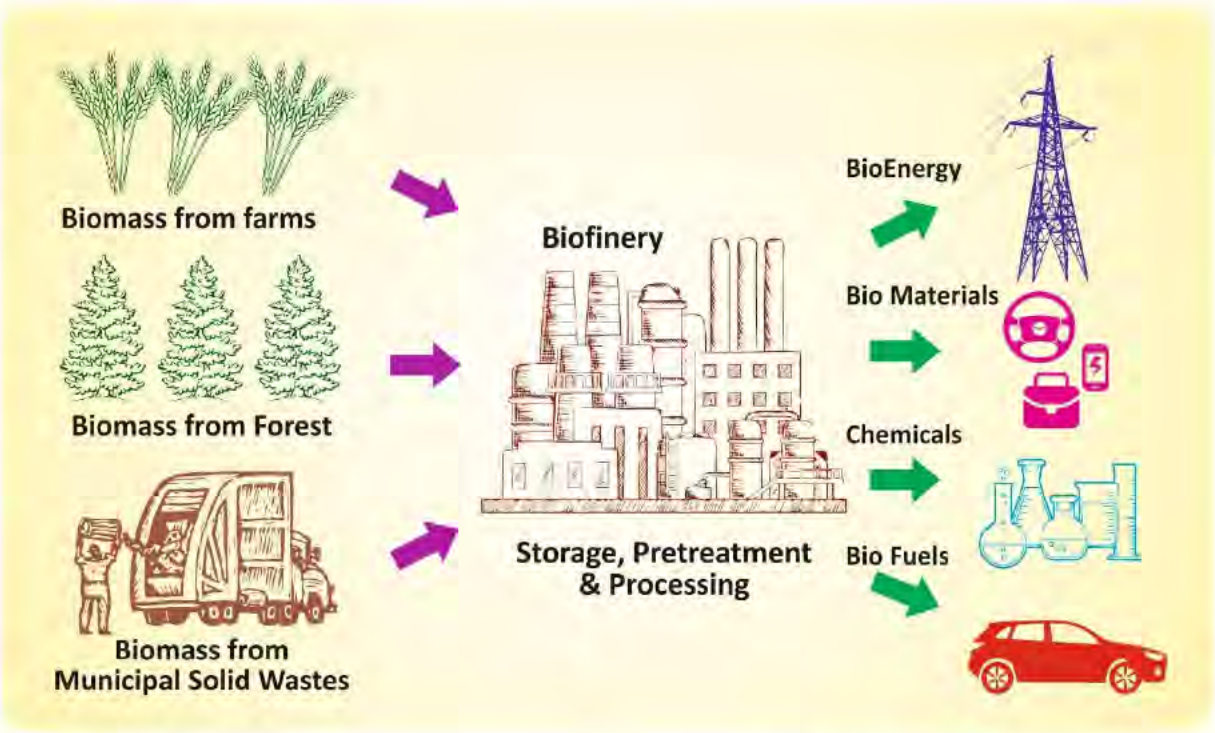

Figure 1.5 Diagrammatic representation of the strategy for a biorefinery approach (adapted from Kurian *et al.*, 2013). Biomass from various sources are processed through a biorefinery system and converted into value-added products.

1.4 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass has a complex construction which makes it very rigid and not easily amenable to bioconversion process for making value-added products. Because of the recalcitrant nature of lignocelluloses, a pretreatment step is required for microorganisms to access and ferment the sugars present. Various pretreatment options are available to fractionate, solubilize, hydrolyze and separate cellulose, hemicellulose, and lignin components (Saha, 2003). The main reason to employ physical and chemical pretreatments is to disrupt the complex structure of lignocellulose before the enzymatic hydrolysis of the holocellulose fraction (Kumar *et al.*, 2009a). However, the pretreatment process is one of the expensive steps in the conversion of lignocellulosic feedstocks to value-added products.

Pretreatment should possess minimal capital cost, biomass cost, downstream processing and operational cost. For making the process cost effective, pretreatment should be applicable for a wide range of lignocellulosic material with unlimited quantity and be able to recover most of the lignocellulosic components in a usable form. It should be able to release sugars or to consequently form sugars by hydrolysis without degradation and loss of carbohydrates. However, for this to materialise, one of the requirement is that it should avoid the formation of any inhibitory by-products that affect subsequent hydrolysis and fermentation (De Bhowmick *et al.*, 2018).

1.4.1 Physical pretreatments

1.4.1.1 Comminution

Comminution is the combination of several mechanical processes such as chipping, shredding, coarse size reduction, grinding, milling, etc., which have been used to improve the digestibility of lignocellulosic biomass. The purpose of these treatments is to increase the surface area and reduce both the chain length and crystallinity of cellulose (Agbor *et al.*, 2011). For mechanical pretreatment, capital costs, operating costs, scale-up possibilities and depreciation of equipment are the very important factors to get economically feasible processing (Agbor *et al.*, 2011; Harmsen *et al.*, 2013). Mechanical pretreatment is usually carried out prior to the processing step, and the defined particle size is relying on the succeeding steps. A series of approaches starting from harvesting and preconditioning of lignocellulosic biomass, chipping, grinding and milling is applied to convert the biomass into a final particle size of 0.2–2.0 mm (Oh *et al.*, 2015). The shear forces generated during milling and grinding resulted in an effective reduction of particle size and cellulose crystallinity than chipping. Factors such as milling type and duration along with the type of biomass regulated the increase in specific surface area, final DP and cellulose crystallinity reduction (Agbor *et al.*, 2011). It was noted that vibratory ball milling effectively reducing the cellulose crystallinity of spruce and aspen chips than ordinary ball milling (Kumar and Sharma, 2017). Disk milling (to produce fibres) is more effective than

hammer milling in boosting cellulose hydrolysis, which generates finer bundles (Zhu *et al.*, 2009).

1.4.1.2 Ultrasonic pretreatment

Ultrasound pretreatment is the process to alter the surface structure of the biomass and to produce oxidizing radicals that chemically attack the lignocellulosic matrix (Baruah *et al.*, 2018). Moreover, ultrasound can disrupt the linkages between polysaccharides and lignin (Shirkavand *et al.*, 2016) which results in the formation of small cavitation bubbles (Kumar and Sharma, 2017). This method is considered as an effective green technology to pretreat the lignocellulosic biomass. The ultrasound damages the lignin hemicellulose complex which caused the increased accessibility and easy extraction of hemicellulose. A combination of ultrasound and alkali pretreatment results in improved lignin degradation and enzymatic saccharification rates and lowering its crystallinity (Hassan *et al.*, 2018).

1.4.2 Chemical pretreatments

1.4.2.1 Acid hydrolysis

One of most effective pretreatment methods conducted to breakdown the lignocellulosic biomass is acid hydrolysis. Depends on the requirement, acids can be used in various concentrations. By altering the treatment temperature, dilute acid treatment can be performed either as continuous flow process (T>160°C, 5–10 wt% substrate concentration) or batch process (T≤160°C, 10–40 wt% substrate concentration). Inorganic acids such as H_2SO_4 and HCl are added to the raw material and the mixture is held at elevated temperature for a short period. As an alternative to inorganic acids, organic acids (e.g. acetic acid, formic acid, citric acid, maleic acid and fumaric acid) can also be used for dilute acid pretreatment (Kootstra *et al.*, 2009). Acid treatment offers good performance in terms of recovering hemicellulosic sugars but there are also some drawbacks. Sugars released by acid hydrolysis might be further converted to furfural and HMF, which strongly inhibits the microbial fermentation. Additionally, acids

can be corrosive on any material such as wood, metal, and neutralization results in the formation of solid waste (Baruah *et al.*, 2018).

H_2SO_4 and HCl are also used in their concentrated form for treating lignocellulosic materials because they are powerful agents for cellulose hydrolysis (Sun and Cheng, 2002). Advantages of concentrated acid hydrolysis are the flexibility in terms of feedstock choice, low concentration of inhibitors, high monomeric sugar yield as well as mild temperature conditions that are needed. Drawbacks of using concentrated acids are the corrosive nature of the acids and the need to recycle acids in order to lower the cost. However, the biomass should have a high dry matter content to recover the acid used, otherwise the water content will dilute the acid used (Harmsen *et al.*, 2013).

1.4.2.2 Alkaline hydrolysis

Alkaline pretreatment is mainly performed to remove the lignin from biomass, which enables the accessibility of the residual polysaccharides (Oh *et al.*, 2015). For alkaline hydrolysis calcium hydroxide, sodium hydroxide, ammonium hydroxide, and potassium hydroxide can be used (Naidu *et al.*, 2018). The substantial amount of hemicellulose removal is depending on the severity of alkali. It is reported that the mechanism of alkaline hydrolysis is depends on the intermolecular linkage breakdown between the hemicellulose and lignin (Sun and Cheng, 2002). Moreover, alkali pretreatments removes the substitutions such as acetyl and few uronic acid substitutions which interferes with the accessibility of enzymes to hemicellulose and cellulose fragments (Zheng *et al.*, 2009). The alkali-treated material is amenable to enzymatic hydrolysis for the release of simple sugars and this pretreatment method is particularly suitable for microbial fermentation where both C6- and C5-sugars are converted to products.

1.4.2.3 Organosolv

Organosolv process refers to the use of an organic solvent or mixtures of organic solvents with water for the removal of lignin which increases the pore volume and accessible surface area of

lignocellulose (Baruah *et al.*, 2018). Other than lignin removal, hemicellulose also be hydrolysed which results in the enhanced enzymatic breakdown of the cellulose fraction. The solvents for such as ethanol, methanol, formic acid, acetic acid ethylene glycol and acetone are commonly used in this method (Sun *et al.*, 2016). The maximum temperature used for the process can be up to 200°C, but the process can be performed at lower temperatures, depending on various factors e.g., biomass type and catalyst usage (Ghose and Bisaria, 1987; Huijgen *et al.*, 2011). Possible catalysts include acids, alkalis and salts which can improve the fractionation and enzyme digestibility of lignocellulosic biomass (Huijgen *et al.*, 2011; Sun *et al.*, 2016).

The main advantages of using organosolv process include high-quality lignin yield, which can be further used for value added chemical production; recoverability of solvents by distillation; lowered enzyme costs as a result of removal of lignin before the enzymatic hydrolysis of the cellulose portion. Removal of lignin in advance could also improve the cellulose accessibility and minimize the absorption of cellulase enzymes to lignin (Harmsen *et al.*, 2013).

1.4.2.4 Oxidative delignification

Delignification of lignocellulose can be achieved by treatment with an oxidizing agent such as H_2O_2, O_3, O_2, NaClO, and Cl_2 (Sun *et al.*, 2016). The effectiveness in delignification depend on the reactivity of oxidizing chemicals with the aromatic ring of lignin. Oxidation reagents help in lignin degradation by attacking and cleaving the aromatic ring structures, while hemicellulose and cellulose are hardly decomposed (Qi *et al.*, 2009; Sun *et al.*, 2016). Oxidising agents can be used to rupture the structure of several lignocellulosic biomass in which the conversion of lignin into carboxylic acids. As the resultant acids can act as inhibitors during fermentation, they must be removed or neutralized. Apart from lignin degradation, this treatment also disturbs the hemicellulose fraction. A significant portion of the hemicellulose might be degraded into toxic products which ends up as waste (Harmsen *et al.*, 2013).

1.4.2.5 Ionic liquids pretreatment

Salts that are present in liquid form at low temperatures are called ionic liquids. There is a broad range of ionic liquids, however, all of them contained an organic cation and inorganic anion as the common characteristic with varied molecular structure (Yang *et al.*, 2019). The variation on the molecular structure renders the linkage between ions very weak and makes the salt to appear as a liquid at room temperature (Baruah *et al.*, 2018). Ionic liquids are different from conventional molecular solvents which possess many appealing properties that makes the thermodynamics and kinetics of chemical reactions carried out in ionic liquids distinctive. Usually ionic liquids comprised of a salt where one or both the ions are large, and the cation with lower degree of symmetry (Earle and Seddon, 2000). In the last decade, ionic liquids employed for dissolution of lignocellulosic biomass such as wood, corn stover, wheat straw, switchgrass, etc and confirmed as efficient (Chen *et al.*, 2017).

1.4.3 Physico-chemical pretreatments

1.4.3.1 Liquid hot water

Liquid hot water (LHW) process is the treatment of biomass with water at high pressure and temperature (Yang *et al.*, 2019). During this pretreatment, hot compressed water is in contact with biomass for 15 min at high temperatures (200–230°C). This process results in the dissolution of 40–60% of the total biomass, with less cellulose removal (4–22%), intermediate lignin removal (35–60%) and complete removal of hemicellulose (Baruah *et al.*, 2018). When the resulting liquid is hydrolysed with acid, over 90% of the hemicellulose can be recovered as monomers. During the course of process, acetic acid is formed, which also catalyse the polysaccharide hydrolysis. These result in the monomeric sugars generation that may further degrade to furfural that may act as an inhibitor of fermentation (Agbor *et al.*, 2011). Variability in results may occur and are dependent on the biomass type with high lignin solubilization impeding the recovery of hemicellulose sugars. The use of no chemicals and low water (>30 wt%) are the main advantage of this pretreatment (Harmsen *et al.*, 2013).

1.4.3.2 Steam explosion

Steam explosion is one of the most employed pretreatment processes owing to its low use of chemicals and limited energy consumption (Harmsen *et al.*, 2013). This method results in complete sugar recovery with low capital investment and low environmental impact concerning the chemicals and conditions being applied and holds great promise for efficiency (Nguyen *et al.*, 2014b; Chen *et al.*, 2017). During steam explosion, the biomass is treated with high-pressure saturated steam, followed by a sudden reduction in pressure, which makes the materials undergo explosive decompression. Steam explosion is typically initiated at a temperature of 160–260°C (corresponding pressure, 0.69–4.83 MPa) for several seconds to a few minutes, before the material is exposed to atmospheric pressure. The biomass-steam mixture is held to promote hemicellulose hydrolysis, and the process is terminated by an explosive decompression (Chen *et al.*, 2017). The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (Menon and Rao, 2012).

1.4.3.3 Ammonia fibre explosion (AFEX) and ammonia recycle percolation (ARP)

Ammonia fibre explosion is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a short time, with a sudden reduction in pressure (De Bhowmick *et al.*, 2018). The AFEX process is very similar to the steam explosion (Menon and Rao, 2012). In a typical AFEX process, the dosage of liquid ammonia used is 1–2 kg of ammonia/kg of dry biomass, with a temperature of 90°C, and a residence time of 30 min (Chen *et al.*, 2017). AFEX pretreatment can significantly improve the fermentation rate of various herbaceous crops and grasses (Zheng *et al.*, 2009). The AFEX technology has been used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, and wheat chaff (Baruah *et al.*, 2018). AFEX pretreatment leads to the decrystallization of cellulose, partial breakdown of hemicellulose, removal of acetyl groups from hemicellulose, cleavage of lignin-carbohydrate complex linkages, increase in accessible surface

area due to structural disruption, and increased wettability of the treated biomass (Menon and Rao, 2012).

An advantage of AFEX is the recovery and reusability of ammonia used during the process. Also, downstream processing is simpler compared to other pretreatment processes (Chen *et al.*, 2017). Over 90% hydrolysis of cellulose and hemicellulose was obtained after AFEX pretreatment of Bermuda grass (approximately 5% lignin) (Agbor *et al.*, 2011) and bagasse (15% lignin). However, AFEX might not be very efficient in case of hardwoods and nutshells which have relatively high lignin content (Taherzadeh and Karimi, 2007).

Another type of process utilizing ammonia is the ammonia recycled percolation (ARP) method. In this process, aqueous ammonia (10–15 wt%) passes through biomass at elevated temperatures (150–170°C) with a fluid velocity of 1cm/min and a residence time of 14 min, after which the ammonia is recovered and recycled (Peral, 2016). Under these conditions, aqueous ammonia reacts primarily with lignin and causes its depolymerization and cleavage of lignin-carbohydrate linkages. The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so a water wash is not necessary (De Bhowmick *et al.*, 2018).

1.4.3.4 CO_2-explosion

In CO_2-explosion, high-pressure CO_2 is introduced into a batch reactor and then released by an explosive decompression (Chen *et al.*, 2017). It is considered that CO_2 reacts with steam and forms carbonic acid (H_2CO_3), and thus improving the rate of hydrolysis. Comparatively, the yields of CO_2 explosion are lower than those obtained from the steam or ammonia explosion (Sun and Cheng, 2002). Even though carbonic acid is a weak acid, it may offer the benefits of acid catalysts without using any strong acids. The partial pressure of CO_2 in water determines the pH of H_2CO_3 and can be neutralized when the pressure of the reactor gets released. It is reported that capital and operational costs of the carbonic acid system are slightly higher than a sulphuric acid-based system (Chen *et al.*, 2017). The efficiency of the treatment is highly susceptible to the pressure of the reactor and solids concentration.

1.4.3.5 Microwave-chemical pretreatment

The microwave-chemical pretreatment results in a more effective pretreatment than the conventional heating chemical pretreatment by increasing the speed of reactions (Zhu *et al.*, 2005, 2006). Treatment with acid and alkali removes hemicellulose and lignin respectively. With microwave-assisted alkali treatment released more lignin than alkali alone. Either shorter time with higher microwave power or longer time with low microwave power result in similar effects on the lignin removal and biomass composition (Kumari and Singh, 2018). A study was conducted by treating bagasse using microwave where the effect of acid, alkali and acid-alkali treatments were investigated for improved fermentable sugar yield (Binod *et al.*, 2012). The combined treatments incorporate the benefits of all treatments and efficiently improve the enzymatic hydrolysis (Chen *et al.*, 2017).

1.4.4 Biological pretreatments

Biological pretreatment is the modification of the chemical composition and/or degradation of the structure of lignocellulosic materials by applying direct microorganisms or enzymes as catalysts (Cavinato *et al.*, 2017). Biological treatment using various types of rot fungi (brown-, red-, white-), a safe and environmentally friendly method, is increasingly being advocated as a process that does not require high energy for lignin removal from lignocellulosic biomass, despite extensive lignin degradation (Kumar *et al.*, 2009b; Menon and Rao, 2012). Although it has so far attracted little attention due to many inherent limitations, biological pretreatment has multiple benefits.

Fungi have distinct degradation characteristics on lignocellulosic biomass. In general, brown and soft rot fungi mainly attack cellulose while imparting minor modifications to lignin, while white-rot fungi more actively degrade the lignin component (Sun and Cheng, 2002). Engineered microbes seem to tackle the problem of bioconversion of substrates that are otherwise non-convertible by conventional wild strains. Current trends are being directed to

nanobiotechnology and genetic engineering for improved processes and products (Chen *et al.*, 2017).

The carbohydrate degrading enzymes are the biocatalysts that could breakdown the polysaccharides into simple sugars. Enzymatic hydrolysis is often preferable because it not only requires less energy due to the relatively mild reaction conditions but also avoids the use of toxic and corrosive chemicals (Xu *et al.*, 2007). Nevertheless, the rate of biological hydrolysis is usually very low, so this pretreatment requires long residence times (Sun and Cheng, 2002; Tengerdy and Szakacs, 2003; Cardona and Sánchez, 2007). Enzymatic systems for lignocellulose hydrolysis are much more complicated than those required for starch hydrolysis. Multiple enzymes, having different substrate specificities (e.g., cellulases, xylanases and other hemicellulases) and catalytic mechanisms (i.e., exoglucanases, endoglucanases, processive endoglucanases, and β-glucosidases), are required to catalyze synergistically for efficient lignocellulose hydrolysis (Wilson, 2011; Bayer *et al.*, 2013).

1.5 LIGNOCELLULOSE DEGRADING ENZYMES

Enzymes are the simplest, cost-effective and convenient tools in the biological conversions of biopolymers. However, it is necessary to produce specific enzymes for the conversion of complex materials into simple sugars (Atalah *et al.*, 2019). Increasing awareness of environmental pollution has enforced the pulp and paper industries to strive for alternate greener technology which will replace the use of harsh chemicals in their processes with microbial enzymes. The application of biocatalysts not only makes the process less toxic but also decreases costs associated with the production and utilization of biomass (Birijlall *et al.*, 2011).

Lignocellulose is a complex molecule that mainly contains cellulose, hemicellulose and lignin. Therefore, to deconstruct the whole structure, a group of cellulases, hemicellulases and ligninolytic enzymes are required. Cellulose breakdown is carried out by cellulases which cleaves the β-1,4 bond in the cellulose chain. It includes endocellulases, exocellulases or

cellobiohydrolases and β-glucosidases. Lignin degradation is a challenging process due to its recalcitrant nature. Some of the important ligninolytic enzymes mainly due to their high activity are lignin peroxidases, manganese peroxidase, versatile peroxidases, and laccases (López-Mondéjar *et al.*, 2019). In contradiction to cellulose and lignin, hemicellulose composes a heterogeneous structure and requires an array of enzymes for complete degradation (Heinen *et al.*, 2018).

1.5.1 Hemicellulases

Hemicelluloses have multiple sugars and more branched structures. Due to its heterogeneity and complexity, the complete hydrolysis of hemicellulose into xylose, arabinose, mannose and galactose requires hydrolytic enzymes such as endo 1,4-β xylanase, β-D-xylosidase, and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylans (Yang *et al.*, 2018).

Over the past several years, the isolation and characterization of hemicellulases producing bacteria and fungi have been investigated extensively (Atalah *et al.*, 2019; Chadha *et al.*, 2019). Among them, *Bacillus* species (with production and secretion of large quantities of extracellular enzymes) continue to be a dominant bacterial workhorse in microbial fermentation (Kazemi *et al.*, 2014). Compared to enzymes from mesophilic sources, thermophilic enzymes tend to be more useful, a trait believed to be achieved through minor alterations in protein structure (Sharma *et al.*, 2019).

1.5.2 Xylanase

Xylanase is the enzyme capable of degrading xylans into xylose and has the potential in producing prebiotic xylooligosaccharides from biomass (Kumar *et al.*, 2017). It also acts as an auxiliary enzyme in the production of biofuels and chemicals from biomass (Binod *et al.*, 2019). They are often produced by microorganisms surviving on plant sources, *viz.*, bacteria,

actinomycetes and fungi (Singh *et al.*, 2019). Endoxylanases have also been reported to catalyze intermolecular transglycosylation in the presence of high concentrations of xylooligomers (Masui *et al.*, 2012).

Industrially important enzymes have a huge global market which reached 5.6 billion US Dollars in 2018 (Global industrial enzymes market overview, 2018). Xylanase, pectinase and cellulase enzymes shares nearly 20% of the total world's enzyme market (M'hamdi *et al.*, 2014; Sahay *et al.*, 2017). Due to high industrial demands, xylanases exhibited revolutionary increase in the financial market which is expected to reach 35 million US Dollars in 2024 (Ahuja and Rawat, 2017). The leading producers of xylanases have been listed in Table 1.2.

Table 1.2 Leading manufacturers of xylanases in the world

Manufacturer	Trade name of xylanase	Country
Danisco Ingredients	Grindazym PF, GP 5000	Denmark
Genencor International	Multifect XL	USA
ABF Group	Rohalase SEP-VISCO, Econase XT	UK
Adisseo	Rovabio Advance	France
Novozymes	Novozym 431, Pulpzyme	Denmark
Enzyme Development Corporation	Enzeco xylanase AN900, S 200	USA
DSM	Ronoxyme WX	Netherlands
Alltech Inc.	Allzym PT, Fibrozyme	USA
BASF Enzymes LLC	Luminase PB 100, PB 200	USA
Takabio	Xylanase	France

(The details were acquired from List of companies, 2019).

Although many xylanases used in industry are from mesophiles, xylanases from thermophilic sources may be of tremendous use in several biotechnological applications (Birijlall *et al.*, 2011). Potentially, xylanase treatment of polysaccharides at high temperatures leads to reduced reaction time and enzyme dosage (Wang *et al.*, 2012). Application of xylanase in pulp and paper

industries has urged considerable research efforts towards producing more thermophilic and alkalophilic xylanases by screening for naturally occurring xylanases. Apart from pulp and paper industries, they also have applications in the bioconversion of agricultural biomass to fermentable sugars and clarification of fruit juices and wines (Nieto-Domínguez *et al.*, 2017). Efforts are being made to produce cellulase-free xylanases from thermophilic/thermotolerant microorganisms which can retain their activity at alkaline pH and high temperatures (Nigam, 2013). Cellulase-free, alkali and thermo-stable microbial xylanases are ideal for bio pulping and bleaching processes (Birijlall *et al.*, 2011). Kaushik *et al.* (2014) produced cellulase-free xylanase from *A. lentulus* that exhibited alkaline stability and thermostability.

From an industrial standpoint, filamentous fungi are particularly interesting as xylanase producers as they excrete substantially greater amounts of xylanolytic enzymes into the culture medium than bacteria or yeast (Haltrich *et al.*, 1996). Many filamentous fungi also produce xylanases with valuable properties, such as thermostability and high-temperature optima. Many industrial processes operate at high temperatures, which makes the use of thermostable enzymes highly attractive (Atalah *et al.*, 2019). A thermostable, cellulase-free xylanase from a filamentous fungus *Thermomyces lanuginosus* has been reported (Singh *et al.*, 2000, 2003) with remarkably good thermostability and wide pH range. A cellulase-free xylanase preparation, from *T. lanuginosus* grown on corncobs medium, has been successfully used to enhance the bleaching of kraft pulp with no adverse effect on the final paper strength (Sinner *et al.*, 1991).

1.5.2.1 Sources of xylanases

Xylanases are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and arthropods (Knob and Carmona 2010) and some members of higher animals, including freshwater mollusks (Yamura *et al.*, 1997). Most of the microbial xylanases have been reported from bacteria and fungi, however, many of them are mesophilic in nature (Chavez *et al.*, 2006). Several bacterial and fungal strains have been studied for the production of xylanase enzymes (Table 1.3) that are stable in harsh environments and have been found more suitable

Table 1.3 Recent reports on the production and properties of xylanases from fungi and bacteria.

Fungal strain (xylanase activity)	**Gene**	**MW (kDa)**	**Optimum pH**	**Optimum temp**	**K_m (mg/ml) / V_{max} (µmol/mg/ min)**	**GH Family**	**Reference**
Aspergillus tamarii Kita (1,215.89 U/mg)	NR	19.5	5.5	60°C	8.13 / 1,330.20	11	Heinen *et al.*, 2018
Bactriola antennata CBS 126.38 (248.2 U/mg)	Xyn11	21.0	5.5	35°C	1.65 / 236.3	11	Liu *et al.*, 2015
Cladosporium neopsychrotolerans SL-16 (453.0 U/mg)	Xyn10A	34.5	6.5-7.0	40°C	1.87 / 331.6	10	Ma *et al.*, 2018
(357.3 U/mg)	Xyn11A	23.5	6.5-7.0	40°C	2.81 / 298.1	11	
Cladosporium sp. (729 U/mg)	XynA	-	6.0	50°C	NR	10	Gil-Durán *et al.*, 2018
Humicola insolens Y1 (382.0 U/mg)	Xyn11B	29.0	6.0	50°C	2.2 / 462.8	11	Shi *et al.*, 2015
Malbranchea pulchella (5.87 U/ml)	MpXyn10A[a]	40.7	5.5	80°C	4.6 / 82	10	Ribeiro *et al.*, 2014
Myceliophthora thermophila BF1-7 (2.31 U/mg)	NR	14.0	12.0	50°C	9.67 / 5.38	NR	Boonrung *et al.*, 2016
Neosartorya tatenoi KKU-CLB-3-2-4-1 (16.07 U/mg)	NR	20.0	10.0	45°C	10.34 / 45.66	NR	Seemakram *et al.*, 2016
Penicillium purpurogenum (37 U/mg)	XynC	49.2	3.5-5	45°C	NR	30	Espinoza and Eyzaguirre, 2018
Trichoderma reesei XYN VI (6-7 U/mg)	XYN VI	47.0	4-5	50°C	NR	30	Biely *et al.*, 2014

Table 1.3 Production and properties of purified xylanases from fungi and bacteria based on recent reports (Continued).

Bacterial strain (xylanase activity)	**Gene**	**MW (kDa)**	**Optimum pH**	**Optimum Temp**	**K_m (mg/ml) / V_{max} (µmol/mg/ min)**	**GH Family**	**Reference**
Caldicellulosiruptor bescii DSM 6725 (497 U/mg)	CbXyn10B[a]	-	7.2	70°C	1.90 / NR	10	An *et al.*, 2015
C. lactoaceticus (6A 44.6 U/mg)	Xyn10A[a]	47.0	6.5	80°C	NR	10	Jia *et al.*, 2014
Caldicoprobacter algeriensis sp. *nov.*, strain TH7C1^T (603.88 U/mg)	XYN35[a]	35.07	11.0	70°C	1.33 / 595	11	Amel *et al.*, 2016
Gloeophyllum trabeum (721.21 U/mg)	Xyl10g[a]	50.0	4.0–7.0	70°C	101.91 / 1820	10	Kim *et al.*, 2014
Herbinix hemicellulosilytica (66 U/mg)	XynA[a]	77.6	6.0	65°C	NR	10	Mechelke *et al.*, 2017
(2.5 U/mg)	XynB[a]	47.6	6.0	45°C	NR	10	
(160 U/mg)	XynC[a]	58.9	6.0	62°C	NR	10	
(271 U/mg)	XynD[a]	47.7	6.0	60°C	NR	10	
(645 U/mg)	XynE[a]	38.7	5.5	57°C	NR	11	
(43 U/mg)	XynF[a]	21.4	5.5	60°C	NR	11	

NB: a-recombinant xylanases; NR- not reported.

for industrial applications as compared to their mesophilic counterparts (Uday *et al.*, 2016). Xylanases with high thermostability could be the better candidates for industrial applications, particularly for enzymatic hydrolysis at elevated temperatures (e.g., bio pulping) where mesophilic xylanases fail to meet desired results (Patel and Savanth, 2015). A number of thermophilic (optimal growth at 50–80°C) and hyperthermophilic (optimal growth at >80°C) xylanase producing microorganisms have been isolated from a variety of sources, including terrestrial and marine solfataric fields, thermal springs, hot pools, volcanic islands, composts and self-heating decaying organic debris (Haki and Rakshit 2003; Singh *et al.* 2003; Cannio *et al.* 2004; Bouacem *et al.* 2014; Elleuche *et al.* 2015; Palavesam 2015; Kumar *et al.* 2018). Among the reported xylanase producers, strains of *Thermomyces* have been found to produce high titres of thermostable xylanolytic enzymes (Kumar *et al.*, 2009a).

There are also reports on the existence of multiple forms of xylanases from different microorganisms (Turner *et al.*, 2007). Elegir *et al.* (1994) reported that *Streptomyces* sp. B-12–2 produced five endoxylanases when grown on oat-spelts xylan. Around 15 xylanases have been reported from the culture filtrates of *A. niger* and 13 xylanases from *T. viride* (Biely *et al.*, 2016). The most outstanding case of the existence of multiple forms of xylanases was the separation of more than 30 different protein bands by analytical electro focusing from *Phanerochaete chrysosporium* grown on Avicel (Dobozi *et al.*, 1992). Heteroxylans having a complex structure require the action of multiple xylanases with overlapping but different specificities as all of the xylosidic linkages in the substrates are not equally accessible to xylan-degrading enzymes (Elleuche *et al.*, 2015).

1.5.2.2 Xylanase production

Different enzyme production levels have been reported to date, which largely vary with the type of microbes, substrate and culture conditions (Patel and Savanth, 2015). *Thermobacillus xylanilyticus* produced xylanase with a specific activity of 480.66 U/mg (Rakotoarivonina *et al.*, 2015). Xylanase produced by *Stenotrophomonas maltophilia* strain X6 had a specific activity of

313.38 U/mg (Raj *et al.*, 2013). Xylanase produced by *Halomonas meridiana* APCMST-KS4 has 26.13 U/mg specific activity (Palavesam, 2015). Family 10 xylanase produced by *C. thermocellum* has a specific activity of 93 U/mg and family 11 xylanase by *Streptomyces lividans* has 119.5 U/mg specific activity (Gonçalves *et al.*, 2015). Xylanase produced by *Streptomyces* sp. CS428 has 926,103 U/mg of specific activity (Pradeep *et al.*, 2013). *Scytalidium thermophilum* produced xylanase with a specific activity of 841.1 U/mg (Kocabaş *et al.*, 2015). *P. janczewskii* produced xylanase with a specific activity of 179.1 U/mg (Terrasan *et al.*, 2013).

A xylanase gene from the extremely thermophilic bacterium *Geobacillus thermoleovorans* was cloned and expressed in *E. coli* BL21 (DE3) with a specific activity of 270 U/mg (Verma and Satyanarayana, 2012). Endo-xylanase gene from a thermophilic bacteria *Geobacillus* sp. WSUCF1 was cloned and expressed heterologously in *E. coli* with good expression level and a specific activity of 461 U/mg (Bhalla *et al.*, 2014). The xylanase gene from *Thermotoga thermarum* was cloned and expressed in *E. coli* BL21 (DE3) with high thermostability and had a specific activity of 145.8 U/mg (Shi *et al.*, 2013). Recombinant xylanase from *Neocallimastix patriciarum* was expressed in *E. coli* and *P. pastoris* with a specific activity of 5778.3 U/mg and 7995.3 U/mg respectively (Cheng *et al.*, 2014). The overexpression of xylanase from *Penicillium occitanis* Pol6 in *P. pastoris* was found to have a specific activity of 8549.85 U/mg (Uday *et al.*, 2016).

1.5.2.3 Xylanases from various glycoside hydrolase families

Xylanases have been grouped into different glycoside hydrolase (GH) families depending on their substrate preference and specific mode of action (López-Mondéjar *et al.*, 2019). The most common xylanase families are GH10, GH11 and GH30 (Biely *et al.*, 2016). Irrespective of their source, whether it is from different bacteria or fungi, xylanases from each family comprise a similar mode of action. Xylanase belongs to the same family produces similar types of products.

1.5.2.4 GH10 xylanase- mode of action

GH10 xylanases require two consecutive unsubstituted xylopyranosyl residues apart from the reducing end to break down the main chain of xylan and they can cleave the glycosidic linkage to xylopyranosyl residue substituted with MeGlcA (Figure 1.6) (Biely *et al.*, 2016). Aldotetraouronic acid, in which 4-*O*-methyl-D-glucuronic acid is linked to the non-reducing end of xylotriose, cannot be hydrolyzed by any of the GH10 enzymes. Aldohexaouronic acid, with 4-*O*-methyl-D-glucuronic acid attached to the middle xylopyranosyl unit of xylopentaose, served as a substrate for the xylanases of GH10 with xylobiose and aldotetraouronic acid ($MeGlcA^3Xyl_3$) as their hydrolysis products.

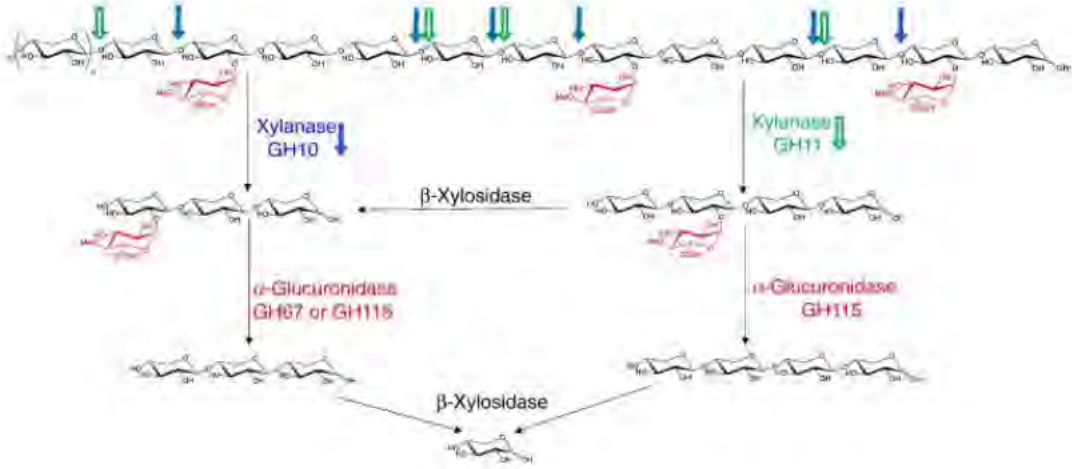

Figure 1.6 Mode of action of GH10 and GH11 xylanases on alkali extracted glucuronoxylan (adapted from Biely *et al.*, 2016). The smallest XOS released by GH10 xylanase is $MeGlcAXyl_3$, whereas $MeGlcAXyl_4$ is the product released by GH11 xylanase, which is further converted into single units by the action of glucuronidase and β-xylosidase.

Further hydrolysis with β-xylosidase and α-glucuronidase confirms the structure of oligosaccharides. β-Xylosidase liberates only non-substituted xylopyranosyl residues from the non-reducing end of xylooligosaccharides (Tenkanen *et al.*, 1996). Thus, products of the action of β-xylosidase are xylose and xylooligosaccharides with glucuronosyl residue attached to the non-reducing end. On the other hand, α-glucuronidase can release only 4-*O*-methyl-D-

glucuronic acid attached to the non-reducing end of xylooligosaccharides (Biely *et al.*, 2000). So, the products of α-glucuronidase action are 4-*O*-methyl-D-glucuronic acid and homoxylooligosaccharides (Figure 1.6) (Biely *et al.*, 2016).

1.5.2.5 GH11 xylanase- mode of action

GH11 xylanases possess a different mode of action compared to GH10 xylanases. They recognize three unsubstituted xylopyranosyl residues in a row as a cleavage site and attack xylan chain one linkage before the Xyl*p*, substituted with MeGlcA (Figure 1.6) (Biely *et al.*, 2016). Since these enzymes release aldopentaouronic acid as the shortest acidic fragment from glucuronoxylan, it is not surprising that aldotetraouronic and aldopentaouronic acids are not hydrolyzed. The low extent of hydrolysis of aldohexaouronic acid reflects a generally low rate of hydrolysis of short oligosaccharides by GH11 xylanases which is further impeded with their substitution by MeGlcA (Patel and Savanth, 2015).

1.5.2.6 GH30 xylanase- mode of action

The GH30 family includes xylanases which perform the controlled hydrolysis of glucuronoxylan. Traditionally these enzymes were classified in GH5 family. MeGlcA or GlcA substituents in the glucuronoxylan determine their mode of cleavage of the main chain. The very first report on this type of enzyme was published even before the CAZy classification has been established (Nishitani and Nevins, 1991). Majority of the bacterial GH30 xylanases (St John *et al.*, 2014) and some fungal species, e.g. *T. reesei* XYNVI (Biely *et al.*, 2014), attack glucuronoxylan main chain at the second glycosidic linkage to the MeGlcA substituents towards the reducing end (St. John *et al.*, 2006; Vršanská *et al.*, 2007; Gallardo *et al.*, 2010). MeGlcA or GlcA thus determines the sites of polysaccharide cleavage (Figure 1.7). The enzymes show extremely low specific activities on unsubstituted xylan chain, linear xylooligosaccharides or xylan that does not contain uronic acid side residues. From hardwood glucuronoxylan, GH30 xylanase generate a mixture of aldouronic acids of general formula $MeGlcA^{2}Xyl_{n}$ where 'n' is dependent on the distribution of uronic acid side residues on the main chain (Figure 1.7).

All these aldouronic acids produced by GH30 xylanase can be theoretically debranched by GH115 α-glucuronidase and then degraded to xylose by other types of xylanases and β-xylosidase or converted by the action of β-xylosidase to $MeGlcA^2Xyl_2$ which would serve as a substrate for GH67 α-glucuronidase (Figure 1.7). More densely MeGlcA-substituted glucuronoxylan regions may be more recalcitrant to the action of GH30 enzymes. There are reports that GH30 xylanases can also cleave neutral xylooligosaccharides (Gallardo *et al.*, 2010) and acetylated xylan chain not substituted by MeGlcA (Busse-Wicher *et al.*, 2014). This activity could be just a side activity of GH30 xylanases observed at a very high enzyme load. The enzymes undoubtedly recognize the COO^- group of the uronic acid residue. The elimination of the carboxyl group by esterification with methanol or conversion of MeGlcA to 4-*O*-methyl-D-glucose leads to an enormous decrease in the specific activity of GH30 xylanases. Interestingly, the cleavage of the modified glucuronoxylan takes place again at the second glycosidic linkage following the branch towards the reducing end (Biely *et al.*, 2015).

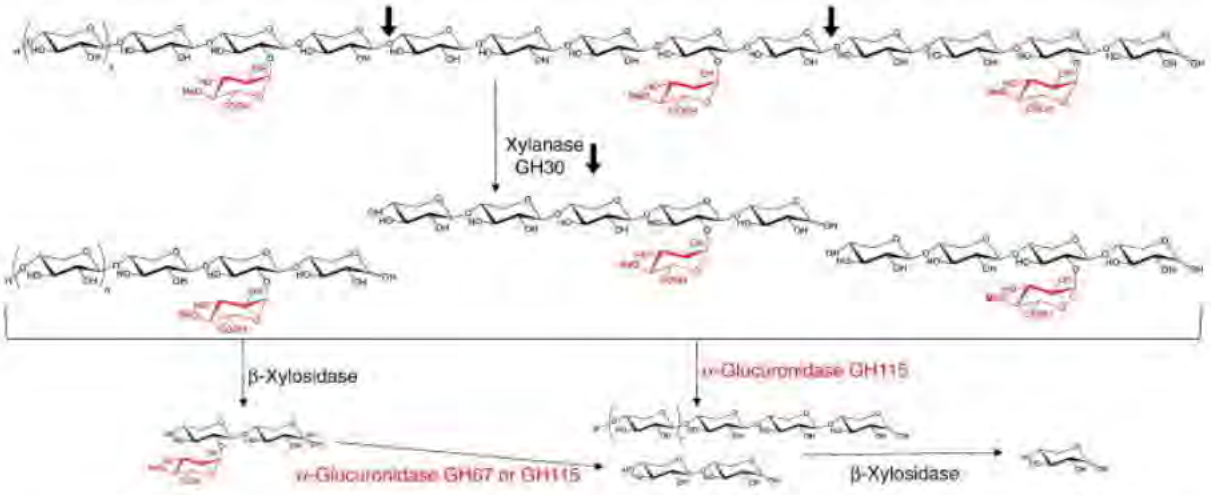

Figure 1.7 Mode of action of GH30 xylanase on alkali extracted glucuronoxylan (adapted from Biely *et al.*, 2016). The basic XOS produced by GH30 xylanase depend on the MeGlcA substitution on the xylan main chain.

1.5.3 Accessory enzymes for xylan degradation

1.5.3.1 Xylosidase

β-Xylosidases (EC 3.2.1.37) are exo-type glycosidases that catalyze the hydrolysis of 1,4-β-D-xylooligosaccharides by removing successive xylose residues from the nonreducing termini. It also releases xylose from branched or substituted xylooligosaccharides produced by the action of endo-1,4-β-xylanases (Subramaniyan and Prema, 2002; Biely, 2003; Terrasan *et al.*, 2013). The systematic name is 1,4-β-D-xylan xylohydrolase; however, commonly known as β-xylosidase and is found in GH families 3, 39, 43, 52, and 54 (Shallom and Shoham, 2003). For many industrial applications such as improving bread dough, production of xylitol, and deinking of recycled paper, β-xylosidases are used in combination with xylanases (Jordan and Wagschal, 2010).

Various microorganisms including bacteria and fungi are reported to produce β-xylosidase, however, very few yeast strains are known to produce the enzyme (Basaran and Ozcan, 2008). *B. subtilis* M015 was reported to produce intracellular β-xylosidase (Banka *et al.*, 2014). β-Xylosidase production has been documented from fungal species such as *Aspergillus awamori* (Paredes *et al.*, 2015), *A. terricola* and *A. ochraceus* (Michelin *et al.*, 2012a, 2012b), *Neocallimastix* sp. M2 (Comlekcioglu *et al.*, 2011), *P. janthinellum* (Kundu and Ray, 2013) and *P. janczewskii* (Terrasan *et al.*, 2013).

1.5.3.2 Arabinofuranosidase

Arabinofuranosidase is the enzyme that attacks arabinoxylan or arabinan and releases arabinofuranoside (Ara*f*) residues (Lagaert *et al.*, 2014). They have been grouped in 3, 43, 51, 54 and 62 GH families. There are two types of α-L-arabinofuranosidases based on their positional specificity and the need for an adjacent hydroxyl group on Xyl*p* residue. The first type of enzyme that acts on singly substituted Xyl*p* with Ara*f* and the second group of enzymes that attacks the doubly arabinosylated Xyl*p* (McCleary *et al.*, 2015). Some of the

α-L-arabinofuranosidases from GH51 showed unique activities on arabinosylated Xyl*p* present in the non-reducing end (Lagaert *et al.*, 2010; Sakamoto *et al.*, 2013; Borsenberger *et al.*, 2014; Koutaniemi and Tenkanen, 2016). GH43 α-L-arabinofuranosidases from *B. adolescentis* and *H. insolens* liberated Ara*f* from double substitution of Xyl*p* in arabinoxylan and ara*f* residue in arabinan (Lagaert *et al.*, 2010; Cartmell *et al.*, 2011). Most importantly, these enzymes cannot liberate Ara*f* residue that has any type of substitution or esterification (Biely *et al.*, 2011).

1.5.3.3 Glucuronidase

Glucuronidase cleaves the linkage between MeGlcA and Xyl*p* and plays a vital role in xylan degradation where α-1,2-linkage is very stable for acid hydrolysis (Biely *et al.*, 2016). To date, only two types of α-glucuronidases have been recognized and were grouped in GH families; GH67 and GH115. GH67 α-glucuronidases can release MeGlcA substituted on the Xylp residue at non-reducing end and are unable to liberate internal Xyl*p* linked MeGlcA (Biely *et al.*, 2000; Zaide *et al.*, 2001; Nurizzo *et al.*, 2002; Nagy *et al.*, 2003). On the other hand, α-glucuronidases that belong to GH115 can liberate MeGlcA from Xyl*p* residues present at both internal and non-reducing sugars (Tenkanen and Siika-aho, 2000; Ryabova *et al.*, 2009).

Unlike GH67 enzymes, GH115 enzymes have a cleft that holds the Xyl*p* at both sides of MeGlcA substituent (Golan *et al.*, 2004; Kolenová *et al.*, 2010; Rogowski *et al.*, 2014; Martínez *et al.*, 2016; Wang *et al.*, 2016). Therefore, theoretically, a cocktail of GH10 and GH11 xylanase with β-xylosidase and α-glucuronidase (GH67/GH115) should completely debranch the alkali extracted glucuronoxylan. If the MeGlcA is further substituted with other residues, the action of α-glucuronidase is limited. Such glucuronoxylan is found in Eucalyptus where 30% of glucuronic acid substitutions have been further substituted with galactose residues (Shatalov *et al.*, 1999; Teleman *et al.*, 2000; Kabel *et al.*, 2002; Magaton *et al.*, 2013).

1.5.4 Application of xylanases

Xylanases constitute the major commercial proportion of hemicellulases but represent only a small percentage of the total enzyme market. Xylanolytic enzymes from microorganisms have attracted a great deal of attention owing to their biotechnological potential in various industrial processes, such as food, feed, and pulp and paper industries (Chavez *et al.*, 2006; Knob *et al.*, 2014; Patel and Savanth, 2015). Other less well documented putative applications include: brewing, to increase wort filterability and reduce haze in the final product (Tikhomirov *et al.*, 2003; Raj *et al.*, 2013; Elleuche *et al.*, 2015); in coffee extraction and in the preparation of soluble coffee (Wong *et al.*, 1988); in detergents (Kumar *et al.*, 2004); in the protoplastation of plant cells (Kulkarni *et al.*, 1999); in the production of pharmacologically active polysaccharides having antimicrobial (Christakopoulos *et al.*, 2003) or antioxidant properties (Katapodis *et al.*, 2003); in the production of alkyl glycosides having surfactant properties (Matsumura *et al.*, 1999); and in the washing of precision devices and semiconductors (Imanaka and Sakurai 1992). Other potential applications include the conversion of xylan in wastes from food and agricultural industry into xylose and xylooligosaccharides (Bhalla *et al.*, 2013), and in the bioconversion of lignocellulosic materials to fuels and chemical feedstocks (Banka *et al.*, 2014; Thomas *et al.*, 2014; Palavesam, 2015).

Xylanases are used in the food industry in several applications including baking, juice preparation, and starch processing (MacCabe *et al.*, 2002). Xylanases together with amylolytic enzymes and proteases play a pivotal role in bread-making (Pillai, 2010). The xylanases, like the other hemicellulases, break down the hemicellulose in wheat-flour, helping in the redistribution of water and leaving the dough softer and easier to knead (Passarinho *et al.*, 2019). Synergistic action of xylanase and cellulase mixtures could result in the efficient release of sugars from lignocelluloses (Harris *et al.*, 2014; Gonçalves *et al.*, 2015). Degradation of lignocellulosic biomass by xylanase in synergism with other enzymes can be used for the generation of biofuels (ethanol), xylitol (sweetener), and organic acids, via fermentation (Ahmed *et al.*, 2012; Raj *et al.*, 2013; Bibi *et al.*, 2014).

In juice making processes, xylanases are applied for juice clarification (Atalah *et al.*, 2019). Xylanases are also used in the animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytases, galactosidases and lipases. These enzymes breakdown xylans in the ingredients of the feed, reducing the viscosity of the raw material (Polizeli *et al.*, 2005; Knob *et al.*, 2014). Xylanases have also significant applications in the paper and pulp industry. Hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the level of chlorine used as the bleaching agent (Morais *et al.*, 2011; Ellis and Magnuson, 2012).

The commercial and biotechnological usage of xylanases have increased over the past few decades. Due to their great biotechnological potential, xylanases grab attention in food industries, feed industries, paper industries, pharmaceutical industries and biofuel production (Kalim *et al.*, 2015; Jemli *et al.*, 2016). The major commercial xylanase producing countries are USA, Canada, Denmark, Germany, Ireland, Finland and Japan (Bajpai, 2014). *Humicola insolens, Aspergillus niger* and *Trichoderma* sp. are some of the commonly used microorganisms for commercial xylanase production (Polizeli *et al.*, 2005; Harris and Ramalingam, 2010). Formerly, xylanase was only used to prepare the animal feeds. Nowadays, industries are focusing to develop acid-free hydrolysis using various enzymatic processes for the treatment of hemicellulose-included materials (Singh *et al.*, 2019).

The biotechnological potential of xylanase also aroused a huge interest in industrial areas such as ethanol and xylitol production in paper and pulp industry; fuel, protein from cell and platform chemical production in food industry (Guimaraes *et al.*, 2013; Irdawati *et al.*, 2018). The abundant production of food, agricultural and forest waste materials should be processed with proper processing and technologies. Since the xylan is the second most polymer in the plant cell wall, microbial xylanases and xylanase producers can be modified to utilize in the food, sugar, ethanol, paper and agro-industries (Sridevi *et al.*, 2016; Walia *et al.*, 2017).

In the pulp industry, xylanases are employed in the bleaching of wood pulp, where they remove the xylan and function as an alternative for chlorine treatment which reduces the environmental

impact (Walia *et al.*, 2017). In combination with ligninolytic enzymes, xylanases can efficiently degrade the apparent xylan and make the fibre more accessible. On the other hand, xylanase has been applied to convert plant biomass into simple fermentable sugars or other anticipated products. A cocktail of several hydrolytic enzymes can be applied to the complex lignocellulosic biomass to get monosaccharides which further can be used as substrates for bioethanol production (Bibra *et al.*, 2018). Recently, xylanases were used in the mucilage extraction from fern fronds where they showed positive effects (Chiang and Lai, 2019).

Controlled hydrolysis of plant-derived polymers has also been practiced nowadays where the intermediate products and their features are suitable for various desirable properties. This includes prebiotic oligosaccharides which are selectively produced by enzymatic hydrolysis. Due to their various mode of action, xylanases applied on xylan which resulted in the production of selective oligosaccharides that provide beneficial effects in human intestinal tract (Karlsson *et al.*, 2018). Xylanase also plays an important role in the partial degradation of plant biomass for poultry and cattle feed production which resulted in enhanced digestibility and increased nutritional value (Juturu and Wu, 2012). The usage of thermophilic xylanase is very important especially in the processing of cellulose and wood fibre which takes place at higher temperatures.

1.6 VALUE-ADDED PRODUCTS FROM LIGNOCELLULOSE

The basic composition of lignocellulose consists of biopolymers that have the potential to be converted to value-added chemicals and fuels. New technologies that promote environmentally friendly and cost-effective processing are the key factors for the conversion of renewable and biodegradable lignocellulosic biomass into value-added products (Hassan *et al.*, 2018). A large number of products can be derived from the processing of lignocellulose (Ye *et al.*, 2014). Such products are mainly grouped into biofuels and biochemicals. Examples of biofuels include bioethanol, biobutanol, biohydrogen, and biochemicals such as elastomers, fibres, resins, sugars, oligosaccharides, antibiotics, flavours, dyes, vitamins, polyols, surfactants, oils, dextrin,

ethyl ester, organic acids and biomaterials. These are used in industries such as transportation, energy, pharmaceutical, chemical, paper, food, wood, pollution treatment and cleaning (Chadha *et al.*, 2019).

1.6.1 Bioethanol

Bioethanol is currently one of the most promising alternatives to conventional petroleum-based transport fuels. It can be produced either from starchy materials or cellulose and hemicellulosic sources (first-generation ethanol). It is an effective alternative for fossil fuels that causes pollution (Hasunuma *et al.*, 2013). As a potential biofuel, it is ideally suited to today's automobile engines as it exhibits high energy density, requires little energy in their production, nontoxic, and most importantly result in reduced emissions during combustion (Dutta and Pal, 2014). There are many ongoing efforts devoted towards the use of engineered and native microorganisms for use as industrial producers of second-generation ethanol (Balusu *et al.*, 2004; Jarboe *et al.*, 2007; Olofsson *et al.*, 2008; Chen *et al.*, 2009; Mazzoli *et al.*, 2014). In contrast to fossil fuels, cellulosic ethanol produced through the fermentation of sugars is a renewable energy source. It is well documented that cellulosic ethanol offers greater environmental benefits and sustainability; however, the concern is the economic viability of the process. To implement successfully the bioethanol production process, the first impediment to be resolved is the efficient removal of lignin and hemicellulose through a cost-effective pretreatment process (Menon and Rao, 2012).

The biofuels from biomass are more expensive and harder to extract. Researchers have tried to simplify the production of biofuels to construct the process easier and convenient at a low cost. The large differences in optimum temperatures between saccharification and fermentation during the simultaneous saccharification and fermentation process, is a drawback of second-generation bioethanol production (Hasunuma and Kondo, 2012). Ethanol production is mainly thought to occur when hexose sugars are fermented, due to the reduction of acetyl-CoA to ethanol by two extra NADPH molecules that are produced (Mazzoli *et al.*, 2014). Another

approach for increasing ethanol production could be the selection of enrichment process for more ethanologenic lactic acid bacteria in which a *Lactobacillus pentosus* strain was isolated through a series of selection and enrichment procedures (Kim *et al.*, 2010).

1.6.2 1, 2 and 1, 3-Propanediol

1, 2-Propanediol (propylene glycol) is a major commodity chemical with a variety of uses. It is produced by a synthetic process from propylene oxide, a non-renewable petrochemical derivative (Corma *et al.*, 2007). Lactic acid has both carboxylic and hydroxyl groups and can be converted into 1,2-propanediol (Fan *et al.*, 2009). Production of 1,2-propanediol by direct hydrogenation of bio-based lactic acid can be an alternative route to the petroleum-based process (Gao *et al.*, 2011). Generally, hydrogenation of lactic acid by chemical catalysts requires esterification of lactic acid and successive hydrogenation.

A valuable bio-functional molecule, 1,3-propanediol can be produced from renewable resources like bagasse using microorganisms. It has several promising properties for many synthetic reactions, particularly for polymer and cosmetic industries. Glycerol is a natural substrate for microbial production of 1,3-propanediol (Tan *et al.*, 2010). The utilization of crude glycerol in the fermentation medium offers a remarkable advantage against the traditional use of pure glycerol as substrate, in some cases, the observed final 1,3-propanediol concentration was higher than 100 g/l and the productivity was about 3.0 g/l/h (Tan *et al.*, 2010).

1.6.3 Biohydrogen

Hydrogen is considered as the most promising alternative energy since it produces only water during combustion and has a higher energy density (122 kJ/g) than hydrocarbon fuels (Anburajan *et al.*, 2019). It is the only fuel that does not produce CO_2 as a by-product when used in fuel cells for electricity generation (Florio *et al.*, 2019). Fermentative hydrogen production from organic substrates has the advantage of simultaneous clean energy production

and waste reduction (Ghosh and Roy, 2019). To reduce the production cost, it is preferable to produce biohydrogen from cheap and renewable lignocellulosic materials (Guo *et al.*, 2010).

Biohydrogen production of sugars through anaerobic fermentation is recognized as a very promising, environmentally friendly and feasible process (Hawkes *et al.*, 2007). The dark fermentation for biological hydrogen production has gained enormous attention due to its inherent advantages over the photo fermentation process. The solid waste and wastewaters with high biodegradable organic content from food processing industries, sugarcane and/or lignocellulose-based refineries, have high biohydrogen production potential (Das and Veziroğlu, 2001; Ntaikou *et al.*, 2010). Several studies on the utilization of C5 and C6 sugars for biohydrogen production have been reported (Khamtib and Reungsang, 2012; Gadhe *et al.*, 2014; Nayak *et al.*, 2014; Reilly *et al.*, 2014; Anburajan *et al.*, 2019). The microorganisms used for hydrogen production include cyanobacteria, photosynthetic bacteria, and other bacteria such as facultative anaerobes (*Enterobacter* sp. and *Citrobacter* sp.) and strict anaerobes (*Clostridium* sp. and rumen bacteria) (Ramprakash and Muthukumar, 2014).

1.6.4 Lactic acid

Lactic acid is a versatile chemical used widely in the food, cosmetic, pharmaceutical, chemical, leather, and textile industries mainly as an acidulant, preservative and flavour compound (Tan *et al.*, 2010; Fan *et al.*, 2011). Search for diverse sources for lactic acid production has surged due to the increasing demand for eco-friendly polylactic acid (Zhang *et al.*, 2014a). Lactic acid has been produced from renewable materials such as corncob, molasses, rice and wheat bran, Jerusalem artichoke tubers, cassava bagasse, sugarcane bagasse derived cellulose, and many woody residues (Wang *et al.*, 2015). However, complete utilization of both cellulosic and hemicellulosic sugars is essential for the efficient and cost-effective conversion of lignocellulose to lactic acid (Ye *et al.*, 2014).

Commercial production of lactic acid is carried out by either chemical synthesis or by biological fermentation (Wang *et al.*, 2015). The synthetic production route has several limitations which

include limited yield (because it is a by-product of biochemical process), and inability to make the desirable L-lactic acid isomer coupled with high manufacturing costs. During the last decade, lactic acid is manufactured mainly by carbohydrate fermentation. Lactic acid bacteria and *A. niger* are the predominant organisms that are used for the commercial production of lactic acid (Datta and Henry, 2006). Thermotolerant *B. coagulans* strain has been reported to be able to ferment pentose sugars completely to L-lactic acid via the pentose phosphate pathway (Ye *et al.*, 2013). Sugars are also catabolized through the Embden–Meyerhof pathway in bacteria, and its end product, i.e., pyruvic acid, is enzymatically reduced either by D-lactate dehydrogenase or L-lactate dehydrogenase, giving rise to the two lactic acid enantiomers (Mazzoli *et al.*, 2014). In order to reduce the fermentation cost for the production of lactic acid, much effort has been made on the development of thermophilic lactic acid bacteria and cheaper feedstocks (Tan *et al.*, 2010). Along with high product specificity, fermentative production of lactic acid tenders several advantages compared to chemical syntheses, such as the low cost of substrates, low production temperature requirement, and low energy consumption (John *et al.*, 2009). However, lactic acid bacteria with industrial potential should be homofermentative and produce optically pure isomers of lactic acid. The bacteria should be highly tolerant to inhibitors, lactic acid and salt concentrations, high temperature and low pH (Nguyen *et al.*, 2012).

1.6.5 Succinic acid

Succinic acid naturally occurs in humans, animals, plants, and microorganisms and plays a pivotal role in biological metabolism (Jiang *et al.*, 2013). As a promising platform chemical, succinic acid has been extensively used as an acidulant, flavouring agent or as a preservative in the food industry. In the pharmaceutical industry, it is mainly used to control acidity (Diaz *et al.*, 2018). Moreover, it serves as a building block for many industrially valuable chemicals such as 1,4 butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid as well as for biodegradable polymers such as polybutrate succinate (McKinlay *et al.*, 2007).

Currently, the commercial succinic acid production is mostly chemically synthesized from butane, derived from petroleum (Morales *et al.*, 2016). In order to produce succinic acid in a bio-based industrial process, the medium used must be of low-cost and succinic acid could be separated easily. In comparison to the petroleum-based process, renewable biological resources used for succinic acid fermentation has gained the focus of research (Jiang *et al.*, 2013). In biological production, succinic acid can be produced during anaerobic fermentation by some anaerobic and facultative anaerobic microorganisms (Song and Lee, 2006). Shi *et al.* (2014) reported that an immobilized fermentation system is more efficient, economically stable and suitable for large-scale production of succinic acid.

1.6.6 Furfural

Furfural is a biofuel precursor and a very versatile and promising bio-based platform chemical with wide applications in several industries including plastics, pharmaceuticals, agrochemicals, etc. (Zhang *et al.*, 2014b). It is produced from lignocellulosic biomass by dehydrating pentoses which are abundantly present in hemicellulose (Mao *et al.*, 2012). Mainly, xylan served as the source that is hydrolyzed to xylose and other monosaccharides, and further dehydration reactions of the pentoses yield furfural.

In the last 2 decades, plenty of efforts have been made to improve the furfural yield. Although the furfural yield of more than 80% was achieved when pure xylose was used as a substrate, the yield of furfural rarely exceeded 60%, when it was produced from lignocellulosic biomass via the one-step process. Therefore, with focussed research, the furfural production was increased in acetic acid and $FeCl_3$ co-catalyzed hydrolysis of corncob up to 67.89% (Mao *et al.*, 2012). An efficient method was developed for furfural production with a yield of 79.6% from untreated corncob using $FeCl_3.6H_2O$ as catalyst (Zhang *et al.*, 2014c). In a different approach, the furfural yield was achieved up to 75% with butyrate/water at 200°C without the addition of an acid catalyst (Hua *et al.*, 2016). Recently, a vapor-releasing reactor system was employed in the absence of catalyst which yielded 73% of furfural (Liu *et al.*, 2018).

1.6.7 Oligosaccharides

Oligosaccharides are sugar molecules with a DP between 2 and 10 and can be classified as digestible or non-digestible based on the physiological properties (Koga and Fujikawa, 1993; Samala *et al.*, 2014). Plant biomass is rich in hemicellulose and cellulose, and can be further broken down into oligosaccharides by hydrolysis methods (Samala *et al.*, 2014). Currently, scientific attention has been directed towards finding and producing non-digestible oligosaccharides, which exhibit the physicochemical and physiological properties beneficial to human health (Olmos and Hansen, 2012). Mussatto and Mancilha (2007) reported that non-digestible oligosaccharides are fermentable substances by gut bacteria in the large intestine that benefit human health and are termed as prebiotics. The modulation of the immune system by oligosaccharides and their role in the reduction of lifestyle-related diseases as well as the maintenance and improvement of human health has also been cited as an area of growing importance. With the increasing health consciousness among consumers and the rapid progress of physiologically active functional foods, the future profile of products containing oligosaccharides with biological activities acquires great attention (Córdova *et al.*, 2019).

1.6.7.1 Oligosaccharides from lignocellulose

Various types of oligosaccharides can be produced from lignocellulose depending on the depolymerization of suitable raw materials. Most commonly cellooligosaccharides (COS), xylooligosaccharides (XOS), arabinooligosaccharides (AOS), galactooligosaccharides (GOS), mannooligosaccharides (MOS), arabino xylooligosaccharides (AXOS), galacturono oligosaccharides and rhamnogalacturono oligosaccharides are produced from the lignocellulosic biomass (Zhao *et al.*, 2017). Unlike other oligosaccharides, xylooligosaccharides gain more attention due to its high range acid stability, organoleptic properties, multidimensional benefits on human health and it is the only nutraceutical produced from lignocellulosic biomass (Amorim *et al.*, 2019a).

1.6.7.2 Xylooligosaccharides

Xylooligosaccharides are xylose-based oligomers containing two to ten xylose molecules linked by β 1–4 bonds (Ferrão *et al.*, 2018). They are decorated with a diversity of substituents, such as acetyl groups, uronic acids and arabinose units (Rahmani *et al.*, 2019). Many agricultural residues have been explored for the production for XOS (Table 1.4). XOS can be produced by the breakdown of xylan chains using different methods, such as chemical processing, enzymatic hydrolysis or a combination of both methods (Samala *et al.*, 2014; Álvarez *et al.*, 2017).

Xylanases attack the xylan backbone randomly to produce both substituted and non-substituted shorter chain oligomers, xylobiose and xylose. However, xylosidases are essential for the complete breakdown of xylan as they hydrolyze xylobiose and xylooligosaccharides to xylose (Zhang *et al.*, 2014c). The enzymes arabinosidase, α-glucuronidase and acetyl xylan esterase act in synergy with the xylanases and xylosidases by releasing the substituents on the xylan backbone to achieve total hydrolysis of xylan to monosaccharides (Bosetto *et al.*, 2016). XOS stabilities can differ greatly depending on the types of oligosaccharide and sugar residues, linkages, ring forms and anomeric configurations. Generally, β-linkages are stronger than α-linkages, and hexoses are more strongly linked than pentoses (Carvalho *et al.*, 2013).

Most of the oligosaccharides are susceptible to pH <4.0, higher temperatures for short time or prolonged storage at room conditions and resulting in the loss of nutritional and physicochemical properties. Nevertheless, the XOS are stable over a wide range of pH (2.5–8.0), such as the relatively low pH value of gastric juice and can also withstand at higher temperatures (100°C) (Carvalho *et al.*, 2013; Amorim *et al.*, 2019a).

Table 1.4 Production of XOS from different agricultural residues via biological conversion.

Substrate	Pretreatment (PTT)	Biocatalyst	Time (h)	$Y_{XOS/xylan}$[a] (mg/g)	DP[c]	Reference
Arecanut husk	Two-stage alkali PTT	endo-1, 4-β-xylanase M1 from *Trichoderma viride*	24	351.0	X_2-X_4	Singh *et al.*, 2018b
Brewers' spent grain	No PTT	Commercial xylanase from *Trichoderma longibrachiatum*	12	444.3	X_2-X_5	Amorim *et al.*, 2019d
		Trichoderma reesei	72	326.2	X_2-X_5	
		Recombinant *B. subtilis* 3610	12	463.41	X_2-X_6	Amorim *et al.*, 2018
Corncobs	Steam explosion using acidic electrolyzed water	*Paenibacillus barengoltzii* (PbXyn10A) xylanase	12	750.0	X_2-X_4	Liu *et al.*, 2018
Fresh whole kenaf stem	Milled, 7% $Ca(OH)_2$ (1.5 h) and 20% peracetic acid (PPA) (2 h)	Recombinant Xyn2 from *Trichoderma reesei* ATCC 58350	48	294.92	X_2-X_5	Azelee *et al.*, 2016
Finger millet seed coat	Defatted, de-starched and water-extracted (25 °C during 8 h)	Commercial xylanase from *Thermomyces lanuginosus*	5	720.0	X_2, X_3	Palaniappan *et al.*, 2017
Mahogany	Thermal PTT (121 °C for 15 min) with 0.05 N NaOH	*Clostridium* sp. BOH3 xylanase	12	572.0[b]	X_2-X_5	Rajagopalan *et al.*, 2017
Mango xylan				504.0[b]	X_2-X_3	
Pretreated corn cob	Ultra-high-pressure PTT	*Streptomyces thermovulgaris* TISTR1984 xylanase	18	106.6[b]	X_2-X_4	Seesuriyachan *et al.*, 2017
Quinoa stalks	Akaline extraction (0.5 M NaOH at 80 °C)	*Rhodothermus marinus Rm*Xyn10ACM	12	12.6	X_2-X_6	Salas-Veizaga *et al.*, 2017

Table 1.4 Production of XOS from different agricultural residues via biological conversion (continued).

Substrate	Pretreatment (PTT)	Biocatalyst	Time (h)	$Y_{XOS/xylan}$[a] (mg/g)	DP[c]	Reference
Rice straw Corn cobs	Milled at particle size < 2 mm	Magnetic cross-linked xylanase aggregate developed from *Acinetobacter pittii* MASK-25 xylanase	1	841.0[b] 691.0[b]	X_2, X_3, X_5/X_6	Purohit *et al.*, 2017
Rice husk	Milled	*Aspergillus nidulans* XynC A773	24	690.0	X_3-X_6	da Silva Menezes *et al.*, 2018
Sugarcane bagasse	Aqueous ammonia PTT	β-xylosidase-free xylanase of *B. subtilis* KCX006	30	670.0[b]	X_2-X_4	Reddy and Krishnan, 2016
Sugarcane bagasse		Gluconic acid pre-hydrolysis, 150°C	1	532.0	X_2-X_6	Zhou *et al.*, 2019
Wheat bran	Washing with 50 mM sodium acetate buffer at pH 5.5 and alkali extraction	Recombinant *Bacillus amyloliquefaciens* xylanase A	24	161.4	X_2-X_6	Liu *et al.*, 2017
Wheat bran	Enzymatic and thermal PTT	Pentopan (1,4-β- xylanase from *Thermomyces lanuginosus)*	24	228.0[b]	X_2-X_6	Mathew *et al.*, 2018
		Neocallimastix patriciarum NpXyn11A	8	186.0[b]	X_3-X_4	
Wheat straw	Alkaline extraction (2% NaOH at 80 °C, 90 min)	*Bacillus halodurans* S7 endoxylanase A mutated at K80R	7	397.7[b]	X_2, X_3	Faryar *et al.*, 2015

NB: a- yields are represented in terms of amount of XOS per amount of xylan, $Y_{XOS/xylan}$ (mg/g); b- calculated from text information and converted to appropriate units; c- degree of polymerization.

XOS are known to have beneficial health properties and are considered to be functional food ingredients. XOS are considered prebiotics and soluble fibre because they are not degraded in the stomach as the humans lack the enzymes required to hydrolyze the β-links and reach the large intestine intact (Carvalho *et al.*, 2013). The prebiotic properties of XOS can promote the growth of beneficial microbiota in the animal gut (Crittenden and Playne, 2008; Hoseinifar *et al.*, 2014). They are known to positively impact the physiological functions of human health among which are reducing cholesterol level, improving the biological availability of calcium (Mussatto and Mancilha, 2007), reducing the risk of colon cancer (Swennen *et al.*, 2006) and having beneficial effect on type II diabetes mellitus (Otieno and Ahring, 2012).

Currently, XOS derived from carbohydrate-rich lignocellulose show wide applications in chemical, food, nutraceutical and pharmaceutical industries (Xiao *et al.*, 2013). XOS can also be used as low-calorie sweeteners and antioxidant additives, especially XOS with a low degree of polymerization (Chen *et al.*, 2016). Additionally, XOS also has a potential application in agriculture as fodder additives to improve the growth of fish and to increase the liver function of livestock and pets (Gullón *et al.*, 2009; Hoseinifar *et al.*, 2014). Regarding its efficiency towards the improvement of gastrointestinal health, XOS are reported to be way better than fructooligosaccharides and galactooligosaccharides (Sun *et al.*, 2015; Mohanty *et al.*, 2018). XOS can also be used to produce biodegradable plastics or nanoparticles (Patel and Goyal, 2011). Ando *et al.* (2004) reported that XOS extracted by the hydrolysis of bamboo have a cytotoxic effect on human leukemia cells.

1.6.7.3 Benefits of oligosaccharides as a prebiotic

Prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit to hosts" (Gibson *et al.*, 2017). Prebiotics has applications in pet foods, human foods, and animal feeds (Samala *et al.*, 2014). Prebiotics supplemented with polyphenolics (antioxidants) simulate a crucial part in the innate immunity and micro and macronutrient absorption of the human gut (Reddy and Krishnan 2010). Short-chain low molecular weight

carbohydrates which are non-digestible are considered as prebiotic. However, there are no clear physical or chemical properties to define this limit (Álvarez *et al.*, 2017; Zhao *et al.*, 2017).

XOS are considered as prebiotics which helps in the proliferation of *Bifidobacteria* and *Lactobacillus*, which act as probiotics and prevent the growth of harmful bacteria, thereby maintaining the healthy gut microflora (Reddy and Krishnan, 2010). The human gastrointestinal tract represents a complex ecosystem where the available carbohydrates influence the growth of the gut microbiota (Manisseri and Gudipati, 2010). XOS exhibit prebiotic effect when consumed as a part of the diet. They are neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract (Chapla *et al.*, 2012). The non-degradable property allows XOS to be used as dietary sweeteners for low-calorie diet foods and for consumption by individuals with diabetes (Choque Delgado *et al.*, 2011). Prebiotics not only used to maintain gastrointestinal health but also used as therapeutic agents against several diseases as well as additives for many food products (Ohbuchi *et al.*, 2010; Samal and Behura, 2015). They also have potential effects on calcium and other mineral absorption, (Scholz-Ahrens and Schrezenmeir, 2007) immune modulation (Lomax and Calder, 2009), bowel pH, reduction of colon cancer risk (Singh *et al.*, 2016), inflammatory bowel disorders (Crohn's disease and ulcerative colitis; Finegold *et al.*, 2014) hypertension and intestinal regularity, lowering of serum cholesterol, decreased risk of colon cancer, improved lactose digestion, reduced allergies, and effect on intestinal microbiota. Human trials have provided further evidence for the potential role of prebiotics in lowering risk of colon cancer (Serban, 2014). By observing the growing demand for XOS in food, feed and pharmaceutical sectors, XOS has been chosen as the value-added product produced from the agricultural residues in this study.

1.7 AIM OF THE STUDY

To characterize enzymatically produced xylooligosaccharides from selected lignocellulosic biomass and to evaluate their application as a prebiotic

1.7.1 Objectives

- To optimize pretreatment methods for xylan extraction from lignocellulosic biomass
- To evaluate the efficacy of GH10, GH11, GH30 xylanases on xylooligosaccharide production and their characterization
- To analyse the structure of xylanases and their mechanisms of action *in silico*
- To evaluate the potential of xylooligosaccharides as a prebiotic in newly formulated food products

CHAPTER TWO

EXTRACTION OF XYLAN FROM BIOMASS AND THEIR CHARACTERIZATION

2.1 INTRODUCTION

Lignocellulosic biomass, the residual waste materials accumulated from the agricultural sector primarily after harvest and processing, poses several disposal challenges. Though some of the biomass is used as a source of energy, the majority of the biomass is stockpiled, which leads to several environmental challenges (Menon and Rao, 2012; Ouyang *et al.*, 2013; Liu *et al.*, 2014). The environmental problems associated with the stockpiling of lignocellulosic biomass can be resolved by employing biomass conversion processes to produce value added products such as bioethanol, biohydrogen, organic acids, platform chemicals, oligosaccharides, etc. The lignocellulosic wastes serve as raw material for the production of these value-added products. Viewed from this perspective, utilization of biomass presents an important opportunity for innovation and business opportunities. In the last two decades, production of bioethanol, biohydrogen and organic acids from lignocellulosic biomass has been extensively studied and the potential of biomass as a carbon source has been proved (Diaz *et al.*, 2018). Considering the potential applications of oligosaccharides, this work is more focussed on the production and application of oligosaccharides from various lignocellulosic sources. Oligosaccharides are considered as dietary fibre and are produced from biomass by the breakdown of polysaccharides present in them. In this cluster, xylooligosaccharides (XOS) are gaining importance and their multifarious bioactive properties such as antioxidant, antimicrobial and prebiotic properties provide an opportunity for its use in the food and pharmaceutical industries.

Biomass from sugarcane, maize, rice and wheat have been extensively studied for value addition and some of the products have been commercialized. However, little attention has been paid to

indigenous crops which are only present in specific geographic locations and contain numerous carbohydrates Sorghum and peanuts are cultivated commercially in certain areas of Africa, whereas, among the different under-utilized crops, bambara and cowpea are cultivated as subsistence crops (Taylor, 2016). The production of peanuts in South Africa is approximately 110,000 tons annually (Meyer and van der Burgh, 2012), whereas sorghum production (170,000 tons per annum) is slightly higher (Agricultural Research Council, 2017). There is no clear data available on the production of bambara and cowpea in South Africa as they are being used for domestic consumption and not for commercial purposes. However, the production of bambara groundnuts in the African continent is estimated as 330,000 tons annually (Agricultural Research Council, 2016). Similarly for cowpea, the global production is estimated to be approximately 3 million tons per annum (Agricultural Research Council, 2014).

Lignocellulose has a complex structure is made of cellulose, hemicellulose and lignin. The lignin covers the cellulose microfibril and provides rigidity to the cell wall (Naidu *et al.*, 2018). Hemicellulose is rich in xylan, a polysaccharide, whose main constituent is the xylose monomer, linked by β-1,4 glycosidic bonds, and could serve as a source of xylooligosaccharides. However, due to the complex hierarchical structure and recalcitrant nature of lignocellulosic biomass, pretreatment steps are mandatory and presents the most critical challenge for effective biomass utilization (Hassan *et al.*, 2018).

The extraction of xylan from lignocellulose has several difficulties, which include non-digestibility of lignocellulosic biomass due to its complex structure and the release of inhibitors during pretreatment procedures (Jönsson and Martín, 2016). Furthermore, pretreatment the formation of soluble inhibitory compounds such as furfural, HMF and carboxylic acids (formic, levulinic and acetic acid) affects the subsequent hydrolysis and fermentation steps (Diaz *et al.*, 2018). Single pretreatment methods are usually less efficient and affect extraction procedures low yields. Usually single pretreatments fail to cleave the complex binding between the lignocellulosic materials resulting in shortened polysaccharides, and the complete breakdown of biomass cannot be achieved. A combination of physicochemical or chemical methods coupled with biological treatment provide maximum breakage of the complex lignocellulose

structure and aid in the easy separation of components (Sindhu *et al.*, 2015). Therefore, a series of chemical pretreatments were selected and tested to extract the xylan from biomass without any change in the polymer length. Xylan obtained via the best extraction method was chosen for XOS production.

2.2 MATERIALS AND METHODS

2.2.1 Selection of raw materials

The lignocellulosic biomass selected for the study were peanut shell (from a peanut processing plant, Durban), bambara, cowpea (supplied by ARC, Pretoria) and sorghum biomass (UKZN, Pietermaritzburg campus). The samples were washed with tap water to remove soil particles followed by several washings with distilled water, chopped and dried at 60°C overnight in a drier, until a constant weight was reached. The dried samples were powdered using a hammer mill (Retsch GmbH, Germany) and sieved to a uniform particle size of <500 µm. The powdered samples were stored in an airtight container until further use.

2.2.2 Extraction of xylan from biomass

Two methods were used for the extraction of xylan from lignocellulosic biomass. In the first method (adapted from Chapla *et al.* (2012) with slight modification), the powdered biomass (50 g) was soaked in 125 ml of 1% sodium hypochlorite (NaClO) solution at room temperature for 1 h to remove lignin. After washing with distilled water, the solid material was dewatered by filtration through a wet muslin cloth. The delignified wet material was soaked in 15% NaOH for 24 h at room temperature, to extract the xylan. The mixture was filtered through a muslin cloth and the filtrate was neutralized and precipitated simultaneously by a mixture of 1% glacial acetic acid in ice-cold 95% ethanol (3 volumes). The neutralized precipitate was centrifuged at 8800 x g for 10 min at 4°C and washed with 75–80% ethanol. The final precipitate was washed with 250 ml of acetone and air-dried. The dried precipitate was reconstituted in distilled water, freeze-dried and stored at room temperature until further use.

In the second method, xylan from milled biomass was extracted according to the procedure of Ebringerová *et al.* (1967). Powdered biomass (50 g) was soaked in 500 ml of distilled water and heated to 50°C on a hot plate stirrer. Glacial acetic acid (7 ml) was then added to the mixture followed by the gradual addition of sodium chlorite ($NaClO_2$, 35 g) and heated at 70°C on hot plate stirrer for 1 h in a fume hood to liberate lignin and pigmented materials. This step was followed by extensive washing with distilled water to remove the lignin and chlorides formed during the reaction. The neutral wet cake thus obtained was suspended in 350 ml of 1% NH_4OH at room temperature for 2 h. The extract was then filtered, and the solid material was further extracted with 5% NaOH at room temperature for 2 h intermittent stirring. The slurry was filtered through a muslin cloth, the extraction procedure was repeated, and the filtrates were pooled and mixed with 3 volumes of 95% ethanol to precipitate the xylan polysaccharide. The precipitate was then collected by filtration, washed with 75–80% ethanol containing 2% acetic acid to neutralize the residual alkali and subsequently washed several times with 80% ethanol. Finally, the precipitated polysaccharide was mixed with distilled water and freeze-dried.

2.2.3 Quantification of total sugars

The phenol-sulphuric acid method (DuBois *et al.*, 1956) was used for the determination of total sugars present in the extracted xylan. The optical density of the reaction mixture was measured at 490 nm using a spectrophotometer. Reference solutions were prepared using xylose as the standard, and distilled water served as a control.

2.2.4 Fourier transform infrared spectroscopy (FTIR)

FTIR spectral profile of the polysaccharide samples were obtained on a Varian 800 FTIR Fourier transform infrared spectrophotometer (Varian, USA) operating at 4000–400 cm^{-1} spectral range with a resolution of 0.9 cm^{-1} using a KBr beam splitter and DTGS detector (7800–350 cm^{-1}). Five mg of finely ground xylans (peanut shell, bambara, cowpea and sorghum biomass) were used for FTIR analysis (Ruzene *et al.*, 2008).

2.2.5 Monosaccharide analysis

Neutral sugars were quantified as alditol acetates after hydrolysis with 2 M trifluoroacetic acid (TFA) for 1 h at 120°C (Englyst and Cummings, 1984). In this method, 3 mg of the polysaccharide sample were hydrolysed in 2 ml of 2 M TFA for 1 h at 120°C. After cooling, the sample was vacuum dried and dissolved in 1 ml of distilled water and vacuum dried again for three more times. The resultant residue was dissolved in 2 ml of 1 M NH_4OH followed by the addition of 10 mg $NaBH_4$. After incubating for 3 h at room temperature, the mixture was treated with a cation exchanger in H^+ form (to decompose excess of $NaBH_4$ and remove sodium ions) and evaporated to dryness. The residue was dissolved in 1 ml of methanol, vacuum dried and this step was repeated thrice to remove boric acid, and finally dried in a vacuum concentrator at 45°C. The dried residue was dissolved in 0.6 ml of pyridine and acetylated after the addition of 0.5 ml of acetic anhydride. After 1 h, the mixture was evaporated and dried at 90°C. The remaining residue was dissolved in chloroform and was analyzed by Gas-Liquid Chromatography (GLC). GLC was performed using a TRACE Ultra Gas Chromatograph coupled with TSQ Quantum XLS weight spectrometer (Thermo Scientific, USA) under the following conditions: Rtx-2330 column (0.32 mm×30 m; Restek) with a temperature program of 80°C for 12 min followed by gradual increase of 8°C/min to reach 160°C and then 4°C/min to reach 250°C and was maintained at 250°C for 25 min; the flow rate of helium was 0.4 ml/min; the injector temperature was 240°C and the detector temperature was 200°C. Detector response of monosaccharide derivatives was evaluated based on the areas of a standard mixture of alditol acetates.

2.2.6 Determination of uronic acids in the extracted polysaccharides

Total uronic acid content was determined by the 3-hydroxy diphenyl assay (Blumenkrantz and Asboe-Hansen, 1973) using D-glucuronic acid as the standard. The meta-hydroxy diphenyl solution was prepared by dissolving 0.15% meta-hydroxy diphenyl in 0.5% NaOH solution. H_2SO_4-tetraborate solution was prepared by mixing 0.0125 M solution of sodium tetraborate in concentrated H_2SO_4.

Calibration standards of 0.2 ml (0.5–20 µg glucuronic acid) were mixed with 1.2 ml of H_2SO_4-tetraborate solution. The enzyme hydrolysate was also prepared in the same manner and was refrigerated in an ice bath. The reaction mixture was vortexed thoroughly and the tubes were heated in a boiling water bath for 5 min followed by cooling in an ice bath and 20 µl of the meta-hydroxy diphenyl reagent was added. The tubes were stirred, and the pink colour was measured at 520 nm using a visible spectrophotometer.

2.2.7 Enzymatic removal of contaminating polysaccharides

In accordance with the monosaccharide results, the extracted xylans were treated with some glycoside hydrolase enzymes to remove the glucose-containing materials. *Clostridium thermocellum* GH16 endo-1,3(4)-β-glucanase and *Paenibacillus* sp. GH5 endo-xyloglucanase were purchased from Megazyme (Bray, Ireland). *Bacillus subtilis* α-amylase was procured from Sigma-Aldrich (St. Luis, MO, USA). To remove the contamination of starch and xyloglucan from peanut shell xylan, it was treated with α-amylase, β-1,3(4)-glucanase and xyloglucanase. The hydrolysates were further analyzed by TLC and MALDI-TOF MS to identify the degradation products. Whereas, bambara, cowpea and sorghum xylans were treated only with α-amylase and analyzed by TLC since they were contaminated with starch.

2.2.8 Thin layer chromatography (TLC) analysis of the hydrolysed products

Qualitative analysis of hydrolysates obtained by the enzymatic hydrolysis of xylans was done on TLC plates. Samples (2 µl) were spotted on silica gel F 60 (Merck, Germany) plates and the chromatograms were developed in either ethyl acetate: acetic acid: 2-propanol: formic acid: water (25:10:5:1:15, v/v) according to the method of Kumar *et al.* (2009) or in a solvent system of n-butanol/ethanol/water (10:8:5, v/v). Sugars were detected by spraying orcinol reagent (1% orcinol in 10% H_2SO_4 in ethanol) on the plates followed by heating at 100°C for 5 min in an oven. Xylose, glucose (Sigma-Aldrich, USA), xylobiose, xylooligosaccharides and maltooligosaccharides (Megazyme, Ireland) were used as standards.

2.2.9 MALDI-TOF mass spectrometry

Oligosaccharides were also analysed by MALI-TOF MS according to Biely *et al.* (2013). The sample plate was prepared by applying 2 µl of a 9 mg/ml mixture of 2,5-dihydroxybenzoic acid (DHB) in 30% acetonitrile to an MTP 384 ground steel target plate TF (Bruker Daltonics). The sample (1 µl) was then mixed with the DHB droplet and was crystallized under a stream of air and analyzed with an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics GmbH, Bremen, Germany) equipped with a nitrogen 337 nm laser beam. The instrument was controlled by the Flex Control 3.3 software package and operated in positive acquisition mode. All spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26 kV, and pulsed ion extraction of 40 ns in the positive ion mode. The acquisition range used was from m/z 0 to 7000. The data was collected from the average of 400 laser shots, with the lowest laser energy necessary to obtain sufficient signal to noise ratios. Peak lists were generated from the MS spectra using Bruker FlexAnalysis software (version 3.3). Ions of sodium adducts of XOS fragments were marked using monosaccharide codes.

2.2.10 NMR spectroscopy

For NMR analysis, all samples were freeze-dried twice from D_2O. ^{1}H-NMR spectra were measured at 25°C using automatic chemical shift calibration in D_2O on either AVANCE III HDX 600 MHz equipped with a Triple inverse TCI H-C/N-D-05-Z cryo probe, or AVANCE III HD 400 MHz with a broad band BB-(H-F)-D-05-Z liquid N_2 Prodigy probe (all from Bruker BioSpin, Rheinstetten, Germany), using a pre-saturation zgpr sequence, with a pre-saturation delay of 2 s, a r.f. 90° pulse and an acquisition time of 2.5 s.

2.3 RESULTS AND DISCUSSION

2.3.1 Delignification of biomass by NaClO and NaOH extraction

Peanut shell, bambara, cowpea, and sorghum biomass were subjected to various pretreatments for the extraction of xylan. The NaClO and NaOH extraction of peanut shell (xylan I) yielded

14.8% of hemicellulose (Table 2.1) which contained predominantly xylan, along with few other polysaccharides which were confirmed by further analyses. Direct extraction of ground peanut shell with NaOH or KOH solutions yielded much lower xylan recovery, which prompted alkaline extraction of biomass in the presence of a delignification agent, e.g., NaClO. The high lignin content of peanut shell (being a dicot) makes the biomass more recalcitrant (Martín *et al.*, 2006). The action of NaClO disrupts the lignin component and its linkages with the cellulosic material, while NaOH causes swelling of cellulose (Meryandini *et al.*, 2008; Singh *et al.*, 2014). In general, alkali treatments are more suitable for xylan extraction since they dissolve the xylan easily and also results in deacetylation of the polysaccharide (Biely *et al.*, 2016).

During the extraction process, bambara and cowpea yielded lower levels of xylan, 11.4% and 12.2% respectively, in comparison to peanut shell (14.8%), which could be due to the lower content of xylan in those biomasses. Another possible reason could be due to the fact that bambara and cowpea are dicots with hard stems and are more recalcitrant than monocots. However, in the case of sorghum, the xylan yield was as high as 30.1%, which showed the maximum hemicellulose content, among the biomass tested. It is apparent that the recalcitrance in monocots would be low due to the lower lignin content and could be easily subjected to alkali treatment.

Oliveira *et al.* (2010) reported a modified alkali extraction method for xylan from corn cob using the NaClO and NaOH which yielded 11%. Another report on the extraction of xylan from corn cob by Gowdhaman and Ponnusami (2015) using similar extraction parameters used in this study yielded 14.2%. Xylan was also extracted from cassava peel and cassava waste using NaClO and NaOH method, and yielded 4.83% and 6.23% respectively (Ratnadewi *et al.*, 2016). However, during our extraction procedures of xylan by the NaClO and NaOH method, some contamination by other polysaccharides was detected. Hence, to increase the xylan yield and to eliminate the co-extraction of other polysaccharides, another extraction process was tested.

2.3.2 Acidic delignification of biomass by $NaClO_2$ and NaOH

The $NaClO_2$ delignification was more effective than NaClO, where most of the lignin portion was eliminated from the biomass. The addition of 1% acetic acid and incubation at 70°C certainly influenced the relaxation the lignocellulosic structure by catalyzing the fast breakdown of linkages between lignin and holocellulose. Using this extraction procedure, a xylan yield of 15.5% was achieved, which is the highest recovery of xylan from peanut shell, to date. Furthermore, the purity of xylan obtained by $NaClO_2$ and NaOH extraction from peanut shell (xylan II) was found to be much higher compared to the NaClO and NaOH extraction process. There has been limited research undertaken on the isolation of xylan from peanut shells and a previous report on xylan extraction from peanut shells yielded only 3.5% xylan through NaOH treatment (Yang *et al.*, 2007).

The same procedure was employed for the extraction of xylan from bambara, cowpea and sorghum, which showed a marginal increase in the xylan yield (12.3%, 13.6% and 32.2%, respectively) compared to the NaClO and NaOH method (Table 2.1). It was also noticed that the colour of the extracted xylan was much lighter than the NaClO and NaOH extracted materials. This could be due to the biomass being completely bleached when most of the lignin was removed by $NaClO_2$ treatment (Hubbell and Ragauskas, 2010; Liu *et al.*, 2016; Kim *et al.*, 2017). The most attractive feature of xylan extraction using this method is that the solubility of xylan in water and buffer was increased which subsequently improved the effective hydrolysis of xylan by xylanases. Some other studies have reported xylan yields of 18.5% from mahogany wood, 15.1% from mango wood (Rajagopalan *et al.*, 2017) and 18.6% from corn cob (Wu *et al.*, 2019) using the same extraction method. From the two methods tested, it was apparent that $NaClO_2$ and NaOH extraction yielded better results and was used for further analyses. In addition to this, the extracted xylan from peanut shells, bambara, cowpea, and sorghum was quantitatively measured by the phenol-sulphuric acid method which showed the presence of 75%, 65.8%, 66.6%, and 75.4% of total sugars (w/w), respectively. The 2-step extraction process resulted in the highest recovery of polysaccharides.

Table 2.1 Recovery of xylan by one-step and two-step alkali extraction methods from agricultural biomass.

Lignocellulosic material used	Xylan recovery (%)	
	NaClO and NaOH extraction	Acidic delignification and NaOH extraction
Peanut shells	14.8±0.30	15.5±0.35
Bambara biomass	11.4±0.40	12.3±0.20
Cowpea biomass	12.2±0.25	13.6±0.40
Sorghum stover	30.1±0.30	32.2±0.50

NB: Value represents the mean of three replicates and the standard error reported.

2.3.3 Fourier transform infrared spectroscopy analysis of extracted xylans

Fourier transform infrared spectroscopy (FTIR) enables the biochemical identification of a sample in a quick and non-destructive way (Wang *et al.*, 2010). The extracted xylans were analyzed by FTIR spectroscopy to reveal their chemical structures based on functional groups. Commercial beechwood xylan was used for comparison (Figure 2.1). The IR spectra of extracted xylans showed the characteristic bands at 1465, 1383, 1248, 1158, 1045, 989 and 897 cm^{-1}, which indicated the presence of the main chain of β-1,4-xylan. The broad and strong stretching observed between 3500 and 3200 cm^{-1} corresponded to the vibrations of hydroxyl groups in the xylan structure. Specifically, the signals at 3455 and 3345 cm^{-1} were due to the stretching vibrations of OH group whereas, 2925 and 2918 cm^{-1} corresponded to CH_2 groups (Figures 2.2, 2.3, 2.4 and 2.5). The abundance of β-glycosidic linkages between xylose units was also indicated by the presence of a sharp band at 897 cm^{-1}. The strong absorption peaks between 1700 and 1600 cm^{-1} could be due to the H-O-H angle vibration of water since hemicellulosic polymers are usually reported to contain bound water. A sharp band at 1045 (Figures 2.1 and 2.2) and 1084 cm^{-1} (Figures 2.3, 2.4 and 2.5) confirmed the presence of C-O, C-C stretch or C-

OH bending in the xylose units. Most of the absorption bands observed in the spectra of all the four xylans were correlated with previous literature (Samanta *et al.* 2012; Zhang *et al.* 2016).

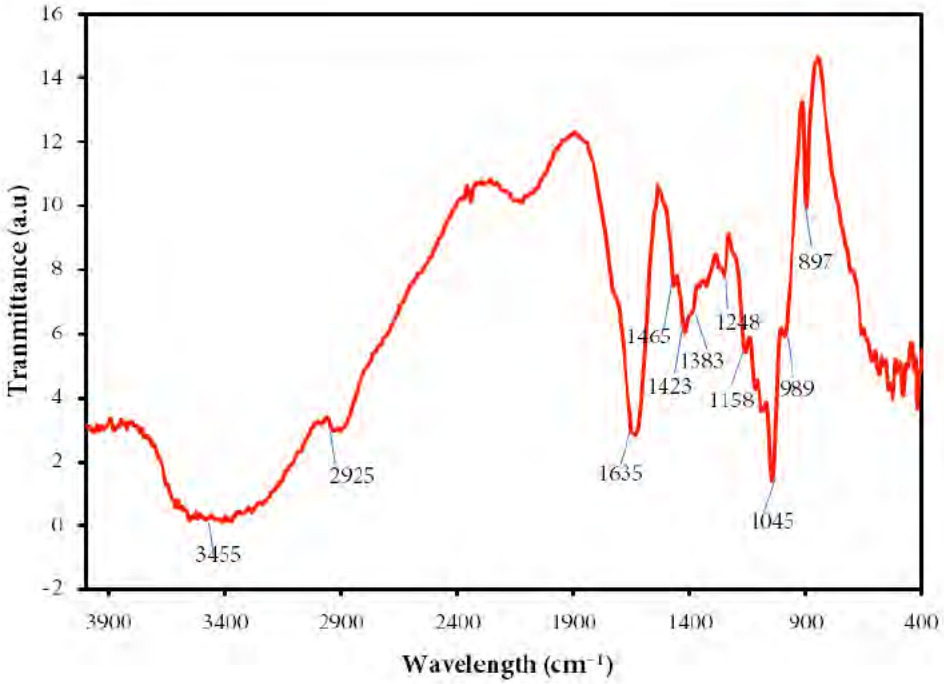

Figure 2.1 FTIR spectrum of beechwood xylan (control).

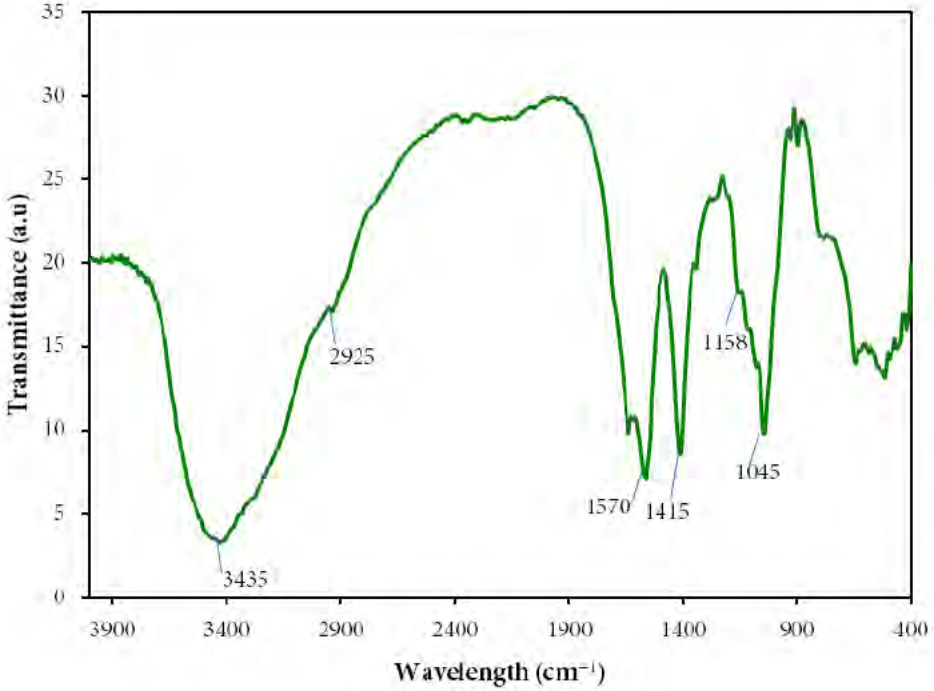

Figure 2.2 FTIR spectrum of peanut shell xylan.

There was a striking similarity between the spectra of beechwood xylan and peanut shell xylan, where a comparable signal pattern was observed, which could be due to the presence of glucuronoxylan in both xylans. However, the spectra of xylans from bambara, cowpea and sorghum had similar signal patterns though deemed different from peanut shell. Strong bands obtained around 1415 and 1610 cm^{-1} denote the presence of COO^- groups of glucuronic acid in the xylan (Sousa *et al.*, 2016). Arabinose side chains attached at *O*-3 positions of the xylopyranosyl backbone were detected by weak signals around 1158 (the C-O-C vibration) and 989 cm^{-1}. Similar FTIR spectrum was also reported for 4-*O*-methylglucuronoxylan from *Fagus sylvatica* sawdust (Sivová *et al.*, 2015). A comparable spectrum was found for glucuronoxylan extracted from corn cob (Oliveira *et al.*, 2010) and corn stover (Rowley *et al.* 2013) which possessed signals for the functional groups (COO^-, C-O, C-C, OH, CH_2 and H-O-H) of xylan backbone and glucuronic acid substitution.

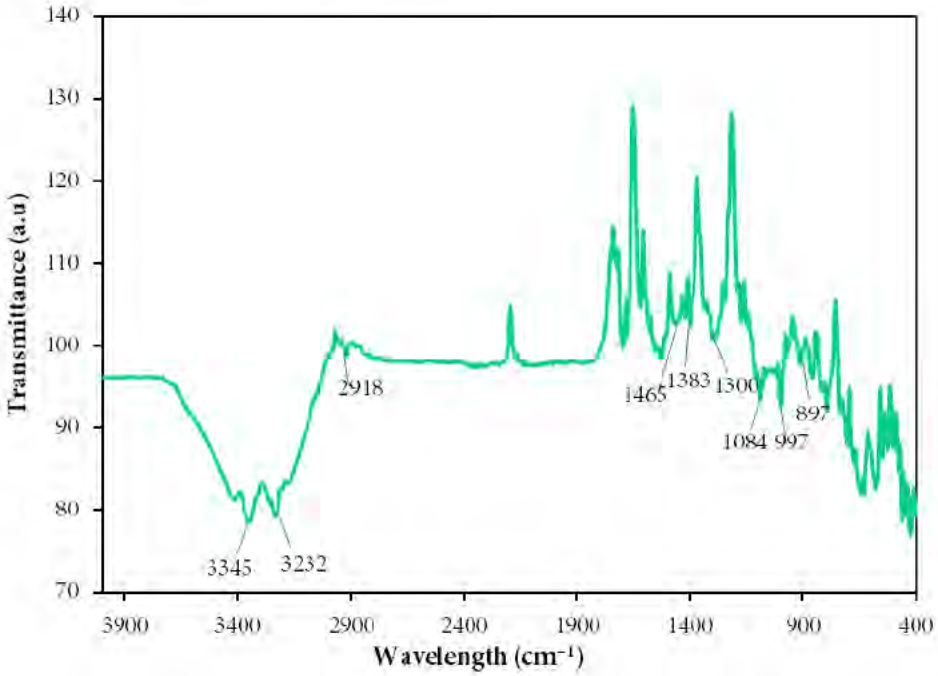

Figure 2.3 FTIR spectrum of bambara xylan.

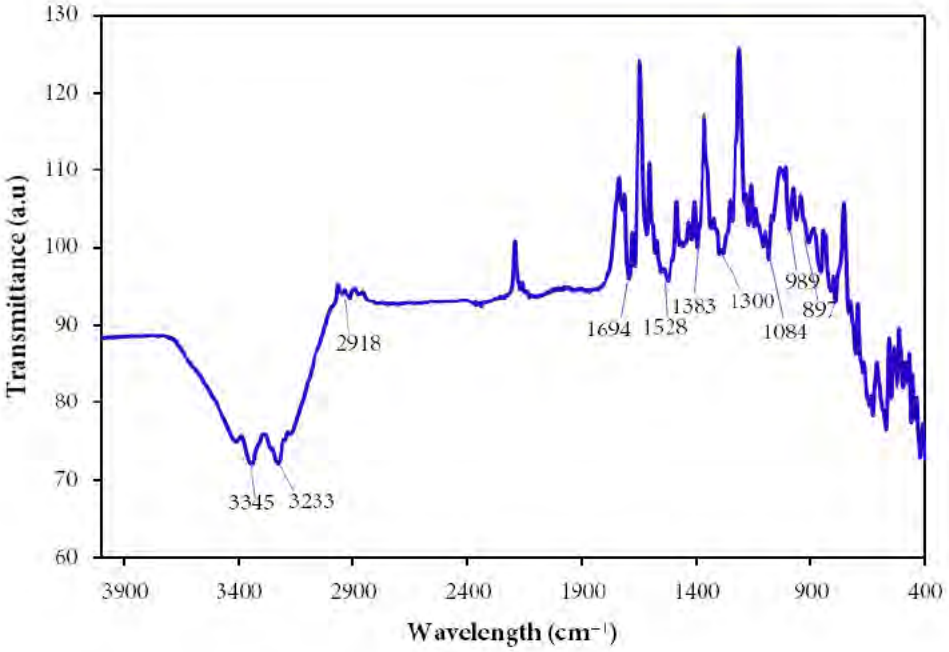

Figure 2.4 FTIR spectrum of cowpea xylan.

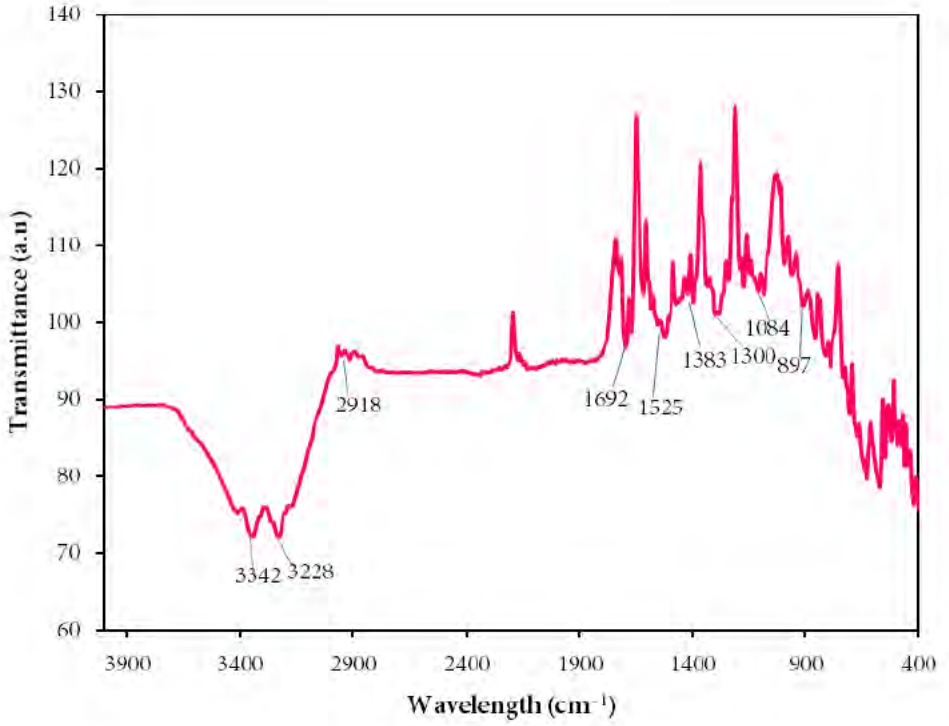

Figure 2.5 FTIR spectrum of sorghum xylan.

2.3.4 Monosaccharide analysis of isolated xylans and starch removal

Relative monosaccharide composition of extracted xylan was determined by the alditol acetate method to study the building blocks of xylan and to confirm the type of xylans which were extracted. As expected, the predominant sugar obtained was xylose in all the four xylans tested (Table 2.2). The ratio of xylose: glucose in peanut shell xylan was 1:0.14, whereas, in bambara and cowpea xylans, a notable amount of glucose was detected during the preliminary analysis. As evident from the monosaccharide analysis, the peanut shell xylan isolated in the one-step procedure (NaClO and NaOH extraction) was contaminated with other hemicelluloses, and particularly with glucose-containing polysaccharides (starch and xyloglucan). The occurrence of glucose could be partially due to the presence of starch co-extracted from the biomass or could be attributed to the biomass being contaminated by starch-containing materials (Ghosh *et al.*, 1995). Arabinose and galactose were the other sugars detected in all the four xylans (Table 2.2). Bambara xylan contained a larger amount of arabinose (1:0.31 ratio to xylose) which indicates more arabinose substitutions than in the other xylans. However, cowpea and sorghum xylans comprised 1:0.23 and 1:0.28 of arabinose to xylose respectively, confirming substitution of arabinose in their xylan. Peanut shell xylan contained a very low content of arabinose and this may suggest that the arabinose substitution was limited in the case of peanut shells.

In addition to neutral monosaccharides, the presence of sugar acids such as glucuronic acids were also expected in xylan (Cantu-Jungles *et al.*, 2017) which was confirmed in this study by uronic acid analysis. The glucuronic acid content of the extracted xylans were as follows: peanut shell- 171 mg/g, bambara- 164 mg/g, cowpea- 197 mg/g and sorghum- 158 mg/g. These results are consistent with previous studies which reported the presence of arabino-4-*O*-methylglucuronoxylans in the leaves of Algerian *Argania spinosa* (Hachem *et al.*, 2016). Acetylglucuronoxylans from aspen wood (Puchart *et al.*, 2019) and acetylarabinoglucuronoxylan form corn cob (Arai *et al.*, 2019) were also reported recently which contained 85 mg/g and 36 mg/g uronic acids, respectively. In comparison with the results

Table 2.2 Monosaccharide analysis of extracted xylans (sugars are estimated as molar ratios in comparison to xylose).

Sample	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	MeGlcA
Peanut shell xylan I†	0.027	0.020	0.120	1.00	0.016	0.086	0.144	0.13
Peanut shell xylan II‡	0.010	0.001	0.040	1.00	0.003	0.020	0.009	0.14
Bambara xylan	0.048	0.013	0.310	1.00	0.047	0.223	0.140	0.06
Cowpea xylan	0.040	0.009	0.232	1.00	0.031	0.190	0.350	0.09
Sorghum xylan	0.014	–	0.285	1.00	0.033	0.082	0.150	0.06

NB: †- xylan isolated by one-step NaClO and NaOH extraction procedure; ‡- xylan isolated by NaOH extraction of $NaClO_2$-delignified and NH_4OH pretreated peanut shells.

reported by Arai *et al.* (2019), the glucuronic content in this study was much higher and prove the higher glucuronic substitution. To confirm whether the extracted xylans were contaminated with other sugars, they were treated with pure α-amylase, β-1,3(4)-glucanase and xyloglucanase, followed by TLC analysis of the released products (Figure 2.6).

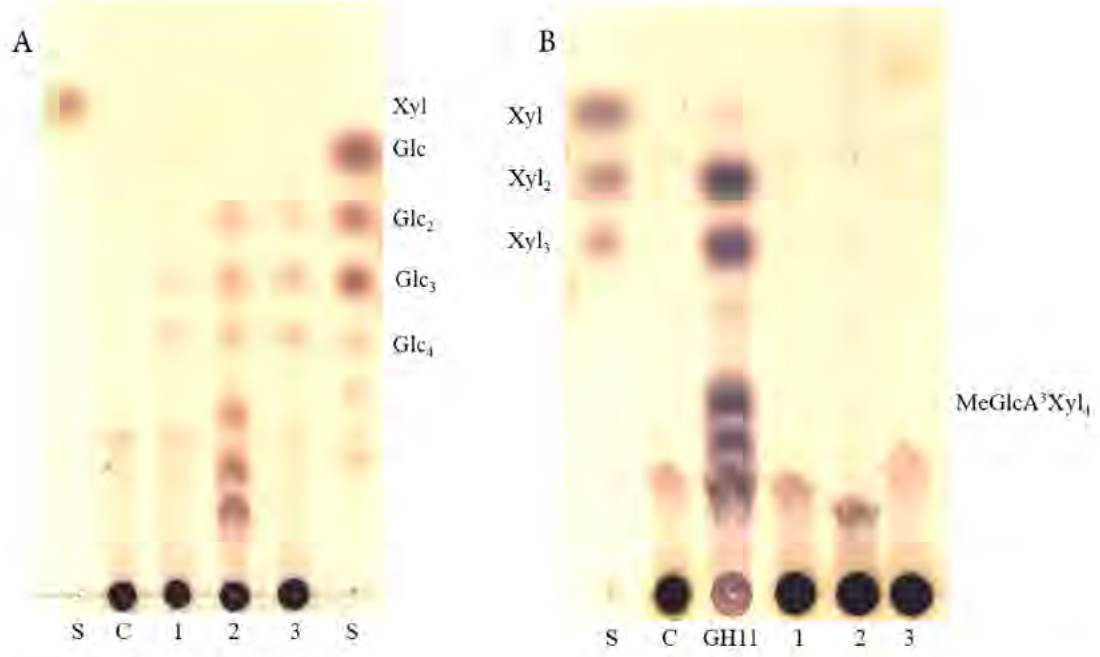

Figure 2.6 TLC analysis of the soluble fractions of peanut shall xylan, xylan I (NaClO and NaOH extraction, A) and xylan II (acidic delignification and NaOH extraction, B) treated with α-amylase (1), xyloglucanase (2), β-1,3(4)-glucanase (3) and GH11 xylanase (GH11). S, standards of xylose and maltooligosaccharides; C, control (xylan incubated without enzyme); Xyl, xylose; Glc_1, glucose; Glc_2, maltose; Glc_3, maltotriose; Glc_4, maltotetraose.

Of the three endoglycanases tested on peanut shell xylan, only xyloglucanase generated a significant amount of oligosaccharides from xyloglucan, whereas α-amylase and β-1,3(4)-glucanase released traces of oligosaccharides. From the TLC results, it is apparent that the major source of glucose in xylan I was xyloglucan (Figure 2.6A). This was further confirmed as typical xylosylated glucooligosaccharides by MALDI-TOF MS analysis (Figure 2.7) with three types of side chains: Xyl- (X), Gal-Xyl- (L) and Fuc-Gal-Xyl- (F). Fucose occurs in the xyloglucan side chains as the non-reducing sugar linked to galactose, attached to side chain

xylose residues (Mishra and Malhotra, 2009). The presence of fucose serves as evidence that some of the oligomers also contained galactose residues which cannot be differentiated from glucose by MALDI-TOF MS analysis since glucose and galactose have the same molecular weight. The presence of xylose-free hexooligosaccharides in the hydrolysate produced by xyloglucanase suggests that peanut shell xyloglucan may have unsubstituted or less substituted regions of the main β-1,4-glucan chain. The identification of xyloglucan as an admixture also means that a small portion of the overall xylose is a constituent of this minor polysaccharide. The molar ratios of sugars in xylan I of peanut shell suggest that xyloglucan admixture could account for 5 to 10%.

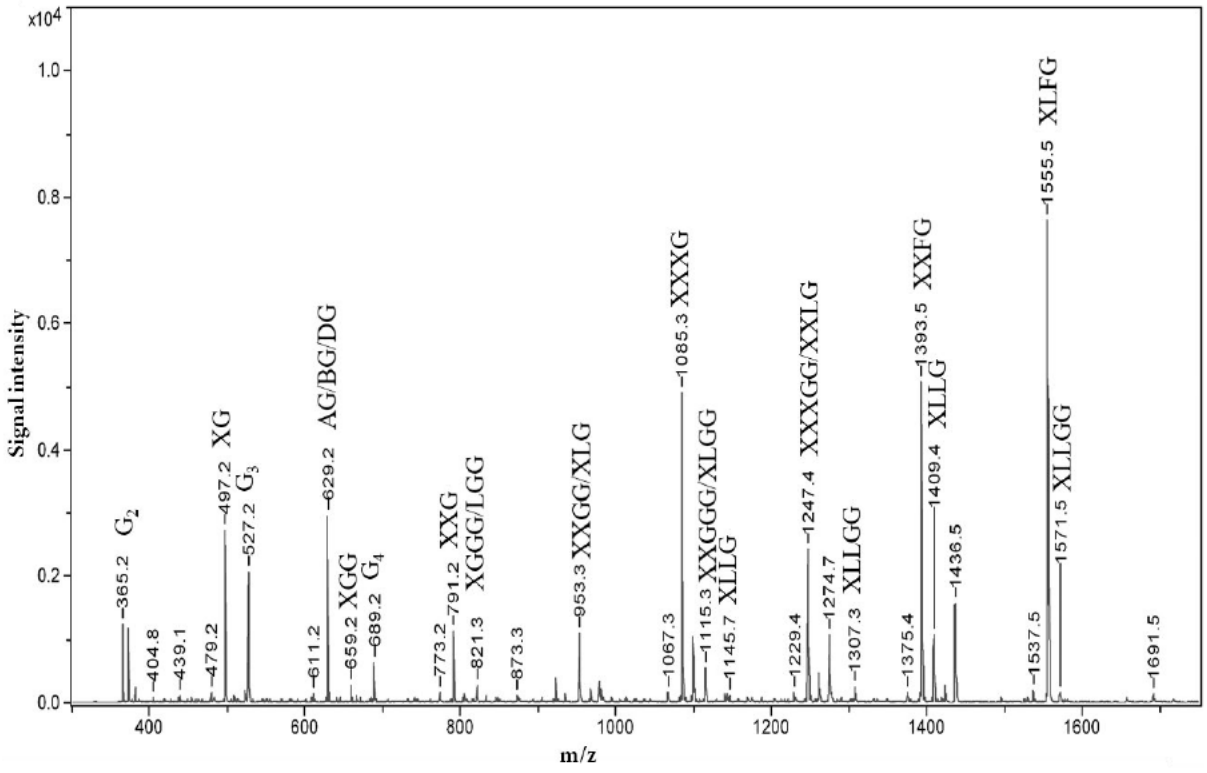

Figure 2.7 MALDI-TOF MS spectrum of xyloglucan oligosaccharides released from peanut shell xylan (NaClO and NaOH extraction) by xyloglucanase. Abbreviations: G_x, ions of neutral cellooligosaccharides; Explanation for the coding: X, Glc-Xyl; L, Glc-Xyl-Gal; F, Glc-Xyl-Gal-Fuc; A, B, D, Disubstituted backbone Glc by two pentoses or side chain Xyl substituted with another pentose.

One of the common plant materials that possess high content of xyloglucan is tamarind seed (yield 46–48% based on dehulled seed), which is also used for the commercial production of xyloglucan (Mishra and Malhotra, 2009; Farias *et al.*, 2019). Xyloglucan was also found in plants such as jojoba seeds, *Arabidopsis thaliana,* African clubmoss, rough horsetail, various lamiids, *Physcomitrella patens*, argan tree, bilberry (Tuomivaara *et al.*, 2015), sycamore, jatoba and *Nasturtium* seeds (Pauly and Keegstra, 2016). Since the xyloglucan was not the product of interest in this study, the content of xyloglucan in peanut shell was not quantified.

The second extraction procedure that involved acid delignification with $NaClO_2$ and pre-extraction with NaOH (Ebringerová *et al.*, 1967) resulted in much purer peanut shell xylan, assigned as xylan II (yield, 15.5%), composed mainly of two components, neutral xylose and uronic acid (Table 2.2). It should be noted that the method used for the analysis of uronic acids (Blumenkrantz and Asboe-Hansen, 1973) does not discriminate between D-glucuronic acid and 4-*O*-methyl-D-glucuronic acid, however, it has indicated a higher degree of branching with uronic acid in peanut shell xylan than in hardwood xylan (St. John *et al.*, 2006; Li *et al.*, 2007). This is significant as peanut shell, could exhibit some interesting substitutions in the xylan structure.

The molar ratio of MeGlcA to xylose in peanut shell glucuronoxylan was more than 7 (Table 2.2). The polysaccharides, starch and xyloglucan that contaminated xylan I (obtained by one-step extraction) was obviously eliminated during the two-step extraction procedure, preceding the NaOH extraction. This is clearly evident from the chromatogram (Figure 2.6B). In contrast to the xylan I preparation, the xylan II was essentially free of other polysaccharide admixtures. Since the xylan II preparation does not contain any substrate for amylase such as xyloglucanase or β-1,3(4)-glucanase, no products were released after hydrolysis. This demonstrates that xylan II was purer than xylan I.

Unlike peanut shell, the other three xylans tested, did not show any traces of xyloglucan. Therefore, only starch removal was performed on bambara, cowpea and sorghum xylans using α-amylase which resulted in the liberation of glucose and maltooligosaccharides

(Figure 2.8). Cowpea xylan had more maltooligosaccharides followed by bambara which contained Glc, Glc_2 and Glc_3 as the predominant products. Sorghum had a negligible amount of maltooligosaccharides released during the amylase treatment.

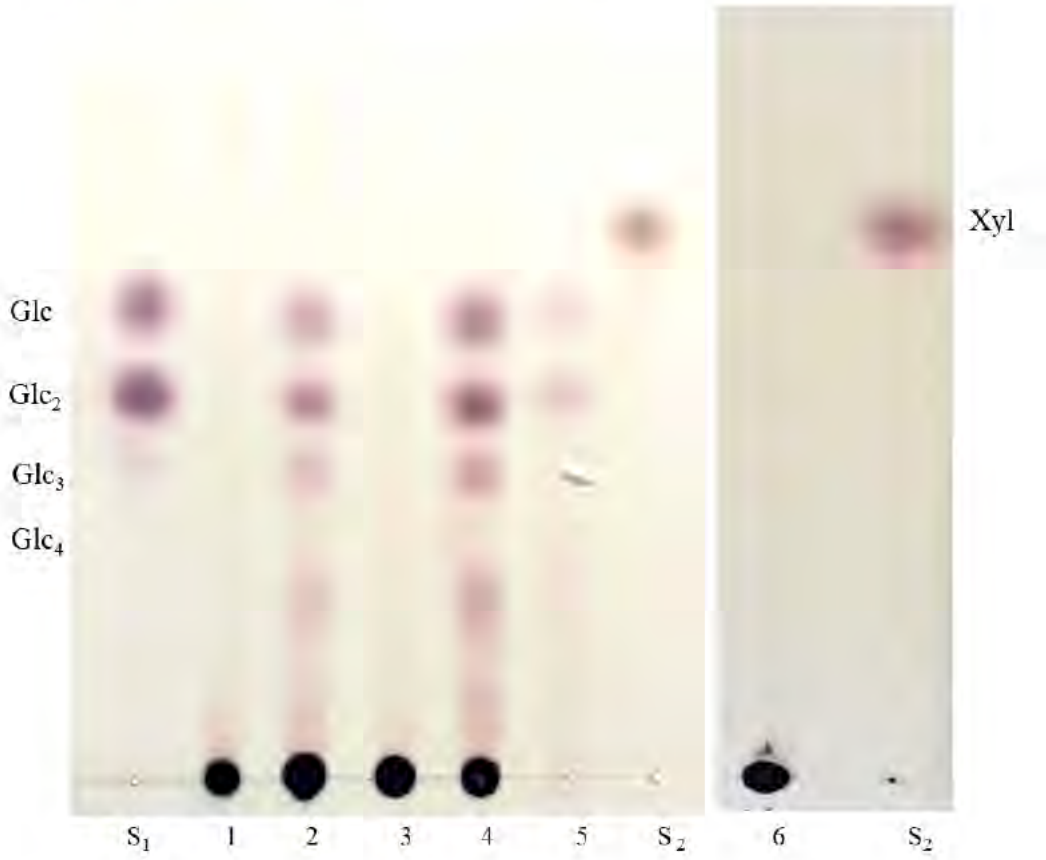

Figure 2.8 TLC analysis of products after α-amylase treatment of xylans from bambara, cowpea and sorghum biomass; S_n, standards (S_1-Glucose and maltooligosaccharides, maltose to maltotetraose; S_2- Xyl, xylose); 1, control bambara xylan; 2, products released by α-amylase from bambara xylan; 3, control cowpea xylan; 4, products released by α-amylase from cowpea xylan; 5, products released by α-amylase from commercial starch 6, products released by α-amylase from sorghum xylan.

2.3.5 NMR analysis of isolated xylans

To identify the functional groups and structure of extracted xylan, NMR analysis was performed. The ^{1}H-NMR spectrum of peanut shell xylan was compared with birchwood xylan (Figure 2.9) which has a Xyl: MeGlcA ratio of 10:1 and has been used as a reference to estimate

the MeGlcA content. The ^{1}H-NMR spectrum of peanut shell xylan extracted by both extraction procedures (Figure 2.10) showed unusually high signals of H-1 for MeGlcA at 5.28 ppm and for the 4-*O*-methyl ether group of MeGlcA at 3.45 ppm. This corresponded to a higher uronic acid content than that observed in hardwood xylans (Verma and Goyal, 2016). The ratio of the above-mentioned peak areas in birchwood xylan (having Xyl:MeGlcA ratio, 10:1) was 4:1 and the ratio of the area in peanut shell xylan was 2.5:1 which indicated a higher MeGlcA content in the corresponding xylan; suggesting the ratio of Xyl:MeGlcA could be approximately 7:1. The purity of xylan II is evident from the ^{1}H-NMR spectroscopy results. The spectrum of xylan II (Figure 2.10B) is sharper than that of xylan I (Figure 2.10A) and does not contain a signal at 5.38 ppm which confirms the absence of H-1 of α-1,4-linked Glc*p* or α-1,3-L-Ara*f*. Based on the signals observed from Figure 2.10B, the xylan II extracted from $NaClO_2$-delignified peanut shell was chosen for further experiments.

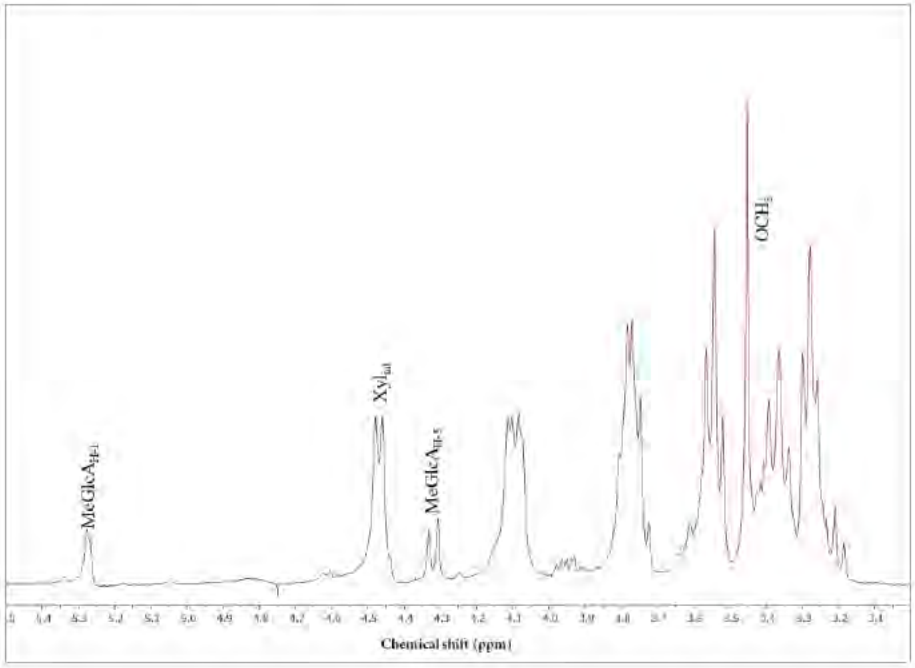

Figure 2.9 ^{1}H-NMR spectrum of birchwood xylan. Description of signals: OCH_3, methyl ether group; $MeGlcA_{H\text{-}1}$ and $MeGlcA_{H\text{-}5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose.

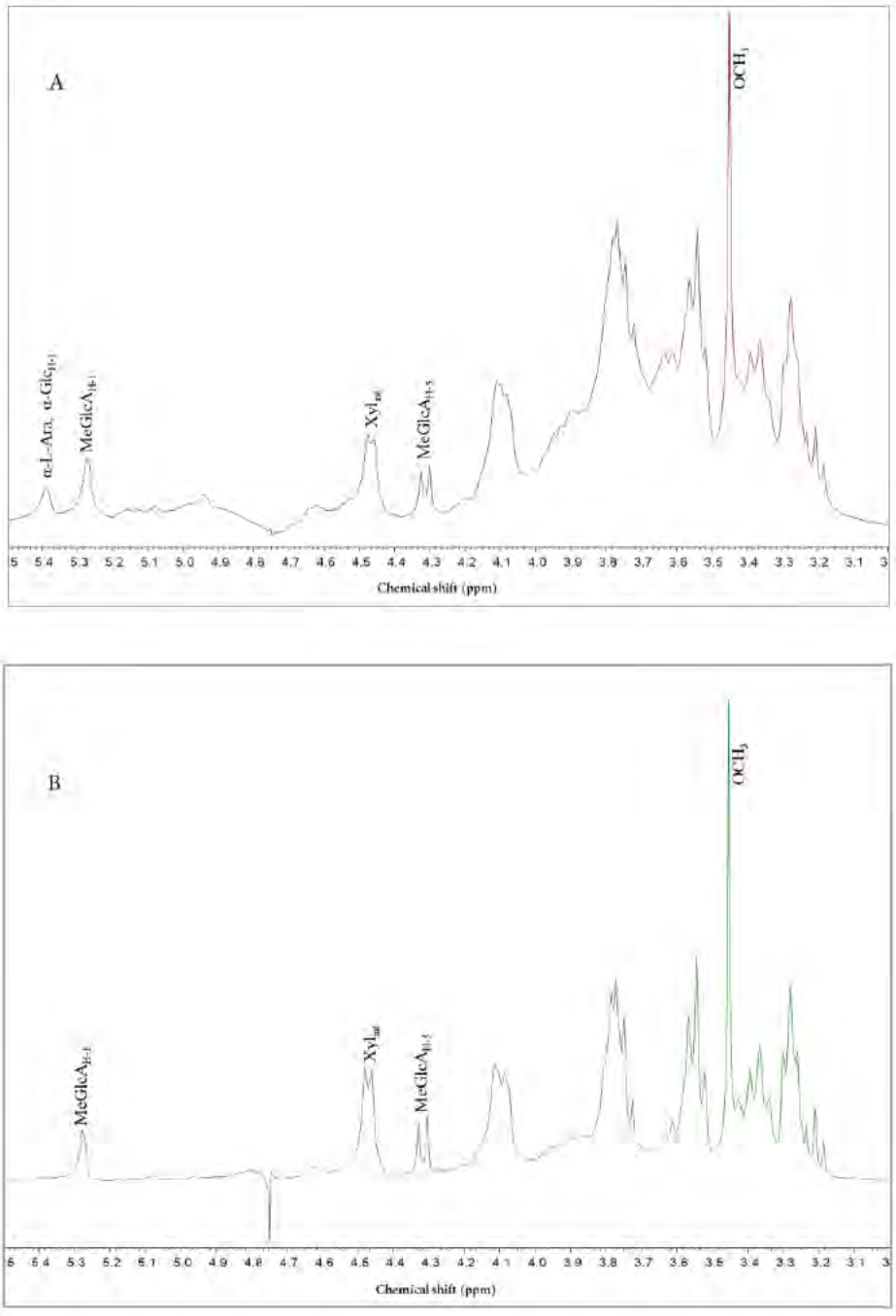

Figure 2.10 ^{1}H-NMR spectra of peanut shell xylan isolated by (A) one-step NaClO and NaOH extraction procedure and, (B) NaOH extraction after $NaClO_2$-delignification and NH_4OH pretreatment. Description of signals: OCH_3, methyl ether group; $MeGlcA_{H\text{-}1}$ and $MeGlcA_{H\text{-}5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; α-L-Ara, H-1 signal of α-1,3-linked arabinose; $\alpha\text{-}Glc_{H\text{-}1}$, H-1 signal of α-1,3-linked glucose.

The absence of signals for acetyl and feruloyl groups in the cowpea xylan confirmed that their ester linkages were destroyed during the alkali extraction. The xylan extraction process and/or the portion of plant material used might have affected the substitutions in the xylose backbone (Biely *et al.*, 2016). The ^{1}H-NMR spectrum of bambara and cowpea xylans contained peaks for MeGlcA at 5.27 ppm and 3.46 ppm (4-*O*-methyl ether group) respectively, (Figures 2.11 and 2.12A), suggesting the presence of glucuronoxylan. These results were corroborated by a previous report on beechwood xylan which showed the corresponding signals in close proximity for xylose, GlcA and/or MeGlcA confirming the presence of xylan (Sousa *et al.*, 2016). In comparison to peanut shell and cowpea xylan, bambara xylan contained similar signals with varied intensity.

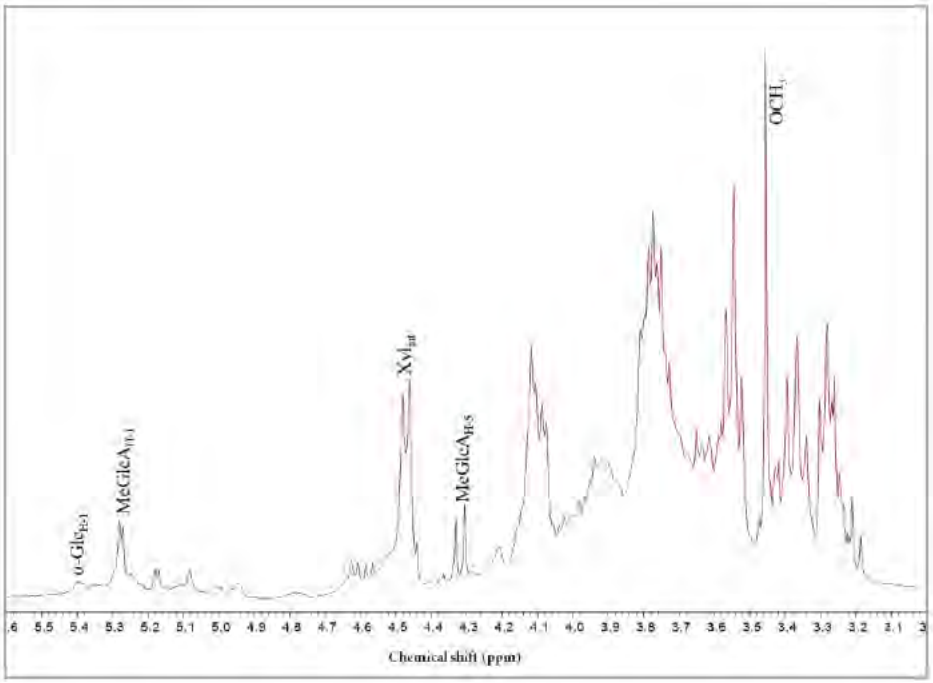

Figure 2.11 ^{1}H-NMR spectrum of xylan isolated by NaOH extraction of $NaClO_2$-delignified and NH_4OH pretreated bambara biomass. Description of signals: OCH_3, methyl ether group; $MeGlcA_{H\text{-}1}$ and $MeGlcA_{H\text{-}5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; $\alpha\text{-}Glc_{H\text{-}1}$, 3-linked- H-1 signal of α-1,3-linked glucose.

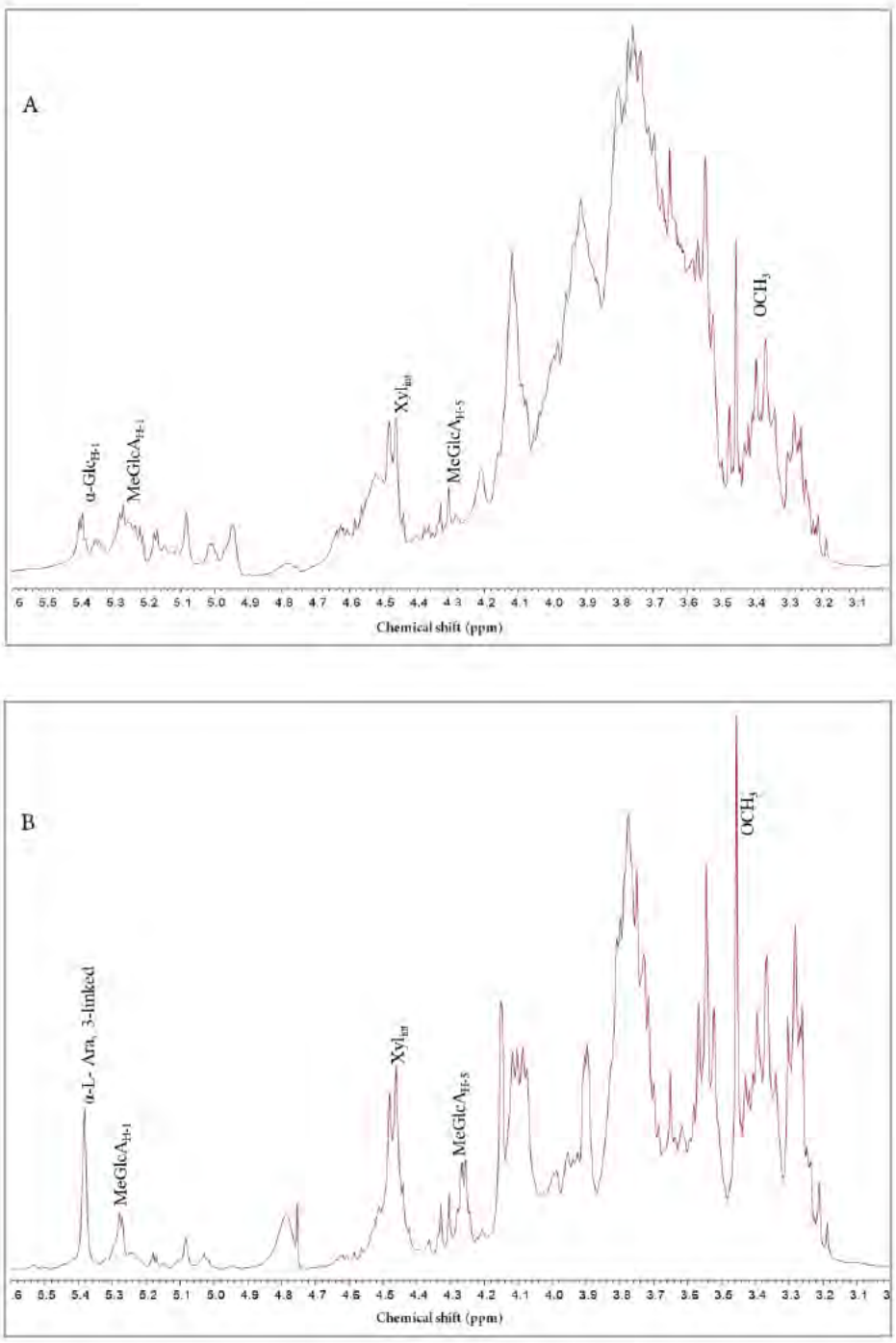

Figure 2.12 ^{1}H-NMR spectra of xylan isolated by NaOH extraction of $NaClO_2$-delignified and NH_4OH pretreated cowpea (A) and sorghum biomass (B). Description of signals: OCH_3, methyl ether group; $MeGlcA_{H-1}$ and $MeGlcA_{H-5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; Ara, 3-linked, H-1 signal of α-1,3-linked arabinose.

The ^{1}H-NMR spectrum of sorghum xylan (Figure 2.12B) contained basic signals for glucuronoxylan similar to the previous xylans with minor differences the peak heights. The intensity of the side chain signals showed that, in comparison to hardwood xylan, the extracted bambara xylan contained smaller amounts of 2-*O*-α-linked MeGlcA or GlcA side residues whereas, cowpea xylan possessed a slightly higher GlcA content. In addition, the peaks at 5.38 ppm (H-1 of α-L-Ara*f* residues) in bambara, cowpea and sorghum xylan indicated the presence of arabinose in the xylan. All the xylan preparations did not show any significant H-1α signal for the reducing-end Xyl (at 5.17 ppm) which confirmed the polymeric nature of the isolated material. These results confirmed the fact that the extraction process did not disturbed the polysaccharide nature of xylan.

2.4 CONCLUSION

In this chapter, detailed structural characterization of xylans isolated from peanut shells, bambara, cowpea and sorghum biomass was performed. Xylan is one of the most complex polysaccharides containing multiple substitutions of different side groups. The heterogenicity of the xylan in different biomass makes it difficult to study and understand their structure. In addition, the identification of complete side chain residues in xylan is very challenging because the chemical treatments during xylan extraction might have influenced the removal of side chains such as acetyl and feruloyl groups. The alkali-extracted polysaccharide from delignified peanut shell is very similar to hardwood glucuronoxylan but contained a much higher degree of substitution with MeGlcA. On the other hand, the delignified and alkali extracted xylan from the other three sources comprised glucuronoxylan with moderate MeGlcA substitution. The presence of arabinose in the extracted xylans could also suggest the presence of traces of arabinan or arabinosylated polysaccharides. It also showed that the biomass from these underutilized crops could be a potential source for glucuronoxylan.

CHAPTER THREE

PRODUCTION AND CHARACTERIZATION OF XYLOOLIGOSACCHARIDES, AND THE INTERACTION STUDIES OF XYLANASE-XYLAN COMPLEX

3.1 INTRODUCTION

Hemicelluloses account for 25–30% of all lignocellulose, which is the second most abundant biopolymer after cellulose. One of the main hemicellulosic polymer found in hardwoods and agro-industrial by-products is xylan, which possesses the xylose backbone. This include cereal straws, sugarcane bagasse, corn stover, sisal etc., (Aracri and Vidal, 2011; Valls *et al.*, 2013, 2018) whereas, mannans are more prominent in softwoods (Scheller and Ulvskov, 2010). The xylan present in cereals and grasses is termed as arabinoxylan, which has abundant substitutions of arabinose and ferulic acid in them (Ebringerová and Heinze, 2000). On the other hand, xylan from hardwood species is called as glucuronoxylan, which is highly substituted with glucuronic acid or 4-*O*-methyl-D-glucuronic acid (MeGlcA) substitutions, and can be heavily acetylated, while it does not contain arabinose and ferulic acid.(Valls *et al.*, 2018). Since xylan is a multibranched polysaccharide, its structural composition is not uniform and mostly complex, since the monomers present in them might have isomers, for example, xylose and arabinose (Naidu *et al.*, 2018).

The deconstruction of xylan can be directed towards a particular end-product using xylanases (EC 3.2.1.8), which randomly cleave the β-(1,4) glycosidic bonds of the xylose backbone of xylan (Xu *et al.*, 2018). These enzymes are glycoside hydrolases (GH), grouped in families such as GH5, GH8, GH10, GH11 and GH30, GH43, GH51, GH98 and GH141 based on their hydrolysis profile and binding ability to the substrate, xylan (Terrapon *et al.*, 2017). Xylanases, which are grouped under each GH family possess different mode of action based on their amino acid sequence and the binding pattern with the substrate (Motta *et al.*, 2013). The major end

products derived from hemicelluloses by these enzymes are xylooligosaccharides (XOS), with a wide degree of polymerization (Moniz *et al.*, 2016). XOS comprises oligomers with a structure of around 2–20 xylose units linked together by β-(1,4) bonds, variably substituted with rich patterns of side groups depending on the source (Mano *et al.*, 2018). For instance, acetyl groups and MeGlcA units are the major substituents of XOS from hardwoods, whereas, XOS produced from softwoods comprise mainly arabinose and ferulic acid as substituents. These XOS are low molecular weight carbohydrates, and are classified into non-digestible oligosaccharides (Mano *et al.*, 2018). These oligosaccharides could be produced from xylan by several methods which include mild acid or alkali treatments, and enzymatic hydrolysis (Jönsson and Martín, 2016) whereas, physical methods mostly breakdown and degrade the polysaccharides severely and results in undesirable products. During acid hydrolysis, the end-products are mostly xylose monomers, which is not desirable during XOS production. Conversely, enzymatic hydrolysis is more preferable than other methods, primarily due to its specificity, high efficiency, and non-production of undesirable by-products (Ma *et al.*, 2017).

To establish a better understanding of the enzyme-substrate complex, an in-depth structural analysis is required. The *in silico* analysis such as enzyme structure prediction, molecular dynamics and docking etc. facilitates the understanding of the structural complexity and interaction between the substrate and the enzyme (Dutta *et al.*, 2018). To perform the interaction studies between an enzyme and substrate, the availability of a good quality three-dimensional structure of the target protein is essential. There are different approaches used to generate the 3D structure of a protein which includes homology modelling, threading (Dorn *et al.*, 2014) and the Ab-initio method (Samudrala *et al.*, 1999). One of the most reliable and widely used methods for interpreting a protein's 3D structure is homology modelling, which builds the structure based on the homology of the query sequence with the target protein, whose 3D structure is already available (Meier and Söding, 2015). The protein models generated using the modelling techniques are stable, however, all proteins are dynamic in nature. To study the dynamics of the protein, molecular dynamic studies of the given model should be performed

which allows the understand how a protein model behaves structurally, dynamically and thermodynamically in a force field (Pandit *et al.*, 2016).

Thus far, several XOS producing strategies have been applied on various lignocellulosic residues, however, very little attention has been given to XOS production from biomass such as peanut shell and sorghum. Furthermore, there are no reports on XOS production from underutilised biomass such as bambara and cowpea. This chapter therefore focuses on the extraction and effective hydrolysis of xylan from the above-mentioned sources by optimizing the conditions. Selected xylanases have been tested and the products (XOS) were observed and characterized. Furthermore, *in silico* analyses of xylanase sequences from *T. lanuginosus*, *C. mixtus* and *E. chrysanthemi* were done to characterize the sequential properties, to analyse the domain and topological architecture, to model the protein 3D structure and its docking with xylan fragments.

3.2 MATERIALS AND METHODS

3.2.1 *Thermomyces lanuginosus* SSBP

Thermomyces lanuginosus SSBP (Singh *et al.*, 2003) from the culture collection of the Department of Biotechnology and Food Technology, Durban University of Technology, was used in this study. The culture was grown on potato dextrose agar (PDA, Oxoid, UK) plates at 50°C for 5 days and was sub-cultured every 4 weeks.

3.2.2 Production of xylanase

For xylanase production, an agar disc (9 mm diameter) of an actively growing 5-day old *T. lanuginosus* culture was inoculated to 500 ml of a production medium (pH 6.5) containing (g/l) corn cob (31.2), yeast extract (30.2), KH_2PO_4 (5.0) and incubated at 50°C in a shaker incubator (150 rpm) for 5 days. After incubation, the culture broth was centrifuged (10,000 x g for 10 min), and the supernatant was used to determine the xylanase activity. All experiments were performed in triplicate and the standard deviation was calculated.

3.2.3 Other enzymes used in the study

GH10 xylanase from *Cellvibrio mixtus*, GH16 endo-1,3(4)-β-glucanase from *Clostridium thermocellum* and GH5 endo-xyloglucanase from *Paenibacillus* sp., GH51 endo-L-1,5-arabinanase and GH43 α-L-arabinofuranosidase were purchased from Megazyme (Bray, Ireland). GH30 xylanase from *Erwinia chrysanthemi* was supplied by Prof. James F. Preston from the University of Florida, Gainesville, FL, USA. α-amylase from *Bacillus licheniformis* was procured from Sigma-Aldrich, USA.

3.2.4 Protein estimation and enzyme assay

The concentration of protein present in the culture supernatant was determined by Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard. Xylanase assay was carried out according to Bailey *et al.* (1992) using 1% beechwood xylan (Sigma-Aldrich, USA) as the substrate. The reaction mixture consisted of 0.9 ml (1%, w/v) substrate in 0.05 M citrate buffer (pH 6.5) and 0.1 ml culture filtrate. After incubation at 50°C for 5 min, the reaction was stopped by the addition of 1.5 ml dinitrosalicylic acid (DNS) solution followed by boiling in a water bath at 100°C for 5 min (Miller, 1959). After cooling the reaction mixture to room temperature, the absorbance values were read at 540 nm using a spectrophotometer (Biochrom Libra S21). Xylose (Sigma-Aldrich, USA) served as the standard. Enzyme activity was expressed as International Units (U) which is defined as the amount of enzyme that produces one μ mole of xylose equivalents per minute (Kaushik *et al.*, 2014). Xylanase activity was also determined on beechwood glucuronoxylan (Sigma-Aldrich, USA) following the formation of reducing groups quantified by the Somogyi-Nelson procedure (Paleg, 1959). Commercial enzymes were used according to the units reported by the supplier.

3.2.5 Partial purification of xylanase

The crude xylanase obtained from *T. lanuginosus* SSBP was partially purified by ammonium sulphate precipitation. The crude enzyme (100 ml) was subjected to ammonium sulphate precipitation (0%–80% saturation) with constant stirring at refrigeration temperature (4–8°C).

The precipitate was then collected by centrifugation (10,000 x g at 4°C) for 5 min and the pellet was dissolved in 20 mM phosphate buffer (pH 6.0). The dissolved protein was desalted by dialysis using a 10 kDa cut off membrane (Sigma-Aldrich, USA) with 20 mM phosphate buffer (pH 6.0) and stirred at 4°C for 24 h. The dialysed preparation was further purified by passing through 10 and 30 kDa MWCO membranes. The active fractions were collected, and purity was checked on SDS-PAGE. Enzyme activity and protein concentrations were estimated after each purification step. The specific activity, yield, and fold purification were also calculated (Chapla *et al.*, 2012).

3.2.6 Xylooligosaccharide production by enzymatic hydrolysis

Xylan extracted from different biomass (bambara, cowpea, sorghum and peanut shell) was hydrolysed with xylanase from three hydrolase families *viz.*, GH10, GH11 and GH30. Xylan (1%, w/v) dissolved in 50 mM sodium citrate buffer (pH 6.5) was treated with *T. lanuginosus* GH11 xylanase (10 U/g) and incubated at 50 °C for 24 h in a water bath. The citrate buffer was replaced with sodium acetate buffer (pH 5.5) for reaction mixtures of GH10 and GH30 xylanases (10 U/g) and was incubated at 35 °C for 24 h. Xylan solution without the enzyme served as control for each reaction. The samples were collected every 4 h and analysed for reducing sugars by DNS method (Miller, 1959). The hydrolysate was separated by centrifugation at 10000 x g at room temperature for 10 min (Chapla, 2012).

Effect of various biochemical parameters on the enzymatic hydrolysis of xylan was studied in order to maximize the production of XOS. Effect of enzyme dosage on XOS release was determined by performing enzymatic hydrolysis at varying xylanase concentrations (1–20 U) on 1% xylan at 50°C, with mild shaking. The effect of substrate concentration was determined in a similar manner by varying the substrate concentration (0.2–2.0%) with 10 U/ml of xylanase. The hydrolysis products were analysed by TLC (Section 2.2.7) and MALDI-TOF MS (Section 2.2.8).

3.2.7 Sequence retrieval and analysis

The xylanase sequences from three different organisms, *C. mixtus* (*Cm*GH10), *T. lanuginosus* (*Tl*GH11) and *E. chrysanthemi* (*Ec*GH30) were retrieved in FASTA format from NCBI (http://www.ncbi.nlm.nih.gov) with the GenBank IDs AAD09439.3, AEH57194.1 and AAB53151.1 respectively. The three sequences were individually analysed for secondary structure and topological studies using PDBsum server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/).

3.2.8 Phylogenetic analysis

Multiple sequence analyses were conducted on GH10, GH11 and GH30 xylanases using the Clustal Omega program. The phylogenetic tree was calculated with the neighbour-joining method implemented in the Clustal Omega package using the final alignment. Approximately 80 sequences from different eukaryotes and prokaryotes were retrieved from CAZy database (http://www.cazy.org/) for the analysis. An attempt was also made to find sequences pattern or conserved regions within the domain, through multiple sequence analyses. The investigation was also carried out to study and characterize other functional domains of these xylanase families.

3.2.9 Protein 3D structure modelling and validation

Modelling of all the three xylanase sequences was done by Modeller 9.20 (Webb and Sali, 2016) software that relies on the principles of homology modelling. Templates of the query sequences (xylanase) were selected based on their highest percentage of similarity and coverage area. Validation and verification studies were also carried out after modelling, with Ramachandran's plot, Verify3D (http://servicesn.mbi.ucla.edu/Verify3D/) and ProsA (https://prosa.services.came.sbg.ac.at/prosa.php) analyses.

3.2.10 Docking studies

The best structure for xylanase from three different sources was selected and prepared for docking and was further implemented using AutoDock Vina (http://autodock.scripps.edu/) package (Trott and Olson, 2010). Prior to AutoDock suite, the PDB file of receptor xylanase was prearranged by removing all water (HETATM) coordinates and then the PDB file was converted to PDBQT format. Binding sites on the receptor were analysed from InterProScan and ScanProsite (de Castro *et al.*, 2006; Dutta *et al.*, 2018) and CASTp tool (http://sts.bioe.uic.edu/castp/index.html?2was) which helped in the gridbox generation. A grid box was created with dimension 30 × 35 × 40 Å along the XYZ direction with a grid spacing of 1 Å, using the AutoGrid module for the *T. lanuginosus* xylanase structure. Similar steps were implemented for the other two xylanase modelled structures, with slightly modified grid box sizes, 28 × 38 × 36 Å and 32 × 44 × 40 Å for *C. mixtus* and *E. chrysanthemi* respectively. The dimensions were adjusted by AutoDockTools (ADT) and finally docking was performed with exhaustiveness set to 100. The PDB files of modelled structures of xylan (ligand) were saved as AutoDock suitable coordinate files (PDBQT) using ADT (Forli *et al.*, 2016). The docking results (pose and energy values) were saved in CSV format for every 6 modes.

3.3 RESULTS AND DISCUSSION

3.3.1 Production and purification of GH11 xylanase from *T. lanuginosus* SSBP

The xylanase from *T. lanuginosus* SSBP was produced in corn cob medium. The crude culture filtrate contained xylanase and other accessory enzymes. The GH family of the enzyme (GH11) was confirmed by comparing the mode of action of this enzyme with other known GH11 xylanases and comparing the products (XOS) released from glucuronoxylan (Kolenová *et al.*, 2006). The enzyme was purified by the method of Lin *et al.* (1999). SDS-PAGE analysis was performed to check the purity of xylanase (Figure 3.1) and a single band was observed at ~24 kDa.

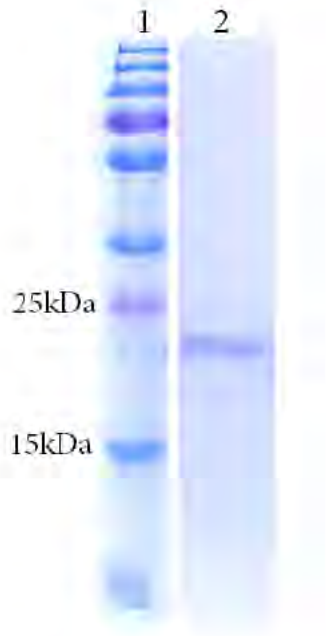

Figure 3.1 SDS-PAGE profile of GH11 xylanase produced from *T. lanuginosus* SSBP. Lane 1, protein marker; lane 2, purified xylanase.

3.3.2 Optimization of xylan concentration and enzyme dosage for XOS production

Production of XOS from xylan by xylanase is cost-effective does not require extreme conditions. To maximise the release from xylan, the enzyme dosage and operational conditions must be optimised. Preliminary studies on the optimization of XOS production was carried out using Beechwood xylan at various concentrations ranging from 0.2–2.0% in sodium citrate buffer, pH 6.5. At 1.0% xylan, the amount of reducing sugar released was 28.22 µmol/ml, which continued to be stable in that range (27–28 µmol/ml) even at a higher xylan concentration of 2.0% (Figure 3.2). Therefore, the lowest xylan concentration (1.0%) which obtained maximum sugar release was chosen as the optimum concentration for XOS production. A further increase in xylan concentration did not significantly increase the release of sugar.

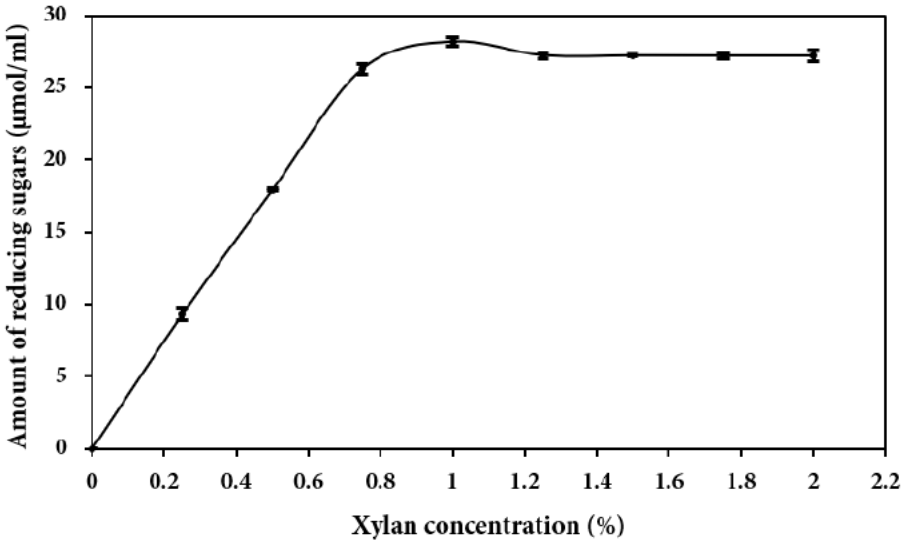

Figure 3.2 Optimization of xylan concentration for XOS production. Each point represents the mean (n=3) ± SD.

The optimum enzyme concentration for the release of sugars from xylan (1%) was determined. Since *Tl*GH11 xylanase breaks down xylan efficiently, low concentrations (1–20 U/mg) was used for optimization. The products released were quantified as reducing sugars, as each of the XOS contains a reducing group at their terminal xylose residue. It is assumed that one mol of xylose is equivalent to one mole of XOS (Valls *et al.*, 2018). An increase in the XOS release was observed until 10 U/mg of enzyme and thereafter no further release of sugars was observed with increased dosages. (Figure 3.3). This could be due to feedback inhibition of xylanase by xylose released in the solution. The feedback inhibition of xylose was also observed by Bibi *et al.* (2014). For this reason, the xylanase dose of 10 U/mg was chosen as the optimal enzyme dosage for XOS production.

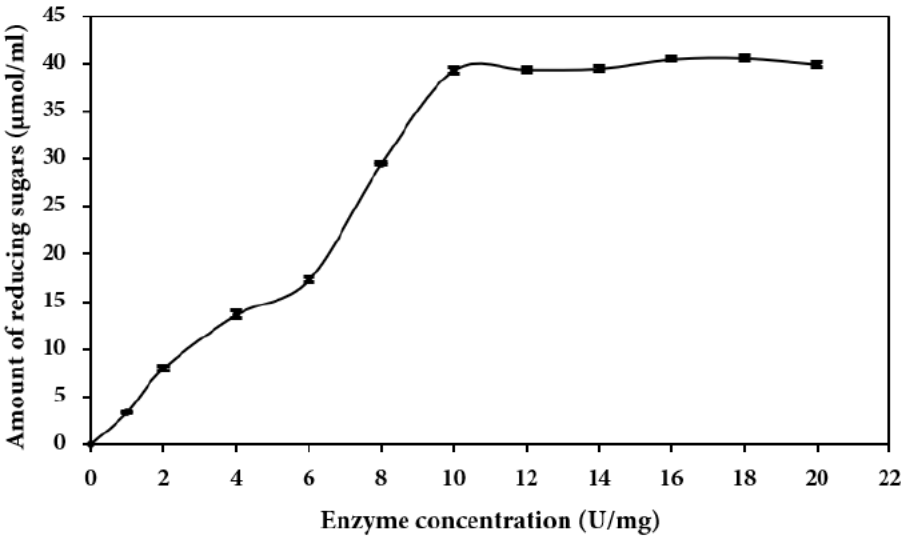

Figure 3.3 Optimization of enzyme dosage for XOS production. Each point represents the mean (n=3) ± SD.

3.3.3 Hydrolysis of peanut shell, bambara, cowpea and sorghum xylan by GH10, GH11 and GH30 xylanases

Xylan hydrolysis was done on the extracted xylans with xylanases from three different GH families and the hydrolysis products were analyzed by TLC (Figures 3.4 and 3.5). Xylanases from distinct GH families exhibit different modes of action (Biely *et al.* 2016) by which the heterogenicity and substitution of the substrate (xylan) could be deduced. The products generated by the action of xylanases from different families may reveal the type of xylan present in each biomass. With respect to the mode of action, GH10 and GH11 xylanases release XOS from glucuronoxylans which are a mixture of linear (neutral) and MeGlcA substituted (acidic) oligomers, whereas GH30 xylanases release only MeGlcA substituted xylooligomers (Puchart *et al.*, 2019).

There is a striking similarity between the products generated by the three types of xylanases from peanut shell xylan and from beechwood glucuronoxylan (Figure 3.4), whereas, the bambara, cowpea and sorghum xylans yielded similar XOS (Figure 3.5) which were slightly lower than peanut shell xylan. The main hydrolysis products of *Cm*GH10 xylanase from each xylan were Xyl, Xyl_2, Xyl_3, $MeGlcA^3Xyl_3$ and $MeGlcA^3Xyl_4$ (Figures 3.4 and 3.5). In agreement

with the previous literature, *Cm*GH10 xylanase produced shorter oligosaccharides than *Tl*GH11 xylanase (Li *et al.*, 2016; Moreira and Filho, 2016; de Freitas *et al.*, 2019).

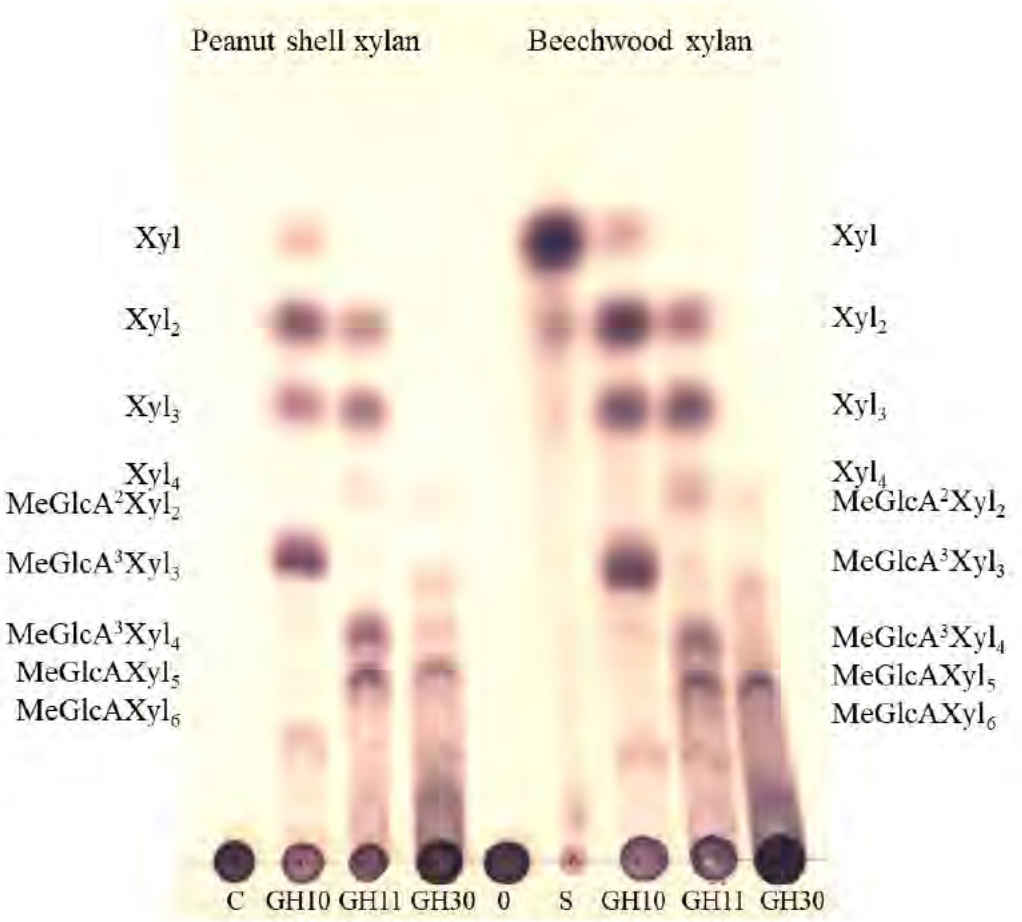

Figure 3.4 TLC analysis of XOS produced from peanut shell xylan and beechwood xylan by GH10, GH11 and GH30 xylanases; C, control (xylan incubated without enzyme); S, standards; Xyl, Xylose; Xyl_2, Xylobiose; Xyl_3, Xylotriose; Xyl_4, Xylotetraose; $MeGlcA^2Xyl_2$, aldotriouronic acid; $MeGlcA^3Xyl_3$, aldotetraouronic acid; $MeGlcA^3Xyl_4$, aldopentaouronic acid; $MeGlcAXyl_5$, aldohexaouronic acid; $MeGlcAXyl_6$, aldoheptaouronic acid.

Due to the typical mode of action, *Cm*GH10 xylanase released more xylose and aldotetraouronic acid than other xylanases as the final acidic product of hydrolysis of glucuronoxylan which was confirmed by TLC. It should be noted that aldopentaouronic acid, where the MeGlcA located at the second xylopyranosyl unit from the non-reducing end of xylotetraose, was also resistant to the action of GH10 xylanases (Kolenová *et al.*, 2006). Among

the four xylans tested, peanut shell xylan and sorghum xylan showed significantly more products released by GH10 xylanase, whereas bambara and cowpea xylans released less XOS which was confirmed by the intensity of the spots on TLC. This was also similar to the observations of Puchart *et al.* (2019), where XOS produced from Aspenwood xylan hydrolysis had Xyl-Xyl_3, $MeGlcA^3Xyl_3$ and $MeGlcA^3Xyl_4$ as the main products.

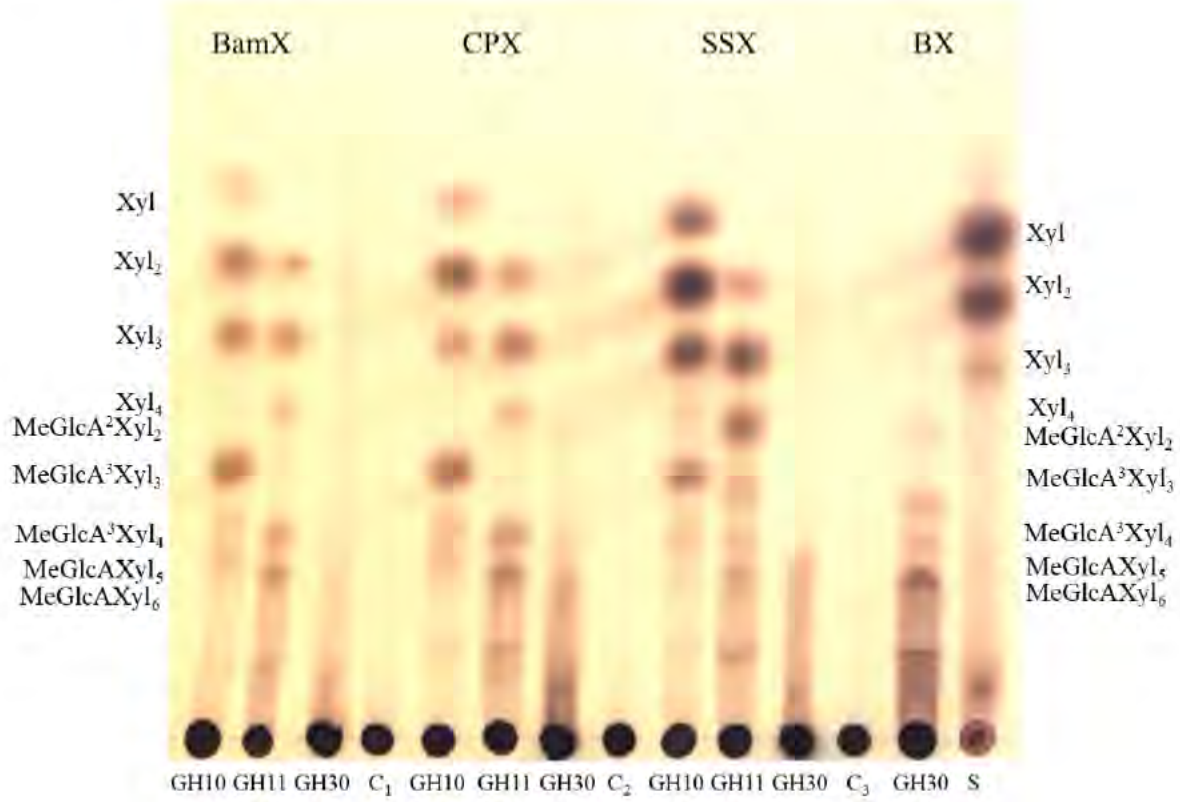

Figure 3.5 TLC analysis of XOS produced from bambara (BamX), cowpea (CPX), sorghum (SSX) and beechwood xylans (BX) by GH10, GH11 and GH30 xylanases; C_1, C_2 and C_3, Controls (xylan incubated without enzyme); S, oligosaccharide standards; Xyl, xylose; Xyl_2, xylobiose; Xyl_3, xylotriose; Xyl_4, xylotetraose; $MeGlcA^2Xyl_2$, Aldotriouronic acid; $MeGlcA^3Xyl_3$, Aldotetraouronic acid; $MeGlcA^3Xyl_4$, Aldopentaouronic acid; $MeGlcAXyl_5$, Aldohexaouronic acid; $MeGlcAXyl_6$, Aldoheptaouronic acid.

*Tl*GH11 xylanase released Xyl_2 and Xyl_3 as linear oligosaccharides in addition to the release of some aldouronic acids such as $MeGlcAXyl_4$, $MeGlcAXyl_5$ and $MeGlcAXyl_6$, during glucuronoxylan hydrolysis. Xyl_2 and Xyl_3 were observed in all the four xylan hydrolysates

whereas Xyl_4 was observed with varying intensities from each xylan. However, unlike other xylanases, *Tl*GH11 xylanase did not produce any free xylose units during the xylan hydrolysis. Based on the mechanism of action of GH11 xylanase, the shortest aldouronic acid released was $MeGlcAXyl_4$, whereas the largest XOS varied according to the substitution behaviour of the xylan used. A wide range of XOS production was perceived form sorghum and peanut shell xylan compared to other xylans tested. All these fragments were typical hydrolysis products of hardwood glucuronoxylan generated by GH11 xylanases. Similar findings were reported from birchwood xylan hydrolysed by GH11 xylanase from *Ophiostoma piliferum*, *Coprinopsis cinereal* (Sydenham *et al.*, 2014) and *Talaromyces amestolkiae* (Nieto-Domínguez *et al.*, 2017). Conversely, *Ec*GH30 xylanase could only act on uronic acid containing xylan (Biely *et al.*, 2016), generating a series of aldouronic acids essentially free of linear XOS. A detectable amount of aldouronic acids such as $MeGlcAXyl_3$, $MeGlcAXyl_4$ and $MeGlcAXyl_5$ were found in peanut shell xylan hydrolysate followed by cowpea xylan hydrolysate. Bambara and sorghum xylan contained small amounts of aldouronic acids after GH30 hydrolysis. A slightly higher proportion of aldouronic acids was found in the peanut shell xylan hydrolysate than the other xylans. This was also found to be higher than the previously isolated xylans from sweetgum wood (Rhee *et al.*, 2014) and beechwood (Biely *et al.*, 2015). These results strongly support the preliminary observations made on different xylans by monosaccharide and NMR analyses. It is noteworthy that the degradation products of xylan resembles those of typical hardwood glucuronoxylan. As shown in the MALDI-TOF MS analysis of the products (refer section 3.3.4), the enzyme also generates a small amount of linear xylooligosaccharides which are not visible on the TLC chromatogram.

3.3.4 MALDI-TOF MS analysis of XOS from different xylans

The MALDI-TOF MS data established the presence of oligosaccharides made up of substituted and unsubstituted pentose sugars in the xylans (Figures 3.6; 3.7; 3.8 and 3.9). The sodium adducts of aldouronic acids in MS spectra correlates well with the TLC observations. TLC and NMR results confirmed that peanut shell xylan has slightly different structural and hydrolysis patterns in comparison to other xylans.

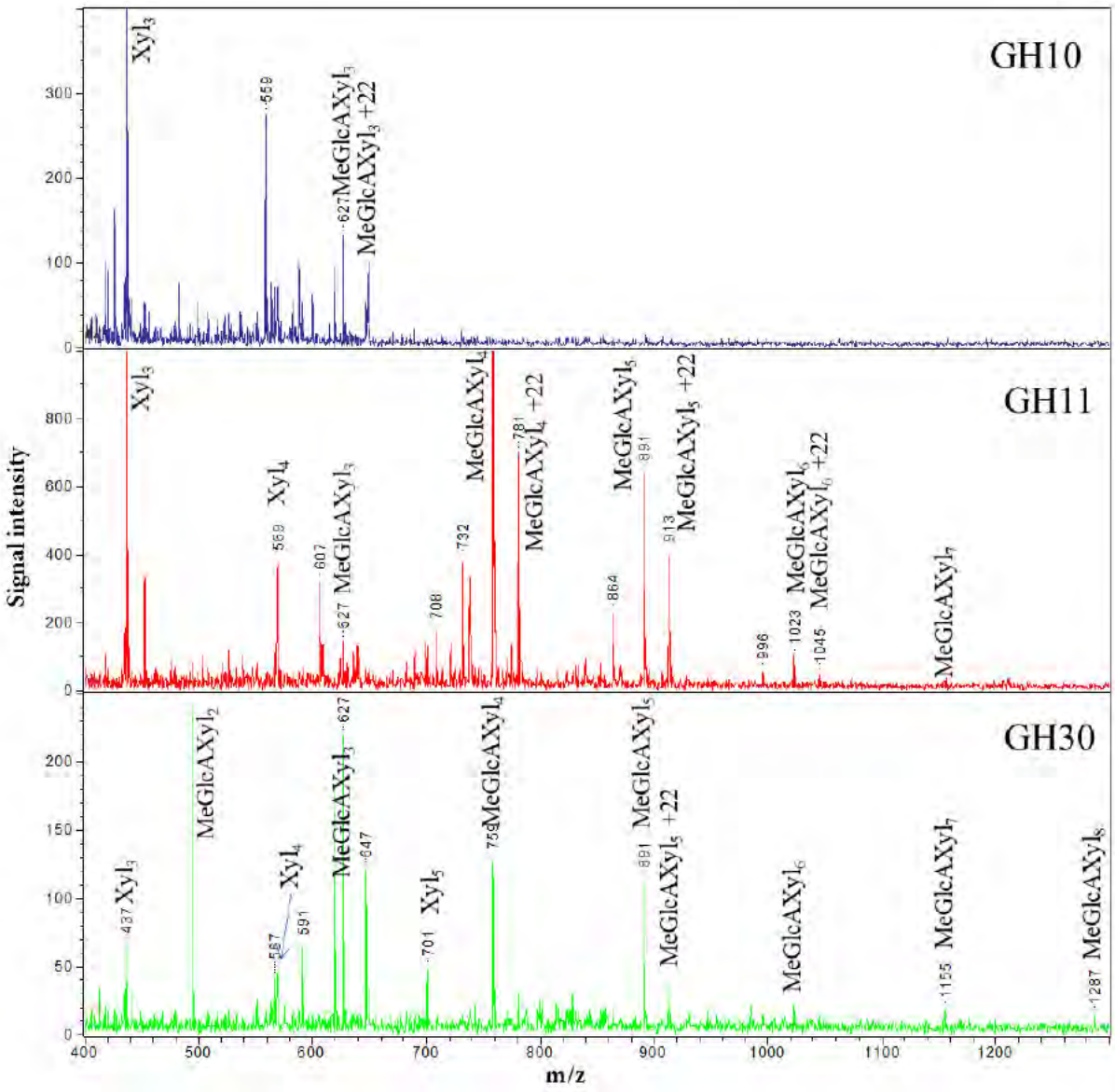

Figure 3.6 MALDI-TOF MS spectra of hydrolysates of peanut shell xylan by GH10, GH11 and GH30 xylanases. The numbers are m/z values of Xyl_n, ions of neutral xylooligosaccharides; $MeGlcA^iXyl_n$, ions of acidic oligosaccharides; +22, Disodium salts of acidic oligosaccharides.

The evidence for the glucuronoxylan nature of the peanut shell xylan was provided by MALDI-TOF MS analysis of the XOS generated from xylan by the three xylanases (Figure 3.6). GH10 xylanases produced shorter XOS than GH11 whereas GH30 released a series of aldouronic acids conferring to the MeGlcA distribution in xylan. Since the molecular mass of both xylose and arabinose is same (150 g/mol), they cannot be distinguished. GH10 xylanase released only Xyl_2 (not shown), Xyl_3 and $MeGlcAXyl_3$ as the main products which were detected by MS. In the

GH11 hydrolysate, neutral XOS (Xyl_3 and Xyl_4) along with some aldouronic acids were detected that also include the signals for disodium ions (+22). The aldouronic acids detected ranged from $MeGlcAXyl_3$ to $MeGlcAXyl_7$. Products released from peanut shell xylan by GH30 xylanase mostly comprised of aldouronic acids with ≥10 xylose units and few uronic acid substitutions.

The products released from bambara xylan by GH10 xylanase mimicked the signal pattern of peanut shell xylan, mainly composed of Xyl_3, Xyl_4 and $MeGlcAXyl_3$ (Figure 3.7). Whereas, GH11 hydrolysates showed signals of XOS from Xyl_3–Xyl_7 along with some aldouronic acids such as $MeGlcAXyl_4$, $MeGlcAXyl_5$ and $MeGlcAXyl_6$. In the case of GH30 xylanase, there was no significant product formation from bambara xylan. The products from cowpea xylan correlate well with the previous observations made from monosaccharide and TLC analyses. Considering the length of the hydrolysed products from cowpea xylan, it can be assumed that the principal ions in the hydrolysate of *Cm*GH10 xylanase correspond to Xyl_3 and aldouronic acids- $MeGlcAXyl_3$ and $MeGlcAXyl_4$ (the ion of the main neutral product, Xyl_2, is not shown) (Figure 3.8). In the case of *Tl*GH11, two longer neutral oligosaccharides were detected from cowpea xylan hydrolysate which had a mass-to-charge ratio values of 569 and 701; the former was presumably xylotetraose, as well as aldouronic acids comprised of one MeGlcA and 4 to 5 pentose residues. Since these ions were not observed in the GH10 xylanase hydrolysate, the larger aldouronic acids from $MeGlcAXyl_5$ and above must have been hydrolysed by GH10 xylanase. In general, GH10 xylanases require only two unsubstituted residues between two substituted xyloses for the cleavage of the main xylan chain and can act on linkages closer to the substituent (Moreira and Filho, 2016). However, GH11 xylanase requires three unsubstituted residues (Wan *et al.*, 2014; Biely *et al.*, 2016). Moreover, some aldouronic acids produced from the enzymatic hydrolysis of these xylans also contained ions of their sodium adducts (m/z 22). GH30 xylanase released a wide range of aldouronic acids starting from $MeGlcAXyl_2$ to $MeGlcAXyl_8$.

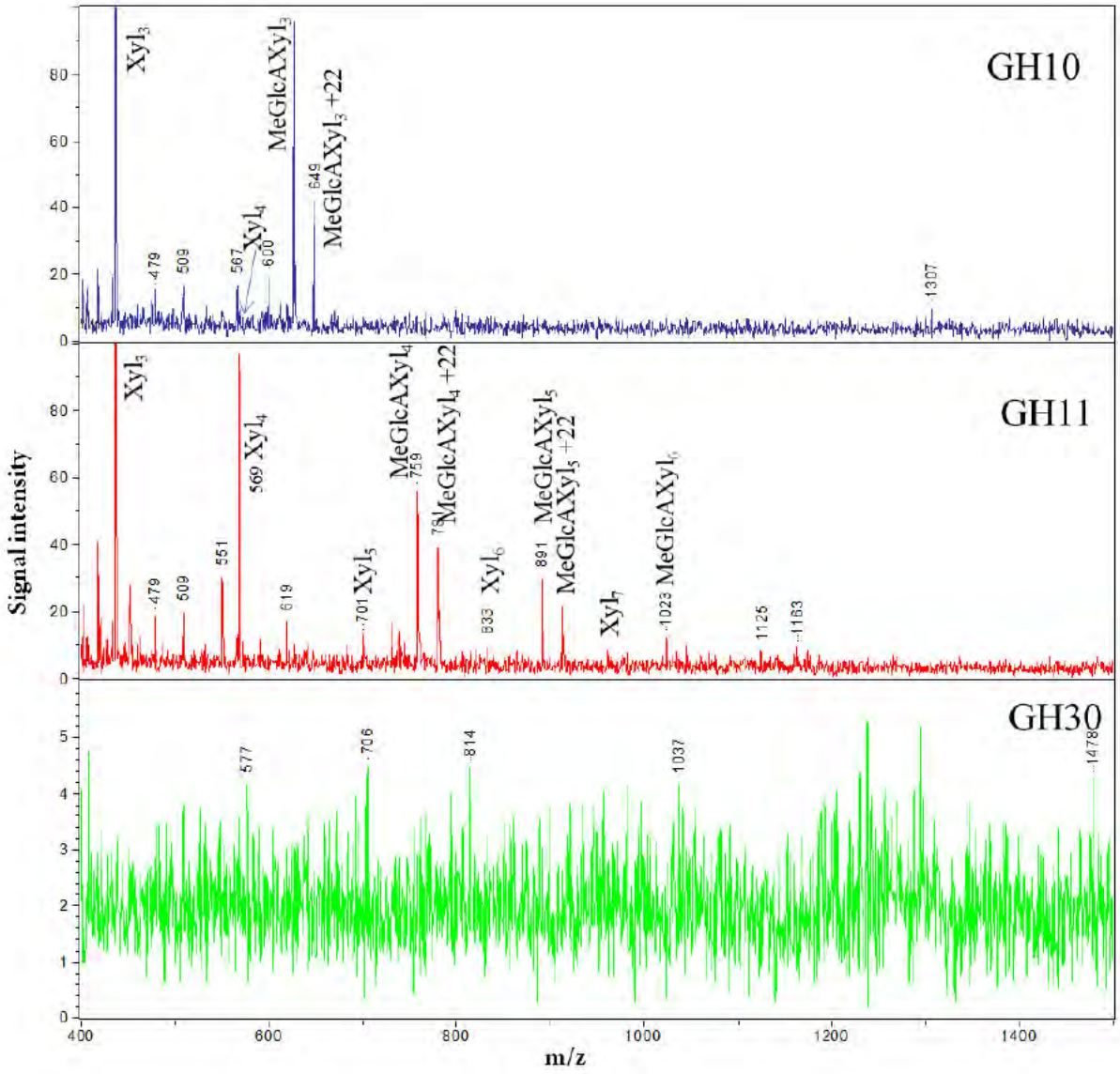

Figure 3.7 MALDI-TOF MS spectrum of xylooligosaccharides produced from bambara xylan by GH10, GH11 and GH30 xylanases. Abbreviations: Xyl_x, ions of neutral xylooligosaccharides; $MeGlcAXyl_x$, ions of acidic oligosaccharides; $MeGlcAXyl_x$ +22, ions of sodium salts of acidic oligosaccharides.

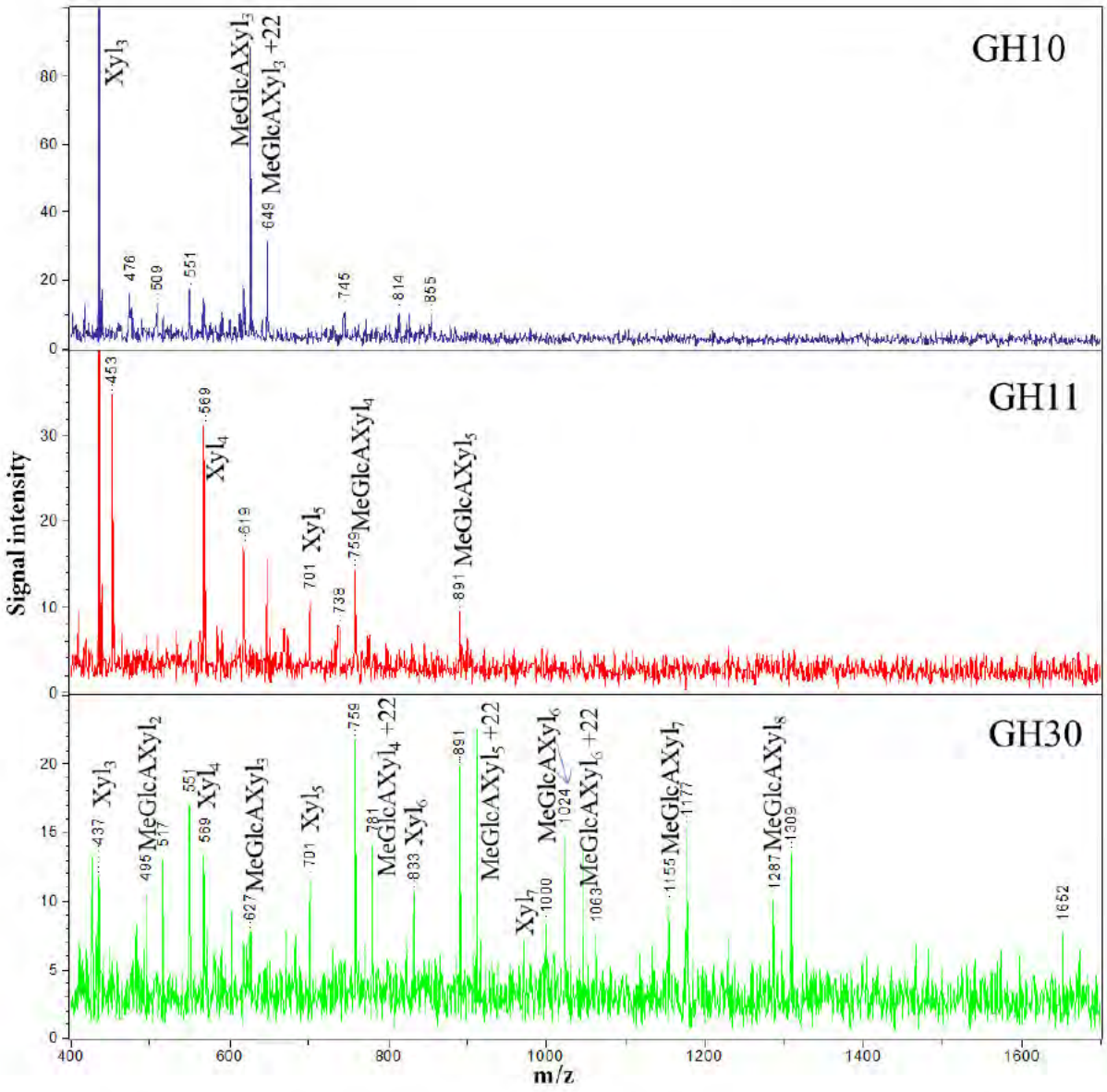

Figure 3.8 MALDI-TOF MS spectrum of xylooligosaccharides produced from cowpea xylan by GH10, GH11 and GH30 xylanases. Abbreviations: Xyl_x, ions of neutral xylooligosaccharides; $MeGlcAXyl_x$, ions of acidic oligosaccharides; $MeGlcAXyl_x$ +22, ions of sodium salts of acidic oligosaccharides.

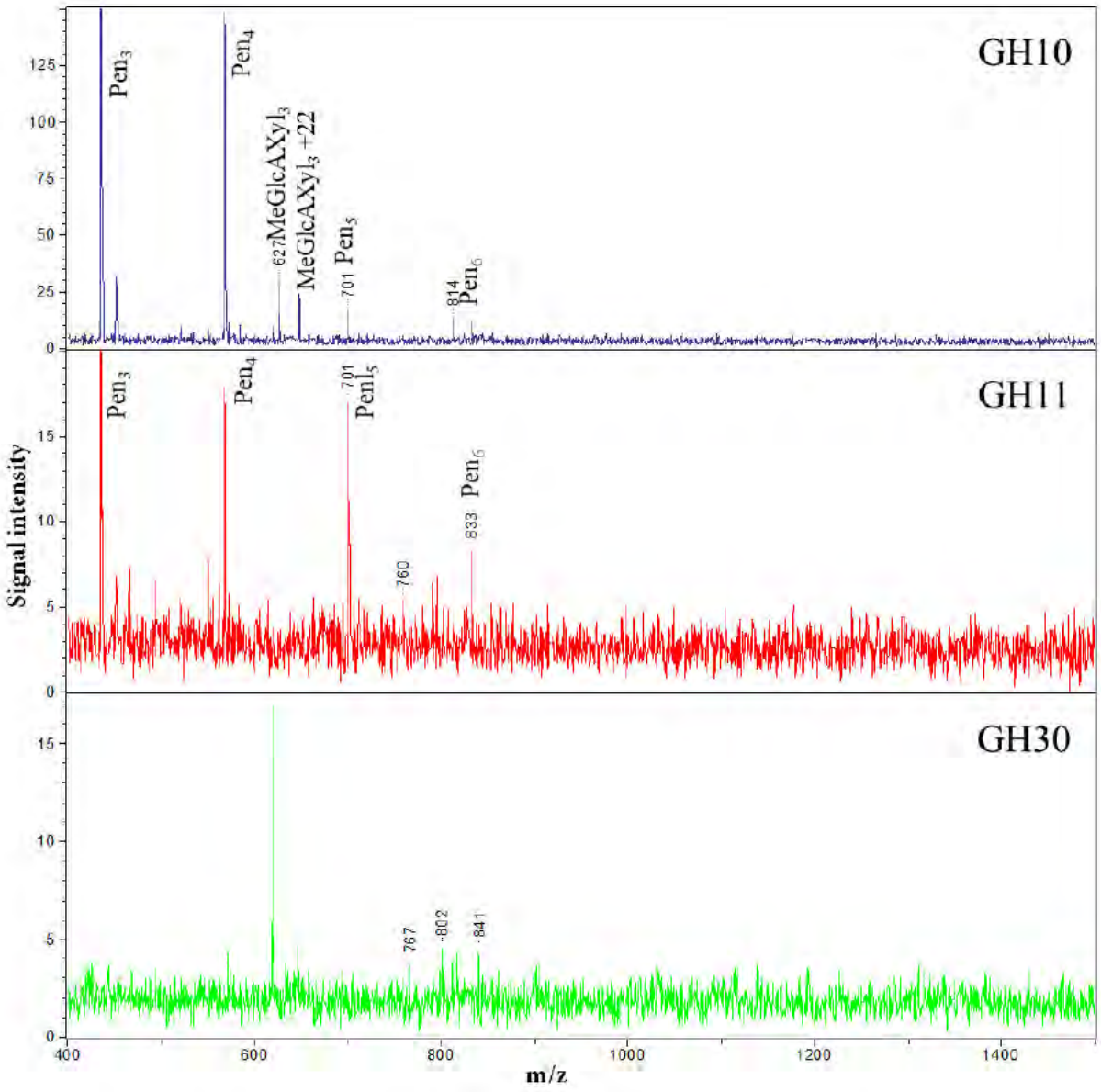

Figure 3.9 MALDI-TOF MS spectrum of xylooligosaccharides produced from sorghum xylan by GH10, GH11 and GH30 xylanases. Abbreviations: Pen_x, ions of neutral and/or arabinosylated xylooligosaccharides; $MeGlcAXyl_x$, ions of acidic oligosaccharides; $MeGlcAXyl_x$ +22, ions of sodium salts of acidic oligosaccharides.

Treatment of with GH10 xylanase produced a series of linear XOS and few short aldouronic acids (Figure 3.9). However, the GH11 and GH30 hydrolysates had very weak signals for aldouronic acids, which is in line with a lower content of MeGlcA (Figure 3.9). A similar trend was also observed during the TLC analysis (Figure 3.5) and the products from GH11 hydrolysate had more intensive bands. Since the molecular weight of xylose and arabinose is

the same, it made them difficult to distinguish and the signals in the spectra were denoted as Pen_x.

The catalytic mechanism of *Cm*GH10, *Tl*GH11 and *Ec*GH30 was shown in both TLC and MALDI-TOF MS analyses. The product release pattern by three xylanases from all the four xylans were comparable to each other and their signal intensities varied based on the concentration of XOS. As expected, the GH10 xylanase released shorter oligosaccharides compared to GH11 xylanase, whereas, GH30 xylanase underperformed on bambara and sorghum xylan, due to the poor substitution of MeGlcA. This data and the NMR spectra confirmed that all the xylans were glucuronoxylan similar to that found in hardwood. There could be a possibility of the existence of another type of hemicellulose in the extracted xylans. A similar study was done with the xylan extracted from Aspenwood treated with the GH10, GH11 and GH30 xylanases showed the release of acetylglucurono XOS and some key signals similar to our results (Puchart *et al.*, 2019).

3.3.5 Sequential analysis of xylanases for structural elucidation

Xylanase sequences from the three different GH families used in this study were retrieved from NCBI and *in silico* studies were done for the expansion and validation of xylan hydrolysis patterns. To gain insights into the sequential arrangement of xylanase, analyses were done using PDBsum, ScanProsite (Dutta *et al.*, 2018) and InterProScan server. The results from PDBsum illustrated the placement of different secondary structural elements (Figures 3.10, 3.11 and 3.12). It also showed the topological orientation of these elements with N and C terminal positions. The PDBsum analysis shows key structural features with their annotation and summary immediately (Laskowski *et al.*, 2018). The xylanase sequence from *T. lanuginosus* consists of 13 helices, 4 β-hairpin loop and 1 disulphide bond (Figure 3.11A), whereas *C. mixtus* and *E. chrysanthemi* xylanases had 17 helices, 1 β -hairpin, 20 strands, 9 γ-turns, 2 disulphides (Figure 3.10A) and 1 helix, 5 β-hairpin loops, 13 strands, 1 disulphide (Figure 3.12A) respectively.

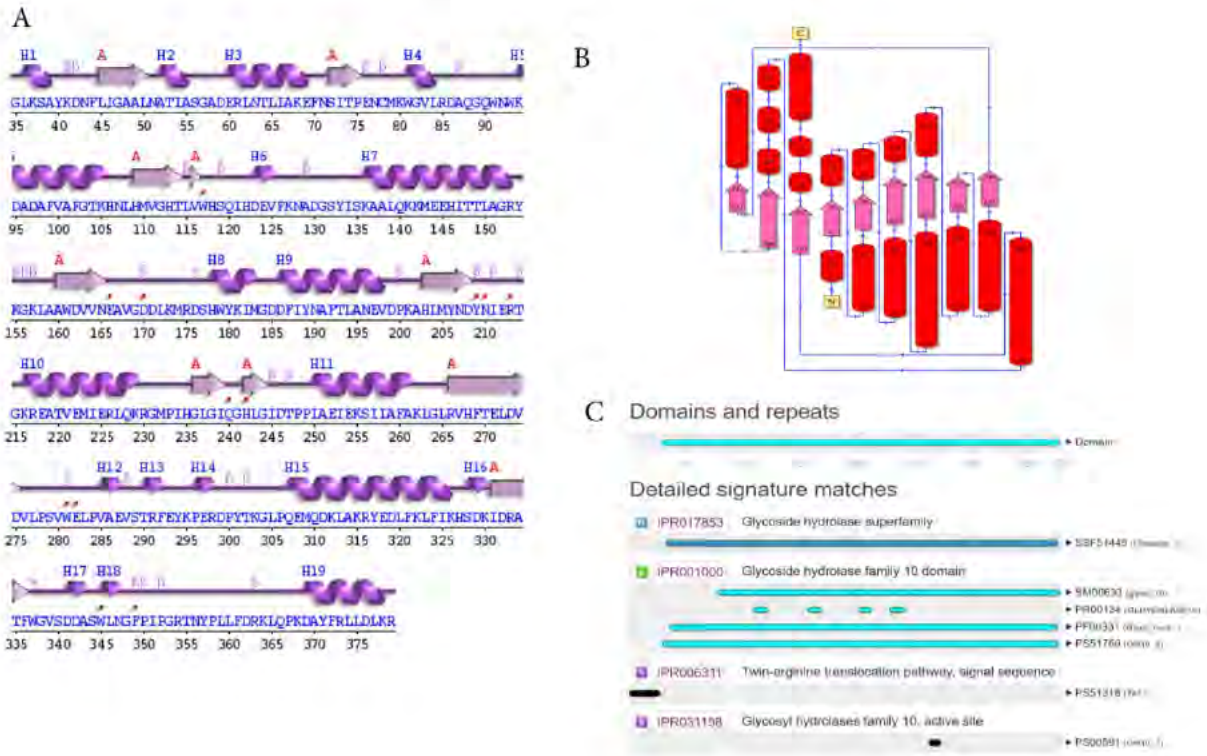

Figure 3.10 Schematic diagrams from the PDBsum for xylanase of *C. mixtus*. (A) The wiring diagram shows the protein's secondary structure (α-helices and β-sheets) together with various structural motifs such as β and γ turns. (B) Topology diagram of the structural domain illustrating the β-strands (large arrows) and, locations of the α-helices (red cylinders). The small arrows indicate the direction of the protein chain, from N- terminus to C- terminus. (C) Insight of *Cm*GH10 xylanase domains identified using the InterProScan server.

The xylanase sequence of *T. lanuginosus* (Figure 3.11B) only had one domain structure, however, the same protein from the other two sources, *C. mixtus* and *E. chrysanthemi* had two domain structures (Figures 3.10B and 3.12B), which were the hydratase and catalytic binding domains. It was further confirmed from the InterProScan result that all the three xylanases had hydrolase domain along with a catalytic domain, residing near the hydrolase domain. The position of the domains and their length are shown in Figures 3.10C, 3.11C and 3.12C. The results also confirmed that the three xylanase sequences belong to the GH10, GH11 and GH30 families. A similar study was done on xylanases from 36 different bacterial sources by Dutta *et al.* (2018) who showed that these xylanases were highly stable, extracellular and contained more

α-helix regions. The structure of *T. lanuginosus* SSBP was also reported after the treatment with chitinase (Khan *et al.*, 2015) and β-xylosidase (Gramany *et al.*, 2015).

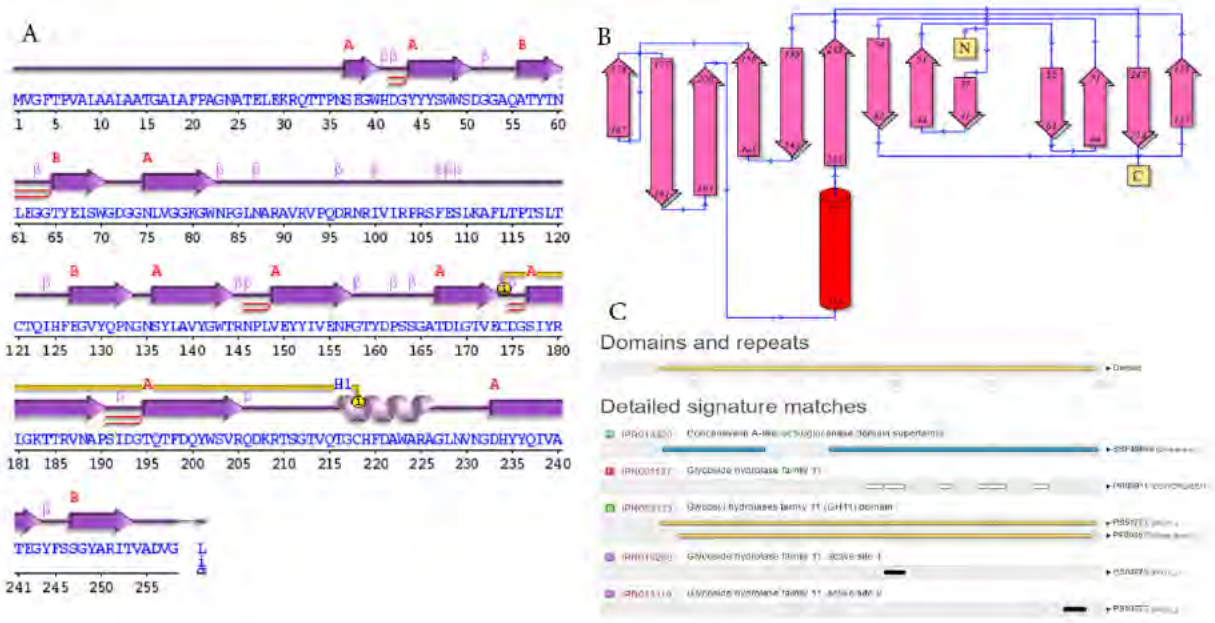

Figure 3.11 Schematic diagrams from the PDBsum for xylanase of *T. lanuginosus*. (A) The wiring diagram shows the protein's secondary structure (α-helices and β-sheets) together with various structural motifs such as β and γ turns, and β hairpins. The yellow linking bars labelled H1 represent disulphide bond. (B) Topology diagram of the structural domain illustrating the linkages of the β-strands (large arrows) the locations of the α-helices (red cylinder). The small arrows indicate the directionality of the protein chain, from the N- terminus to the C- terminus. (C) Insight of *Tl*GH11 xylanase domains identified using the InterProScan server.

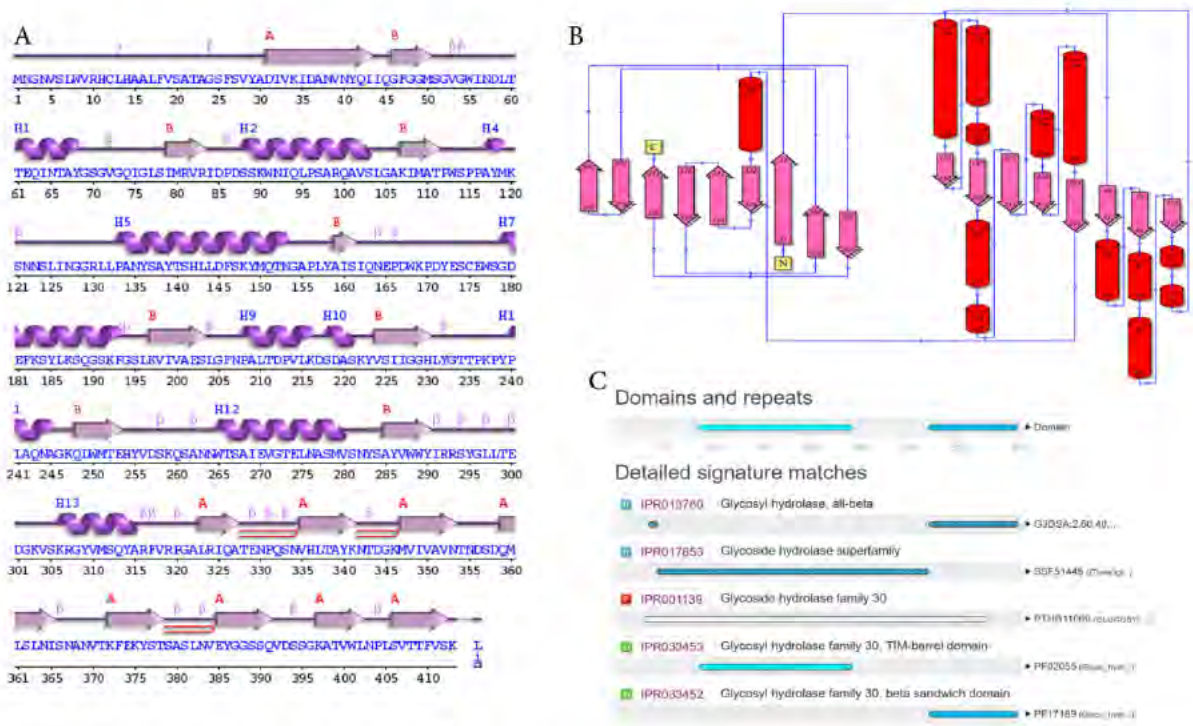

Figure 3.12 Schematic diagrams from the PDBsum for xylanase of *E. chrysanthemi*. (A) The wiring diagram shows the protein's secondary structure (α-helices and β-sheets) together with various structural motifs such as β and γ turns, and β hairpins. (B) Topology diagram of the structural domain illustrating the linkages of the β-strands (large arrows), the location of the α-helices (red cylinders). The small arrows indicate the direction of the protein chain, from the N- terminus to the C- terminus. (C) Insight of *Ec*GH30 xylanase domains identified using the InterProScan server.

3.3.6 Phylogenetic analysis

Based on the information from the CAZy database, 39 xylanase sequences similar to the xylanase from *C. mixtus* of the GH10 family were selected. The sequence length for all the sequences was approximately 379 amino acids. A cladogram type of phylogenetic tree was constructed (Figure 3.13).

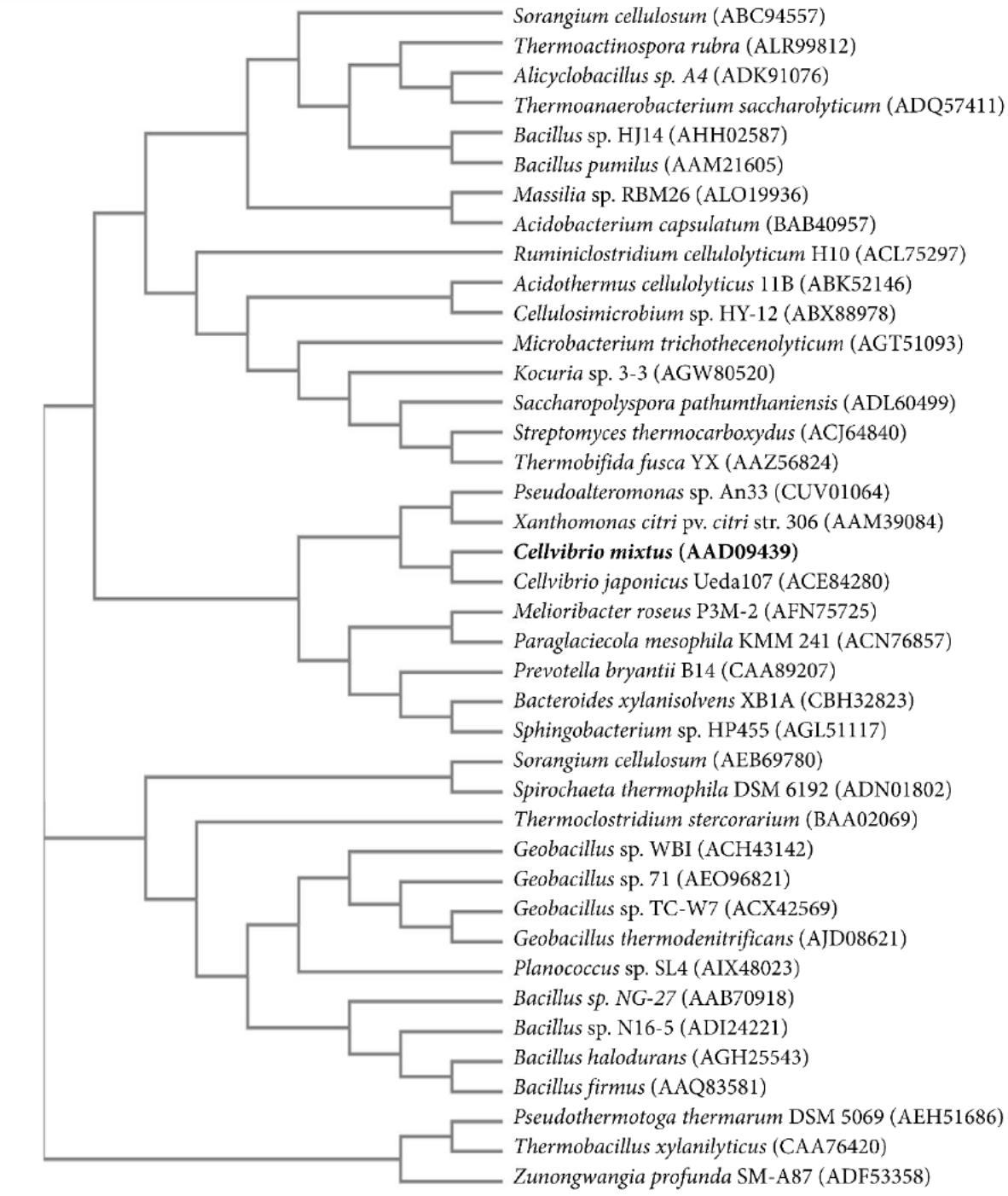

Figure 3.13 Phylogenetic analysis of the amino acid sequences of xylanases from family GH10. *C. mixtus* is highlighted in bold letters.

The phylogenetic tree had three groups at the bottom of the tree which were seperated from the main cluster. The first clade had only three sequences (accession no. AEH51686, CAA76420 and ADF53358), whereas the second clade contained 12 sequences were mainly from *Bacillus*

and *Geobacillus* genera. The third group was the biggest of the three clades and contained *C. mixtus*. It was also noted that *C. mixtus* xylanase had maximum similarity with the neighbouring sequence *C. japanicus* Ueda107 (ACE84280) which indicated that they belong to the same genus. This neighbouring sequence could be used as a template for modelling the protein of interest. E181, N182, K185 are the amino acids within the active site doamin which shared 90% consensus with the other sequences. Xylanase from *G. thermoleovorans* shared highest homology with *Geobacillus* sp. TC-W7 xylanase having E187 and E239 as catalytically important amino acids (Verma and Satyanarayana, 2012). A GH10 xylanase from *Thermotoga thermarum* had a closer relationship with *P. mobilis* and contained E525 and E726 which were more conserved among the diverse members of GH10 xylanase (Shi *et al.*, 2013). *Caldicellulosiruptor lactoaceticus* xylanase possessed E161 and E266 as the conserved amino acids (Jia *et al.*, 2014). The deduced sequence of xylanase from *Streptomyces thermovulgaris* TISTR1948 contained one putative conserved GH 10 domain (from Y71 to V359) and the putative catalytic residues of E169, D212, and E277 showed high identity with *S. lividans* 1326 (Boonchuay *et al.*, 2016).

In the case of GH11 xylanase, a total of 50 sequences were retrieved from the CAZy database for the GH11 family, having sequence similarity with the reference sequence, *T. lanuginosus*. The cladogram represented three main clusters, where the reference sequence belonged to cluster 1. All the three clusters were sharing a common ancestor (root), however, the cluster 1, 2 and 3 in Figure 3.14 refers to a minimum amount of sequential or structural similarity among the three clusters.

The *T. lanuginosus* xylanase was most closely related to *T. dupontii* (AGI02590) and *Paecilomyces thermophila* (ACS26244) (Figure 3.14). The overall clade formation and branching in the phylogenetic tree elucidated the relationship of *T. lanuginosus* xylanase to the other xylanases from the GH11 family (Álvarez-Cervantes *et al.*, 2016). From the multiple sequence analysis, it was observed that *T. lanuginosus* shares conserved amino acids with other input sequences as well.

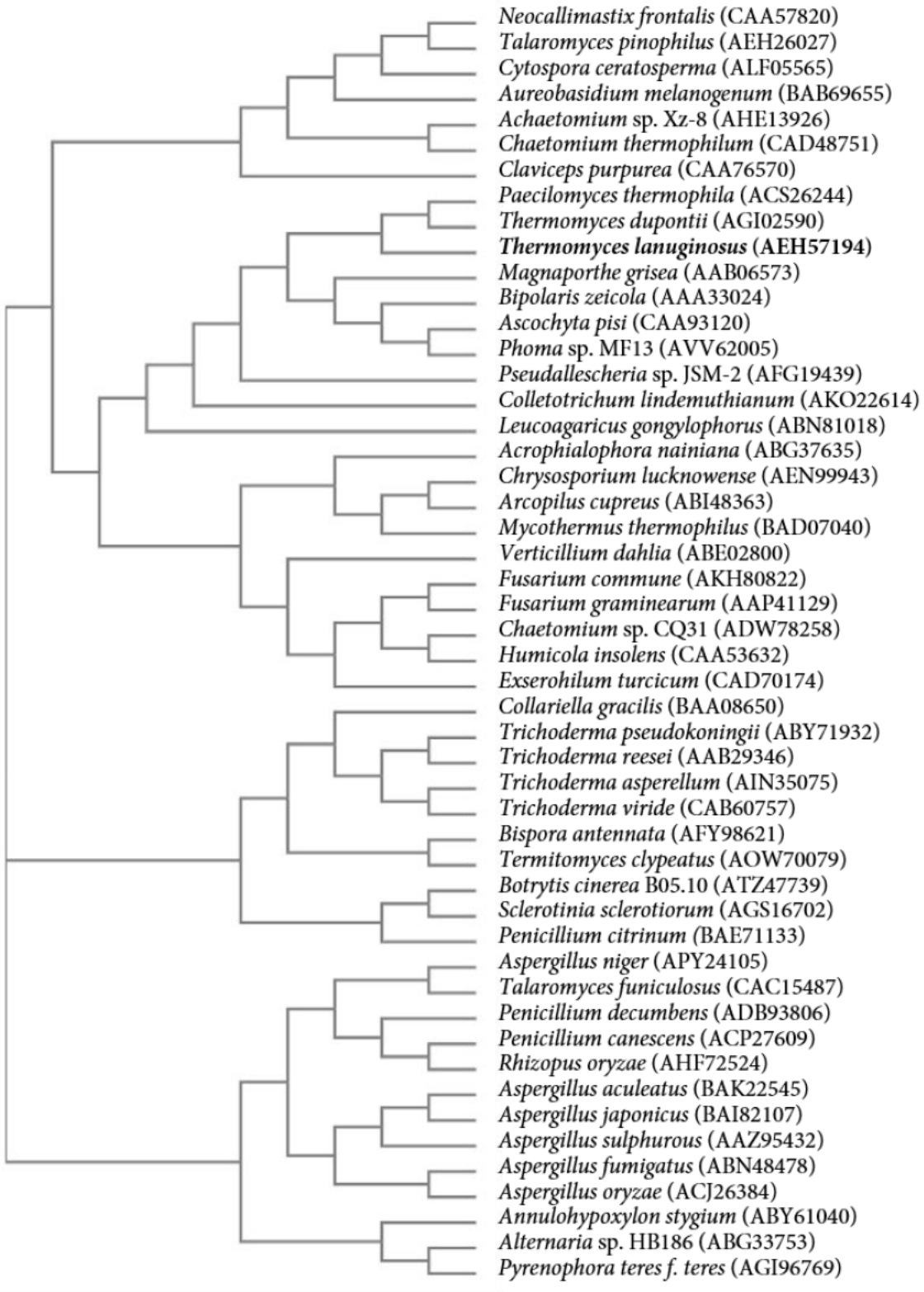

Figure 3.14 Phylogenetic analysis of the amino acid sequences of xylanases from GH11 family. *T. lanuginosus* is highlighted with bold letters.

Similarly, G118, Y180, G181, W182, P186, E189, Y191, P202, G214, Y224, S236, G239, F244, Q246, R251, W274, G278 and G305 showed 100% consensus with other input sequences. A GH11 xylanase from *Thermobacillus xylanilyticus* contained eight aromatic residues namely Y6, Y13, Y42, Y97, W102, W109, Y172, and Y176 and two conserved residues W4 and W161 (Rakotoarivonina *et al.*, 2015).

Twenty protein sequences of GH30 xylanase similar to *E. chrysanthemi* xylanase were selected. The sequence length of the *E. chrysanthemi* was approximately 440 amino acids. From the cladogram of *E. chrysanthemi*, the closest neighbour was *Dickeya dadantii* (AIU97348) as they shared a common origin of divergence. The clade containing *E. chrysanthemi* represented 13 sequences which showed the proximity of the sequences (Figure 3.15). Multiple sequence analyses showed a conserved area for the sequences of GH30 family xylanases and the residues which were in the region of the active site was selected for the docking studies. The maximum sequence similarly is often associated with structural similarity while multiple sequence alignment identifies the conserved region in such similar sequences. Multiple sequence alignment results showed that residues R142, L143, A150, L155, D156, F157, M162, N165 and Y170 were within the active site domain having 100% consensus with other protein sequences. In a previous study, the GH30 xylanase from *Penicillium purogenum* showed a maximum of 53% similarity with *Bispora* sp. xylanase and multiple sequence alignment identified the catalytic residues at E209 and E301 (Espinoza and Eyzaguirre, 2018).

The phylogenetic analysis assisted the identification of the ancestral roots of xylanase from all three GH families and they were further aligned with the sequences that showed the maximum similarity among xylanases from other organisms. By using these observations, docking analysis was carried out where active sites serve as the most important factor. Sequence analysis assisted in the identification of the conserved regions which may act as active sites in the docking study.

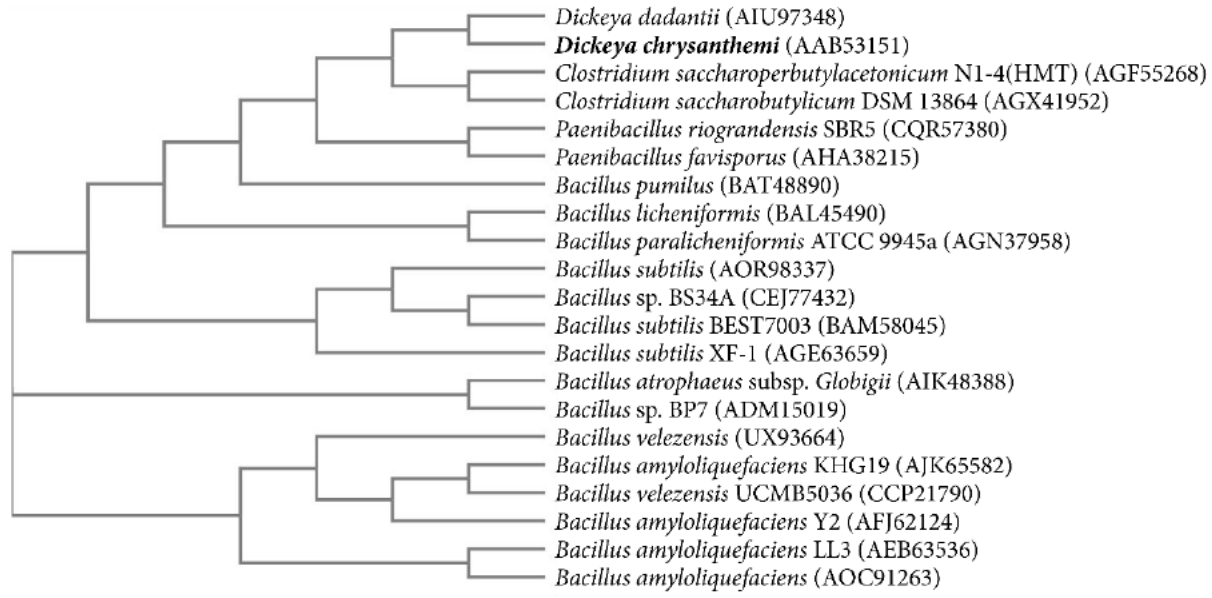

Figure 3.15 Phylogenetic analysis of the amino acid sequences xylanases from GH30 family. *E. chrysanthemi* is represented as *Dickeya chrysanthemi* in bold letters.

3.3.7 Docking studies on xylan and xylanase

Modeller software generated five models for each xylanase sequence from *C. mixtus, T. lanuginosus* and *E. chrysanthemi* (Table 3.1). The best template with percentage sequence similarity and DOPE score values for all the models represented in Table 3.1. The models with a lowest DOPE score (with a negative value) −55808.89 (model 2 of *C. mixtus*), −22966.22 (model 1 of *T. lanuginosus*) and −48628.11 (model 1 of *E. chrysanthemi*) (Figure 3.16) were selected for further analysis. The selection was carried out based on the previous report (Roy and Mukherjee, 2015).

Table 3.1 Sequence and structural information of xylanase from different organisms. DOPE score for all five models are provided along with the lowest score in bold letters using Modeller 9.20. Verification results of the models with the lowest DOPE scores were presented as Verfy3D and Z score.

Name of organism	Sequence length (amino acid)	Template with %similarity	DOPE score	Verify 3D score	ProsA value (Z score)
C. mixtus	379	1UQZ, 83%	−55690.26 **−55808.89** −54721.53 −55075.13 −55028.68	81.13%	5.99
T. lanuginosus	258	1YNA, 99.7%	**−22966.22** −22363.17 −22792.88 −22473.98 −22669.17	85.58%	5.39
E. chrysanthemi	413	1NOF, 99%	**−48628.11** −48144.49 −47930.91 −48123.65 −48035.16	92.01%	8.31

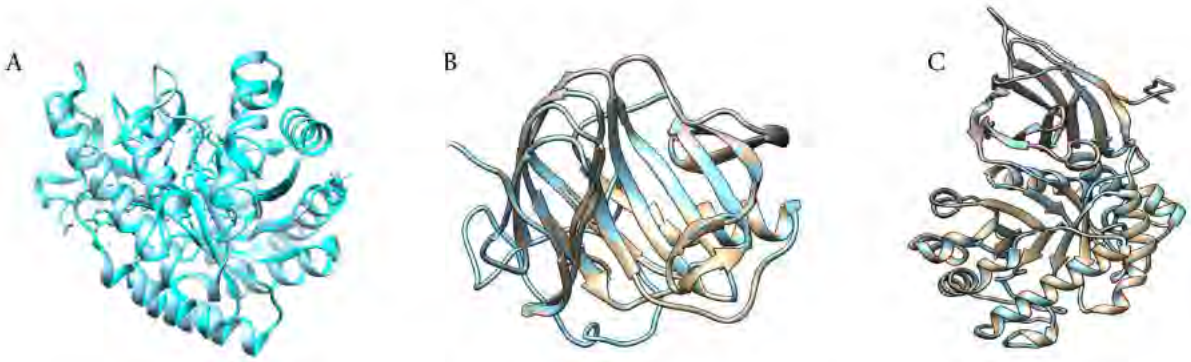

Figure 3.16 Stereo view of the modelled structure of xylanase from (A) *C. mixtus*, (B) *T. lanuginosus* and (C) *E. chrysanthemi*. Cyan and grey colour represent the modelled and template structures.

Ramachandran plot (Figure 3.17) confirmed that the models were stereo-chemically stable (Laskowski *et al.*, 1993) as a high percentage residues were in the favoured region 91.9% (*C. mixtus*), 90.02% (*T. lanuginosus*) and 92% (*E. chrysanthemi*). An endoxylanase from *Coprinus cinereus* (Kumar *et al.*, 2013) and *Aspergillus tamarii* Kita (Heinen *et al.*, 2018) showed 99.4% and 87.9% of residues respectively, in the favoured region. Validation of modelled structures was done by calculating the Verify3D score (81.13%, 85.58% and 92.01% respectively) and Z score (5.99, 5.39 and 8.31 respectively) (Table 3.1). Estimation of the environment of each residue in the model with respect to the expected environment in the high-resolution X-ray structures carried out by Verify3D analysis (Pawlowski *et al.*, 2008). Z-score represented the absolute quality of a model by providing an estimate of the 'degree of nativeness' of the structural features and its likelihood (Benkert *et al.*, 2011). Verify3D and Z scores confirmed that the 3D structure of all the three proteins were of good quality. The 3D structure of *C. cinereus* demonstrated 66% identity and a Z score value of 35.2 with template 1XNK-A (Kumar *et al.*, 2013). The deviation studies between two structures helps in deducing the similarities and dissimilarities of protein folds, therefore root-mean-square deviation (RMSD) was also done for the modelled structures and their best templates by Chimera software. Super-imposed structures with RMSD values of 0.704 Å (*C. mixtus*), 0.092 Å (*T. lanuginosus*) and 0.166 Å (*E. chrysanthemi*) indicated a lower deviation between the structures (Figure 3.16).

Generally, the lower RMSD values result in the enhancement of thermostability of the enzyme (Xie *et al.*, 2018), which was confirmed in case of *T. lanuginosus* xylanase which possessed the lowest score among the other enzymes used.

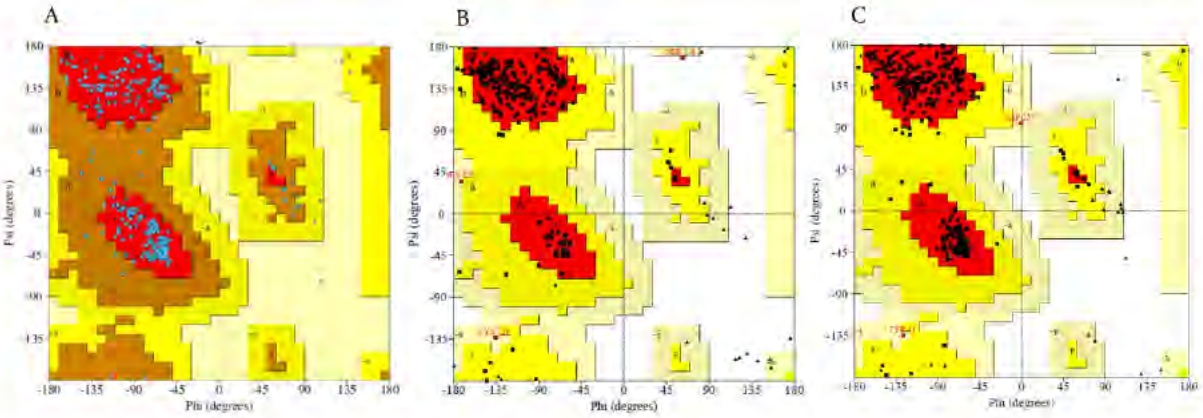

Figure 3.17 Ramachandran plot of xylanase generated from Procheck server (A) *C. mixtus*, (B) *T. lanuginosus* and (C) *E. chrysanthemi*. Percentage of residues falling in the favoured region for three organisms is 90.02%, 91.9% and 92% respectively.

Docking studies of xylanase from three GH families with aldopentaouronic acid were done which unravelled the best-docked pose (a candidate binding mode) of the strongest complex to check the structural characteristics. Aldopentaouronic acid was chosen as a substrate since it is one of the simple ligands that can bind the xylanase. The interaction studies performed in this work supports the current understanding of the nature of xylanase-ligand interactions, and it can, therefore, be used to guide other computationally driven enzyme substrate investigations. Docking result in the form of scoring values indicated that the best complex of aldopentaouronic acid with xylanase was *T. lanuginosus*, since it had the lowest binding affinity of −10.24 kcal/mol (Pose 2).

The docked structure of *T. lanuginosus* (Pose 2) was viewed using PyMOL plugin to understand the binding. *T. lanuginosus* xylanase had a beta-barrel type super-secondary structure that acts as a nice cleft for the aldopentaouronic acid (Figures 3.18A and B). It is also apparent that neighbouring residues such as valine, lysine, glycine, tyrosine, asparagine,

tryptophan and isoleucine were making bonds with hydrogen and hydroxyl groups of aldopentaouronic acid (Figure 3.18C).

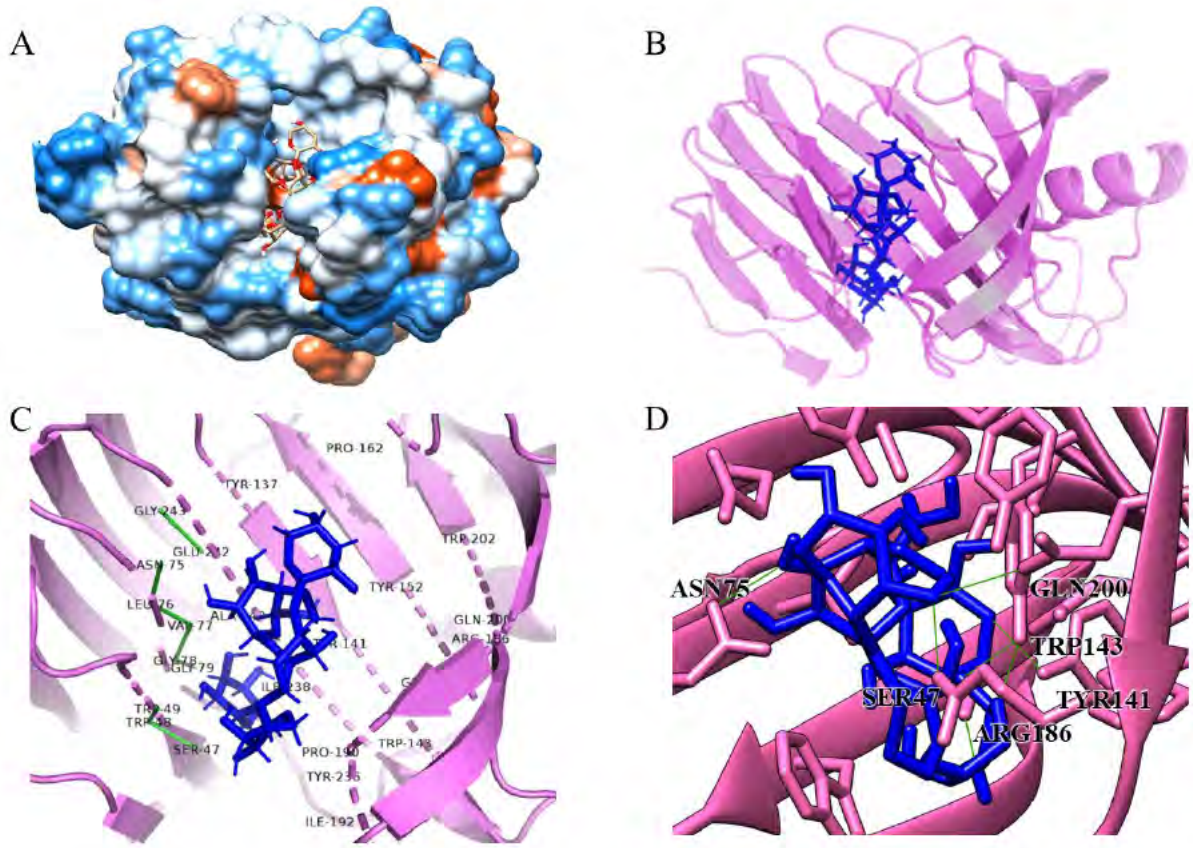

Figure 3.18 Schematic illustration of the binding of *Tl*GH11 xylanase (*T. lanuginosus*) and aldopentaouronic acid MeGlcA^{3}Xyl$_{4}$. (A) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (B) Cartoon view of the complex; (C) Three letter code of the residues of the enzyme interacting with the ligand (D) Inter-molecular H-bonds are represented by green bonds. In (B) and (D) the ligand is shown in blue sticks and the enzyme in pink colour.

Table 3.2 Description of Docking input and output results. Pose and scoring values are for the best docking regions (active sites) which are highlighted in Bold letters.

Enzyme	Receptor	Ligand	Active Sites[a]	Pose	Scoring values[b] (kcal/mol)
*Cm*GH10				1	−9.40
				2	−9.53
	Xylanase-model2.pdbqt (GH10)	Xylan.pdbqt	165–200, **309–319**	3	−9.38
				4	−9.09
				5	−8.80
				6	−8.44
*Tl*GH11				1	−9.0
				2	−10.24
	Xylanase-model1.pdbqt (GH11)	Xylan.pdbqt	**147–157**, 239–250	3	−7.97
				4	−8.49
				5	−8.89
				6	−9.30
*Ec*GH30				1	−9.93
				2	−8.85
	Xylanase-model1.pdbqt (GH30)	Xylan.pdbqt	**145–168,** 330–350	3	−8.56
				4	−7.24
				5	−8.44
				6	−8.01

Tyrosine and tryptophan have aromatic side chains whereas lysine and asparagine encountered as positively charged amino acids which represent the strength of binding and affinity between the xylanase- aldopentaouronic acid complex. Multiple binding poses of the complex structure were created to identify the key residues responsible for ligand (aldopentaouronic acid) recognition in the binding site. Scoring values of similar poses suggested that the xylan opted for its best binding mode inside the cleft of xylanase. Seven H-bonds were formed between aldopentaouronic acid with the following amino acids Ser47, Asn75, Tyr141, Tyr141, Arg186, Gln200 and Tyr236 (Figure 3.18D). The docking energy (−10.24 kcal/mol) and the number of H-bonds formed clearly indicated a good complex formation (Manimekalai *et al.*, 2015; Sarkar *et al.*, 2018) between the receptor (xylanase) and ligand molecule (aldopentaouronic acid).

The other two xylanase-ligand complex structures derived from *C. mixtus* and *E. chrysanthemi* were also studied. It was observed that Pose 2 with −9.53 kcal/mol (*C. mixtus)* and Pose 1 with −9.93 kcal/mol (*E. chrysanthemi)* had the highest binding affinity (Table 3.2) and their docked structures shown in Figures 3.19A and 3.20A. Interacting amino acids, H bond and bond formation were different for individual complexes. Xylanase of *C. mixtus* developed interactions for the neighbouring amino acids Asn264, Trp265, Thr266, Asp356, Ser357, Pro404 and Leu405 (Figure 3.19C). Likewise, *E. chrysanthemi* xylanase formed interactions between Asn264, Trp265, Thr266, Ile269, Asn355, Asp356, Ala380, Pro404 and Leu405 (Figure 3.20C). Thr266 participated in one of the H bond formations whereas the other bond was formed with a bond length of 2.9 Å. Docking was done in this study to ensure the docking nature of aldopentaouronic acid to its receptor (xylanase) that could endure large scale processing during product (xylose) formation. A comparison of molecular dynamics and changes observed between wild type and mutated genes from *T. lanuginosus* DSM 5826 produced xylanase variants after DNA shuffling (Stephens *et al.*, 2014).

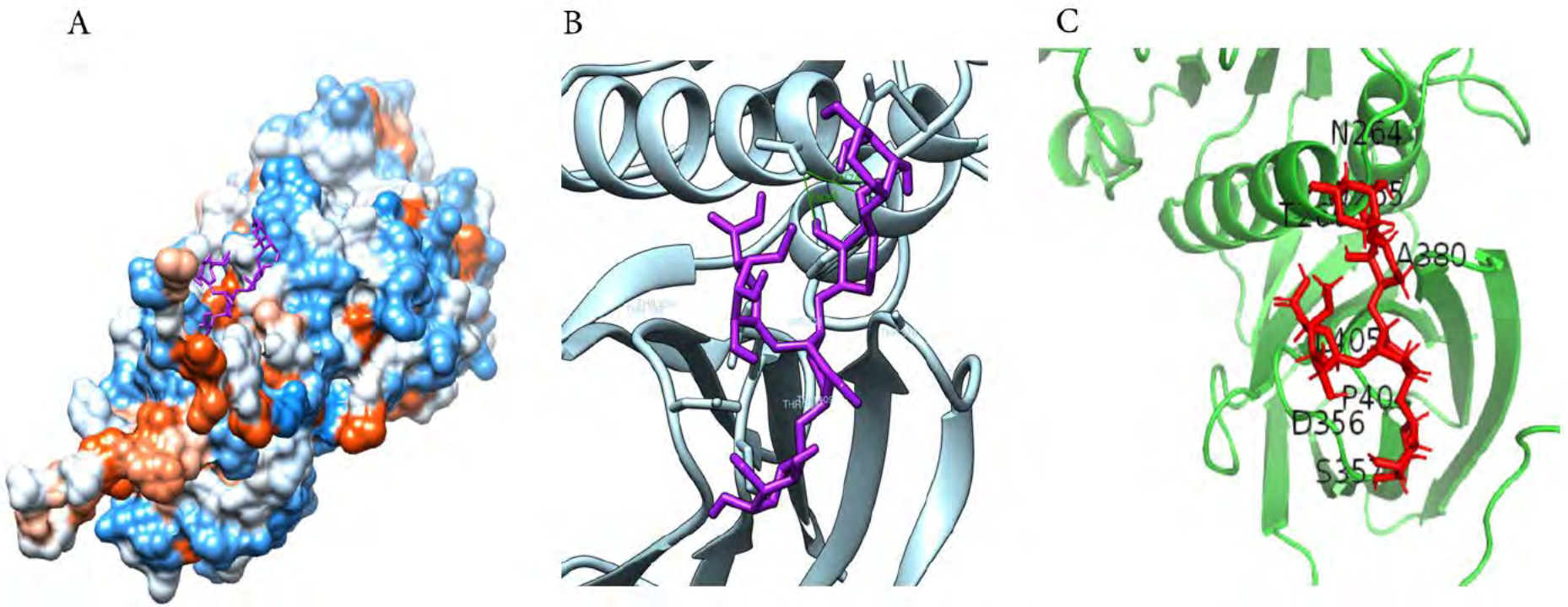

Figure 3.19 Schematic illustration of complex structure of *Cm*GH10 xylanase (*C. mixtus*) and aldopentaouronic acid. (A) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (B) Cartoon view of xylanase (grey) and aldopentaouronic acid (purple) (C) Three letter code of the residues of the enzyme interacting with the ligand.

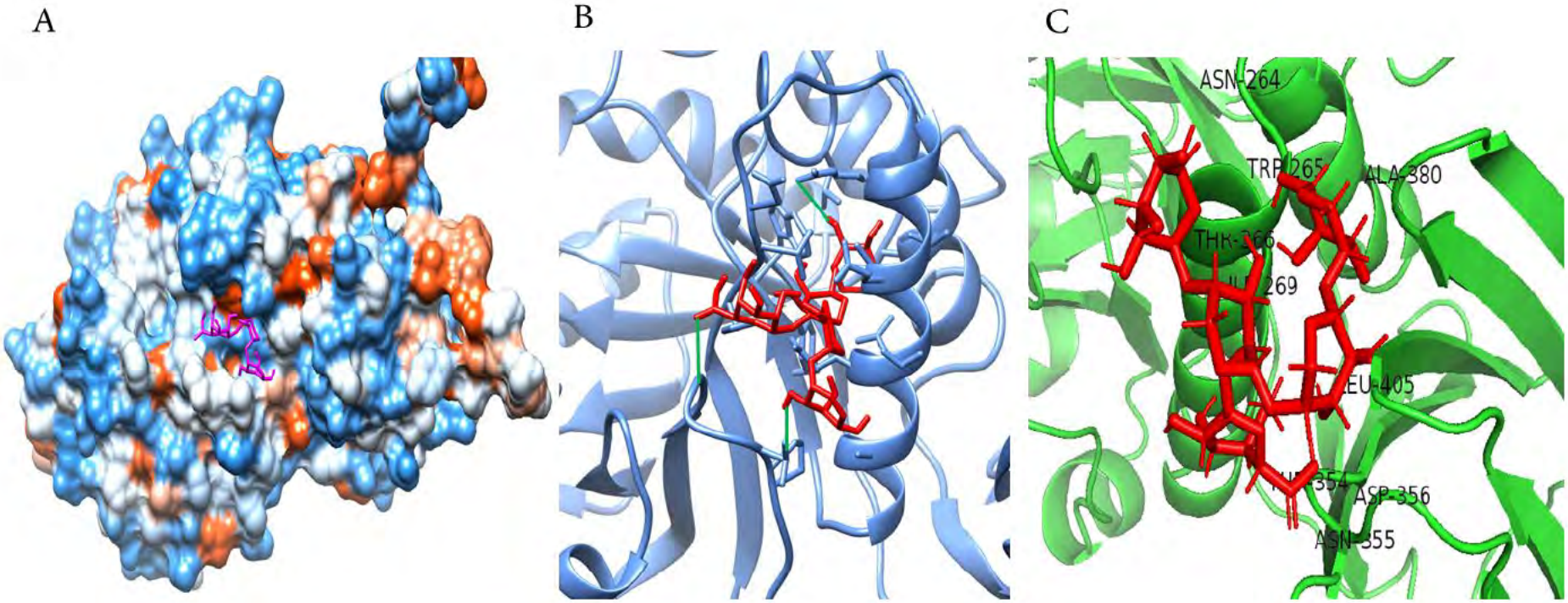

Figure 3.20 Schematic illustration of complex structure of *Ec*GH30 xylanase (*E. chrysanthemi*) and aldopentaouronic acid. (A) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (B) Cartoon view of xylanase (blue-grey) and aldopentaouronic acid (red) (C) Three letter code of the residues of the enzyme interacting with the ligand.

3.4 CONCLUSION

In this chapter, the efficiency of xylan hydrolysis by *Tl*GH11 xylanase was improved by the optimization of substrate concentration and enzyme dosage. The analysis of the enzymatic hydrolysates of peanut shells, bambara, cowpea and sorghum biomass showed that xylanases from three GH families had unique mode of action towards the substrate. The shorter oligosaccharides produced from these xylans had a DP of 2–6 which could comply for value addition. TLC results showed a clear hydrolysis pattern of xylanases on all the four xylans. MALDI-TOF MS analysis revealed the identity of XOS produced in the xylan hydrolysates by their molecular masses. It was therefore concluded that XOS produced from all the xylans comprise mainly glucuronoxylan. The *in silico* study summarizes of all the three xylanase sequences showed a good percentage of consensus in multiple sequence analysis thereby forming clusters in the cladogram. This observation helped in unravelling the xylanase protein with maximum sequential and structural similarities which can be applied as a reference model for homology modelling in future. The structure modelling and interaction study by docking also aided in identification the residues, amino acids and active sites in the enzymes that are involved in the breakdown of xylan. In addition, this study also provided the information to prove that *T. lanuginosus* xylanase was the most suitable enzyme for XOS production.

CHAPTER FOUR

APPLICATION OF OLIGOSACCHARIDES AS A PREBIOTIC IN FOOD PRODUCTS

4.1 INTRODUCTION

With increasing consumer's interest in health and wellness, the demand for functional foods has increased in the last decade. This has propelled the growth of functional foods market, subsequently promoting the discovery of new functional food constituents from different natural sources (Gurpilhares *et al.*, 2019). The non-conventional biomolecules present in food which can modulate metabolic pathways in the body, promoting the health and well-being of humans are termed as functional components. Carotenoids, non-starchy polysaccharides, fatty acids, phenolics, plant sterols and prebiotic oligosaccharides are some of the examples of such functional food components (Abuajah *et al.*, 2015). Despite the presence of various dietary supplements, the search for newer sources is desirable to improve the efficiency of functional foods. In recent years, research on prebiotics has gained much interest due to its selectivity in stimulating the growth of beneficial microbes in the gut microbiota that confer health benefits to the host (Singh *et al.*, 2015). Normal gut microbiota play an important role in the metabolism of host nutrients, maintaining the integrity of the gut mucosal barrier, metabolism of drugs and xenobiotic compounds, protection against pathogens and immunomodulation (Jandhyala *et al.*, 2015).

Prebiotics are non-digestible food ingredients classified as dietary fibres which are rare in natural foods but can be synthesized from carbohydrate sources. Milk, honey, banana, onions, garlic, wheat, oats, chicory, leeks, asparagus, Jerusalem artichokes, bamboo shoots and soybeans are some of the natural sources of prebiotics (Petrova and Petrov, 2017). Prebiotics play a vital role in improving the growth of gut microbiota in humans (Goulet *et al.*, 2019). However, the

consumption of prebiotics via natural food products is limited, due to their low concentration. This could be overcome by supplementing prebiotics in the diet to maintain good intestinal health.

Among the different prebiotics, inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and isomaltooligosaccharides are well studied, and have a good share of the commercial market (Brownawell *et al.*, 2012). Xylooligosaccharides are a new class in this cluster and are gaining immense attention due to their specific metabolic function as a prebiotic in the human intestinal system (Dávila *et al.*, 2019). XOS are reported to improve probiotic levels in the intestine at the lowest concentration (Amorim *et al.*, 2019b). XOS dosage of 2 g/day is adequate to improve the intestinal probiotic levels, whereas, oligosaccharides such as GOS and FOS are required in higher concentrations (up to 10 g/day and 4 g/day, respectively) (Mohanty *et al.*, 2018). Several biological functions of XOS in the human body have been studied in the last two decades. It has been noted in a recent study that XOS supplemented soluble fibre diet helped in reducing adiposity and fat synthesis along with improved intestinal microbial count (Long *et al.*, 2019). XOS are also reported to possess antioxidant, antidiabetic, antitumor, anti-inflammatory and immunostimulatory activities (Singh *et al.*, 2015). All these remarkable properties of XOS make them a strong competitor to the oligosaccharides currently available in the commercial market. This has stimulated researchers globally to study newer and novel sources of XOS.

Previous studies have shown that various prebiotics can be produced from different waste materials and agricultural biomass. In South Africa, biomass from several crops are underutilized and still need to be explored for value addition. These crops are not commercialized globally and due to their indigeneity, they are not sufficiently explored. Peanut shell, bambara, cowpea and sorghum biomass are some of the underexplored crop residues in South Africa which have potential to be used as a source for oligosaccharide production. Preliminary studies conducted in our laboratory have shown that all the four biomass have abundant xylan which could be explored for XOS production. Few studies have been reported

on the production of XOS from peanut shells, cowpea and sorghum, however, production of XOS from bambara biomass has never been attempted. On the same note, similar to agricultural biomass, many of the food crops with high nutrition profiles, such as amadumbe and bambara groundnut have not been explored well. Both amadumbe and bambara groundnut are rich in carbohydrates (67% and 80% respectively), while protein (15% and 12% respectively) and fat (10% and 1% respectively) contents are comparatively low (Mawoyo *et al.*, 2017; Oyeyinka and Oyeyinka, 2018).

In recent times, the demand for fat-based spreads has declined considerably due to the fact that fat has being linked to obesity-related diseases. However, carbohydrate-based low-fat spreads are gaining wide attention due to their health benefits (Do *et al.*, 2016). Amadumbe and bambara groundnut flours have great demand in the spread industry due to their high carbohydrate and low-fat content (Mukurumbira, 2017; Oyeyinka, 2017). Yogurt is another important food ingredient included in almost all diets. It is a probiotic rich food that improves the commensal bacteria of the gut (Pei *et al.*, 2017).

In this chapter, XOS produced from peanut shells, bambara, cowpea and sorghum biomass were evaluated for their potential as prebiotics via *in vitro* fermentation. A novel spread formulation was developed using amadumbe and bambara groundnut flours and prebiotic supplementation of bambara XOS in spreads and yogurt was studied.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of probiotic bacteria

Probiotic bacteria were isolated from fermented Mahewu drink and yogurt samples collected from the local market, Durban, South Africa. Probiotic bacteria were screened and isolated using MRS agar plates and incubated anaerobically at 37°C for 48 h. For the isolation of *Bifidobacteria*, cysteine hydrochloride was used as a selective agent. The Gram-positive and catalase-negative bacteria were further identified by ribotyping.

4.2.2 Identification and phylogenetic analysis of the isolated bacteria

The isolated probiotic microorganisms were identified by a ribotyping method involving 16S rDNA sequencing at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. DNA was extracted from the bacterial cultures using the ZR Fungal/Bacterial DNA Kit (Zymo Research, USA), and the 16S rDNA gene was amplified by polymerase chain reaction using the primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The PCR reactions were performed in a final volume of 25 µl having approximately 1.0 µl of DNA, 12.5 µl of OneTaq Quick-Load 2X Master Mix (NEB, Catalogue No. M0486), 0.5 µl of each primer and 10.5 µl of sterile nuclease-free water. The DNA amplification conditions were: initial denaturation at 94°C for 30 s, 30 cycles of 94°C for 15 s, 45°C for 15 s 68°C for 1 min, and a final extension step at 68°C for 5 min. PCR products were gel extracted (Zymo Research, Zymoclean Gel DNA Recovery Kit, D4001), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser (Applied Biosystems Inc., Foster City, CA, USA). Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit, D4050) were analyzed using CLC Main Workbench 7 followed by a BLAST search (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). The resultant homologous sequences of other species from BLAST tool were used for phylogenetic analysis. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and were in the units of the number of base substitutions per site. Codon positions included were of 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted using MEGAX software (Kumar *et al.*, 2018).

4.2.3 Measurement of bacterial growth on XOS

Three selected bacterial cultures, *viz.*, *Lactobacillus plantarum* NS6, *Lactococcus lactis* NS9 and *Bifidobacterium animalis* NS11 were used for the prebiotic study. *In vitro* fermentation of XOS by lactic acid bacteria and *Bifidobacterium* strains was studied by supplementing XOS as the carbon source in the culture media. The modified MRS media contained 10.0 g/l protease peptone, 10.0 g/l beef extract, 5.0 g/l yeast extract, 1.0 g/l Tween 80, 2.0 g/l ammonium citrate, 5.0 g/l sodium acetate, 0.1 g/l magnesium sulfate, 0.05 g/l manganese sulfate, 2.0 g/l dipotassium sulfate, 0.05 g/l cysteine hydrochloride (before inoculation in the case of *Bifidobacterium* strain) and 1.0% (w/v) XOS (Chapla *et al.*, 2012). XOS derived by the enzymatic hydrolysis of xylan from peanut shell, bambara, cowpea and sorghum biomass were tested in the *in vitro* fermentation experiments.

The medium containing XOS was inoculated with 5% (v/v) *L. plantarum, L. lactis* and *B. animalis* in separate screw cap bottles and incubated anaerobically at 37°C for 48 h. The growth of bacteria and the utilization of XOS were monitored at 24 and 48 h by measuring the pH and absorbance of the culture broth. Positive control fermentations were carried out by substituting XOS with glucose in the media and uninoculated media served as the blank. For negative controls, media without a carbon source was used for fermentation. The culture broths were centrifuged at 5000 x g for 15 min and the cell pellet was washed with sterile distilled water and oven-dried at 85°C to determine the dry cell mass (Chapla *et al.*, 2012).

4.2.4 Development of prebiotic functional foods

4.2.4.1 Preparation of amadumbe and bambara groundnut flour

Amadumbe and bambara groundnut were collected from local farmers in the KwaZulu Natal province, South Africa. Freshly harvested amadumbe corms were washed, peeled, sliced into a thickness of 3.0 mm and dried at 60°C in an air dryer (United Scientific, South Africa) until a constant weight was obtained. Dried flakes were milled into flour using a grinder (Model:

8010S, Torrington, USA) and sieved (screen size: 180 mm) to obtain fine flour. The flour was then mixed with water and gelatinized by heating to modify the starch. The gelatinized starch was dried using a drum dryer, powdered and stored at room temperature prior to analysis. In the case of bambara, the groundnuts were roasted in an oven, dehulled and ground using a grinder. The fine flour was collected by sieving (180 mm) and stored in an airtight container.

4.2.4.2 Development of amadumbe-bambara spread

A new product (amadumbe-bambara spread) was formulated with amadumbe and bambara flour as the base material, using a modified method from Do *et al.* (2016). The ground bambara flour (70%) was mixed with 30% gelatinized amadumbe flour to serve as the basic ingredient for the spread preparation. Other ingredients in the formulation included vegetable oil, lecithin, salt and sugar. XOS (2.5%) was added as a dietary supplement. The XOS dosage was finalized based on earlier reports (Al-Sheraji *et al.*, 2013; Mohanty *et al.*, 2018). All the ingredients except flour were mixed with hot water and homogenized. The flour was added slowly to the mixture and blended thoroughly to form a semi-solid paste.

4.2.4.3 Texture analysis of spread samples

Petri dishes (100 mm x 15 mm) were filled with the spread and levelled for texture analysis. The spread sample was divided into 4 quadrants and the texture analysis was carried out on each portion. A texture analyzer (Shimadzu Corporation, Japan) equipped with a load cell of 30 kg was used to obtain the force-time curves. A 2.5 cm diameter acrylic cylindrical probe was pushed into the spread up to 4 mm at a speed of 0.5 mm/s and was then retracted until the sample column completely broke from the probe. All measurements were performed at ambient temperature (Tanti *et al.*, 2016).

4.2.4.4 Accelerated stability tests

The stability of the formulated samples was evaluated by measuring water (and/or oil) separation after centrifugation. A known amount of the spread sample (1.0 g) was added to

2.0 ml Eppendorf tubes and centrifuged at 12000 x g for 10 min. Water (and/or oil) syneresis was measured as the percentage of the amount of free water (or oil) separated to the total amount of sample (Do *et al.*, 2016).

4.2.4.5 Nutritional analysis of the spread samples

The total fat, protein, ash and moisture content of the spread samples were measured by AOAC methods (AOAC, 2016). The samples were freeze-dried prior to analysis and the total carbohydrates were estimated by calculating the difference in protein, lipid, fibre and ash in the total composition.

4.2.4.6 Sensory analysis of the spread samples

Sensory evaluation was performed to assess the consumer acceptability of the newly formulated spread samples. The following five parameters were assessed: appearance, aroma, taste, texture and overall acceptability. The analysis was done by 30 untrained panel members who were randomly selected from the postgraduate students of the Department of Biotechnology and Food Technology, Durban University of Technology. The samples were evaluated based on a 9-point hedonic scale (Pimentel *et al.*, 2015). The tests were conducted in separate cabins with coded samples.

4.2.4.7 Preparation of XOS enriched yogurt

To prepare the prebiotic yogurt, 1% skim milk powder and 0.5% gelatin were added to fresh milk, heated at 65°C for 15 min and cooled to 46–48°C. Yogurt starter culture (1.0%) and XOS (2.5%) were added to the milk and were incubated at ambient temperature. The pH of the sample was monitored every 1 h until it dropped to 4.5 where the fermentation was arrested. A control yogurt sample was prepared without the addition of XOS and both the samples were refrigerated for further analysis (Mousavi *et al.*, 2019).

4.2.4.8 Physicochemical analysis of yogurt samples

pH and titratable acidity of the prebiotic yogurt was determined by the AOAC method (AOAC, 2016) and viscosity was measured using a Haake Visoctester C (Thermo Electron GmbH, Karlsruhe, Germany). Syneresis of the yogurt was determined by centrifugation according to Aryana (2003).

4.2.4.9 Microbial analysis of yogurt samples

The viability of probiotic organisms during the storage period was assessed by microbial counting. The yogurt samples were stored at 4°C and samples were collected weekly for 4 weeks. The samples were then serially diluted, inoculated on MRS plates and incubated at 37°C for 48 h. After incubation, the microbial content was estimated.

4.2.4.10 Nutritional and sensory analysis of yogurt samples

Nutritional and sensory analysis of plain and XOS enriched yogurt was carried out by the methods mentioned in section 4.2.3.4 and 4.2.3.5.

4.2.5 Statistical analysis

Data obtained from the above experiments were analyzed using the SPSS software (IBM corporations, USA). Means and standard deviations were calculated; analysis of variance and Duncan test were used to detect significant differences ($\alpha = 0.05$) among the various data analyzed.

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation and identification of probiotic bacteria

Forty bacterial colonies were isolated from 8 Mahewu samples on MRS agar plates and 10 bacteria were isolated from 2 yogurt samples on MRS media supplemented with cysteine

hydrochloride, used for the selection of *Bifidobacteria*. Bacterial colonies obtained from Mahewu samples were mostly white cream coloured while colonies of the bacteria isolated from yogurt samples were white coloured. Among these, 12 colonies that have distinctive morphological features were further screened and identified. Gram staining confirmed that all the bacteria were gram-positive among which 10 were rod-shaped and the remaining 2 were cocci shaped. All the 12 colonies were negative for catalase which confirmed both the strict and facultative anaerobic nature of the bacteria, which is a characteristic feature of probiotic microorganisms. Ribotyping is a widely accepted method to identify the bacteria and its phylogenetic analysis. The 16S rDNA sequencing mediated identification showed that majority of the isolates (8 isolates) obtained from Mahewu were *Lactobacillus plantarum*, and 2 isolates were identified as *Lactococcus lactis*, while the 2 bacterial colonies isolated from yogurt were identified as *Bifidobacterium animalis*. All the isolates selected belonged to the probiotic community.

Probiotic bacteria have been isolated from many sources. In contrast to the natural sources, fermented products have been observed as one of the best sources of probiotic bacteria. Probiotic bacteria such as *L. plantarum* and *L. lactis* have been isolated from fruits and cattle milk, respectively (da Costa *et al.*, 2018; Yerlikaya, 2019). Natural sources do have large number of microorganisms other than probiotics, whereas, the microbial composition of fermented products is dominated by probiotic microorganisms. Many of the earlier studies have reported the isolation of potent probiotics from fermented foods. *L. plantarum* and *L. lactis* were isolated from fermented porridge "Mahewu" (Pswarayi and Gänzle, 2019); *L. plantarum* from yogurt (Nami *et al.*, 2019) and *B. animalis* from dairy products (Alhudhud *et al.*, 2014). Our results are in agreement with the above reports.

4.3.2 Phylogenetic analysis of probiotic microorganisms

The phylogenetic tree of the 16S rDNA sequences of bacteria was constructed using MEGA-X software by the neighbor-joining method (Figure 4.1, 4.2 and 4.3) and showed the detailed

evolutionary relationships between the strains under study, and other closely related species. Three bacteria from different genus were selected for phylogenetic analysis viz., *L. plantarum* NS6, *L. lactis* NS9 and *B. animalis* NS11 and their evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree obtained from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and were in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences which were carried out on the selected bacteria. All ambiguous positions were removed for each sequence pair using the pairwise deletion option. For *L. plantarum* NS6 and *L. lactis* NS9, a total of 1563 and 1536 positions were in the final dataset with the optimal tree having total branch lengths of 31.86 and 12.36, respectively. In the case of *B. animalis* NS11, there were a total of 1549 positions in the final data set which showed the optimal tree with 72.28 as the sum of the branch length. The 16S rDNA based phylogenetic analysis demonstrated 99.66% sequence similarity of *L. plantarum* NS6 with closely related *Lactobacillus plantarum* WGX143, whereas, *L. lactis* NS9 and *B. animalis* NS11 showed 99.93% sequence similarity with *Lactococcus lactis* LLY003 and *Bifidobacterium animalis* FC13646, respectively.

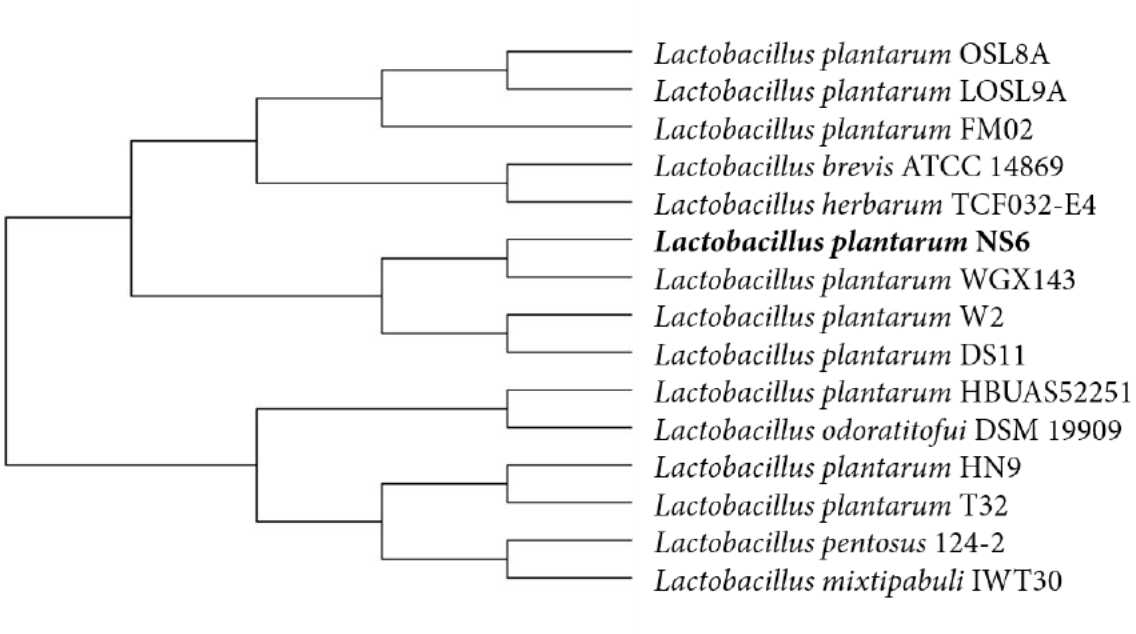

Figure 4.1 Phylogenetic tree of *L. plantarum* NS6 and other closely related *Lactobacillus* species based on 16S rRNA sequences. The tree was generated using the neighbour-joining method.

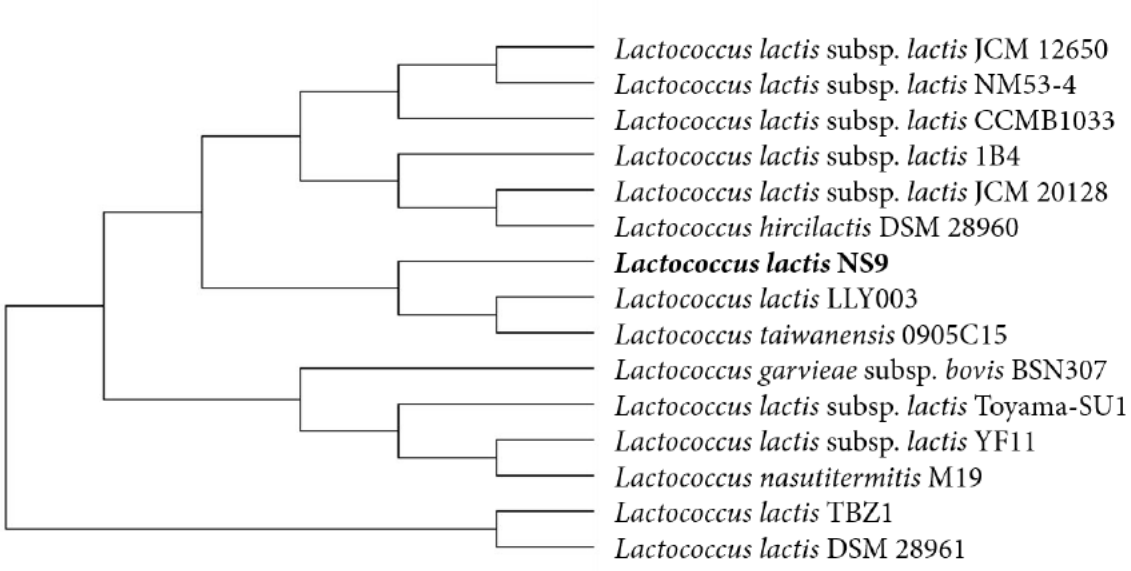

Figure 4.2 Phylogenetic tree of *L. lactis* NS9 and other closely related *Lactococcus* species based on 16S rRNA sequences. The tree was generated using the neighbour-joining method.

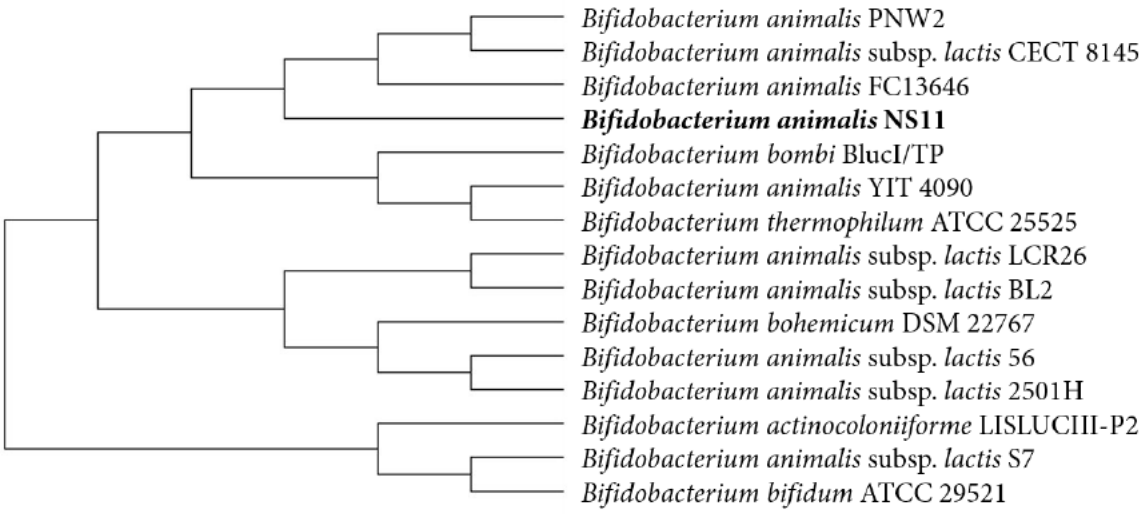

Figure 4.3 Phylogenetic tree of *B. animalis* NS11 and other closely related *Bifidobacterium* species based on 16S rRNA sequences. The tree was generated using the neighbour-joining method.

4.3.3 Measurement of bacterial growth on XOS derived from lignocellulosic materials

The prebiotic potential of XOS was determined by growing the probiotic organisms on XOS supplemented media. Some of the well-established genera of probiotics include *Bifidobacterium*, *Lactobacillus* and *Lactococcus* which have a pivotal role in improving human health (de Melo Pereira *et al.*, 2018). The modified MRS media supplemented with XOS from peanut shells, bambara, cowpea and sorghum xylans were inoculated with *L. plantarum* NS6, *L. lactis* NS9 and *B. animalis* NS11. pH, optical density, and dry cell mass were monitored to determine the prebiotic potential of XOS.

All the three probiotic bacteria were able to utilize XOS from peanut shells, bambara, cowpea and sorghum biomass. This was evident from the increase in absorbance and biomass after 48 h incubation (Table 4.1). XOS were able to enhance the growth of all the three probiotic bacteria which was substantiated by the acidic conditions in the growth medium. In all the XOS supplemented media, the pH decreased to 4.1, whereas the control medium with glucose had a

pH of 5.3 to 5.4. It was noted that the substrates having a higher cell biomass had lower pH values compared to substrates with low cell growth. This indicates that the decrease in pH is directly proportional to the growth of probiotic bacteria (Sims *et al.*, 2014). The hydrolytic enzymes produced by the probiotic microorganisms help in the digestion of non-digestible oligosaccharides (NDOs) and produce short-chain fatty acids (SCFA) resulting in the reduction of pH (Ho *et al.*, 2018). That could be one of the reasons for the reduction of pH which was observed in all the media supplemented with XOS in our study. A recent report has showed a significant drop in pH after supplementing XOS from beechwood to faecal inoculum as a result of the production of short-chain fatty acids (Amorim *et al.*, 2019a).

The maximum cell biomass of *L. plantarum* NS6 was observed in the media containing peanut shell XOS (0.79±0.03 mg/ml) followed by cowpea XOS (0.72±0.04 mg/ml) and bambara (0.71±0.02 mg/ml). In the case of *L. lactis* NS9, the growth on all the four XOS media showed a similar trend (~0.65±0.03 to 0.69±0.04 mg/ml). The highest growth (0.82±0.01 mg/ml) of *B. animalis* NS11 was observed with the media containing XOS from peanut shell (Table 4.1). The purity of the XOS produced from peanut shell could be the reason for the maximum growth of the probiotics compared to other XOS from other sources (MALDI-TOF MS data; Figure 3.6).

XOS obtained from all the four xylans (peanut shells, bambara, cowpea and sorghum) were utilized efficiently by *B. animalis* NS11, *L. plantarum* NS6 and *L. lactis* NS9 than the control media which had glucose as the main carbon source. In the presence of glucose, cell biomass was 0.47±0.02 mg/ml for *B. animalis* NS11, 0.49±0.03 mg/ml for *L. plantarum* NS6 and 0.60±0.01 mg/ml for *L. lactis* NS9 after 48 h of incubation. This could be due to the efficiency of some probiotic bacteria in utilizing pentose sugars over hexoses (Finegold *et al.*, 2014). Therefore, it can be concluded that XOS could be a better carbon source for growth of probiotics than glucose.

Table 4.1 Growth characteristics of *L. plantarum* NS6, *L. lactis* NS9 and *B. animalis* NS11 on XOS from different sources and glucose.

Bacterial strain	Substrates*	24 h			48 h		
		Absorbance (600 nm)	Dry cell mass (mg/ml)	pH	Absorbance (600 nm)	Dry cell mass (mg/ml)	pH
L. plantarum NS6	PS XOS	0.45±0.02	0.42±0.01	5.3	0.62±0.02	0.79±0.03	4.2
	Bam XOS	0.39±0.01	0.40±0.02	5.2	0.56±0.01	0.71±0.02	4.6
	CP XOS	0.41±0.01	0.45±0.02	5.4	0.63±0.03	0.72±0.04	4.4
	SS XOS	0.45±0.02	0.50±0.04	5.3	0.62±0.02	0.68±0.03	4.6
	Glu	0.31±0.02	0.32±0.01	5.6	0.52±0.02	0.49±0.03	5.3
L. lactis NS9	PS XOS	0.39±0.03	0.40±0.01	5.6	0.68±0.02	0.66±0.04	4.3
	Bam XOS	0.35±0.02	0.41±0.01	5.5	0.67±0.03	0.65±0.03	4.2
	CP XOS	0.39±0.02	0.46±0.03	5.5	0.68±0.03	0.66±0.02	4.6
	SS XOS	0.39±0.03	0.43±0.01	5.1	0.67±0.02	0.69±0.04	4.2
	Glu	0.31±0.03	0.35±0.02	5.8	0.59±0.02	0.53±0.01	5.4
B. animalis NS11	PS XOS	0.55±0.03	0.41±0.02	5.4	0.81±0.03	0.82±0.01	4.2
	Bam XOS	0.47±0.01	0.39±0.01	5.6	0.71±0.02	0.66±0.02	4.4
	CP XOS	0.51±0.03	0.32±0.01	5.2	0.72±0.04	0.63±0.03	4.3
	SS XOS	0.50±0.02	0.36±0.03	5.7	0.69±0.04	0.67±0.03	4.1
	Glu	0.34±0.01	0.29±0.02	5.7	0.55±0.03	0.47±0.02	5.3

NB: Value represent the mean of three replicates and the standard error is reported.

*PS (peanut shell), Bam (Bambara), CP (Cowpea), SS (Sorghum) and Glu (Glucose).

Many Bifidobacteria and lactic acid bacteria were shown to catabolize a variety of mono and oligosaccharides released by glycosyl hydrolases from non-digestible plant polysaccharides. Studies have shown the ability of selected strains of *Lactobacillus* and *Bifidobacterium* to ferment XOS (Moure *et al.*, 2006). Furthermore, positive effects of XOS on the growth of probiotic bacteria are also documented (Buruiana *et al.*, 2017; Dávila *et al.*, 2019). Moura *et al.* (2007) studied *in vitro* fermentation of XOS from corncob using *B. adolescentis* and *L. brevis* and reported that better growth was observed with XOS than control. Similarly, a study was reported on the *in vitro* fermentation of XOS from Bengal gram husk and wheat bran xylan by *B. adolescentis* NDRI 236 which readily utilized the oligosaccharide (Madhukumar and Muralikrishna, 2010). In another study, XOS extracted from sugarcane bagasse has been reported to promote the growth of *Bifidobacteria* (Reddy and Krishnan, 2016). Rajagopalan *et al.* (2017) highlighted the positive effect of XOS from hardwood on the growth of *Bifidobacteria* and *Lactobacilli* strains. Similar results have been observed in our study where XOS from peanut shells, bambara, cowpea and sorghum biomass enhanced the growth of *L. plantarum* NS6, *L. lactis* NS9 and *B. animalis* NS11.

4.3.4 Development of prebiotic functional foods

4.3.4.1 Textural properties of the formulated spread

In this study, a new spread was developed using amadumbe and bambara flour as the base and the impact of bambara XOS supplementation on the spread's quality was assessed using texture analysis. Textural differences between plain and XOS enriched spread samples were significant. XOS enriched spread had less adhesive and was stretchy than the control spread (Table 4.2). The force applied to penetrate and retrieve the probe in XOS enriched spread was less than the force required for the control spread. The plain spread (2.75 N) was harder than the XOS enriched spread (2.59 N). This clearly indicates that the addition of XOS lowered the hardness moderately, which aids in spreading. Adhesiveness was not detected in both the spread samples

and could be easily applied on bread or crackers and did not adhere to teeth or the palate. Low adhesiveness is considered a desirable textural quality of spreads (Radoĉaj *et al.*, 2011).

Table 4.2 Texture analysis of plain spread and XOS enriched spread.

Spread type	Parameters tested		
	Hardness (N)	Adhesive force (N)	Adhesiveness (J)
Plain spread	2.75±0.05	0.01168±0.0006	0.0000
XOS enriched spread	2.59±0.12	0.01047±0.0003	0.0000

NB: Value represents the mean of three replicates and the standard error is reported.

4.3.4.2 Accelerated phase separation of plain and XOS enriched spreads

Accelerated phase separation is a useful technique to determine the stability and water holding capacity of a food product. Syneresis is the oozing of the liquid portion from the semi-solid foods (Wolfschoon-Pombo *et al.*, 2018). This property makes the food undesirable which subsequently affects its market and production. The spreads formulated with bambara XOS showed lower water and/or oil syneresis than the plain spread which contained slightly more oil and water (Figure 4.4). The water content in bambara XOS enriched spread was 22.7% whereas the plain spread comprised 23.4% water. Compared to water, the oil content in XOS enriched spread (11.6%) was marginally lower than the plain spread (12%). As bambara XOS supplemented spread had low oil and water syneresis, they could be efficiently utilized in making various other food products. Conventional emulsion instability processes such as flocculation, coalescence and phase separation can be efficiently reduced by increasing the viscosity of the continuous phase to block the movement of droplets in any semi-solid samples (McClements, 2015). In this study, bambara XOS increased the viscosity of the spread, which was confirmed by accelerated phase separation, where the XOS enriched spread showed a slight increase in the solid portion in comparison to the control.

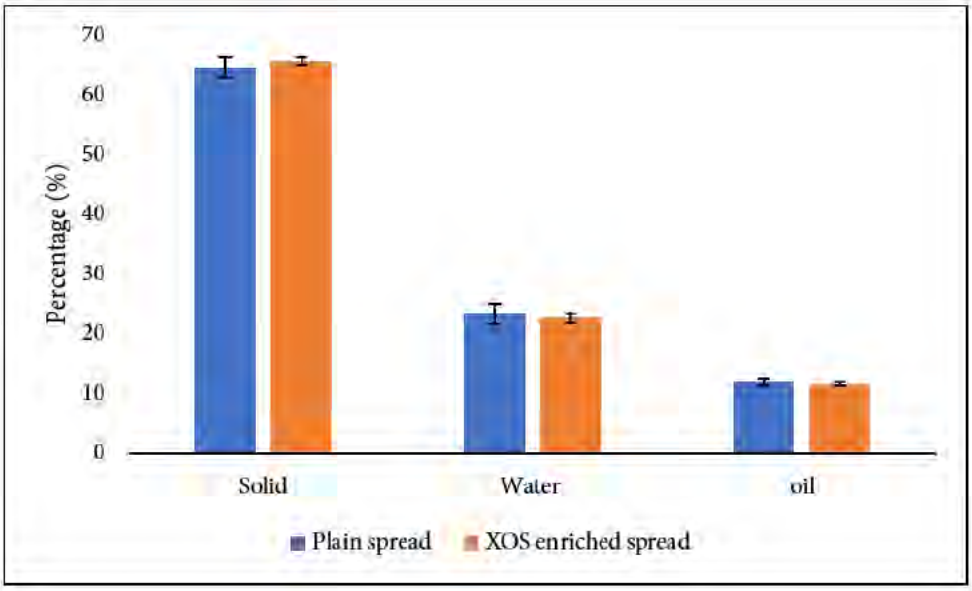

Figure 4.4 Accelerated phase separation after centrifugation of plain and XOS enriched spread samples.

4.3.4.3 Physiochemical properties of plain and prebiotic yogurts

An attempt was made to produce a prebiotic yogurt by adding XOS from bambara to the yogurt. Plain yogurt without the addition of bambara XOS, served as control. Physiochemical properties such as pH, titratable acidity, viscosity and syneresis could influence the quality and acceptance of yogurt. The pH of plain and XOS enriched yogurt decreased marginally during the first week of storage at 4°C from 4.61 to 4.59 and 4.73 to 4.70, respectively. Subsequently, the pH dropped gradually in the 2nd and 3rd weeks from 4.59 to 4.35 in plain yogurt and 4.70 to 4.48 in XOS enriched yogurt, while in the 4th week no significant changes were observed in both the samples (Table 4.3). It seems that the addition of prebiotics does not have any significant difference in the buffering capacity of the product during fermentation, because both the samples showed similar pH decline rates. The reduction in pH during storage (up to 21 days) could be correlated with the metabolic activity of probiotic bacteria. A further pH drop was not observed in 4th week which could be due to the cessation of metabolic activity in 4th week of storage. A similar trend to the above results was observed by Shaghaghi *et al.* (2013) with a pH drop of 0.4 in both the oligofructose supplemented yogurt and the control. In a different study,

yogurt containing barley-based β-glucan showed a slight pH drop (0.1) during 28 days of storage (Ozcan and Kurtuldu, 2014).

In food analysis, pH and titratable acidity are two interrelated concepts that deal with the measurement of acidity. A change in the titratable acidity over the storage period was similar to that of the pH change. A steady increase in the titratable acidity was observed in both plain yogurt and XOS enriched yogurt, where it increased in the first three weeks from 0.78% to 0.95% for plain yogurt and 0.82% to 0.96% for XOS enriched yogurts respectively. No significant change was observed in titratable acidity as well as pH during the last week of storage. Our results agree with a previous study where titratable acidity of yogurt supplemented with β-glucan was slightly increased (0.88%) during the 4-week storage (Ozcan and Kurtuldu, 2014). Lactic acid bacteria produce both acetic acid and lactic acid as the by-products when grown on xylose. In general, lactic acid is a strong acidifier that leads to a quicker drop in pH than acetic acid. This suggests that more acetic acid was produced in XOS supplemented yogurt which had high pH in comparison to plain yogurt. Similar results were observed where arabinoxylooligosaccharide (AXOS) supplementation produced more acetic acid (Gullón *et al.*, 2014).

Syneresis of yogurt is an important characteristic as the whey separation affects the texture of yogurt and decreases the consumer acceptability (Akgun *et al.*, 2016). The syneresis values of prebiotic yogurt samples are presented in Table 4.3. The syneresis values obtained throughout the storage period were slightly decreased from 29.89% to 26.96% for XOS enriched yogurt and 30.67% to 27.54% for plain yogurt. The decrease in syneresis of prebiotic yogurt could be due to the increase in texture consistency. The structural complexity of XOS prevented syneresis by increasing the water-binding capacity. Table 4.3 shows that on day 1, a higher syneresis value was observed for the control sample and the lower value was recorded for the XOS supplemented sample. The syneresis decreased until day 21 and then it showed a slight increase on day 28. At the end of the storage period, plain yogurt had a higher syneresis than XOS supplemented yogurt, suggesting the potential of XOS to minimize the syneresis.

Table 4.3 The physicochemical properties of plain and XOS enriched yogurt.

	Plain yogurt				XOS enriched yogurt			
Storage time (Days)	pH	Titratable acidity (%LA)	Viscosity (cP)	Syneresis (%)	pH	Titratable acidity (%LA)	Viscosity (cP)	Syneresis (%)
1	4.61±0.16	0.78±0.02	19.34±0.65	30.67±0.71	4.73±0.12	0.82±0.00	20.16±0.12	29.89±0.92
7	4.59±0.21	0.85±0.01	19.39±0.77	29.95±0.88	4.70±0.17	0.87±0.01	20.72±0.11	28.67±0.73
14	4.43±0.19	0.92±0.03	19.42±0.58	28.83±0.73	4.52±0.22	0.94±0.02	20.81±0.29	27.56±0.84
21	4.35±0.13	0.95±0.02	19.56±0.87	27.28±0.97	4.48±0.15	0.96±0.01	21.05±0.23	26.12±0.81
28	4.35±0.18	0.96±0.01	19.55±0.59	27.54±0.63	4.47±0.13	0.98±0.03	21.10±0.42	26.96±0.72

NB: Value represents the mean of three replicates and the standard error reported.

A similar result has been reported by Shaghaghi *et al.* (2013) where yogurt supplemented with oligofructose showed a syneresis reduction from 30.65% to 26.47% until the 21st day of storage which increased to 27.10% on further storage. This may be due to the depletion of oligosaccharides in yogurt sample which could have led to an increase in syneresis.

Viscosity increased gradually in both plain yogurt and XOS enriched yogurt during the 28 days of storage. In the case of plain yogurt, it increased from 19.34 cP to 19.55 cP, whereas, for XOS enriched yogurt, the viscosity increased from 20.16 cP to 21.10 cP. The increased acidity affects the formation of the yogurt which eventually leads to the decrease in its firmness. The decrease in pH was directly proportional to the increase in viscosity. This was corroborated by an earlier report which observed the same trend where yogurt supplemented with oligofructose showed a decrease in pH from 4.71 to 4.34 and an increase in viscosity from 21.77 cP to 23.09 cP (Shaghaghi *et al.*, 2013).

4.3.4.4 Viability test of yogurt samples

Milk-based products provide a favourable environment for probiotics to grow. Viability of probiotic bacteria in fermented food products throughout their shelf life is an important factor which fulfils the necessity of functional foods. The yogurts were tested for the viability of probiotic bacteria during the 28 days of storage. The initial population of lactic acid bacteria on XOS enriched yogurt sample on day 1 was higher than the plain yogurt sample. The viable count observed for plain yogurt was 7.49 log CFU/g while it was slightly higher in the case of XOS supplemented yogurt (7.65 log CFU/g). This could be correlated with the positive effects of XOS on the growth of lactic acid bacteria. During storage at 4°C, the bacterial count declined in both the samples on day 14 and was stagnant from the 21st to 28th day of storage (Figure 4.5). On day 28, the bacterial count in plain yogurt was 6.69 log CFU/g and 6.87 log CFU/g in XOS enriched yogurt; which is higher than the minimum desirable count of 6 log CFU/g (Sah *et al.*, 2015). The probable reason for the decline of lactic acid bacteria is the increase in acidity in both yogurt samples during storage, which could have adversely affected its growth. Our results

corroborates with the findings of Shaghaghi *et al.* (2013) where the oligofructose supplemented yogurt had a viable count of *L. rhamnosus* of 6.15 log CFU/g on 28th day of storage. A study conducted with β-glucan in probiotic yogurt had a viability proportion index of 0.83 (yogurt containing *B. bifidum* and barley-based β-glucan) and 0.85 (yogurt containing *B. bifidum* and oat-based β-glucan) respectively (Ozcan and Kurtuldu, 2014). Similar results were obtained in our study where a viability proportion index of 0.78 was observed for XOS enriched yogurt.

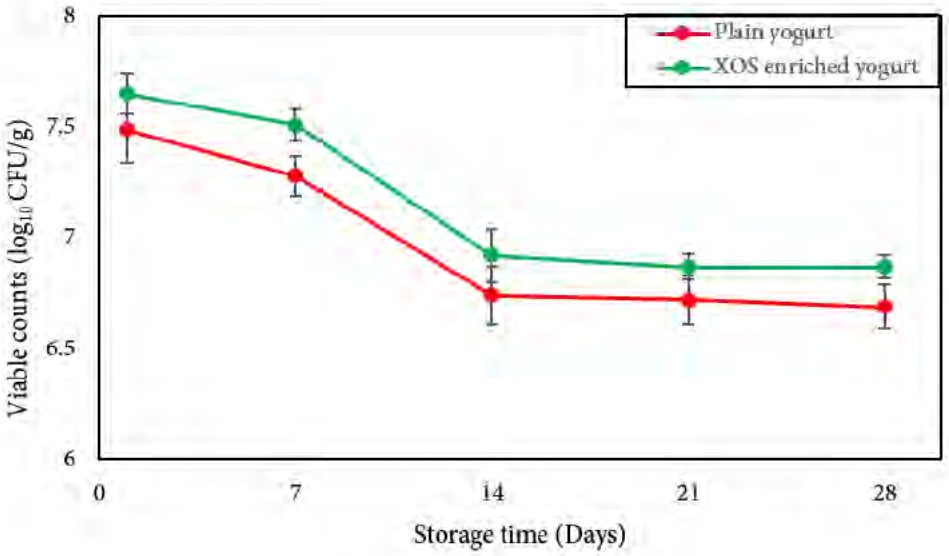

Figure 4.5 Viability of bacteria in plain and XOS enriched yogurt.

4.3.4.5 The nutritional profile of XOS enriched spread and yogurt

XOS are reported to have multifarious bioactive properties and supplementation of XOS in food products will apparently improve their prebiotic potential and further improve the health of the consumer (Singh *et al.*, 2015; Yang *et al.*, 2015). To check the nutritional efficacy of the XOS enriched spread and yogurt, the samples were freeze-dried prior to analysis and their nutritional profiling was performed following the methods of AOAC (AOAC, 2016). XOS are a pure source of dietary fibres and therefore XOS enriched product will have a higher dietary fibre content. This was confirmed by dietary fibre analysis where XOS supplemented spread showed increased dietary fibre content of 12.4% in comparison to 11.6% in the plain spread (Table 4.4).

Carbohydrate content was higher in XOS supplemented spread (24.7%) than plain spread (24.0%). XOS enriched spread generated a slightly higher amount of energy (1709 kJ) as compared to plain spread (1699 kJ). A slight decrease in protein, fat, moisture and ash content was observed in the case of XOS enriched spread which could be correlated with the replacement of spread with bambara XOS at 2.5% level, which subsequently decreased the mentioned composition in the spread.

Table 4.4 Nutritional profile of plain spread and XOS enriched spread.

Composition	Plain spread (%)	XOS enriched spread (%)
Protein	28.5±0.95	28.2±0.81
Fat	19.3±0.68	19.2±0.73
Dietary fibre	11.6±0.43	12.4±0.46
Carbohydrates	24.0±1.02	24.7±0.94
Energy (kJ)	1699±64	1709±58
Moisture	11.3±0.46	10.4±0.37
Ash	5.30±0.16	5.10±0.09

NB: Value represents the mean of three replicates and the standard error is reported.

Nutritional analysis of the freeze-dried yogurt samples was showed in Table 4.5. Dietary fibre increased in XOS enriched yogurt (5.95%) in comparison to plain yogurt (5.57%); a similar trend was observed with the carbohydrate content where plain yogurt (55.9%) had lower levels than XOS enriched yogurt (56.3%). The increase in both the dietary fibre and carbohydrate can be related to the supplementation of XOS in yogurt. Protein, moisture and ash content also declined in XOS enriched yogurt. In contrast to the above trend, the fat content was slightly higher in XOS enriched yogurt (16.6%) than the plain yogurt (16.2%). The reason behind elevated levels of fat can be correlated with the ability of lactic acid bacteria to produce short chain fatty acids during XOS fermentation. The estimated output in terms of energy from XOS enriched yogurt was slightly higher (1842 kJ) than the plain yogurt (1822 kJ) and hence could be more preferable.

Table 4.5 Nutritional profiles of freeze-dried plain and prebiotic yogurt.

Composition	Plain yogurt (%)	XOS enriched yogurt (%)
Protein	13.4±0.64	12.9±0.34
Fat	16.2±0.73	16.6±0.51
Dietary fibre	5.57±0.08	5.95±0.13
Carbohydrates	55.9±1.31	56.5±1.56
Energy (kJ)	1822±61.0	1842±52.0
Moisture	6.24±0.09	5.59±0.15
Ash	2.69±0.08	2.46±0.12

NB: Value represents the mean of three replicates and the standard error is reported.

4.3.5 Sensory evaluation

Over the past 6 decades, consumer assessment has been extensively used to evaluate the quality and acceptability of food products. Prominent product properties can be effectively assessed with the help of sensory analysis which subsequently determines the product acceptability. Sensory properties of spread have a large effect on consumer acceptability, and especially, the appearance which is the first sensory characteristic, perceived by the consumer (Figure 4.6). The mean value for appearance of XOS enriched spread samples was 7.27 which was greater than the plain spread (7.13) which suggested the positive effect of XOS on the appearance of the spread. There was a similar score (~7.0) for the aroma of the samples which indicated that it does not impart any aroma or odour to the product. In the case of taste, the XOS enriched spread was preferred (7.03) to the plain spread (6.3). The texture of XOS enriched spread was 7.1 which was slightly higher than plain spread (6.8). The sensory properties of the XOS enriched spread are essential before commercialization and they should display similar sensorial acceptance and sensory attributes (texture, aroma, and flavour) of traditional spreads (Cruz *et al.*, 2010). All the five aspects clearly indicated that XOS enriched spread was more preferential than plain spread (Figure 4.6). A similar trend was observed by Prakash and Priya (2016) who studied the incorporation of FOS in a blueberry jelly. The addition of FOS in

improved the overall acceptability of the jelly, but in contrast to our results, FOS enriched jelly scored a lower score for texture. The lowered textural attributes can be correlated with the low levels of viscosity of oligosaccharides which in turn provides good mouthfeel of the food products. Similar results were found in the gelatin gel added with β-glucan which lowered the viscosity providing good mouthfeel of the gel (Sinthusamran and Benjakul, 2018).

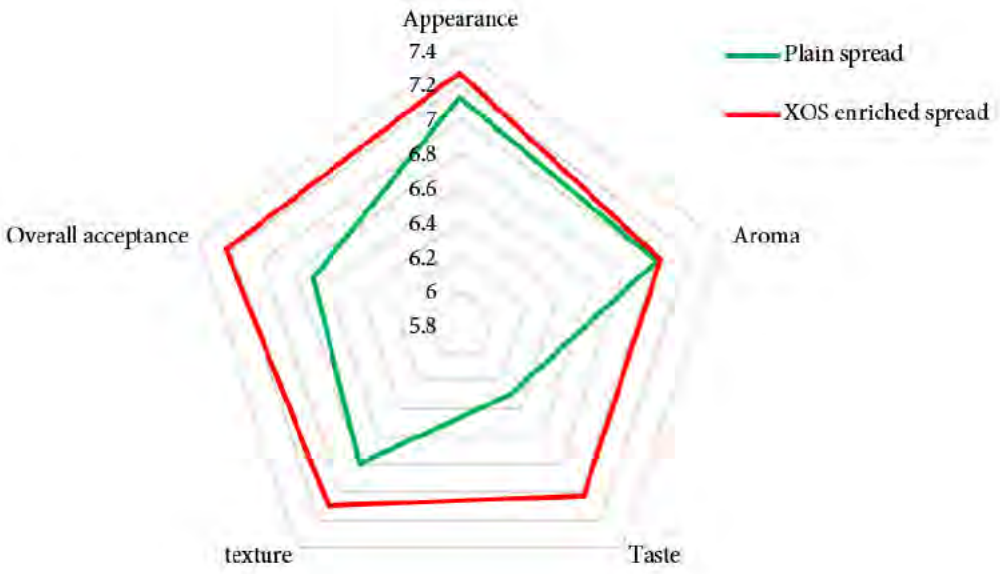

Figure 4.6 Radar plot for consumer acceptability of plain spread and XOS enriched spread.

Unlike the results of the spread, XOS supplemented yogurt showed contrary results for its sensorial properties. The overall acceptance, texture and taste were marginally improved in the case of XOS supplemented yogurt while appearance and aroma obtained a slightly higher score for plain yogurt. The appearance and aroma for plain yogurt was 7.9 and 7.77, respectively whereas these parameters were slightly lower (7.77 and 7.43, respectively) for XOS supplemented yogurt. Yogurt is a milk-based product that has a characteristic white colour; addition of XOS could have altered the colour of the yogurt and the appearance had slightly lower scores. Yogurt also has a unique aroma which was altered due to addition of XOS. Better taste, texture and overall acceptance was observed in XOS supplemented yogurt (6.87, 7.33 and 7.33, respectively) than plain yogurt (6.83, 7.07 and 7.2, respectively). Hence overall acceptance

values suggested XOS enriched yogurt was more preferable than plain yogurt. In contrast to our results, a recent study reported that XOS supplementation in yogurt did not affect the colour, flavour and odour of the product, however, the flavour becomes intense in the case of higher concentration (5%) of XOS addition (Penksza *et al.*, 2018).

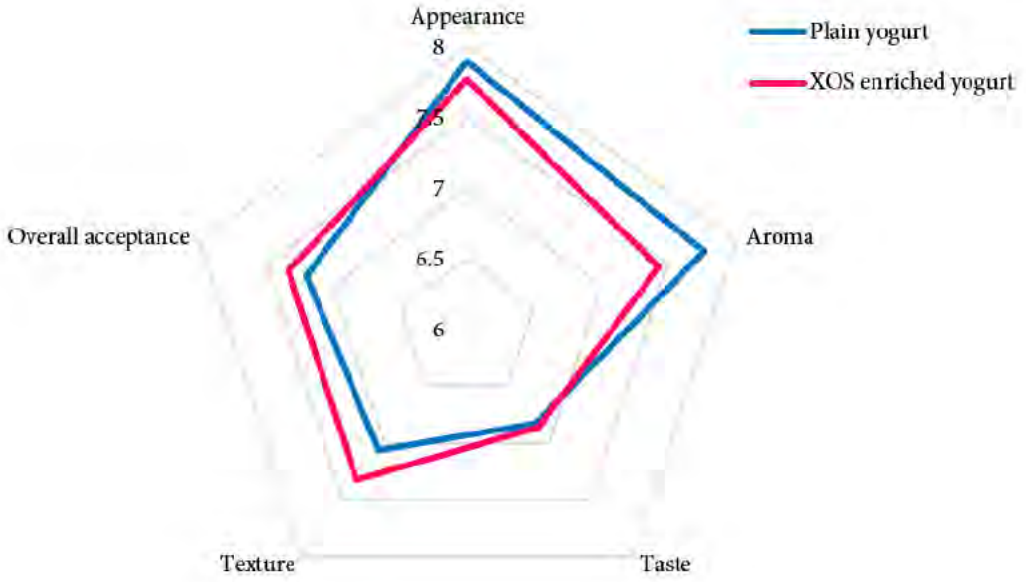

Figure 4.7 Radar plot for consumer acceptability of plain yogurt and XOS enriched yogurt.

4.4 CONCLUSION

In this chapter, the effectiveness of XOS as a prebiotic was examined and their potential as a food supplement was studied. During *in vitro* fermentation, *L. plantarum* NS6 and *B. animalis* NS11 grew well on XOS media whereas *L. lactis* NS9 had limited growth. Amadumbe and bambara which are underutilized crops were used to formulate carbohydrate-rich low-fat spread and this is the first report on the utilization of these flours for the preparation of spread. The newly formulated spread showed similar characteristics to traditional spreads in terms of texture, stability and overall acceptability. Therefore, the formulation is suggested to be sustainable product in the market for spreads. The XOS enriched yogurt showed an excellent

probiotic growth and maintained a good microbial count during storage than plain yogurt. In both spread and yogurt samples, the addition of XOS did not affect their sensory characteristics which is an advantage for using them in any kind of food formulation. The XOS enriched spread and yogurt samples were well accepted by the consumers which substantiates that the trails were successful. The prebiotic supplementation could offer more underlying health benefits to the consumers and hence the formulated products are alternatives to the products already present in the market.

5 CONCLUDING REMARKS

Global lignocellulosic biomass production exceeds 220 billion dry tons annually (Fang *et al.*, 2019) due to the massive generation of agricultural and forest biomass. However, most of this energy rich biomass remains unutilized or dumped as waste or burnt. Such biomass has various biomolecules in them which could be exploited for value addition. Conversion of these biomolecules, however, requires greater attention and the concept of valorization of lignocellulosic biomass to value-added products is an innovative approach in this regard. Products such as bioethanol, biohydrogen, fibres, oligosaccharides, resins, sugars, antibiotics, dyes, vitamins, polyols, flavours, surfactants, organic acids, and biomaterials could be produced by the effective processing of lignocellulosic biomass through chemical and biological methods. Hence, recent research has focused more on converting these waste materials into useful products in a cost-effective and eco-friendly manner to meet the market demands.

Though lignocellulose has great potential, due to its complex matrix, the biomass needs to be derivatised into simpler forms employing different pretreatment techniques. The primary motive of pretreatment is to alter their chemical composition, acetyl and methyl ester groups, crystalline nature, degree of polymerization and accessibility to different polymers present in them. However, each pretreatment method affects and modifies the structure of lignocellulose in a different manner. In the current scenario, where stringent laws on waste clearance are implemented globally, there is a scope for developing novel pretreatment techniques for exploring agriculture-derived lignocellulose. The pretreatment developed must be based on the physical and chemical composition of biomass, targeting the separation of each component of the lignocellulosic framework.

Currently, several pretreatments are available for the deconstruction of the lignocellulosic framework which includes physical, chemical, physicochemical and biological methods. In general, physical pretreatment reduces the particle size and crystallinity and expands the surface area of materials, whereas, chemical treatments breakdown the bonding between hemicellulose

and lignin, resulting in the debranching of the lignocellulosic matrix. The chemicals used for the breakdown and extraction of lignocellulosic components will be left as waste after the process and must be treated prior to release in the environment. Such limitations of chemical treatment could be overcome by employing biological methods that are mostly targeted to achieve desired product formation rather than the random approach. Although individual methods were effective to a certain extent, a combined pretreatment deemed more efficient in the breakdown of the lignocellulosic material.

One of the principal components of the lignocellulosic matrix is hemicellulose, that possesses potential applications in platform chemical production, drug delivery, nutraceuticals, and biomedical applications. Besides their application, processing of hemicellulose for value addition holds promise for the management of agricultural residues that are presently underutilized. Xylan is the most widely studied hemicellulose, with a xylose backbone in the main chain and could be used as a substrate for many value-added products. Xylooligosaccharides (XOS) is one of the major products of commercial importance that can be obtained from xylan. Being associated with biostimulatory activity and a positive effect on human health as a potent prebiotic, XOS engross a considerable share in the prebiotic market.

Several techniques have been employed to produce XOS from polymeric xylan. Among these, the enzymatic conversion is one of the most sustainable and environmentally friendly processes. One of the main advantages of employing enzymatic hydrolysis is the absence of toxic by-products released from xylan. Therefore, extraction of xylan from underutilized sources and their bioconversion to XOS were attempted in this study. This work provides insights into the structural features of xylan isolated from peanut shells, bambara, cowpea, and sorghum biomass, for the first time. Various pretreatment techniques including acid, alkali and physical methods were employed to extract the xylan. During acid treatments, the release of monosaccharides was observed which affected the production of oligosaccharides, therefore, the method was excluded. During alkali treatment, the direct extraction of xylan using alkali yielded a significant amount of xylan, however, more xylan was trapped in the lignocellulosic matrix due to its rigidity. Hence, an alternate method with a preceding delignification step was

tested, where sodium hypochlorite was used as a bleach, followed by NaOH extraction. Xylan extracted by this method contained some co-extracted carbohydrates, specifically of starch origin. The presence of starch was confirmed by treating the extracted polysaccharide with α-amylase which released few maltooligosaccharides. Surprisingly, the glucose content observed in the extracted polysaccharide from peanut shell accounted for the presence of xyloglucan which was confirmed by xyloglucanase treatment. However, only starch contamination was observed for xylans extracted from the other three biomass. To acquire purer xylan during extraction, another approach was carried out using sodium chlorite as the delignification agent. This time, the co-extraction of other polysaccharides was eliminated yielding much purer xylan. The functional groups of extracted xylans were confirmed by FTIR spectroscopy. Further, the xylans were characterized by ^{1}H-NMR spectroscopy and confirmed that all the four xylans contained typical glucuronoxylan similar to hardwood glucuronoxylan. These observations were supported by monosaccharide analysis.

In the next step, enzymatic hydrolysis of xylan was optimized to yield maximum XOS. Enzymatic hydrolysis of xylans was conducted using the GH11 xylanase from *T. lanuginosus* SSBP and was compared with GH10 and GH30 xylanases. Hydrolysis of the extracted xylans by the three different xylanases resulted in the release of neutral XOS (2–7 DP) and acidic oligosaccharides differing in the degree of polymerization. TLC observations showed that GH10 xylanase produced shorter oligosaccharides along with xylose. The hydrolysate also contained few acidic oligosaccharides, since the longer oligosaccharides were cleaved by the action of GH10 xylanase. On the contrary, the GH11 xylanase from *T. lanuginosus* SSBP was found to be very efficient in hydrolysing xylans from different biomass into XOS with essentially no release of free xylose. The acidic oligosaccharides produced from all the four xylans by GH11 xylanase contained larger aldouronic acids, along with their sodium adducts, whereas, GH30 xylanase produced only aldouronic acids. The hydrolysates produced by the enzymatic action were further analyzed by MALDI-TOF MS. The MALDI-TOF MS signals showed that the products generated by the action of GH10 and GH11 xylanases supported the TLC results. However,

GH30 xylanase products were below the detection limits in the case of bambara and sorghum xylan.

The *in silico* analysis was performed to understand the hydrolytic mechanism of xylanases, with respect to their structural and functional characteristics. The amino acid sequence of GH10, GH11, and GH30 xylanases showed maximum similarity to the xylanases from closely related species. Further, these sequences were analyzed by the PDBsum server that revealed the secondary structure, topology and conserved regions of the enzymes. Using the structural data, 3D structures were modelled, and their stability was verified. The docking of these enzymes with aldopentaouronic acid was performed which showed that *Tl*GH11 xylanase had the best docked pose with a binding energy of −10.24 kcal/mol. The *in silico* study suggested that *Tl*GH11 xylanase was more appropriate than GH10 and GH30 xylanases for binding to xylan.

Application of acidic XOS (aldouronic acids) as prebiotics is an emerging area of application than the use of conventional neutral XOS. Aldouronic acids derived from hardwood xylan have shown to have potential as a bioactive material to be applied in medicine. On experimental animals such as mouse and rat, it has been demonstrated that the aldouronic acids promote recovery from iron deficiency anaemia by enhancing serum ion levels (Kobayashi *et al.*, 2011) and prevent the development of atopic dermatitis-like skin lesions (Ohbuchi *et al.*, 2010). In earlier studies, XOS have been reported to have multifarious bioactive properties which prevents gut infection, diabetes (Yang *et al.*, 2015), neuro-toxicity (Krishna *et al.*, 2015), suppress colon cancer initiation (Aachary *et al.*, 2015), prevention of colon inflammation (Femia *et al.*, 2010; Lin *et al.*, 2016), and improvement of intestinal health (Amorim *et al.*, 2019). They also have various applications in medical, food, pharmaceutical, feed, and cosmetic industries. The ability of dietary XOS to function as prebiotics in humans is governed by their substitution patterns where simple oligosaccharides could be readily utilized by probiotics.

In vitro fermentation of the isolated probiotics on media containing XOS as a sole carbon source has shown that XOS from all the biomass was effective as a substrate for enhancing the growth rate and cell mass of *Lactobacillus plantarum* NS6, *Lactococcus lactis* NS9, and *Bifidobacterium*

animalis NS11. In addition, the observed rate of XOS utilization was found to be strain specific. Results from our study have shown the prebiotic potential of XOS during *in vitro* fermentation, however, further studies are recommended exploring the animal model and human clinical trials which would form a basis for their commercialization.

Indisputably, functional food development is increasing as one of the promising and dynamically growing practices in the food industry. Gradually, novel components have been introduced with potential prebiotic activity. In this context, XOS was supplemented in food products to improve their nutritional efficiency. In this study, a novel XOS enriched spread was formulated using bambara and amadumbe flours with low-fat content. The newly formulated spread mimics the commercial spreads in terms of nutritional and sensory characteristics with increased dietary fibre. A prebiotic yogurt was also prepared which possessed good probiotic activity during storage than control. Considering the profound nutraceutical characteristics of XOS, the spread and yogurt supplemented with XOS offers the potential to be explored in the food sector.

5.1 Future perspectives

The demand for ecofriendly, industrially viable technologies for the bioconversion of raw materials are increasing tremendously. Engineering the microorganisms to convert whole biomass into platform chemicals and biofuels is recommended to reduce the usage of pretreatments and production costs. Though significant progress has been made in the bioprocessing of agricultural residues to higher value-added products, there are still knowledge gaps that need attention. This study provided a scientific insight into the production and characterization of XOS from peanut shell, bambara, cowpea, and sorghum biomass. Further research on the following will pave a new direction for biomass beneficiation and XOS application.

i. Optimizing pretreatment technologies to directly convert biomass into XOS without the formation of monomers and inhibitors

ii. Evaluation of antimicrobial and antioxidant property of XOS

iii. Efficacy of bioactive XOS through animal studies and clinical trials

iv. Further application of XOS in regular food products and their commercialization

REFERENCES

Abuajah, C.I., Ogbonna, A.C., Osuji, C.M., 2015. Functional components and medicinal properties of food: a review. J. Food Sci. Technol. 52, 2522–2529. https://doi.org/10.1007/s13197-014-1396-5

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment: fundamentals toward application. Biotechnol. Adv. 29, 675–85. https://doi.org/10.1016/j.biotechadv.2011.05.005

Agricultural Research Council, 2017. A profile of the South African grain sorghum market value chain. Pretoria, South Africa.

Agricultural Research Council, 2016. Production guidelines for Bambara groundnuts. Pretoria, South Africa.

Agricultural Research Council, 2014. Production guidelines for Cowpea. Pretoria, South Africa.

Ahmed, S., Imdad, S.S., Jamil, A., 2012. Comparative study for the kinetics of extracellular xylanases from *Trichoderma harzianum* and *Chaetomium thermophilum*. Electron. J. Biotechnol. 15. https://doi.org/10.2225/vol15-issue3-fulltext-2

Ahuja, K., Rawat, A., 2017. Global market insights.

Akgun, A., Yazici, F., Gulec, H.A., 2016. Effect of reduced fat content on the physicochemical and microbiological properties of buffalo milk yoghurt. LWT - Food Sci. Technol. 74, 521–527. https://doi.org/10.1016/j.lwt.2016.08.015

Al-Sheraji, S.H., Ismail, A., Manap, M.Y., Mustafa, S., Yusof, R.M., Hassan, F.A., 2013. Prebiotics as functional foods: A review. J. Funct. Foods 5, 1542–1553. https://doi.org/10.1016/J.JFF.2013.08.009

Alhudhud, M., Humphreys, P., Laws, A., 2014. Development of a growth medium suitable for exopolysaccharide production and structural characterisation by *Bifidobacterium animalis* ssp. *lactis* AD011. J. Microbiol. Methods 100, 93–98.

https://doi.org/10.1016/J.MIMET.2014.02.021

Álvarez, C., González, A., Negro, M.J., Ballesteros, I., Oliva, J.M., Sáez, F., 2017. Optimized use of hemicellulose within a biorefinery for processing high value-added xylooligosaccharides. Ind. Crop. Prod. 99, 41–48. https://doi.org/10.1016/j.indcrop.2017.01.034

Amel, B.D., Nawel, B., Khelifa, B., Mohammed, G., Manon, J., Salima, K.G., Farida, N., Hocine, H., Bernard, O., Jean-Luc, C., Marie-Laure, F., 2016. Characterization of a purified thermostable xylanase from *Caldicoprobacter algeriensis* sp. *nov*. strain TH7C1T. Carbohydr. Res. 419, 60–68. https://doi.org/10.1016/j.carres.2015.10.013

Amorim, C., Silvério, S.C., Cardoso, B.B., Alves, J.I., Pereira, M.A., Rodrigues, L.R., 2019a. In vitro assessment of prebiotic properties of xylooligosaccharides produced by *Bacillus subtilis* 3610. Carbohydr. Polym. In press. https://doi.org/10.1016/j.carbpol.2019.115460

Amorim, C., Silvério, S.C., Gonçalves, R.F.S., Pinheiro, A.C., Silva, S., Coelho, E., Coimbra, M.A., Prather, K.L.J., Rodrigues, L.R., 2019b. Downscale fermentation for xylooligosaccharides production by recombinant *Bacillus subtilis* 3610. Carbohydr. Polym. 205, 176–183. https://doi.org/10.1016/j.carbpol.2018.09.088

Amorim, C., Silvério, S.C., Prather, K.L.J., Rodrigues, L.R., 2019c. From lignocellulosic residues to market: Production and commercial potential of xylooligosaccharides. Biotechnol. Adv. In press. https://doi.org/10.1016/J.BIOTECHADV.2019.05.003

Amorim, C., Silvério, S.C., Rodrigues, L.R., 2019d. One-step process for producing prebiotic arabino-xylooligosaccharides from brewer's spent grain employing *Trichoderma* species. Food Chem. 270, 86–94. https://doi.org/10.1016/j.foodchem.2018.07.080

Amorim, C., Silvério, S.C., Silva, S.P., Coelho, E., Coimbra, M.A., Prather, K.L.J., Rodrigues, L.R., 2018. Single-step production of arabino-xylooligosaccharides by recombinant *Bacillus subtilis* 3610 cultivated in brewers' spent grain. Carbohydr. Polym. 199, 546–554. https://doi.org/10.1016/J.CARBPOL.2018.07.017

An, J., Xie, Y., Zhang, Y., Tian, D., Wang, S., Yang, G., Feng, Y., 2015. Characterization of a thermostable, specific GH10 xylanase from *Caldicellulosiruptor bescii* with high catalytic activity. J. Mol. Catal. B Enzym. 117, 13–20. https://doi.org/10.1016/J.MOLCATB.2015.04.003

Anburajan, P., Yoon, J.-J., Kumar, G., Park, J.-H., Kim, S.-H., 2019. Evaluation of process performance on biohydrogen production in continuous fixed bed reactor (C-FBR) using acid algae hydrolysate (AAH) as feedstock. Int. J. Hydrogen Energy 44, 2164–2169. https://doi.org/10.1016/J.IJHYDENE.2018.09.098

Ando, H., Ohba, H., Sakaki, T., Takamine, K., Kamino, Y., Moriwaki, S., Bakalova, R., Uemura, Y., Hatate, Y., 2004. Hot-compressed-water decomposed products from bamboo manifest a selective cytotoxicity against acute lymphoblastic leukemia cells. Toxicol. Vitr. 18, 765–771. https://doi.org/10.1016/J.TIV.2004.03.011

AOAC, 2016. Official Methods of Analysis of AOAC International. AOAC International, Rockville, Maryland.

Aracri, E., Vidal, T., 2011. Xylanase- and laccase-aided hexenuronic acids and lignin removal from specialty sisal fibres. Carbohydr. Polym. 83, 1355–1362. https://doi.org/10.1016/J.CARBPOL.2010.09.058

Arai, T., Biely, P., Uhliariková, I., Sato, N., Makishima, S., Mizuno, M., Nozaki, K., Kaneko, S., Amano, Y., 2019. Structural characterization of hemicellulose released from corn cob in continuous flow type hydrothermal reactor. J. Biosci. Bioeng. 127, 222–230. https://doi.org/10.1016/j.jbiosc.2018.07.016

Arevalo-Gallegos, A., Ahmad, Z., Asgher, M., Parra-Saldivar, R., Iqbal, H.M.N., 2017. Lignocellulose: A sustainable material to produce value-added products with a zero waste approach-A review. Int. J. Biol. Macromol. 99, 308–318. https://doi.org/10.1016/j.ijbiomac.2017.02.097

Aryana, K.J., 2003. Folic acid fortified fat-free plain set yoghurt. Int. J. Dairy Technol. 56, 219–

222. https://doi.org/10.1046/j.1471-0307.2003.00105.x

Atalah, J., Cáceres-Moreno, P., Espina, G., Blamey, J.M., 2019. Thermophiles and the applications of their enzymes as new biocatalysts. Bioresour. Technol. 280, 478–488. https://doi.org/10.1016/J.BIORTECH.2019.02.008

Azelee, N.I.W., Jahim, J.M., Ismail, A.F., Fuzi, S.F.Z.M., Rahman, R.A., Illias, R.M., 2016. High xylooligosaccharides (XOS) production from pretreated kenaf stem by enzyme mixture hydrolysis. Ind. Crops Prod. 81, 11–19. https://doi.org/10.1016/j.indcrop.2015.11.038

Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270. https://doi.org/10.1016/0168-1656(92)90074-J

Bajpai, P., 2014. Xylanolytic Enzymes. Academic Press, Amsterdam.

Balusu, R., Paduru, R.M.R., Seenayya, G., Reddy, G., 2004. Production of ethanol from cellulosic biomass by *Clostridium thermocellum* SS19 in submerged fermentation: screening of nutrients using Plackett-Burman design. Appl. Biochem. Biotechnol. 117, 133–142. https://doi.org/10.1385/ABAB:117:3:133

Banka, A.L., Albayrak Guralp, S., Gulari, E., 2014. Secretory expression and characterization of two hemicellulases, xylanase, and β-xylosidase, isolated from *Bacillus subtilis* M015. Appl. Biochem. Biotechnol. 174, 2702–10. https://doi.org/10.1007/s12010-014-1219-1

Bao, H., Liu, R., Liang, L., Jiang, Y., Jiang, M., Ma, J., Chen, K., Jia, H., Wei, P., Ouyang, P., 2014. Succinic acid production from hemicellulose hydrolysate by an *Escherichia coli* mutant obtained by atmospheric and room temperature plasma and adaptive evolution. Enzyme Microb. Technol. 66, 10–15. https://doi.org/10.1016/j.enzmictec.2014.04.017

Baruah, J., Nath, B.K., Sharma, R., Kumar, S., Deka, R.C., Baruah, D.C., Kalita, E., 2018. Recent trends in the pretreatment of lignocellulosic biomass for value-added products. Front. Energy Res. https://doi.org/10.3389/fenrg.2018.00141

Basaran, P., Ozcan, M., 2008. Characterization of b-xylosidase enzyme from a *Pichia stipitis* mutant. Bioresour. Technol. 99, 38–43. https://doi.org/10.1016/j.biortech.2006.11.056

Bayer, E.A., Shoham, Y., Lamed, R., 2013. Lignocellulose-decomposing bacteria and their enzyme systems, in: The Prokaryotes. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 215–266. https://doi.org/10.1007/978-3-642-30141-4_67

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K.M., Sani, R.K., 2013. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. Bioresour. Technol. 128, 751–759. https://doi.org/10.1016/j.biortech.2012.10.145

Bhalla, A., Bischoff, K.M., Uppugundla, N., Balan, V., Sani, R.K., 2014. Novel thermostable endo-xylanase cloned and expressed from bacterium *Geobacillus* sp. WSUCF1. Bioresour. Technol. 165, 314–318. https://doi.org/10.1016/j.biortech.2014.03.112

Bibi, Z., Ansari, A., Zohra, R.R., Aman, A., Ul Qader, S.A., 2014. Production of xylan degrading endo-1, 4-β-xylanase from thermophilic *Geobacillus stearothermophilus* KIBGE-IB29. J. Radiat. Res. Appl. Sci. 7, 478–485. https://doi.org/10.1016/j.jrras.2014.08.001

Bibra, M., Kunreddy, V., Sani, R., 2018. Thermostable xylanase production by *Geobacillus* sp. strain DUSELR13, and its application in ethanol production with lignocellulosic biomass. Microorganisms 6, 93. https://doi.org/10.3390/microorganisms6030093

Biely, P., 2003. Xylanolytic enzymes, in: Whitaker, J.R., Voragen, A.G.J., Wong, D.W.S. (Eds.), Handbook of Food Enzymology. Marcel Dekker, Inc., New York, USA, pp. 879–915.

Biely, P., Cziszárová, M., Uhliariková, I., Agger, J.W., Li, X.-L., Eijsink, V.G.H., Westereng, B., 2013. Mode of action of acetylxylan esterases on acetyl glucuronoxylan and acetylated oligosaccharides generated by a GH10 endoxylanase. Biochim. Biophys. Acta 1830, 5075–5086. https://doi.org/10.1016/j.bbagen.2013.07.018

Biely, P., de Vries RP, R.V., Vrsanská, M., Visser, J., 2000. Inverting character of alpha-glucuronidase A from *Aspergillus tubingensis*. Biochim. Biophys. Acta 1474, 360–364. https://doi.org/10.1016/s0304-4165(00)00029-5

Biely, P., Malovíková, A., Hirsch, J., Morkeberg Krogh, K.B.R., Ebringerová, A., 2015. The role of the glucuronoxylan carboxyl groups in the action of endoxylanases of three glycoside hydrolase families: A study with two substrate mutants. Biochim. Biophys. Acta 1850, 2246–2255. https://doi.org/10.1016/j.bbagen.2015.07.003

Biely, P., Mastihubová, M., Tenkanen, M., Eyzaguirre, J., Li, X.-L., Vršanská, M., 2011. Action of xylan deacetylating enzymes on monoacetyl derivatives of 4-nitrophenyl glycosides of β-D-xylopyranose and α-L-arabinofuranose. J. Biotechnol. 151, 137–142. https://doi.org/10.1016/J.JBIOTEC.2010.10.074

Biely, P., Puchart, V., Stringer, M.A., Mørkeberg Krogh, K.B.R., 2014. *Trichoderma reesei* XYN VI - a novel appendage-dependent eukaryotic glucuronoxylan hydrolase. FEBS J. 281, 3894–3903. https://doi.org/10.1111/febs.12925

Biely, P., Singh, S., Puchart, V., 2016. Towards enzymatic breakdown of complex plant xylan structures: state of the art. Biotechnol. Adv. 34, 1260–1274. https://doi.org/10.1016/j.biotechadv.2016.09.001

Binod, P., Gnansounou, E., Sindhu, R., Pandey, A., 2019. Enzymes for second generation biofuels: Recent developments and future perspectives. Bioresour. Technol. Reports 5, 317–325. https://doi.org/10.1016/j.biteb.2018.06.005

Binod, P., Satyanagalakshmi, K., Sindhu, R., Janu, K.U., Sukumaran, R.K., Pandey, A., 2012. Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. Renew. Energy 37, 109–116. https://doi.org/10.1016/J.RENENE.2011.06.007

Birijlall, N., Manimaran, A., Kumar, K.S., Permaul, K., Singh, S., 2011. High level expression of a recombinant xylanase by *Pichia pastoris* NC38 in a 5 L fermenter and its efficiency in biobleaching of bagasse pulp. Bioresour. Technol. 102, 9723–9. https://doi.org/10.1016/j.biortech.2011.07.059

Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of

uronic acids. Anal. Biochem. 54, 484–489. https://doi.org/10.1016/0003-2697(73)90377-1

Boonchuay, P., Takenaka, S., Kuntiya, A., Techapun, C., Leksawasdi, N., Seesuriyachan, P., Chaiyaso, T., 2016. Purification, characterization, and molecular cloning of the xylanase from *Streptomyces thermovulgaris* TISTR1948 and its application to xylooligosaccharide production. J. Mol. Catal. B Enzym. 129, 61–68. https://doi.org/10.1016/J.MOLCATB.2016.03.014

Boonrung, S., Katekaew, S., Mongkolthanaruk, W., Aimi, T., Boonlue, S., 2016. Purification and characterization of low molecular weight extreme alkaline xylanase from the thermophilic fungus *Myceliophthora thermophila* BF1-7. Mycoscience 57, 408–416. https://doi.org/10.1016/J.MYC.2016.07.003

Borsenberger, V., Dornez, E., Desrousseaux, M.-L., Massou, S., Tenkanen, M., Courtin, C.M., Dumon, C., O'Donohue, M.J., Fauré, R., 2014. A 1H NMR study of the specificity of α-L-arabinofuranosidases on natural and unnatural substrates. Biochim. Biophys. Acta - Gen. Subj. 1840, 3106–3114. https://doi.org/10.1016/J.BBAGEN.2014.07.001

Bosetto, A., Justo, P.I., Zanardi, B., Venzon, S.S., Graciano, L., dos Santos, E.L., de Cássia Garcia Simão, R., 2016. Research progress concerning fungal and bacterial β-xylosidases. Appl. Biochem. Biotechnol. 178, 766–795. https://doi.org/10.1007/s12010-015-1908-4

Bouacem, K., Bouanane-Darenfed, A., Boucherba, N., Joseph, M., Gagaoua, M., Ben Hania, W., Kecha, M., Benallaoua, S., Hacène, H., Ollivier, B., Fardeau, M.-L., 2014. Partial characterization of xylanase produced by *Caldicoprobacter algeriensis*, a new thermophilic anaerobic bacterium isolated from an Algerian hot spring. Appl. Biochem. Biotechnol. 174, 1969–1981. https://doi.org/10.1007/s12010-014-1153-2

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K.B., Ramakrishnan, S., 2011. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. Enzyme Res. 2011, 1–17. https://doi.org/10.4061/2011/787532

Brownawell, A.M., Caers, W., Gibson, G.R., Kendall, C.W.C., Lewis, K.D., Ringel, Y., Slavin,

J.L., 2012. Prebiotics and the health benefits of fiber: current regulatory status, future research, and goals. J. Nutr. 142, 962–74. https://doi.org/10.3945/jn.112.158147

Buruiana, C., Gomez, B., Vizireanu, C., Garrote, G., 2017. Manufacture and evaluation of xylooligosaccharides from corn stover as emerging prebiotic candidates for human health. LWT - Food Sci. Technol. 77, 449–459. https://doi.org/10.1016/j.lwt.2016.11.083

Buschke, N., Schafer, R., Becker, J., Wittmann, C., 2013. Metabolic engineering of industrial platform microorganisms for biorefinery applications-optimization of substrate spectrum and process robustness by rational and evolutive strategies. Bioresour. Technol. 135, 544–554. https://doi.org/10.1016/j.biortech.2012.11.047

Busse-Wicher, M., Gomes, T.C.F., Tryfona, T., Nikolovski, N., Stott, K., Grantham, N.J., Bolam, D.N., Skaf, M.S., Dupree, P., 2014. The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of *Arabidopsis thaliana*. Plant J. 79, 492–506. https://doi.org/10.1111/tpj.12575

Cannio, R., Di Prizito, N., Rossi Alessandra Morana, M., 2004. A xylan-degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity. Extremophiles 8, 117–124. https://doi.org/10.1007/s00792-003-0370-3

Cantu-Jungles, T.M., Iacomini, M., Cipriani, T.R., Cordeiro, L.M.C., 2017. Isolation and characterization of a xylan with industrial and biomedical applications from edible açaí berries (*Euterpe oleraceae*). Food Chem. 221, 1595–1597. https://doi.org/10.1016/j.foodchem.2016.10.133

Cardona, C.A., Sánchez, Ó.J., 2007. Fuel ethanol production: Process design trends and integration opportunities. Bioresour. Technol. 98, 2415–2457. https://doi.org/10.1016/J.BIORTECH.2007.01.002

Cartmell, A., McKee, L.S., Peña, M.J., Larsbrink, J., Brumer, H., Kaneko, S., Ichinose, H., Lewis, R.J., Viksø-Nielsen, A., Gilbert, H.J., Marles-Wright, J., 2011. The structure and function of an arabinan-specific alpha-1,2-arabinofuranosidase identified from screening the

activities of bacterial GH43 glycoside hydrolases. J. Biol. Chem. 286, 15483–15495. https://doi.org/10.1074/jbc.M110.215962

Carvalho, A.F.A., Neto, P.D.O., da Silva, D.F., Pastore, G.M., 2013. Xylo-oligosaccharides from lignocellulosic materials: Chemical structure, health benefits and production by chemical and enzymatic hydrolysis. Food Res. Int. 51, 75–85. https://doi.org/10.1016/j.foodres.2012.11.021

Cavinato, C., Ugurlu, A., de Godos, I., Kendir, E., Gonzalez-Fernandez, C., 2017. Biogas production from microalgae. Microalgae-Based Biofuels Bioprod. 155–182. https://doi.org/10.1016/B978-0-08-101023-5.00007-8

Celinska, E., Grajek, W., 2009. Biotechnological production of 2,3-butanediol–current state and prospects. Biotechnol. Adv. 27, 715–725. https://doi.org/10.1016/j.biotechadv.2009.05.002

Chadha, B.S., Kaur, B., Basotra, N., Tsang, A., Pandey, A., 2019. Thermostable xylanases from thermophilic fungi and bacteria: Current perspective. Bioresour. Technol. 277, 195–203. https://doi.org/10.1016/j.biortech.2019.01.044

Chandel, A.K., Garlapati, V.K., Singh, A.K., Antunes, F.A.F., da Silva, S.S., 2018. The path forward for lignocellulose biorefineries: Bottlenecks, solutions, and perspective on commercialization. Bioresour. Technol. 264, 370–381. https://doi.org/10.1016/J.BIORTECH.2018.06.004

Chandel, K., Jandaik, S., Kumari, V., Sarswati, S., Sharma, A., Kumar, D., Kumar, N., 2013. Isolation, purification and screening of cellulolytic fungi from mushroom compost for production of enzyme (cellulase). Int. J. Curr. Res. 5, 222–229.

Chapla, D., 2012. Application of xylanase from Aspergillus foetidus MTCC 4898 for production of xylooligosaccharides from agro-residues. Sardar Patel University.

Chapla, D., Divecha, J., Madamwar, D., Shah, A., 2010. Utilization of agro-industrial waste for xylanase production by *Aspergillus foetidus* MTCC 4898 under solid state fermentation and its application in saccharification. Biochem. Eng. J. 49, 361–369.

https://doi.org/10.1016/j.bej.2010.01.012

Chapla, D., Pandit, P., Shah, A., 2012. Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Bioresour. Technol. 115, 215–221. https://doi.org/10.1016/j.biortech.2011.10.083

Chavez, R., Bull, P., Eyzaguirre, J., 2006. The xylanolytic enzyme system from the genus *Penicillium*. J. Biotechnol. 123, 413–433. https://doi.org/10.1016/j.jbiotec.2005.12.036

Chen, H., Liu, J., Chang, X., Chen, D., Xue, Y., Liu, P., Lin, H., Han, S., 2017. A review on the pretreatment of lignocellulose for high-value chemicals. Fuel Process. Technol. 160, 196–206. https://doi.org/10.1016/j.fuproc.2016.12.007

Chen, J., Zhang, W., Tan, L., Wang, Y., He, G., 2009. Optimization of metabolic pathways for bioconversion of lignocellulose to ethanol through genetic engineering. Biotechnol. Adv. 27, 593–598. https://doi.org/10.1016/J.BIOTECHADV.2009.04.021

Chen, W., Guo, C., Hussain, S., Zhu, B., Deng, F., Xue, Y., Geng, M., Wu, L., 2016. Role of xylo-oligosaccharides in protection against salinity-induced adversities in Chinese cabbage. Environ. Sci. Pollut. Res. 23, 1254–1264. https://doi.org/10.1007/s11356-015-5361-2

Cheng, Y.-S., Chen, C.-C., Huang, C.-H., Ko, T.-P., Luo, W., Huang, J.-W., Liu, J.-R., Guo, R.-T., 2014. Structural analysis of a glycoside hydrolase family 11 xylanase from *Neocallimastix patriciarum*: Insight into the molecular basis of a thermophilic enzyme. J. Biol. Chem. 289, 11020–11028. https://doi.org/10.1074/jbc.M114.550905

Cherubin, M.R., Oliveira, D.M. da S., Feigl, B.J., Pimentel, L.G., Lisboa, I.P., Gmach, M.R., Varanda, L.L., Morais, M.C., Satiro, L.S., Popin, G.V., Paiva, S.R. de, Santos, A.K.B. dos, Vasconcelos, A.L.S. de, Melo, P.L.A. de, Cerri, C.E.P., Cerri, C.C., Cherubin, M.R., Oliveira, D.M. da S., Feigl, B.J., Pimentel, L.G., Lisboa, I.P., Gmach, M.R., Varanda, L.L., Morais, M.C., Satiro, L.S., Popin, G.V., Paiva, S.R. de, Santos, A.K.B. dos, Vasconcelos, A.L.S. de, Melo, P.L.A. de, Cerri, C.E.P., Cerri, C.C., 2018. Crop residue harvest for bioenergy production and its implications on soil functioning and plant growth: A review.

Sci. Agric. 75, 255–272. https://doi.org/10.1590/1678-992x-2016-0459

Chiang, C.F., Lai, L.S., 2019. Effect of enzyme-assisted extraction on the physicochemical properties of mucilage from the fronds of *Asplenium australasicum* (J. Sm.) Hook. Int. J. Biol. Macromol. 124, 346–353. https://doi.org/10.1016/j.ijbiomac.2018.11.181

Choque Delgado, G.T., Tamashiro, W.M.D.S.C., Junior, M.R.M.M. roberto M., Moreno, Y.M.F., Pastore, G.M., Delgado, G.T.C., Tamashiro, W.M.D.S.C., Junior, M.R.M.M. roberto M., Moreno, Y.M.F., Pastore, G.M., 2011. The putative effects of prebiotics as immunomodulatory agents. Food Res. Int. 44, 3167–3173. https://doi.org/10.1016/j.foodres.2011.07.032

Christakopoulos, P., Katapodis, P., Kalogeris, E., Kekos, D., Macris, B.J., Stamatis, H., Skaltsa, H., 2003. Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. Interntional J. Biol. Macromol. 31, 171–175. https://doi.org/10.1016/S0141-8130(02)00079-X

Chu, Q., Li, X., Xu, Y., Wang, Z., Huang, J., Yu, S., Yong, Q., 2014. Functional cello-oligosaccharides production from the corncob residues of xylo-oligosaccharides manufacture. Process Biochem. 49, 1217–1222. https://doi.org/10.1016/j.procbio.2014.05.007

Colodel, C., Vriesmann, L.C., de Oliveira Petkowicz, C.L., 2018. Cell wall polysaccharides from Ponkan mandarin (*Citrus reticulata* Blanco cv. *Ponkan*) peel. Carbohydr. Polym. 195, 120–127. https://doi.org/10.1016/J.CARBPOL.2018.04.066

Comlekcioglu, U., Aygan, A., Yazdic, F.C., Ozkose, E., 2011. Effects of various agro-wastes on xylanase and b-xylosidase production of anaerobic ruminal fungi. J. Sci. Ind. Res. (India). 70, 293–299.

Cordeiro, L.M.C., Reinhardt, V. de F., Baggio, C.H., Werner, M.F. de P., Burci, L.M., Sassaki, G.L., Iacomini, M., 2012. Arabinan and arabinan-rich pectic polysaccharides from quinoa (*Chenopodium quinoa*) seeds: Structure and gastroprotective activity. Food Chem. 130,

937–944. https://doi.org/10.1016/J.FOODCHEM.2011.08.020

Córdova, A., Astudillo, C., Illanes, A., 2019. Membrane technology for the purification of enzymatically produced oligosaccharides, in: Separation of Functional Molecules in Food by Membrane Technology. pp. 113–153. https://doi.org/10.1016/B978-0-12-815056-6.00004-8

Corma, A., Iborra, S., Velty, A., 2007. Chemical routes for the transformation of biomass into chemicals. Chem. Rev. 107, 2411–2502. https://doi.org/10.1021/CR050989D

Crittenden, R., Playne, M.J., 2008. Prebiotics, in: Handbook of Probiotics and Prebiotics. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 533–581. https://doi.org/10.1002/9780470432624.ch7

Cruz, A.G., Walter, E.H.M., Cadena, R.S., Faria, J.A.F., Bolini, H.M.A., Pinheiro, H.P., Sant'Ana, A.S., 2010. Survival analysis methodology to predict the shelf-life of probiotic flavored yogurt. Food Res. Int. 43, 1444–1448. https://doi.org/10.1016/J.FOODRES.2010.04.028

da Costa, W.K.A., Brandão, L.R., Martino, M.E., Garcia, E.F., Alves, A.F., de Souza, E.L., de Souza Aquino, J., Saarela, M., Leulier, F., Vidal, H., Magnani, M., 2018. Qualification of tropical fruit-derived *Lactobacillus plantarum* strains as potential probiotics acting on blood glucose and total cholesterol levels in Wistar rats. Food Res. Int. https://doi.org/10.1016/J.FOODRES.2018.08.035

da Silva, A.E., Marcelino, H.R., Gomes, M.C.S., Oliveira, E.E., Nagashima Jr, T., Egito, E.S.T., 2012. Xylan, a promising hemicellulose for pharmaceutical use, in: Products and Applications of Biopolymers. pp. 61–85. https://doi.org/10.5772/33070

da Silva Menezes, B., Rossi, D.M., Squina, F., Ayub, M.A.Z., 2018. Xylooligosaccharides production by fungi cultivations in rice husk and their application as substrate for lactic acid bacteria growth. Bioresour. Technol. Reports 2, 100–106. https://doi.org/10.1016/J.BITEB.2018.05.004

da Silva, S.S., Chandel, A.K., Wickramasinghe, S.R., Dominguez, J.M., 2012. Fermentative production of value-added products from lignocellulosic biomass. J. Biomed. Biotechnol. 2012, 2. https://doi.org/10.1155/2012/826162

Das, D., Veziroğlu, T.N., 2001. Hydrogen production by biological processes: a survey of literature. Int. J. Hydrogen Energy 26, 13–28. https://doi.org/10.1016/S0360-3199(00)00058-6

Datta, R., Henry, M., 2006. Lactic acid: recent advances in products, processes and technologies - a review. J. Chem. Technol. Biotechnol. 81, 1119–1129. https://doi.org/10.1002/jctb.1486

Dávila, I., Gullón, B., Alonso, J.L., Labidi, J., Gullón, P., 2019. Vine shoots as new source for the manufacture of prebiotic oligosaccharides. Carbohydr. Polym. 207, 34–43. https://doi.org/10.1016/J.CARBPOL.2018.11.065

De Bhowmick, G., Sarmah, A.K., Sen, R., 2018. Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products. Bioresour. Technol. 247, 1144–1154. https://doi.org/10.1016/j.biortech.2017.09.163

de Castro, E., Sigrist, C.J.A., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P.S., Gasteiger, E., Bairoch, A., Hulo, N., 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res. 34, 362–365. https://doi.org/10.1093/nar/gkl124

de Freitas, C., Carmona, E., Brienzo, M., 2019. Xylooligosaccharides production process from lignocellulosic biomass and bioactive effects. Bioact. Carbohydrates Diet. Fibre 18, 1–8. https://doi.org/10.1016/J.BCDF.2019.100184

de Melo Pereira, G.V., de Oliveira Coelho, B., Magalhães Júnior, A.I., Thomaz-Soccol, V., Soccol, C.R., 2018. How to select a probiotic? A review and update of methods and criteria. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2018.09.003

Diaz, A.B., Blandino, A., Caro, I., 2018. Value added products from fermentation of sugars derived from agro-food residues. Trends Food Sci. Technol. 71, 52–64.

https://doi.org/10.1016/j.tifs.2017.10.016

Ding, H.H., Cui, S.W., Goff, H.D., Chen, J., Wang, Q., Han, N.F., 2015. Arabinan-rich rhamnogalacturonan-I from flaxseed kernel cell wall. Food Hydrocoll. 47, 158–167. https://doi.org/10.1016/J.FOODHYD.2015.01.011

Do, V.H., Mun, S., Kim, Y.L., Rho, S.J., Park, K.H., Kim, Y.R., 2016. Novel formulation of low-fat spread using rice starch modified by 4-α-glucanotransferase. Food Chem. 208, 132–141. https://doi.org/10.1016/j.foodchem.2016.03.101

Dobozi, M.S., Szakács, G., Bruschi, C. V., 1992. Xylanase activity of *Phanerchaete chrysosporium*. Appl. Environ. Microbiol. 58, 3466 – 3471. https://doi.org/10.1128/aem.58.11.3466-3471.1992

Dorn, M., e Silva, M.B., Buriol, L.S., Lamb, L.C., 2014. Three-dimensional protein structure prediction: Methods and computational strategies. Comput. Biol. Chem. 53, 251–276. https://doi.org/10.1016/J.COMPBIOLCHEM.2014.10.001

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. https://doi.org/10.1021/ac60111a017

Dutta, B., Banerjee, A., Chakraborty, P., Bandopadhyay, R., 2018. In silico studies on bacterial xylanase enzyme: structural and functional insight. J. Genet. Eng. Biotechnol. 16, 749–756. https://doi.org/10.1016/j.jgeb.2018.05.003

Dutta, S., Pal, S., 2014. Promises in direct conversion of cellulose and lignocellulosic biomass to chemicals and fuels: Combined solvent–nanocatalysis approach for biorefinary. Biomass and Bioenergy 62, 182–197. https://doi.org/10.1016/j.biombioe.2013.12.019

Earle, M.J., Seddon, K.R., 2000. Ionic liquids. Green solvents for the future, Chemical and Engineering News.

Ebringerová, A., Heinze, T., 2000. Xylan derivatives - biopolymers with valuable properties. Macromol. Rapid Commun. 21, 542–556.

Ebringerová, A., Kramár, A., Rendoš, F., Domansky, R., 1967. Die Stufenextraktion der Hemicellulosen aus dem Holz der Hagebuche (*Carpinus betulus* L.). Holzforschung 21, 74–77. https://doi.org/10.1515/hfsg.1967.21.3.74

Eckardt, N.A., 2008. Role of xyloglucan in primary cell walls. Plant Cell 20, 1421–1422. https://doi.org/10.1105/tpc.108.061382

Elegir, G., Szakács, G., Jeffries, T.W., 1994. Purification, characterization, and substrate specificities of multiple xylanases from *Streptomyces* sp. strain B-12-2. Appl. Environ. Microbiol. 60, 2609–15.

Elleuche, S., Schäfers, C., Blank, S., Schröder, C., Antranikian, G., 2015. Exploration of extremophiles for high temperature biotechnological processes. Curr. Opin. Microbiol. 25, 113–119. https://doi.org/10.1016/j.mib.2015.05.011

Ellis, J.T., Magnuson, T.S., 2012. Thermostable and alkalistable xylanases produced by the thermophilic bacterium *Anoxybacillus flavithermus* TWXYL3. ISRN Microbiol. 2012, 1–8. https://doi.org/10.5402/2012/517524

Englyst, H.N., Cummings, J.H., 1984. Simplified method for the measurement of total non-starch polysaccharides by gas - liquid chromatography of constituent sugars as alditol acetates. Analyst 109, 937–942. https://doi.org/10.1039/an9840900937

Espinoza, K., Eyzaguirre, J., 2018. Identification, heterologous expression and characterization of a novel glycoside hydrolase family 30 xylanase from the fungus *Penicillium purpurogenum*. Carbohydr. Res. 468, 45–50. https://doi.org/10.1016/J.CARRES.2018.08.006

Fan, F., Zhuo, R., Sun, S., Wan, X., Jiang, M., Zhang, X., Yang, Y., 2011. Cloning and functional analysis of a new laccase gene from *Trametes* sp. 48424 which had the high yield of laccase and strong ability for decolorizing different dyes. Bioresour. Technol. 102, 3126–3137. https://doi.org/10.1016/j.biortech.2010.10.079

Fan, Y., Zhou, C., Zhu, X., 2009. Selective catalysis of lactic acid to produce commodity

chemicals. Catal. Rev. 51, 293–324. https://doi.org/10.1080/01614940903048513

Fang, Z., Smith Jr, R.L., Tian, X.-F., 2019. Production of materials from sustainable biomass resources. Springer Nature, Singapore.

Farias, C.L.A., Martinez, G.R., Cadena, S.M.S.C., Mercê, A.L.R., de Oliveira Petkowicz, C.L., Noleto, G.R., 2019. Cytotoxicity of xyloglucan from *Copaifera langsdorffii* and its complex with oxovanadium (IV/V) on B16F10 cells. Int. J. Biol. Macromol. 121, 1019–1028. https://doi.org/10.1016/J.IJBIOMAC.2018.10.131

Faryar, R., Linares-Pastén, J.A., Immerzeel, P., Mamo, G., Andersson, M., Stålbrand, H., Mattiasson, B., Nordberg Karlsson, E., 2015. Production of prebiotic xylooligosaccharides from alkaline extracted wheat straw using the K80R-variant of a thermostable alkali-tolerant xylanase. Food Bioprod. Process. 93, 1–10. https://doi.org/10.1016/j.fbp.2014.11.004

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution (N. Y). 39, 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Fernández, P. V., Quintana, I., Cerezo, A.S., Caramelo, J.J., Pol-Fachin, L., Verli, H., Estevez, J.M., Ciancia, M., 2013. Anticoagulant activity of a unique sulfated pyranosic (1→3)-β-L-arabinan through direct interaction with thrombin. J. Biol. Chem. 288, 223–233. https://doi.org/10.1074/jbc.M112.386441

Ferrão, L.L., Ferreira, M.V.S., Cavalcanti, R.N., Carvalho, A.F.A., Pimentel, T.C., Silva, R., Esmerino, E.A., Neto, R.P.C., Tavares, M.I.B., Freitas, M.Q., Menezes, J.C.V., Cabral, L.M., Moraes, J., Silva, M.C., Mathias, S.P., Raices, R.S.L., Pastore, G.M., Cruz, A.G., 2018. The xylooligosaccharide addition and sodium reduction in requeijão cremoso processed cheese. Food Res. Int. 107, 137–147. https://doi.org/10.1016/j.foodres.2018.02.018

Finegold, S.M., Li, Z., Summanen, P.H., Downes, J., Thames, G., Corbett, K., Dowd, S., Krak, M., Heber, D., 2014. Xylooligosaccharide increases bifidobacteria but not lactobacilli in human gut microbiota. Food Funct. 5, 436–45. https://doi.org/10.1039/c3fo60348b

FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A., 2010. A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. Bioresour. Technol. 101, 8915–8922. https://doi.org/10.1016/j.biortech.2010.06.125

Florio, C., Nastro, R.A., Flagiello, F., Minutillo, M., Pirozzi, D., Pasquale, V., Ausiello, A., Toscano, G., Jannelli, E., Dumontet, S., 2019. Biohydrogen production from solid phase-microbial fuel cell spent substrate: A preliminary study. J. Clean. Prod. 227, 506–511. https://doi.org/10.1016/J.JCLEPRO.2019.03.316

Forli, S., Huey, R., Pique, M.E., Sanner, M., Goodsell, D.S., Arthur, J., 2016. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. Nat. Protoc. 11, 905–919. https://doi.org/10.1038/nprot.2016.051

Gadhe, A., Sonawane, S.S., Varma, M.N., 2014. Ultrasonic pretreatment for an enhancement of biohydrogen production from complex food waste. Int. J. Hydrogen Energy 39, 7721–7729. https://doi.org/10.1016/j.ijhydene.2014.03.105

Gallardo, O., Fernández-Fernández, M., Valls, C., Valenzuela, S.V., Roncero, M.B., Vidal, T., Díaz, P., Pastor, F.I.J., 2010. Characterization of a family GH5 xylanase with activity on neutral oligosaccharides and evaluation as a pulp bleaching aid. Appl. Environ. Microbiol. 76, 6290–6294. https://doi.org/10.1128/AEM.00871-10

Gao, C., Ma, C., Xu, P., 2011. Biotechnological routes based on lactic acid production from biomass. Biotechnol. Adv. 29, 930–939. https://doi.org/10.1016/j.biotechadv.2011.07.022

Gao, X., Kumar, R., Wyman, C.E., 2014. Fast hemicellulose quantification via a simple one-step acid hydrolysis. Biotechnol. Bioeng. 111, 1088–1096. https://doi.org/10.1002/bit.25174

George, J., Sabapathi, S.N., 2015. Cellulose nanocrystals: Synthesis, functional properties, and applications. Nanotechnol. Sci. Appl. 8, 45–54. https://doi.org/10.2147/NSA.S64386

Ghose, T.K., Bisaria, V.S., 1987. Measurement of hemicellulase activities: Part I Xylanases. Pure Appl. Chem. 59, 1739–1751. https://doi.org/10.1351/pac198759121739

Ghosh, A.K., Naskar, A.K., Jana, M.L., Khowala, S., Sengupta, S., 1995. Purification and characterization if an amyloglucosidase from *Termitomyces clypeatus* that liberates glucose from xylan. Biotechnol. Prog. 11, 453–456. https://doi.org/10.1021/bp00034a013

Ghosh, S., Roy, S., 2019. Novel integration of biohydrogen production with fungal biodiesel production process. Bioresour. Technol. 288, 121603. https://doi.org/10.1016/J.BIORTECH.2019.121603

Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J., Scott, K., Stanton, C., Swanson, K.S., Cani, P.D., Verbeke, K., Reid, G., 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat. Rev. Gastroenterol. Hepatol. 14, 491. https://doi.org/10.1038/nrgastro.2017.75

Gil-Durán, C., Ravanal, M.-C., Ubilla, P., Vaca, I., Chávez, R., 2018. Heterologous expression, purification and characterization of a highly thermolabile endoxylanase from the Antarctic fungus *Cladosporium* sp. Fungal Biol. 122, 875–882. https://doi.org/10.1016/J.FUNBIO.2018.05.002

Gillet, S., Aguedo, M., Petitjean, L., Morais, A.R.C., Da Costa Lopes, A.M., Łukasik, R.M., Anastas, P.T., 2017. Lignin transformations for high value applications: Towards targeted modifications using green chemistry. Green Chem. 19, 4200–4233. https://doi.org/10.1039/c7gc01479a

Global industrial enzymes market overview, 2018. Global industrial enzymes market overview 2018-Forecast to 2024 Press Release.

Golan, G., Shallom, D., Teplitsky, A., Zaide, G., Shulami, S., Baasov, T., Stojanoff, V., Thompson, A., Shoham, Y., Shoham, G., 2004. Crystal structures of *Geobacillus stearothermophilus* alpha-glucuronidase complexed with its substrate and products: mechanistic implications. J. Biol. Chem. 279, 3014–3024. https://doi.org/10.1074/jbc.M310098200

Gonçalves, G.A.L.L., Takasugi, Y., Jia, L., Mori, Y., Noda, S., Tanaka, T., Ichinose, H., Kamiya, N., 2015. Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. Enzyme Microb. Technol. 72, 16–24. https://doi.org/10.1016/j.enzmictec.2015.01.007

Goulet, O., Hojsak, I., Kolacek, S., Pop, T.L., Cokugras, F.C., Zuccotti, G., Pettoello-Mantovani, M., Fabiano, V., 2019. Paediatricians play a key role in preventing early harmful events that could permanently influence the development of the gut microbiota in childhood. Acta Paediatr. 108, 1942–1954. https://doi.org/10.1111/apa.14900

Gowdhaman, D., Ponnusami, V., 2015. Production and optimization of xylooligosaccharides from corncob by *Bacillus aerophilus* KGJ2 xylanase and its antioxidant potential. Int. J. Biol. Macromol. 79, 595–600. https://doi.org/10.1016/J.IJBIOMAC.2015.05.046

Gramany, V., Khan, F.I., Govender, A., Bisetty, K., Singh, S., Permaul, K., 2015. Cloning, expression, and molecular dynamics simulations of a xylosidase obtained from *Thermomyces lanuginosus*. J. Biomol. Struct. Dyn. 1102, 1–12. https://doi.org/10.1080/07391102.2015.1089186

Guimaraes, N.C. de A., Sorgatto, M., Peixoto-Nogueira, S. de C., Betini, J.H.A., Zanoelo, F.F., Marques, M.R., Polizeli, M. de L.T. de M., Giannesi, G.C., 2013. Bioprocess and biotechnology: Effect of xylanase from *Aspergillus niger* and *Aspergillus flavus* on pulp biobleaching and enzyme production using agroindustrial residues as substract. Springerplus 2, 1–7. https://doi.org/10.1186/2193-1801-2-380

Gullón, B., Gullón, P., Tavaria, F., Pintado, M., Gomes, A.M., Alonso, J.L., Parajó, J.C., 2014. Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. J. Funct. Foods 6, 438–449. https://doi.org/10.1016/j.jff.2013.11.010

Gullón, P., Pereiro, G., Alonso, J.L., Parajó, J.C., 2009. Aqueous pretreatment of agricultural wastes: Characterization of soluble reaction products. Bioresour. Technol. 100, 5840–5845.

https://doi.org/10.1016/J.BIORTECH.2009.06.011

Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, J.-P., 2010. Hydrogen production from agricultural waste by dark fermentation: A review. Int. J. Hydrogen Energy 35, 10660–10673. https://doi.org/10.1016/J.IJHYDENE.2010.03.008

Gurpilhares, D. de B., Cinelli, L.P., Simas, N.K., Pessoa Jr., A., Sette, L.D., 2019. Marine prebiotics: Polysaccharides and oligosaccharides obtained by using microbial enzymes. Food Chem. 280, 175–186. https://doi.org/10.1016/J.FOODCHEM.2018.12.023

Hachem, K., Faugeron, C., Kaid-Harche, M., Gloaguen, V., 2016. Structural investigation of cell wall xylan polysaccharides from the leaves of Algerian *Argania spinosa*. Molecules 21, 1–10. https://doi.org/10.3390/molecules21111587

Hagglund, P., 2002. Mannan-hydrolysis by hemicellulases. Lund University.

Haki, G.D., Rakshit, S.K., 2003. Developments in industrially important thermostable enzymes: a review. Bioresour. Technol. 89, 17–34. https://doi.org/10.1016/S0960-8524(03)00033-6

Haldar, D., Purkait, M.K., 2020. Lignocellulosic conversion into value-added products: A review. Process Biochem. In press. https://doi.org/10.1016/j.procbio.2019.10.001

Haltrich, D., Nidetzky, B., Kulbe, K.D., Steiner, W., Župančič, S., 1996. Production of fungal xylanases. Bioresour. Technol. 58, 137–161. https://doi.org/10.1016/S0960-8524(96)00094-6

Harmsen, P., Lips, S., Bakker, R., 2013. Pretreatment of lignocellulose for biotechnological production of lactic acid. AA Wageningen, The Netherlands.

Harris, A.D., Ramalingam, C., 2010. Xylanases and its application in food industry: a review. J. Exp. Sci. 1, 1–11.

Harris, P. V, Xu, F., Kreel, N.E., Kang, C., Fukuyama, S., 2014. New enzyme insights drive advances in commercial ethanol production. Curr. Opin. Chem. Biol. 19, 162–170. https://doi.org/10.1016/j.cbpa.2014.02.015

Hassan, S.S., Williams, G.A., Jaiswal, A.K., 2018. Emerging technologies for the pretreatment of lignocellulosic biomass. Bioresour. Technol. 262, 310–318. https://doi.org/10.1016/j.biortech.2018.04.099

Hasunuma, T., Kondo, A., 2012. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. Process Biochem. 47, 1287–1294. https://doi.org/10.1016/J.PROCBIO.2012.05.004

Hasunuma, T., Okazaki, F., Okai, N., Hara, K.Y., Ishii, J., Kondo, A., 2013. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. Bioresour. Technol. 135, 513–522. https://doi.org/10.1016/j.biortech.2012.10.047

Hawkes, F.R., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D.L., 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int. J. Hydrogen Energy 32, 172–184. https://doi.org/10.1016/J.IJHYDENE.2006.08.014

Heikkinen, S., 2016. Biodegradable films from cereal arabinoxylans. University of Helsinki.

Heinen, P.R., Bauermeister, A., Ribeiro, L.F., Messias, J.M., Almeida, P.Z., Moraes, L.A.B., Vargas-rechia, C.G., Oliveira, A.H.C. De, Ward, R.J., Filho, E.X.F., Kadowaki, M.K., Jorge, J.A., Polizeli, M.L.T.M., 2018. GH11 xylanase from *Aspergillus tamarii* Kita: purification by one-step chromatography and xylooligosaccharides hydrolysis monitored in real-time by mass spectrometry. Int. J. Biol. Macromol. 108, 291–299. https://doi.org/10.1016/j.ijbiomac.2017.11.150

Ho, A.L., Kosik, O., Lovegrove, A., Charalampopoulos, D., Rastall, R.A., 2018. In vitro fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with different molecular weights by human faecal bacteria. Carbohydr. Polym. 179, 50–58. https://doi.org/10.1016/j.carbpol.2017.08.077

Hoch, G., 2007. Cell wall hemicelluloses as mobile carbon stores in non-reproductive plant tissues. Funct. Ecol. 21, 823–834. https://doi.org/10.1111/j.1365-2435.2007.01305.x

Hoseinifar, S.H., Sharifian, M., Vesaghi, M.J., Khalili, M., Esteban, M.Á., 2014. The effects of dietary xylooligosaccharide on mucosal parameters, intestinal microbiota and morphology and growth performance of Caspian white fish (*Rutilus frisii* kutum) fry. Fish Shellfish Immunol. 39, 231–6. https://doi.org/10.1016/j.fsi.2014.05.009

Hua, D.-R., Wu, Y.-L., Liu, Y.-F., Chen, Y., Yang, M.-D., Lu, X.-N., Li, J., 2016. Preparation of furfural and reaction kinetics of xylose dehydration to furfural in high-temperature water. Pet. Sci. 13, 167–172. https://doi.org/10.1007/s12182-015-0069-y

Hubbell, C.A., Ragauskas, A.J., 2010. Effect of acid-chlorite delignification on cellulose degree of polymerization. Bioresour. Technol. 101, 7410–7415. https://doi.org/10.1016/J.BIORTECH.2010.04.029

Huijgen, W.J.J., Smit, A.T., Reith, J.H., Uil, H. Den, 2011. Catalytic organosolv fractionation of willow wood and wheat straw as pretreatment for enzymatic cellulose hydrolysis. J. Chem. Technol. Biotechnol. 86, 1428–1438. https://doi.org/10.1002/jctb.2654

Imanaka, T., Sakurai, S., 1992. Method of washing super precision devices, semiconductors, with enzymes. 5,078,802.

Irdawati, I., Syamsuardi, S., Agustien, A., Rilda, Y., 2018. Screening of thermophilic bacteria produce xylanase from Sapan Sungai Aro hot spring South Solok. IOP Conf. Ser. Mater. Sci. Eng. 335. https://doi.org/10.1088/1757-899X/335/1/012021

Ishii, T., Konishi, T., Yamasaki, T., Enomoto, A., Yoshida, M., Maeda, I., Shimizu, K., 2010. NMR characterization of acidic xylo-oligosaccharides containing two methylglucuronic acid residues from Japanese cedar and Hinoki cypress. Carbohydr. Polym. 81, 964–968. https://doi.org/10.1016/j.carbpol.2010.03.055

Isikgor, F.H., Becer, C.R., 2015. Lignocellulosic biomass: A sustainable platform for production of bio-based chemicals and polymers. Polym. Chem. 6, 4497–4559. https://doi.org/10.1039/C5PY00263J

Jandhyala, S.M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., Nageshwar Reddy,

D., 2015. Role of the normal gut microbiota. World J. Gastroenterol. 21, 8787–803. https://doi.org/10.3748/wjg.v21.i29.8787

Jarboe, L.R., Grabar, T.B., Yomano, L.P., Shanmugan, K.T., Ingram, L.O., 2007. Development of ethanologenic bacteria, in: Biofuels. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 237–261. https://doi.org/10.1007/10_2007_068

Jemli, S., Ayadi-Zouari, D., Hlima, H. Ben, Bejar, S., 2016. Biocatalysts: Application and engineering for industrial purposes. Crit. Rev. Biotechnol. https://doi.org/10.3109/07388551.2014.950550

Jia, X., Mi, S., Wang, J., Qiao, W., Peng, X., Han, Y., 2014. Insight into glycoside hydrolases for debranched xylan degradation from extremely thermophilic bacterium *Caldicellulosiruptor lactoaceticus*. PLoS One 9, e106482. https://doi.org/10.1371/journal.pone.0106482

Jiang, M., Xu, R., Xi, Y.L., Zhang, J.H., Dai, W.Y., Wan, Y.J., Chen, K.Q., Wei, P., 2013. Succinic acid production from cellobiose by *Actinobacillus succinogenes*. Bioresour. Technol. 135, 469–474. https://doi.org/10.1016/j.biortech.2012.10.019

John, R.P., G.S., A., Nampoothiri, K.M., Pandey, A., 2009. Direct lactic acid fermentation: Focus on simultaneous saccharification and lactic acid production. Biotechnol. Adv. 27, 145–152. https://doi.org/10.1016/j.biotechadv.2008.10.004

Johnravindar, D., Murugesan, K., Wong, J.W.C., Elangovan, N., 2017. Waste-to-biofuel: production of biobutanol from sago waste residues. Environ. Technol. (United Kingdom) 38, 1725–1734. https://doi.org/10.1080/09593330.2017.1283362

Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103–112. https://doi.org/10.1016/J.BIORTECH.2015.10.009

Jordan, D.B., Wagschal, K., 2010. Properties and applications of microbial β-D-xylosidases featuring the catalytically efficient enzyme from *Selenomonas ruminantium*. Appl.

Microbiol. Biotechnol. 86, 1647–1658. https://doi.org/10.1007/s00253-010-2538-y

Jung, Y., Kim, H., Song, D.-S., Choi, I.-G., Yang, T., Lee, H., Seung, D., Kim, K., 2014. Feasibility test of utilizing *Saccharophagus degradans* 2-40T as the source of crude enzyme for the saccharification of lignocellulose. Bioprocess Biosyst. Eng. 37, 707–710. https://doi.org/10.1007/s00449-013-1040-1

Juturu, V., Wu, J.C., 2012. Microbial xylanases: Engineering, production and industrial applications. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2011.11.006

Kabel, M.., Schols, H.., Voragen, A.G.., 2002. Complex xylo-oligosaccharides identified from hydrothermally treated Eucalyptus wood and brewery's spent grain. Carbohydr. Polym. 50, 191–200. https://doi.org/10.1016/S0144-8617(02)00022-X

Kajaste, R., 2014. Chemicals from biomass – managing greenhouse gas emissions in biorefinery production chains – a review. J. Clean. Prod. 75, 1–10. https://doi.org/10.1016/j.jclepro.2014.03.070

Kalim, B., Böhringer, N., Ali, N., Schäberle, T., 2015. Xylanases–from microbial origin to industrial application. Br. Biotechnol. J. 7, 1–20. https://doi.org/10.9734/bbj/2015/15982

Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P., Angelidaki, I., 2009. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresour. Technol. 100, 2562–2568. https://doi.org/10.1016/j.biortech.2008.11.011

Karlsson, E.N., Schmitz, E., Linares-Pastén, J.A., Adlercreutz, P., 2018. Endo-xylanases as tools for production of substituted xylooligosaccharides with prebiotic properties. Appl. Microbiol. Biotechnol. 102, 9081–9088. https://doi.org/10.1007/s00253-018-9343-4

Katapodis, P., Vardakou, M., Kalogeris, E., Kekos, D., Macris, B.J., Christakopoulos, P., 2003. Enzymic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan. Eur. J. Nutr. 42, 55–60.

Kaushik, P., Mishra, A., Malik, A., 2014. Dual application of agricultural residues for xylanase production and dye removal through solid state fermentation. Int. Biodeterior.

Biodegradation 96, 1–8. https://doi.org/10.1016/j.ibiod.2014.08.006

Kazemi, A., Rasoul-Amini, S., Shahbazi, M., Safari, A., Ghasemi, Y., 2014. Isolation, identification and media optimization of high level cellulase production by *Bacillus* sp. BCCS A3, in a fermentation system using respnse surface methodology. Prep. Biochem. Biotechnol. 44, 107–118.

Khamtib, S., Reungsang, A., 2012. Biohydrogen production from xylose by *Thermoanaerobacterium thermosaccharolyticum* KKU19 isolated from hot spring sediment. Int. J. Hydrogen Energy 37, 12219–12228. https://doi.org/10.1016/j.ijhydene.2012.06.038

Khan, F.I., Govender, A., Permaul, K., Singh, S., Bisetty, K., 2015. Thermostable chitinase II from *Thermomyces lanuginosus* SSBP: Cloning, structure prediction and molecular dynamics simulations. J. Theor. Biol. 374, 107–114. https://doi.org/10.1016/j.jtbi.2015.03.035

Khokhar, I., Haider, M.S., Mushtaq, S., Mukhtar, I., 2012. Isolation and screening of highly cellulolytic filamentous fungi. J. Appl. Sci. Environ. Manag. 16, 223–226.

Kim, H.M., Choi, Y.-S., Lee, D.-S., Kim, Y.-H., Bae, H.-J., 2017. Production of bio-sugar and bioethanol from coffee residue (CR) by acid-chlorite pretreatment. Bioresour. Technol. 236, 194–201. https://doi.org/10.1016/J.BIORTECH.2017.03.143

Kim, H.M., Lee, K.H., Kim, K.H., Lee, D.S., Nguyen, Q.A., Bae, H.J., 2014. Efficient function and characterization of GH10 xylanase (Xyl10g) from *Gloeophyllum trabeum* in lignocellulose degradation. J. Biotechnol. 172, 38–45. https://doi.org/10.1016/j.jbiotec.2013.12.013

Kim, J.-H., Block, D.E., Shoemaker, S.P., Mills, D.A., 2010. Atypical ethanol production by carbon catabolite derepressed lactobacilli. Bioresour. Technol. 101, 8790–8797. https://doi.org/10.1016/J.BIORTECH.2010.06.087

Knob, A., Carmona, E.C., 2010. Purification and characterization of two extracellular xylanases

from *Penicillium sclerotiorum*: a novel acidophilic xylanase. Appl. Biochem. Biotechnol. 162, 429–443. https://doi.org/10.1007/s12010-009-8731-8

Knob, A., Fortkamp, D., Prolo, T., Izidoro, S.C., Almeida, J.M., 2014. Agro-residues as alternative for xylanase production by filamentous fungi. BioResources 9, 5738–5773.

Kobayashi, Y., Wakasugi, E., Ohbuchi, T., Yokoyama, M., Yasui, R., Kuwahata, M., Nakabou, Y., Kido, Y., 2011. Acidic xylooligosaccharide promotes recovery from iron deficiency anemia by enhancing serum iron level in rats. Biomed. Res. 22, 417–423.

Kocabaş, D.S., Güder, S., Özben, N., 2015. Purification strategies and properties of a low-molecular weight xylanase and its application in agricultural waste biomass hydrolysis. J. Mol. Catal. B Enzym. 115, 66–75. https://doi.org/10.1016/j.molcatb.2015.01.012

Kochumalayil, J.J., Zhou, Q., Kasai, W., Berglund, L. a, 2013. Regioselective modification of a xyloglucan hemicellulose for high-performance biopolymer barrier films. Carbohydr. Polym. 93, 466–72. https://doi.org/10.1016/j.carbpol.2012.12.041

Koga, K., Fujikawa, S., 1993. Xylo-oligosaccharides, in: Nakakuki, T. (Ed.), Oligosaccharides: Production, Properties and Applications, Japanese Technology Reviews Series. Gordon and Breach, Philadelphia, pp. 130–143.

Kolenová, K., Ryabova, O., Vršanská, M., Biely, P., 2010. Inverting character of family GH115 α-glucuronidases. FEBS Lett. 584, 4063–4068. https://doi.org/10.1016/j.febslet.2010.08.031

Kolenová, K., Vršanská, M., Biely, P., 2006. Mode of action of endo-β-1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. J. Biotechnol. 121, 338–345. https://doi.org/10.1016/j.jbiotec.2005.08.001

Kootstra, A.M.J., Beeftink, H.H., Scott, E.L., Sanders, J.P.M., 2009. Comparison of dilute mineral and organic acid pretreatment for enzymatic hydrolysis of wheat straw. Biochem. Eng. J. 46, 126–131. https://doi.org/10.1016/j.bej.2009.04.020

Koutaniemi, S., Tenkanen, M., 2016. Action of three GH51 and one GH54 α-

arabinofuranosidases on internally and terminally located arabinofuranosyl branches. J. Biotechnol. 229, 22–30. https://doi.org/10.1016/J.JBIOTEC.2016.04.050

Krüger, A., Schäfers, C., Schröder, C., Antranikian, G., 2018. Towards a sustainable biobased industry – Highlighting the impact of extremophiles. N. Biotechnol. 40, 144–153. https://doi.org/10.1016/j.nbt.2017.05.002

Kulkarni, N., Shendye, A., Rao, M., 1999. Molecular and biotechnological aspects of xylanases. FEMS Microbiol. Rev. 23, 411–456. https://doi.org/10.1111/j.1574-6976.1999.tb00407.x

Kumar, A.K., Sharma, S., 2017. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. Bioresour. Bioprocess. 4, 7:1-19. https://doi.org/10.1186/s40643-017-0137-9

Kumar, B.K., Balakrishnan, H., Rele, M. V, 2004. Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87-6-10) with commercial detergents and proteases. J. Ind. Microbiol. Biotechnol. 31, 83–87.

Kumar, D., Kumar, S.S., Kumar, J., Kumar, O., Mishra, S.V., Kumar, R., Malyan, S.K., 2017. Xylanases and their industrial applications: A review. Biochem. Cell. Arch. 17, 353–360. https://doi.org/10.1007/s002530100704

Kumar, K.S., Manimaran, A., Permaul, K., Singh, S., 2009a. Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. J. Biosci. Bioeng. 107, 494–498. https://doi.org/10.1016/j.jbiosc.2008.12.020

Kumar, L., Dutt, D., Tapas, S., Kumar, P., 2013. Purification, bio-chemical characterization, homology modeling and active site binding mode interactions of thermo-alkali-tolerant β-1,4 endoxylanase from *Coprinus cinereus* LK-D-NCIM-1369. Biocatal. Agric. Biotechnol. 2, 267–277. https://doi.org/10.1016/J.BCAB.2013.04.004

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P., 2009b. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem.

Res. 48, 3713–3729. https://doi.org/10.1021/ie801542g

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Kumari, D., Singh, R., 2018. Pretreatment of lignocellulosic wastes for biofuel production: A critical review. Renew. Sustain. Energy Rev. 90, 877–891. https://doi.org/10.1016/j.rser.2018.03.111

Kundu, A., Ray, R.R., 2013. Production of intracellular b -xylosidase from the submerged fermentation of citrus wastes by *Penicillium janthinellum* MTCC. 3 Biotech 3, 241–246. https://doi.org/10.1007/s13205-012-0091-3

Kurian, J.K., Nair, G.R., Hussain, A., Raghavan, G.S.V., 2013. Feedstocks, logistics and pre-treatment processes for sustainable lignocellulosic biorefineries: A comprehensive review. Renew. Sustain. Energy Rev. 25, 205–219. https://doi.org/10.1016/j.rser.2013.04.019

Lagaert, S., Pollet, A., Courtin, C.M., Volckaert, G., 2014. β-Xylosidases and α-L-arabinofuranosidases: Accessory enzymes for arabinoxylan degradation. Biotechnol. Adv. 32, 316–332. https://doi.org/10.1016/J.BIOTECHADV.2013.11.005

Lagaert, S., Pollet, A., Delcour, J.A., Lavigne, R., Courtin, C.M., Volckaert, G., 2010. Substrate specificity of three recombinant α-L-arabinofuranosidases from *Bifidobacterium adolescentis* and their divergent action on arabinoxylan and arabinoxylan oligosaccharides. Biochem. Biophys. Res. Commun. 402, 644–650. https://doi.org/10.1016/J.BBRC.2010.10.075

Lagaron, J.M., Lopez-Rubio, A., Fabra, M.J., 2016. Bio-based packaging. J. Appl. Polym. Sci. 133, 1–15. https://doi.org/10.1002/app.42971

Laskowski, R.A., Jabłonska, J., Pravda, L., Rekova, R.S.V., Thornton, J.M., 2018. PDBsum: Structural summaries of PDB entries. Protein Sci. 27, 129—134. https://doi.org/10.1002/pro.3289

Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291. https://doi.org/10.1107/S0021889892009944

Li, H., Xue, Y., Wu, J., Wu, H., Qin, G., Li, C., Ding, J., Liu, J., Gan, L., Long, M., 2016. Enzymatic hydrolysis of hemicelluloses from *Miscanthus* to monosaccharides or xylo-oligosaccharides by recombinant hemicellulases. Ind. Crops Prod. 79, 170–179. https://doi.org/10.1016/j.indcrop.2015.11.021

Li, J., Kisara, K., Danielsson, S., Lindström, M.E., Gellerstedt, G., 2007. An improved methodology for the quantification of uronic acid units in xylans and other polysaccharides. Carbohydr. Res. 342, 1442–1449. https://doi.org/10.1016/j.carres.2007.03.031

Limayem, A., Ricke, S.C., 2012. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Prog. Energy Combust. Sci. 38, 449–467. https://doi.org/10.1016/j.pecs.2012.03.002

Lin, J., Ndlovu, L.M., Singh, S., Pillay, B., 1999. Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP. Biotechnol. Appl. Biochem. 30, 73–79. https://doi.org/10.1111/j.1470-8744.1999.tb01162.x

List of companies, 2019. List of companies [WWW Document]. URL http://www.listofcompaniesin.com/product-s/xylanase-enzymes.html (accessed 9.20.19).

Liu, C.-M., Wu, S.-Y., Chu, C.-Y., Chou, Y.-P., 2014. Biohydrogen production from rice straw hydrolyzate in a continuously external circulating bioreactor. Int. J. Hydrogen Energy 39, 19317–19322. https://doi.org/10.1016/j.ijhydene.2014.05.175

Liu, K.-X., Li, H.-Q., Zhang, J., Zhang, Z.-G., Xu, J., 2016. The effect of non-structural components and lignin on hemicellulose extraction. Bioresour. Technol. 214, 755–760. https://doi.org/10.1016/J.BIORTECH.2016.05.036

Liu, L., Chang, H., Jameel, H., Park, S., 2018a. Furfural production from biomass pretreatment

hydrolysate using vapor-releasing reactor system. Bioresour. Technol. 252, 165–171. https://doi.org/10.1016/j.biortech.2018.01.006

Liu, M. qi, Huo, W. kang, Xu, X., Weng, X. yan, 2017. Recombinant *Bacillus amyloliquefaciens* xylanase A expressed in *Pichia pastoris* and generation of xylooligosaccharides from xylans and wheat bran. Int. J. Biol. Macromol. 105, 656–663. https://doi.org/10.1016/j.ijbiomac.2017.07.073

Liu, Q., Wang, Y., Luo, H., Wang, L., Shi, P., Huang, H., Yang, P., Yao, B., 2015. Isolation of a novel cold-active family 11 xylanase from the filamentous fungus *Bispora antennata* and deletion of its N-terminal amino acids on thermostability. Appl. Biochem. Biotechnol. 175, 925–936. https://doi.org/10.1007/s12010-014-1344-x

Liu, X., Liu, Y., Jiang, Z., Liu, H., Yang, S., Yan, Q., 2018b. Biochemical characterization of a novel xylanase from *Paenibacillus barengoltzii* and its application in xylooligosaccharides production from corncobs. Food Chem. 264, 310–318. https://doi.org/10.1016/J.FOODCHEM.2018.05.023

Lomax, A.R., Calder, P.C., 2009. Prebiotics, immune function, infection and inflammation: a review of the evidence. Br. J. Nutr. 101, 633–658. https://doi.org/10.1017/S0007114508055608

Long, J., Yang, J., Henning, S.M., Woo, S.L., Hsu, M., Chan, B., Heber, D., Li, Z., 2019. Xylooligosaccharide supplementation decreases visceral fat accumulation and modulates cecum microbiome in mice. J. Funct. Foods 52, 138–146. https://doi.org/10.1016/j.jff.2018.10.035

López-Mondéjar, R., Algora, C., Baldrian, P., 2019. Lignocellulolytic systems of soil bacteria: A vast and diverse toolbox for biotechnological conversion processes. Biotechnol. Adv. 37, 107374. https://doi.org/10.1016/j.biotechadv.2019.03.013

Lowry, O.H., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275. https://doi.org/10.1016/0304-3894(92)87011-4

Lupoi, J.S., Singh, S., Parthasarathi, R., Simmons, B.A., Henry, R.J., 2015. Recent innovations in analytical methods for the qualitative and quantitative assessment of lignin. Renew. Sustain. Energy Rev. 49, 871–906. https://doi.org/10.1016/J.RSER.2015.04.091

M'hamdi, N., Darej, C., Jebali, J., Bouraoui, R., Metahni, S., Frouja, I., Singh, D.G., Jarboui, I., Brar, S.K., 2014. Different enzymes and their production, in: Brar, S.K., Verma, M. (Eds.), Enzymes in Value-Addition of Wastes. Nova Science Publishers, Inc, New York, pp. 109–132.

Ma, R., Bai, Y., Huang, H., Luo, H., Chen, S., Fan, Y., Cai, L., Yao, B., 2017. Utility of thermostable xylanases of *Mycothermus thermophilus* in generating prebiotic xylooligosaccharides. J. Agric. Food Chem. 65, 1139–1145. https://doi.org/10.1021/acs.jafc.6b05183

Ma, R., Huang, H., Bai, Y., Luo, H., Fan, Y., Yao, B., 2018. Insight into the cold adaptation and hemicellulose utilization of *Cladosporium neopsychrotolerans* from genome analysis and biochemical characterization. Sci. Rep. 8, 6075. https://doi.org/10.1038/s41598-018-24443-7

MacCabe, A.P., Orejas, M., Tamayo, E.N., Villanueva, A., Ramón, D., 2002. Improving extracellular production of food-use enzymes from *Aspergillus nidulans*. J. Biotechnol. 96, 43–54. https://doi.org/10.1016/S0168-1656(02)00036-6

Madeira, J.V., Contesini, F.J., Calzado, F., Rubio, M.V., Zubieta, M.P., Lopes, D.B., de Melo, R.R., 2017. Agro-industrial residues and microbial enzymes: An overview on the eco-friendly bioconversion into high value-added products, in: Brahmachari, G., Demain, A.L., Adrio, J.L. (Eds.), Biotechnology of Microbial Enzymes. Academic press, pp. 475–511. https://doi.org/10.1016/B978-0-12-803725-6.00018-2

Madhukumar, M.S., Muralikrishna, G., 2010. Structural characterisation and determination of prebiotic activity of purified xylo-oligosaccharides obtained from Bengal gram husk (*Cicer arietinum* L.) and wheat bran (*Triticum aestivum*). Food Chem. 118, 215–223.

https://doi.org/10.1016/j.foodchem.2009.04.108

Magaton, A.S., Silva, T.C.F., Colodette, J.L., Piló-Veloso, D., Milagres, F.R., 2013. Behavior of xylans from *Eucalyptus* species. Part 1. The influence of structural features of *Eucalyptus* xylans on their retention in kraft pulp. Holzforschung 67, 115–122. https://doi.org/10.1515/hf-2012-0034

Mandal, E.K., Mandal, S., Maity, S., Behera, B., Maiti, T.K., Islam, S.S., 2013. Structural studies of an immunostimulating gluco-arabinan from seeds of *Caesalpinia bonduc*. Carbohydr. Polym. 92, 704–711. https://doi.org/10.1016/J.CARBPOL.2012.08.093

Manimekalai, R., Salomi, M.V., Shoba, G., 2015. Insilico docking analysis of carbazole alkaloids from *Murraya koenigii* against PP2A. Int. J. Pharma Bio Sci. 6, 913–928.

Manisseri, C., Gudipati, M., 2010. Bioactive xylo-oligosaccharides from wheat bran soluble polysaccharides. LWT - Food Sci. Technol. 43, 421–430. https://doi.org/10.1016/j.lwt.2009.09.004

Mano, M.C.R., Neri-Numa, I.A., da Silva, J.B., Paulino, B.N., Pessoa, M.G., Pastore, G.M., 2018. Oligosaccharide biotechnology: an approach of prebiotic revolution on the industry. Appl. Microbiol. Biotechnol. 102, 17–37. https://doi.org/10.1007/s00253-017-8564-2

Mao, L., Zhang, L., Gao, N., Li, A., 2012. $FeCl_3$ and acetic acid co-catalyzed hydrolysis of corncob for improving furfural production and lignin removal from residue. Bioresour. Technol. 123, 324–331. https://doi.org/10.1016/j.biortech.2012.07.058

Martín, C., López, Y., Plasencia, Y., Hernández, E., 2006. Characterisation of agricultural and agro-industrial residues as raw materials for ethanol production. Chem. Biochem. Eng. 20, 443–447.

Martínez, P.M., Appeldoorn, M.M., Gruppen, H., Kabel, M.A., 2016. The two *Rasamsonia emersonii* α-glucuronidases, ReGH67 and ReGH115, show a different mode-of-action towards glucuronoxylan and glucuronoxylo-oligosaccharides. Biotechnol. Biofuels 9, 105. https://doi.org/10.1186/s13068-016-0519-9

Masui, D.C., Zimbardi, A.L.R.L., Souza, F.H.M., Guimarães, L.H.S., Furriel, R.P.M., Jorge, J.A., 2012. Production of a xylose-stimulated β-glucosidase and a cellulase-free thermostable xylanase by the thermophilic fungus *Humicola brevis* var. thermoidea under solid state fermentation. World J. Microbiol. Biotechnol. 28, 2689–2701. https://doi.org/10.1007/s11274-012-1079-1

Mathew, S., Aronsson, A., Karlsson, E.N., Adlercreutz, P., 2018. Xylo- and arabinoxylooligosaccharides from wheat bran by endoxylanases, utilisation by probiotic bacteria, and structural studies of the enzymes. Appl. Microbiol. Biotechnol. 102, 3105–3120. https://doi.org/10.1007/s00253-018-8823-x

Matsumura, S., Sakiyama, K., Toshima, K., 1999. Preparation of octyl β-D-xylobioside and xyloside by xylanase catalyzed direct transglycosylation reaction of xylan and octanol. Biotechnol. Lett. 21, 17–22. https://doi.org/10.1023/A:1005464025881

Mawoyo, B., Adebola, P., Gerrano, A.S., Amonsou, E.O., 2017. Effect of genotypes and growth locations on composition and functional properties of amadumbe flours. J. Food Sci. Technol. 54, 3577–3586. https://doi.org/10.1007/s13197-017-2816-0

Mazlan, N.A., Samad, K.A., Wan Yussof, H., Saufi, S.M., Jahim, J., 2019. Xylooligosaccharides from potential agricultural waste: characterization and screening on the enzymatic hydrolysis factors. Ind. Crops Prod. 129, 575–584. https://doi.org/10.1016/j.indcrop.2018.12.042

Mazzoli, R., Bosco, F., Mizrahi, I., Bayer, E.A., Pessione, E., 2014. Towards lactic acid bacteria-based biorefineries. Biotechnol. Adv. 32, 1216–1236. https://doi.org/10.1016/j.biotechadv.2014.07.005

McCleary, B. V., McKie, V.A., Draga, A., Rooney, E., Mangan, D., Larkin, J., 2015. Hydrolysis of wheat flour arabinoxylan, acid-debranched wheat flour arabinoxylan and arabino-xylo-oligosaccharides by β-xylanase, α-L-arabinofuranosidase and β-xylosidase. Carbohydr. Res. 407, 79–96. https://doi.org/10.1016/J.CARRES.2015.01.017

McClements, D.J., 2015. Food emulsions : principles, practices, and techniques, 3rd ed. CRC Press, Boca Raton, FL.

McKinlay, J.B., Vieille, C., Zeikus, J.G., 2007. Prospects for a bio-based succinate industry. Appl. Microbiol. Biotechnol. 76, 727–740. https://doi.org/10.1007/s00253-007-1057-y

Mechelke, M., Koeck, D.E., Broeker, J., Roessler, B., Krabichler, F., Schwarz, W.H., Zverlov, V.V., Liebl, W., 2017. Characterization of the arabinoxylan-degrading machinery of the thermophilic bacterium *Herbinix hemicellulosilytica*—Six new xylanases, three arabinofuranosidases and one xylosidase. J. Biotechnol. 257, 122–130. https://doi.org/10.1016/J.JBIOTEC.2017.04.023

Meier, A., Söding, J., 2015. Automatic prediction of protein 3D structures by probabilistic multi-template homology modeling. PLOS Comput. Biol. 11, e1004343. https://doi.org/10.1371/journal.pcbi.1004343

Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Prog. Energy Combust. Sci. 38, 522–550. https://doi.org/10.1016/j.pecs.2012.02.002

Meryandini, A., Sunarti, T.C., Noami, A., Mutia, F., 2008. Using *Streptomyces* xylanase to produce xylooligosaccharide from corncob. Biotropia (Bogor). 15, 119–128. https://doi.org/10.11598/btb.2008.15.2.71

Meyer, F., van der Burgh, G., 2012. Evaluating the sustainability of the South African groundnut industry, Bereau for Food and Agricultural Policy.

Michelin, M., Peixoto-Nogueira, S.C., Silva, T.M., Jorge, J. a., Terenzi, H.F., Teixeira, J. a., Polizeli, M.D.L.T.M., 2012a. A novel xylan degrading β-D-xylosidase: Purification and biochemical characterization. World J. Microbiol. Biotechnol. 28, 3179–3186. https://doi.org/10.1007/s11274-012-1128-9

Michelin, M., Polizeli, M. de L.T.M., Ruzene, D.S., Silva, D.P., Ruiz, H.A., Vicente, A.A., Jorge, J.A., Terenzi, H.F., Teixeira, J.A., 2012b. Production of xylanase and β-xylosidase from

autohydrolysis liquor of corncob using two fungal strains. Bioprocess Biosyst. Eng. 35, 1185–1192. https://doi.org/10.1007/s00449-012-0705-5

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030

Mishra, A., Malhotra, A.V., 2009. Tamarind xyloglucan: a polysaccharide with versatile application potential. J. Mater. Chem. 19, 8528–8536. https://doi.org/10.1039/b911150f

Mohanty, D., Misra, S., Mohapatra, S., Sahu, P.S., 2018. Prebiotics and synbiotics: Recent concepts in nutrition. Food Biosci. 26, 152–160. https://doi.org/10.1016/J.FBIO.2018.10.008

Moniz, P., Ho, A.L., Duarte, L.C., Kolida, S., Rastall, R.A., Pereira, H., Carvalheiro, F., 2016. Assessment of the bifidogenic effect of substituted xylo-oligosaccharides obtained from corn straw. Carbohydr. Polym. 136, 466–473. https://doi.org/10.1016/j.carbpol.2015.09.046

Morais, S., Barak, Y., Hadar, Y., Wilson, D.B., Shoham, Y., Lamed, R., Bayer, E.A., 2011. Assembly of xylanases into designer cellulosomes promotes efficient hydrolysis of the xylan component of a natural recalcitrant cellulosic substrate. MBio 2, 1–11. https://doi.org/10.1128/mBio.00233-11

Morales, M., Ataman, M., Badr, S., Linster, S., Kourlimpinis, I., Papadokonstantakis, S., Hatzimanikatis, V., Hungerbühler, K., 2016. Sustainability assessment of succinic acid production technologies from biomass using metabolic engineering. Energy Environ. Sci. 9, 2794–2805. https://doi.org/10.1039/c6ee00634e

Moreira, L.R.S., Filho, E.X.F., 2016. Insights into the mechanism of enzymatic hydrolysis of xylan. Appl. Microbiol. Biotechnol. 100, 5205–5214. https://doi.org/10.1007/s00253-016-7555-z

Motta, F.L., Andrade, C.C.P., Santana, M.H.A., 2013. A review of xylanase production by the fermentation of xylan: classification, characterization and applications, in: Chandel, A.K.,

da Silva, S.S. (Eds.), Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization. Intech, Croatia, pp. 251–275. https://doi.org/10.5772/53544

Moura, P., Barata, R., Carvalheiro, F., Gírio, F., Loureiro-Dias, M.C., Esteves, M.P., 2007. In vitro fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by *Bifidobacterium* and *Lactobacillus* strains. LWT - Food Sci. Technol. 40, 963–972. https://doi.org/10.1016/J.LWT.2006.07.013

Moure, A., Gullón, P., Domínguez, H., Parajó, J.C., 2006. Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. Process Biochem. 41, 1913–1923. https://doi.org/10.1016/j.procbio.2006.05.011

Mousavi, M., Heshmati, A., Daraei Garmakhany, A., Vahidinia, A., Taheri, M., 2019. Texture and sensory characterization of functional yogurt supplemented with flaxseed during cold storage. Food Sci. Nutr. 7, 907–917. https://doi.org/10.1002/fsn3.805

Mussatto, S.I., Dragone, G., Guimarães, P.M.R., Silva, J.P.A., Carneiro, L.M., Roberto, I.C., Vicente, A., Domingues, L., Teixeira, J.A., 2010. Technological trends, global market, and challenges of bio-ethanol production. Biotechnol. Adv. 28, 817–830. https://doi.org/10.1016/J.BIOTECHADV.2010.07.001

Mussatto, S.I., Mancilha, I.M., 2007. Non-digestible oligosaccharides: A review. Carbohydr. Polym. 68, 587–597. https://doi.org/10.1016/J.CARBPOL.2006.12.011

Muthaiyan, A., Limayem, A., Ricke, S.C., 2011. Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. Prog. Energy Combust. Sci. 37, 351–370. https://doi.org/10.1016/J.PECS.2010.06.005

Nagy, T., Nurizzo, D., Davies, G.J., Biely, P., Lakey, J.H., Bolam, D.N., Gilbert, H.J., 2003. The alpha-glucuronidase, GlcA67A, of *Cellvibrio japonicus* utilizes the carboxylate and methyl groups of aldobiouronic acid as important substrate recognition determinants. J. Biol. Chem. 278, 20286–20292. https://doi.org/10.1074/jbc.M302205200

Naidu, D.S., Hlangothi, S.P., John, M.J., 2018. Bio-based products from xylan: A review. Carbohydr. Polym. 179, 28–41. https://doi.org/10.1016/J.CARBPOL.2017.09.064

Nami, Y., Bakhshayesh, R.V., Manafi, M., Hejazi, M.A., 2019. Hypocholesterolaemic activity of a novel autochthonous potential probiotic *Lactobacillus plantarum* YS5 isolated from yogurt. LWT- Food Sci. Technol. 111, 876–882. https://doi.org/10.1016/J.LWT.2019.05.057

Naran, R., Black, S., Decker, S.R., Azadi, P., 2009. Extraction and characterization of native heteroxylans from delignified corn stover and aspen. Cellulose 16, 661–675. https://doi.org/10.1007/s10570-009-9324-y

Nayak, B.K., Roy, S., Das, D., 2014. Biohydrogen production from algal biomass (*Anabaena* sp. PCC 7120) cultivated in airlift photobioreactor. Int. J. Hydrogen Energy 39, 7553–7560. https://doi.org/10.1016/j.ijhydene.2013.07.120

Nguyen, C.M., Kim, J.S., Hwang, H.J., Park, M.S., Choi, G.J., Choi, Y.H., Jang, K.S., Kim, J.C., 2012. Production of L-lactic acid from a green microalga, *Hydrodictyon reticulum*, by *Lactobacillus paracasei* LA104 isolated from the traditional Korean food, makgeolli. Bioresour. Technol. 110, 552–559. https://doi.org/10.1016/j.biortech.2012.01.079

Nguyen, C.M., Nguyen, T.N., Choi, G.J., Choi, Y.H., Jang, K.S., Park, Y.J., Kim, J.C., 2014a. Acid hydrolysis of *Curcuma longa* residue for ethanol and lactic acid fermentation. Bioresour. Technol. 151, 227–235. https://doi.org/10.1016/j.biortech.2013.10.039

Nguyen, Q.A., He, W., Holmes, L.D., 2014b. Methods for controlling pretreatment of biomass. US 2014/0083939A1.

Nieto-Domínguez, M., de Eugenio, L.I., York-Durán, M.J., Rodríguez-Colinas, B., Plou, F.J., Chenoll, E., Pardo, E., Codoñer, F., Jesús Martínez, M., 2017. Prebiotic effect of xylooligosaccharides produced from birchwood xylan by a novel fungal GH11 xylanase. Food Chem. 232, 105–113. https://doi.org/10.1016/j.foodchem.2017.03.149

Nigam, P.S., 2013. Microbial enzymes with special characteristics for biotechnological

applications. Biomolecules 3, 597–611.

Nishitani, K., Nevins, D.J., 1991. Glucuronoxylan xylanohydrolase. A unique xylanase with the requirement for appendant glucuronosyl units. J. Biol. Chem. 266, 6539–6543.

Ntaikou, I., Antonopoulou, G., Lyberatos, G., 2010. Biohydrogen production from biomass and wastes via dark fermentation: A review. Waste and Biomass Valorization 1, 21–39. https://doi.org/10.1007/s12649-009-9001-2

Nurizzo, D., Nagy, T., Gilbert, H.J., Davies, G.J., 2002. The structural basis for catalysis and specificity of the *Pseudomonas cellulosa* α-glucuronidase, GlcA67A. Structure 10, 547–556. https://doi.org/10.1016/S0969-2126(02)00742-6

Oh, Y.H., Eom, I.Y., Joo, J.C., Yu, J.H., Song, B.K., Lee, S.H., Hong, S.H., Park, S.J., 2015. Recent advances in development of biomass pretreatment technologies used in biorefinery for the production of bio-based fuels, chemicals and polymers. Korean J. Chem. Eng. 32, 1945–1959. https://doi.org/10.1007/s11814-015-0191-y

Ohbuchi, T., Sakaino, M., Takahashi, T., Azumi, N., Ishikawa, K., Kawazoe, S., Kobayashi, Y., Kido, Y., 2010. Oral administration of acidic xylooligosaccharides prevents the development of atopic dermatitis-like skin lesions in NC/Nga mice. J. Nutr. Sci. Vitaminol. (Tokyo). 56, 54–59. https://doi.org/10.3177/jnsv.56.54

Oliveira, E.E., Silva, A.E., Júnior, T.N., Gomes, M.C.S., Aguiar, L.M., Marcelino, H.R., Araújo, I.B., Bayer, M.P., Ricardo, N.M.P.S., Oliveira, A.G., Egito, E.S.T., 2010. Xylan from corn cobs, a promising polymer for drug delivery: Production and characterization. Bioresour. Technol. 101, 5402–5406. https://doi.org/10.1016/j.biortech.2010.01.137

Olmos, J.C., Hansen, M.E.Z., 2012. Enzymatic depolymerization of sugar beet pulp: Production and characterization of pectin and pectic-oligosaccharides as a potential source for functional carbohydrates. Chem. Eng. J. 192, 29–36.

Olofsson, K., Bertilsson, M., Lidén, G., 2008. A short review on SSF-an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels 1, 1–

14. https://doi.org/10.1186/1754-6834-1-7

Otieno, D.O., Ahring, B.K., 2012. The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: xylooligosaccharides (XOS), arabinooligosaccharides (AOS), and mannooligosaccharides (MOS). Carbohydr. Res. 360, 84–92. https://doi.org/10.1016/j.carres.2012.07.017

Ouyang, J., Ma, R., Zheng, Z., Cai, C., Zhang, M., Jiang, T., 2013. Open fermentative production of L-lactic acid by *Bacillus* sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material. Bioresour. Technol. 135, 475–480. https://doi.org/10.1016/j.biortech.2012.09.096

Oyeyinka, S.A., Oyeyinka, A.T., 2018. A review on isolation, composition, physicochemical properties and modification of Bambara groundnut starch. Food Hydrocoll. https://doi.org/10.1016/j.foodhyd.2017.09.012

Ozcan, T., Kurtuldu, O., 2014. Influence of dietary fiber addition on the properties of probiotic yogurt. Int. J. Chem. Eng. Appl. 5, 397–401. https://doi.org/10.7763/IJCEA.2014.V5.417

Palaniappan, A., Balasubramaniam, V.G., Antony, U., 2017. Prebiotic potential of xylooligosaccharides derived from finger millet seed coat. Food Biotechnol. 31, 264–280. https://doi.org/10.1080/08905436.2017.1369433

Palavesam, A., 2015. Investigation on lignocellulosic saccharification and characterization of haloalkaline solvent tolerant endo-1,4 β-d-xylanase from *Halomonas meridiana* APCMST-KS4. Biocatal. Agric. Biotechnol. 4, 761–766. https://doi.org/10.1016/j.bcab.2015.09.007

Paleg, L.G., 1959. Citric acid interference in estimation of reducing sugars with alkaline copper reagents. Anal. Chem. 31, 1902–1904. https://doi.org/10.1021/ac60155a072

Pandey, A., 2009. Handbook of plant based biofuels. CRC Press, Boca Raton, FL.

Pandit, R.K., Mukherjee, T.K., Kumar, A., Kumar, V., Kaur, P.S., 2016. Structure prediction and assessment of beta-lactamase tem-1 from *S. typhi* using molecular dynamics and

simulation studies. Int. J. Recent Sci. Res. 3, 9509–6513.

Paredes, R. de S., Barros, R. da R.O. de, Inoue, H., Yano, S., Bon, E.P. da S., 2015. Production of xylanase, α-l-arabinofuranosidase, β-xylosidase, and β-glucosidase by *Aspergillus awamori* using the liquid stream from hot-compressed water treatment of sugarcane bagasse. Biomass Convers. Biorefinery 3–11. https://doi.org/10.1007/s13399-015-0159-5

Passarinho, A.T.P., Ventorim, R.Z., Maitan-Alfenas, G.P., de Oliveira, E.B., Guimarães, V.M., 2019. Engineered GH11 xylanases from *Orpinomyces* sp. PC-2 improve techno-functional properties of bread dough. J. Sci. Food Agric. 99, 741–747. https://doi.org/10.1002/jsfa.9242

Patel, S., Goyal, A., 2011. Functional oligosaccharides: production, properties and applications. World J. Microbiol. Biotechnol. 27, 1119–1128. https://doi.org/10.1007/s11274-010-0558-5

Patel, S.J., Savanth, V.D., 2015. Review on fungal xylanases and their applications. Int. J. Adv. Res. 3, 311–315.

Pauly, M., Keegstra, K., 2016. Biosynthesis of the plant cell wall matrix polysaccharide xyloglucan. Annu. Rev. Plant Biol. 67, 26.1-26.25. https://doi.org/10.1146/annurev-arplant-043015-112222

Pawlowski, M., Gajda, M.J., Matlak, R., Bujnicki, J.M., 2008. MetaMQAP: A meta-server for the quality assessment of protein models. BMC Bioinformatics 9, 403. https://doi.org/10.1186/1471-2105-9-403

Pei, R., Martin, D.A., DiMarco, D.M., Bolling, B.W., 2017. Evidence for the effects of yogurt on gut health and obesity. Crit. Rev. Food Sci. Nutr. 57, 1569–1583. https://doi.org/10.1080/10408398.2014.883356

Peng, J., Wang, W., Jiang, Y., Liu, M., Zhang, H., Shao, W., 2011. Enhanced soluble expression of a thermostable cellulase from *Clostridium thermocellum* in *Escherichia coli*. Curr. Microbiol. 63, 523–30. https://doi.org/10.1007/s00284-011-0012-4

Penksza, P., Banka, Z., Szilárd, K., Huszár, K.P., Németh, C., Adrienn Tóth, Réka, J., 2018. Utilization of xylo-oligosaccharides as prebiotics in yogurt. J. Hyg. Eng. Des. 22, 66–71.

Peral, C., 2016. Biomass pretreatment strategies (technologies, environmental performance, economic considerations, industrial implementation), in: Poltronieri, P. (Ed.), Biotransformation of Agricultural Waste and By-Products. Elsevier, pp. 125–160. https://doi.org/10.1016/B978-0-12-803622-8.00005-7

Petrova, P., Petrov, K., 2017. Prebiotic-probiotic relationship: The genetic fundamentals of polysaccharides conversion by *Bifidobacterium* and *Lactobacillus* genera, Food Bioconversion. Elsevier Inc. https://doi.org/10.1016/B978-0-12-811413-1.00007-3

Pillai, S.K.K., 2010. Investigation of bioprocess parameters for the production of hemicellulases by *Thermomyces lanuginosus* strains. Durban University of Technology.

Pimentel, T.C., Madrona, G.S., Garcia, S., Prudencio, S.H., 2015. Probiotic viability, physicochemical characteristics and acceptability during refrigerated storage of clarified apple juice supplemented with *Lactobacillus paracasei* ssp. *paracasei* and oligofructose in different package type. LWT - Food Sci. Technol. 63, 415–422. https://doi.org/10.1016/J.LWT.2015.03.009

Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A., Amorim, D.S., 2005. Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67, 577–591.

Ponnusamy, V.K., Nguyen, D.D., Dharmaraja, J., Shobana, S., Banu, J.R., Saratale, R.G., Chang, S.W., Kumar, G., 2019. A review on lignin structure, pretreatments, fermentation reactions and biorefinery potential. Bioresour. Technol. 271, 462–472. https://doi.org/10.1016/j.biortech.2018.09.070

Pradeep, G.C., Choi, Y.H., Choi, Y., Seong, C.N., Cho, S.S., Lee, H.J., Yoo, J.C., 2013. A novel thermostable cellulase free xylanase stable in broad range of pH from *Streptomyces* sp. CS428. Process Biochem. 48, 1188–1196. https://doi.org/10.1016/j.procbio.2013.06.007

Prakash, N., Priya, S., 2016. Development of novel functional confectionery using low reduced sugar. Indian J. Drugs 4, 141–148.

Prieur, B., Meub, M., Wittemann, M., Klein, R., Bellayer, S., Fontaine, G., Bourbigot, S., 2017. Phosphorylation of lignin: characterization and investigation of the thermal decomposition. RSC Adv. 7, 16866–16877. https://doi.org/10.1039/c7ra00295e

Pswarayi, F., Gänzle, M.G., 2019. Composition and origin of the fermentation microbiota of Mahewu, a Zimbabwean fermented cereal beverage. Appl. Environ. Microbiol. 85, e03130-18. https://doi.org/10.1128/AEM.03130-18

Puchart, V., Mørkeberg Krogh, K.B.R., Biely, P., 2019. Glucuronoxylan 3-O-acetylated on uronic acid-substituted xylopyranosyl residues and its hydrolysis by GH10, GH11 and GH30 endoxylanases. Carbohydr. Polym. 205, 217–224. https://doi.org/10.1016/J.CARBPOL.2018.10.043

Purohit, A., Rai, S.K., Chownk, M., Sangwan, R.S., Yadav, S.K., 2017. Xylanase from *Acinetobacter pittii* MASK 25 and developed magnetic cross-linked xylanase aggregate produce predominantly xylopentose and xylohexose from agro biomass. Bioresour. Technol. 244, 793–799. https://doi.org/10.1016/J.BIORTECH.2017.08.034

Qi, B., Chen, X., Shen, F., Su, Y., Wan, Y., 2009. Optimization of enzymatic hydrolysis of wheat straw pretreated by alkaline peroxide using response surface methodology. Ind. Eng. Chem. Res. 48, 7346–7353. https://doi.org/10.1021/ie8016863

Radoĉaj, O.F., Dimić, E.B., Vujasinović, V.B., 2011. Optimization of the texture of fat-based spread containing hull-less pumpkin (*Cucurbita pepo* L.) seed press-cake. Acta Period. Technol. 42, 131–143. https://doi.org/10.2298/APT1142131R

Rahmani, N., Kahar, P., Lisdiyanti, P., Lee, J., Yopi, Prasetya, B., Ogino, C., Kondo, A., 2019. GH-10 and GH-11 Endo-1,4-B-xylanase enzymes from *Kitasatospora* sp. produce xylose and xylooligosaccharides from sugarcane bagasse with no xylose inhibition. Bioresour. Technol. 272, 315–325. https://doi.org/10.1016/j.biortech.2018.10.007

Raj, A., Kumar, S., Singh, S.K., 2013. A highly thermostable xylanase from *Stenotrophomonas maltophilia*: purification and partial characterization. Enzyme Res. 2013, 429305. https://doi.org/10.1155/2013/429305

Rajagopalan, G., Shanmugavelu, K., Yang, K., 2017. Production of prebiotic-xylooligosaccharides from alkali pretreated mahogany and mango wood sawdust by using purified xylanase of *Clostridium* strain BOH3. Carbohydr. Polym. 167, 158–166. https://doi.org/10.1016/j.carbpol.2017.03.021

Rakotoarivonina, H., Hermant, B., Aubry, N., Rémond, C., 2015. Engineering the hydrophobic residues of a GH11 xylanase impacts its adsorption onto lignin and its thermostability. Enzyme Microb. Technol. 81, 47–55. https://doi.org/10.1016/j.enzmictec.2015.07.009

Ramprakash, B., Muthukumar, K., 2014. Comparative study on the production of biohydrogen from rice mill wastewater. Int. J. Hydrogen Energy 39, 14613–14621. https://doi.org/10.1016/j.ijhydene.2014.06.029

Ratnadewi, A.A.I., Santoso, A.B., Sulistyaningsih, E., Handayani, W., 2016. Application of cassava peel and waste as raw materials for xylooligosaccharide production using endoxylanase from *Bacillus subtilis* of soil termite abdomen. Procedia Chem. 18, 31–38. https://doi.org/10.1016/j.proche.2016.01.007

Reddy, S.S., Krishnan, C., 2016. Production of high-pure xylooligosaccharides from sugarcane bagasse using crude β-xylosidase-free xylanase of *Bacillus subtilis* KCX006 and their bifidogenic function. LWT - Food Sci. Technol. 65, 237–245. https://doi.org/10.1016/j.lwt.2015.08.013

Reddy, S.S., Krishnan, C., 2010. Production of prebiotics and antioxidants as health food supplements from lignocellulosic materials using multi enzymatic hydrolysis. Int. J. Chem. Sci. 8, S535–S549.

Reilly, M., Dinsdale, R., Guwy, A., 2014. Mesophilic biohydrogen production from calcium hydroxide treated wheat straw. Int. J. Hydrogen Energy 39, 16891–16901.

https://doi.org/10.1016/j.ijhydene.2014.08.069

Rhee, M.S., Wei, L., Sawhney, N., Rice, J.D., John, J.S., Hurlbert, J.C., Preston, F., 2014. Engineering the xylan utilization system in *Bacillus subtilis* for production of acidic xylooligosaccharides. Appl. Environ. Microbiol. 80, 917–927. https://doi.org/10.1128/AEM.03246-13

Ribeiro, Liliane FC, De Lucas, R.C., Vitcosque, G.L., Ribeiro, Lucas F, Ward, R.J., Rubio, M. V, Damásio, A.R., Squina, F.M., Gregory, R.C., Walton, P.H., Jorge, J.A., Prade, R.A., Buckeridge, M.S., Polizeli, M. de L.T., 2014. A novel thermostable xylanase GH10 from *Malbranchea pulchella* expressed in *Aspergillus nidulans* with potential applications in biotechnology. Biotechnol. Biofuels 7, 1–11. https://doi.org/10.1186/1754-6834-7-115

Rodionova, M.V., Poudyal, R.S., Tiwari, I., Voloshin, R.A., Zharmukhamedov, S.K., Nam, H.G., Zayadan, B.K., Bruce, B.D., Hou, H.J.M., Allakhverdiev, S.I., 2017. Biofuel production: Challenges and opportunities. Int. J. Hydrogen Energy 42, 8450–8461. https://doi.org/10.1016/J.IJHYDENE.2016.11.125

Rogowski, A., Baslé, A., Farinas, C.S., Solovyova, A., Mortimer, J.C., Dupree, P., Gilbert, H.J., Bolam, D.N., 2014. Evidence that GH115 α-glucuronidase activity, which is required to degrade plant biomass, is dependent on conformational flexibility. J. Biol. Chem. 289, 53–64. https://doi.org/10.1074/jbc.M113.525295

Rowley, J., Decker, S.R., Michener, W., Black, S., 2013. Efficient extraction of xylan from delignified corn stover using dimethyl sulfoxide. 3 Biotech 3, 433–438. https://doi.org/10.1007/s13205-013-0159-8

Roy, D., Mukherjee, K., 2015. Homology modeling and docking studies of human chitotriosidase with its natural inhibitors. J. Proteins Proteomics 6, 183–196.

Ruzene, D.S., Ilva, P.D., Vicente, A.A., Goncalves, A.R., Teixeira, J.A., 2008. An alternative application to the Portuguese agro industrial residue: wheat straw. Appllied Biochem. Biotechnol. 194, 85–96. https://doi.org/10.1007/978-1-60327-526-2_43

Ryabova, O., Vršanská, M., Kaneko, S., van Zyl, W.H., Biely, P., 2009. A novel family of hemicellulolytic α-glucuronidase. FEBS Lett. 583, 1457–1462. https://doi.org/10.1016/j.febslet.2009.03.057

Sah, B.N.P., Vasiljevic, T., McKechnie, S., Donkor, O.N., 2015. Effect of refrigerated storage on probiotic viability and the production and stability of antimutagenic and antioxidant peptides in yogurt supplemented with pineapple peel. J. Dairy Sci. 98, 5905–5916. https://doi.org/10.3168/jds.2015-9450

Saha, B.C., 2003. Hemicellulose bioconversion. J. Ind. Microbiol. Biotechnol. 30, 279–91. https://doi.org/10.1007/s10295-003-0049-x

Sahay, H., Yadav, A.N., Singh, A.K., Singh, S., Kaushik, R., Saxena, A.K., 2017. Hot springs of Indian Himalayas: potential sources of microbial diversity and thermostable hydrolytic enzymes. 3 Biotech 7, 118. https://doi.org/10.1007/s13205-017-0762-1

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sakamoto, T., Inui, M., Yasui, K., Hosokawa, S., Ihara, H., 2013. Substrate specificity and gene expression of two *Penicillium chrysogenum* α-L-arabinofuranosidases (AFQ1 and AFS1) belonging to glycoside hydrolase families 51 and 54. Appl. Microbiol. Biotechnol. 97, 1121–1130. https://doi.org/10.1007/s00253-012-3978-3

Salas-Veizaga, D.M., Villagomez, R., Linares-Pastén, J.A., Carrasco, C., Álvarez, M.T., Adlercreutz, P., Nordberg Karlsson, E., 2017. Extraction of glucuronoarabinoxylan from quinoa stalks (*Chenopodium quinoa* willd.) and evaluation of xylooligosaccharides produced by GH10 and GH11 xylanases. J. Agric. Food Chem. 65, 8663–8673. https://doi.org/10.1021/acs.jafc.7b01737

Samal, L., Behura, N.C., 2015. Prebiotics: An emerging nutritional approach for improving gut health of livestock and poultry. Asian J. Anim. Vet. Adv. 10, 724–739.

https://doi.org/10.3923/ajava.2015.724.739

Samala, A., Srinivasan, R., Yadav, M., 2014. Comparison of xylo-oligosaccharides production by autohydrolysis of fibers separated from ground corn flour and DDGS. Food Bioprod. Process. https://doi.org/10.1016/j.fbp.2014.04.004

Samanta, A.K., Jayapal, N., Kolte, A.P., Senani, S., Sridhar, M., Dhali, A., Suresh, K.P., Jayaram, C., Prasad, C.S., 2015. Process for enzymatic production of xylooligosaccharides from the xylan of corn cobs. J. Food Process. Preserv. 39, 729–736. https://doi.org/10.1111/jfpp.12282

Samanta, A.K., Senani, S., Kolte, A.P., Sridhar, M., Sampath, K.T., Jayapal, N., Devi, A., 2012. Production and in vitro evaluation of xylooligosaccharides generated from corn cobs. Food Bioprod. Process. 90, 466–474. https://doi.org/10.1016/j.fbp.2011.11.001

Samudrala, R., Xia, Y., Huang, E., Levitt, M., 1999. Ab initio protein structure prediction using a combined hierarchical approach. Proteins Struct. Funct. Bioinforma. 37, 194–198. https://doi.org/10.1002/(SICI)1097-0134(1999)37:3+<194::AID-PROT24>3.0.CO;2-F

Sarkar, B., Verma, S.K., Akhtar, J., Netam, S.P., Gupta, S.K., Panda, P.K., Mukherjee, K., 2018. Molecular aspect of silver nanoparticles regulated embryonic development in Zebrafish (*Danio rerio*) by Oct-4 expression. Chemosphere 206, 560–567. https://doi.org/10.1016/j.chemosphere.2018.05.018

Schädel, C., Blöchl, A., Richter, A., Hoch, G., Schadel, C., Blochl, A., Richter, A., Hoch, G., 2009. Short-term dynamics of nonstructural carbohydrates and hemicelluloses in young branches of temperate forest trees during bud break. Tree Physiol. 29, 901–911. https://doi.org/10.1093/treephys/tpp034

Scheller, H., Ulvskov, P., 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61, 263–289. https://doi.org/10.1146/annurev-arplant-042809-112315

Scholz-Ahrens, K.E., Schrezenmeir, J., 2007. Inulin and oligofructose and mineral metabolism: The evidence from animal trials. J. Nutr. 137, 2513S-2523S.

https://doi.org/10.1093/jn/137.11.2513S

Seemakram, W., Boonrung, S., Katekaew, S., Aimi, T., Boonlue, S., 2016. Purification and characterization of low molecular weight alkaline xylanase from *Neosartorya tatenoi* KKU-CLB-3-2-4-1. Mycoscience 57, 326–333. https://doi.org/10.1016/J.MYC.2016.05.001

Seesuriyachan, P., Kawee-ai, A., Chaiyaso, T., 2017. Green and chemical-free process of enzymatic xylooligosaccharide production from corncob: Enhancement of the yields using a strategy of lignocellulosic destructuration by ultra-high pressure pretreatment. Bioresour. Technol. 241, 537–544. https://doi.org/10.1016/J.BIORTECH.2017.05.193

Serban, D.E., 2014. Gastrointestinal cancers: Influence of gut microbiota, probiotics and prebiotics. Cancer Lett. 345, 258–270. https://doi.org/10.1016/J.CANLET.2013.08.013

Shaghaghi, M., Pourahmad, R., Adeli, H.R.M., 2013. Synbiotic yogurt production by using prebiotic compounds and probiotic Lactobacilli. Int. Res. J. Appl. Basic Sci. 5, 839–846.

Shallom, D., Shoham, Y., 2003. Microbial hemicellulases. Curr. Opin. Microbiol. 6, 219–228.

Sharma, Abha, Sharma, Anamika, Singh, S., Kuhad, R.C., Nain, La., 2019. Thermophilic fungi and their enzymes for biorefineries, in: Tiquia-Arashiro, S.M., Grube, M. (Eds.), Fungi in Extreme Environments: Ecological Role and Biotechnological Significance. Springer Nature, pp. 479–502. https://doi.org/10.1007/978-3-030-19030-9

Shatalov, A.A., Evtuguin, D. V, Pascoal Neto, C., 1999. (2-O-α-D-Galactopyranosyl-4-O-methyl-α-D-glucurono)-D-xylan from *Eucalyptus globulus* Labill. Carbohydr. Res. 320, 93–99. https://doi.org/10.1016/S0008-6215(99)00136-6

Shi, H., Zhang, Y., Li, X., Huang, Y., Wang, L., Wang, Y., Ding, H., Wang, F., 2013. A novel highly thermostable xylanase stimulated by Ca2+ from *Thermotoga thermarum*: cloning, expression and characterization. Biotechnol. Biofuels 6, 26. https://doi.org/10.1186/1754-6834-6-26

Shi, P., Du, Y., Yang, H., Huang, H., Zhang, X., Wang, Y., Yao, B., 2015. Molecular

characterization of a new alkaline-tolerant xylanase from *Humicola insolens* Y1. Biomed Res. Int. 2015, 1–7. https://doi.org/10.1155/2015/149504

Shi, X., Chen, Y., Ren, H., Liu, D., Zhao, T., Zhao, N., Ying, H., 2014. Economically enhanced succinic acid fermentation from cassava bagasse hydrolysate using *Corynebacterium glutamicum* immobilized in porous polyurethane filler. Bioresour. Technol. 174, 190–197. https://doi.org/10.1016/j.biortech.2014.09.137

Shirkavand, E., Baroutian, S., Gapes, D.J., Young, B.R., 2016. Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment – A review. Renew. Sustain. Energy Rev. 54, 217–234. https://doi.org/10.1016/J.RSER.2015.10.003

Sims, I.M., Ryan, J.L.J., Kim, S.H., 2014. In vitro fermentation of prebiotic oligosaccharides by *Bifidobacterium lactis* HN019 and *Lactobacillus* spp. Anaerobe 25, 11–17. https://doi.org/10.1016/j.anaerobe.2013.11.001

Sindhu, R., Binod, P., Pandey, A., 2015. Biological pretreatment of lignocellulosic biomass – an overview. Bioresour. Technol. 199, 76–82. https://doi.org/10.1016/j.biortech.2015.08.030

Singh, R., Shukla, A., Tiwari, S., Srivastava, M., 2014. A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential. Renew. Sustain. Energy Rev. 32, 713–728. https://doi.org/10.1016/j.rser.2014.01.051

Singh, R.D., Banerjee, J., Arora, A., 2015. Prebiotic potential of oligosaccharides: A focus on xylan derived oligosaccharides. Bioact. Carbohydrates Diet. Fibre 5, 19–30. https://doi.org/10.1016/j.bcdf.2014.11.003

Singh, S., Singh, G., Arya, S.K., 2018a. Mannans: An overview of properties and application in food products. Int. J. Biol. Macromol. 119, 79–95. https://doi.org/10.1016/J.IJBIOMAC.2018.07.130

Singh, R.D., Banerjee, J., Sasmal, S., Muir, J., Arora, A., 2018b. High xylan recovery using two stage alkali pre-treatment process from high lignin biomass and its valorisation to xylooligosaccharides of low degree of polymerisation. Bioresour. Technol. 256, 110–117.

https://doi.org/10.1016/J.BIORTECH.2018.02.009

Singh, R.S., Singh, R.P., Kennedy, J.F., 2016. Recent insights in enzymatic synthesis of fructooligosaccharides from inulin. Int. J. Biol. Macromol. 85, 565–572. https://doi.org/10.1016/j.ijbiomac.2016.01.026

Singh, S., Madlala, A.M., Prior, B.A., 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. FEMS Microbiol. Rev. 27, 3–16. https://doi.org/10.1016/s0168-6445(03)00018-4

Singh, S., Pillay, B., Dilsook, V., Prior, B.A. a., 2000. Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. J. Appl. Microbiol. 88, 975–982. https://doi.org/10.1046/j.1365-2672.2000.01063.x

Singh, S., Sidhu, G.K., Kumar, V., Dhanjal, D.S., Datta, S., & Singh, J., 2019. Fungal xylanases: sources, types, and biotechnological applications, in: Yadav, A.N. (Ed.), Recent Advancement in White Biotechnology Through Fungi. Springer Nature, Cham, pp. 405–428. https://doi.org/10.1007/978-3-030-10480-1_12

Sinner, M., Ditzelmüller, G., Wizani, W., Steiner, W., Esterbauer, E., 1991. VAI-Biobleiche. Das Pap. 45, 403–410.

Sinthusamran, S., Benjakul, S., 2018. Physical, rheological and antioxidant properties of gelatin gel as affected by the incorporation of β-glucan. Food Hydrocoll. 79, 409–415. https://doi.org/10.1016/j.foodhyd.2018.01.018

Sivová, V., Nosáľová, G., Jureček, L., Turjan, J., Vlčková, S., Bystrický, P., Kantárová, D., Capek, P., 2015. *Fagus sylvatica* glucuronoxylan sulfate–chemical profile and pharmacological view. Starch-Stärke 68, 621–628. https://doi.org/10.1002/star.201500122

Song, H., Lee, S.Y., 2006. Production of succinic acid by bacterial fermentation. Enzyme Microb. Technol. 39, 352–361. https://doi.org/10.1016/J.ENZMICTEC.2005.11.043

Sousa, S., Ramos, A., Evtuguin, D. V., Gamelas, J.A.F., 2016. Xylan and xylan derivatives-their performance in bio-based films and effect of glycerol addition. Ind. Crops Prod. 94, 682–

689. https://doi.org/10.1016/j.indcrop.2016.09.031

Sreedevi, S., Sajith, S., Benjamin, S., 2013. Cellulase producing bacteria from the wood-yards on Kallai river bank. Adv. Microbiol. 3, 326–332. https://doi.org/10.4236/aim.2013.34046

Sridevi, A., Sandhya, A., Ramanjaneyulu, G., Narasimha, G., Devi, P.S., 2016. Biocatalytic activity of *Aspergillus niger* xylanase in paper pulp biobleaching. 3 Biotech 6, 165. https://doi.org/10.1007/s13205-016-0480-0

St. John, F.J., Rice, J.D., Preston, J.F., 2006. Characterization of XynC from *Bacillus subtilis* subsp. *subtilis* strain 168 and analysis of its role in depolymerization of glucuronoxylan. J. Bacteriol. 188, 8617–8626. https://doi.org/10.1128/JB.01283-06

St John, F.J., Dietrich, D., Crooks, C., Pozharski, E., González, J.M., Bales, E., Smith, K., Hurlbert, J.C., IUCr, 2014. A novel member of glycoside hydrolase family 30 subfamily 8 with altered substrate specificity. Acta Crystallogr. Sect. D Biol. Crystallogr. 70, 2950–2958. https://doi.org/10.1107/S1399004714019531

Stephens, D.E., Khan, F.I., Singh, P., Bisetty, K., Singh, S., Permaul, K., 2014. Creation of thermostable and alkaline stable xylanase variants by DNA shuffling. J. Biotechnol. 187, 139–46. https://doi.org/10.1016/j.jbiotec.2014.07.446

Subramaniyan, S., Prema, P., 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit. Rev. Biotechnol. 22, 33–64. https://doi.org/10.1080/07388550290789450

Sun, J.X., Sun, X.F., Sun, R.C., Su, Y.Q., 2004. Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses. Carbohydr. Polym. 56, 195–204. https://doi.org/10.1016/j.carbpol.2004.02.002

Sun, M., Zheng, H., Meng, L., Sun, J., Song, H., Bao, Y., Pei, H., Yan, Z., Zhang, X., Zhang, J., Liu, Y., Lu, F., 2015. Direct cloning, expression of a thermostable xylanase gene from the metagenomic DNA of cow dung compost and enzymatic production of xylooligosaccharides from corncob. Biotechnol. Lett. 37, 1877–1886.

https://doi.org/10.1007/s10529-015-1857-6

Sun, S.L., Wen, J.L., Ma, M.G., Song, X.L., Sun, R.C., 2014. Integrated biorefinery based on hydrothermal and alkaline treatments: Investigation of sorghum hemicelluloses. Carbohydr. Polym. 111, 663–669. https://doi.org/10.1016/j.carbpol.2014.04.099

Sun, Shaoni, Sun, Shaolong, Cao, X., Sun, R., 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Bioresour. Technol. 199, 49–58. https://doi.org/10.1016/j.biortech.2015.08.061

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour. Technol. 83, 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Swennen, K., Courtin, C.M., Lindemans, G.C., Delcour, J.A., 2006. Large-scale production and characterisation of wheat bran arabinoxylooligosaccharides. J. Sci. Food Agric. 86, 1722–1731. https://doi.org/10.1002/jsfa.2470

Sydenham, R., Zheng, Y., Riemens, A., Tsang, A., Powlowski, J., Storms, R., 2014. Cloning and enzymatic characterization of four thermostable fungal endo-1,4-β-xylanases. Appl. Microbiol. Biotechnol. 98, 3613–3628. https://doi.org/10.1007/s00253-013-5244-8

Taherzadeh, M.J., Karimi, K., 2007. Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review. Bioresources 2, 707–738.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. 101, 11030–11035. https://doi.org/10.1073/pnas.0404206101

Tan, T., Shang, F., Zhang, X., 2010. Current development of biorefinery in China. Biotechnol. Adv. 28, 543–555. https://doi.org/10.1016/j.biotechadv.2010.05.004

Tanti, R., Barbut, S., Marangoni, A.G., 2016. Oil stabilization of natural peanut butter using food grade polymers. Food Hydrocoll. 61, 399–408. https://doi.org/10.1016/j.foodhyd.2016.05.034

Taylor, J.R.N., 2016. Grain production and consumption: Africa, in: Wrigley, C.W., Corke, H., Seetharaman, K., Faubion, J. (Eds.), Encyclopedia of Food Grains. Academic Press, Oxford, pp. 359–366. https://doi.org/10.1016/B978-0-12-394437-5.00043-7

Teixeira, R.S., Silva, A.S., Moutta, R.O., Ferreira-Leitão, V.S., Barros, R.R., Ferrara, M., Bon, E.P., 2014. Biomass pretreatment: a critical choice for biomass utilization via biotechnological routes, in: BMC Proceedings. BioMed Central, Florianópolis, Brazil, pp. 1–3. https://doi.org/10.1186/1753-6561-8-S4-O34

Teleman, A., Lundqvist, J., Tjerneld, F., Stålbrand, H., Dahlman, O., 2000. Characterization of acetylated 4-O-methylglucuronoxylan isolated from aspen employing 1H and 13C NMR spectroscopy. Carbohydr. Res. 329, 807–815. https://doi.org/10.1016/S0008-6215(00)00249-4

Tengerdy, R.P., Szakacs, G., 2003. Bioconversion of lignocellulose in solid substrate fermentation. Biochem. Eng. J. 13, 169–179. https://doi.org/10.1016/S1369-703X(02)00129-8

Tenkanen, M., Luonteri, E., Teleman, A., 1996. Effect of side groups on the action of β-xylosidase from *Trichoderma reesei* against substituted xylo-oligosaccharides. FEBS Lett. 399, 303–306. https://doi.org/10.1016/S0014-5793(96)01313-0

Tenkanen, M., Siika-aho, M., 2000. An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. J. Biotechnol. 78, 149–161. https://doi.org/10.1016/S0168-1656(99)00240-0

Terrapon, N., Lombard, V., Drula, E., Coutinho, P.M., Henrissat, B., 2017. The CAZy database/the Carbohydrate-Active Enzyme (CAZy) Database: Principles and usage guidelines, in: A Practical Guide to Using Glycomics Databases. Springer Japan, Tokyo, pp. 117–131. https://doi.org/10.1007/978-4-431-56454-6_6

Terrasan, C.R.F., Temer, B., Sarto, C., Júnior, F.G.S., Carmona, E.C., 2013. Xylanase and β-xylosidase from *Penicillium janczewskii*: production, physico-chemical properties, and

application of the crude extract to pulp biobleaching. BioResources 8, 1292–1305.

Thomas, L., Joseph, A., Gottumukkala, L.D., 2014. Xylanase and cellulase systems of *Clostridium* sp.: an insight on molecular approaches for strain improvement. Bioresour. Technol. 158, 343–50. https://doi.org/10.1016/j.biortech.2014.01.140

Tikhomirov, D.F., Sinitsyn, A.P., Zorov, I.N., Williams, C., 2003. Non-starch polysaccharide hydrolysing microbial enzymes in grain processing, in: Courtin, C.M., Veraverbeke, W.S., Delcour, J.A. (Eds.), Recent Advances in Enzymes in Grain Processing. Katholieke University, Leuven, pp. 423–418.

Trott, O., Olson, A., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31, 455–461. https://doi.org/10.1002/jcc.21334

Tuomivaara, S.T., Yaoi, K., O'Neill, M.A., York, W.S., 2015. Generation and structural validation of a library of diverse xyloglucan-derived oligosaccharides, including an update on xyloglucan nomenclature. Carbohydr. Res. 402, 56–66. https://doi.org/10.1016/j.carres.2014.06.031

Turner, P., Mamo, G., Karlsson, E.N., 2007. Potential and utilization of thermophiles and thermostable enzymes in biorefining. Microb. Cell Fact. 6, 9. https://doi.org/10.1186/1475-2859-6-9

Uday, U.S.P., Choudhury, P., Bandyopadhyay, T.K., Bhunia, B., 2016. Classification, mode of action and production strategy of xylanase and its application for biofuel production from water hyacinth. Int. J. Biol. Macromol. 82, 1041–1054. https://doi.org/10.1016/j.ijbiomac.2015.10.086

United Nations, 2019. World Population Prospects 2019: Highlights (ST/ESA/SER.A/423).

Valls, C., Cadena, E.M., Blanca Roncero, M., 2013. Obtaining biobleached eucalyptus cellulose fibres by using various enzyme combinations. Carbohydr. Polym. 92, 276–282. https://doi.org/10.1016/J.CARBPOL.2012.08.083

Valls, C., Pastor, F.I.J., Vidal, T., Roncero, M.B., Díaz, P., Valenzuela, S. V, 2018. Antioxidant activity of xylooligosaccharides produced from glucuronoxylan by Xyn10A and Xyn30D xylanases and *Eucalyptus* autohydrolysates. Carbohydr. Polym. 194, 43–50. https://doi.org/10.1016/j.carbpol.2018.04.028

van Zyl, W.H., Rose, S.H., Trollope, K., Görgens, J.F., 2010. Fungal β-mannanases: Mannan hydrolysis, heterologous production and biotechnological applications. Process Biochem. 45, 1203–1213. https://doi.org/10.1016/J.PROCBIO.2010.05.011

Verma, A.K., Goyal, A., 2016. A novel member of family 30 glycoside hydrolase subfamily 8 glucuronoxylan endo-β-1,4-*Clostridium thermocellum* orchestrates catalysis on arabinose decorated xylans. J. Mol. Catal. B Enzym. 129, 6–14. https://doi.org/10.1016/j.molcatb.2016.04.001

Verma, D., Satyanarayana, T., 2012. Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. Bioresour. Technol. 107, 333–8. https://doi.org/10.1016/j.biortech.2011.12.055

Vršanská, M., Kolenová, K., Puchart, V., Biely, P., 2007. Mode of action of glycoside hydrolase family 5 glucuronoxylan xylanohydrolase from *Erwinia chrysanthemi*. FEBS J. 274, 1666–1677. https://doi.org/10.1111/j.1742-4658.2007.05710.x

Walia, A., Guleria, S., Mehta, P., Chauhan, A., Parkash, J., 2017. Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. 3 Biotech 7, 11. https://doi.org/10.1007/s13205-016-0584-6

Walton, S.L., 2009. Biological conversion of hemicellulose extract into value-added fuels and chemicals. University of Maine.

Wan, Q., Zhang, Q., Hamilton-Brehm, S., Weiss, K., Mustyakimov, M., Coates, L., Langan, P., Graham, D., Kovalevsky, A., 2014. X-ray crystallographic studies of family 11 xylanase Michaelis and product complexes: Implications for the catalytic mechanism. Acta

Crystallogr. Sect. D Biol. Crystallogr. 70, 11–23. https://doi.org/10.1107/S1399004713023626

Wang, H., Pu, Y., Ragauskas, A., Yang, B., 2019. From lignin to valuable products–strategies, challenges, and prospects. Bioresour. Technol. 271, 449–461. https://doi.org/10.1016/j.biortech.2018.09.072

Wang, J., Kim, K.H., Kim, S., Kim, Y.S., Li, Q.X., Jun, S., 2010. Simple quantitative analysis of *Escherichia coli* K-12 internalized in baby spinach using Fourier Transform Infrared spectroscopy. Int. J. Food Microbiol. 144, 147–151. https://doi.org/10.1016/j.ijfoodmicro.2010.09.013

Wang, W., Yan, R., Nocek, B.P., Vuong, T. V, Di Leo, R., Xu, X., Cui, H., Gatenholm, P., Toriz, G., Tenkanen, M., Savchenko, A., Master, E.R., 2016. Biochemical and structural characterization of a five-domain GH115 α-glucuronidase from the marine bacterium *Saccharophagus degradans* 2-40T. J. Biol. Chem. 291, 14120–14133. https://doi.org/10.1074/jbc.M115.702944

Wang, Y., Fu, Z., Huang, H., Zhang, H., Yao, B., Xiong, H., Turunen, O., 2012. Improved thermal performance of *Thermomyces lanuginosus* GH11 xylanase by engineering of an N-terminal disulfide bridge. Bioresour. Technol. 112, 275–279. https://doi.org/10.1016/j.biortech.2012.02.092

Wang, Y., Tashiro, Y., Sonomoto, K., 2015. Fermentative production of lactic acid from renewable materials: recent achievements, prospects, and limits. J. Biosci. Bioeng. 119, 10–18. https://doi.org/10.1016/j.jbiosc.2014.06.003

Webb, B., Sali, A., 2016. Comparative protein structure modeling using MODELLER. Curr. Protoc. Bioinforma. https://doi.org/10.1002/cpbi.3

Wilson, D.B., 2011. Microbial diversity of cellulose hydrolysis. Curr. Opin. Microbiol. 14, 259–263. https://doi.org/10.1016/J.MIB.2011.04.004

Wolfschoon-Pombo, A.F., Dang, B.P., Chiriboga Chiriboga, B., 2018. Forced syneresis

determination results from commercial cream cheese samples. Int. Dairy J. 85, 129–136. https://doi.org/10.1016/j.idairyj.2018.05.006

Wong, C.-L., Huang, C.-C., Lu, W.-B., Chen, W.-M., Chang, J.-S., 2012. Producing 2,3-butanediol from agricultural waste using an indigenous *Klebsiella* sp. Zmd30 strain. Biochem. Eng. J. 69, 32–40. https://doi.org/10.1016/j.bej.2012.08.006

Wong, K.K.Y., Tan, L.U.L., Saddler, J.N., 1988. Multiplicity of β-1, 4-xylanase in microorganisms: functions and applications. Microbiol. Rev. 52, 305–317.

Wu, B., Yu, Q., Chang, S., Pedroso, M.M., Gao, Z., He, B., Schenk, G., 2019. Expansin assisted bio-affinity immobilization of endoxylanase from *Bacillus subtilis* onto corncob residue: Characterization and efficient production of xylooligosaccharides. Food Chem. 282, 101–108. https://doi.org/10.1016/j.foodchem.2019.01.004

Xia, Y.-G., Liang, J., Yang, B.-Y., Wang, Q.-H., Kuang, H.-X., 2015. Structural studies of an arabinan from the stems of *Ephedra sinica* by methylation analysis and 1D and 2D NMR spectroscopy. Carbohydr. Polym. 121, 449–456. https://doi.org/10.1016/J.CARBPOL.2014.12.058

Xiao, X., Bian, J., Peng, X.-P., Xu, H., Xiao, B., Sun, R.-C., 2013. Autohydrolysis of bamboo (*Dendrocalamus giganteus* Munro) culm for the production of xylo-oligosaccharides. Bioresour. Technol. 138, 63–70. https://doi.org/10.1016/j.biortech.2013.03.160

Xie, D.F., Fang, H., Mei, J.Q., Gong, J.Y., Wang, H.P., Shen, X.Y., Huang, J., Mei, L.H., 2018. Improving thermostability of (R)-selective amine transaminase from *Aspergillus terreus* through introduction of disulfide bonds. Biotechnol. Appl. Biochem. 65, 255–262. https://doi.org/10.1002/bab.1572

Xu, Z., Wang, Q., Jiang, Z., Yang, X., Ji, Y., 2007. Enzymatic hydrolysis of pretreated soybean straw. Biomass and Bioenergy 31, 162–167. https://doi.org/10.1016/J.BIOMBIOE.2006.06.015

Xu, Z., Zhang, S., Mu, Y., Kong, J., 2018. Paenibacillus panacisoli enhances growth of

Lactobacillus spp. by producing xylooligosaccharides in corn stover ensilages. Carbohydr. Polym. 184, 435–444. https://doi.org/10.1016/J.CARBPOL.2017.12.044

Xue, J.-L., Zhao, S., Liang, R.-M., Yin, X., Jiang, S.-X., Su, L.-H., Yang, Q., Duan, C.-J., Liu, J.-L., Feng, J.-X., 2016. A biotechnological process efficiently co-produces two high value-added products, glucose and xylooligosaccharides, from sugarcane bagasse. Bioresour. Technol. 204, 130–8. https://doi.org/10.1016/j.biortech.2015.12.082

Yamura, I., Koga, T., Matsumoto, T., 1997. Purification and some properties of endo-1,4-β xylanase from a fresh water mollusk *Pomacea insularus* (de Oringny). Biosci. Biotechnol. Biochem. 61, 615–620.

Yang, C.-H.H., Yang, S.-F.F., Liu, W.-H.H., 2007. Production of xylooligosaccharides from xylans by extracellular xylanases from *Thermobifida fusca*. J. Agric. Food Chem. 55, 3955–3959. https://doi.org/10.1021/jf0635964

Yang, H., Shi, Z., Xu, G., Qin, Y., Deng, J., Yang, J., 2019. Bioethanol production from bamboo with alkali-catalyzed liquid hot water pretreatment. Bioresour. Technol. 274, 261–266. https://doi.org/10.1016/J.BIORTECH.2018.11.088

Yang, J., Summanen, P.H., Henning, S.M., Hsu, M., Lam, H., Huang, J., Tseng, C.-H., Dowd, S.E., Finegold, S.M., Heber, D., Li, Z., 2015. Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study. Front. Physiol. 6, 216. https://doi.org/10.3389/fphys.2015.00216

Yang, Y., Yang, J., Liu, J., Wang, R., Liu, L., Wang, F., Yuan, H., 2018. The composition of accessory enzymes of *Penicillium chrysogenum* P33 revealed by secretome and synergistic effects with commercial cellulase on lignocellulose hydrolysis. Bioresour. Technol. 257, 54–61. https://doi.org/10.1016/j.biortech.2018.02.028

Ye, L., Hudari, M.S. Bin, Li, Z., Wu, J.C., 2014. Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for L-lactic acid production by *Bacillus coagulans* JI12. Biochem. Eng. J. 83, 16–21.

https://doi.org/10.1016/j.bej.2013.12.005

Ye, L., Zhou, X., Hudari, M.S. Bin, Li, Z., Wu, J.C., 2013. Highly efficient production of L-lactic acid from xylose by newly isolated *Bacillus coagulans* C106. Bioresour. Technol. 132, 38–44. https://doi.org/10.1016/J.BIORTECH.2013.01.011

Yerlikaya, O., 2019. Probiotic potential and biochemical and technological properties of *Lactococcus lactis* ssp. *lactis* strains isolated from raw milk and kefir grains. J. Dairy Sci. 102, 124–134. https://doi.org/10.3168/JDS.2018-14983

Zabed, H.M., Akter, S., Yun, J., Zhang, G., Awad, F.N., Qi, X., Sahu, J.N., 2019. Recent advances in biological pretreatment of microalgae and lignocellulosic biomass for biofuel production. Renew. Sustain. Energy Rev. 105, 105–128. https://doi.org/10.1016/j.rser.2019.01.048

Zaide, G., Shallom, D., Shulami, S., Zolotnitsky, G., Golan, G., Baasov, T., Shoham, G., Shoham, Y., 2001. Biochemical characterization and identification of catalytic residues in α-glucuronidase from *Bacillus stearothermophilus* T-6. Eur. J. Biochem. 268, 3006–3016. https://doi.org/10.1046/j.1432-1327.2001.02193.x

Zhang, L., Yu, H., Wang, P., Li, Y., 2014a. Production of furfural from xylose, xylan and corncob in gamma-valerolactone using $FeCl_3 \cdot 6H_2O$ as catalyst. Bioresour. Technol. 151, 355–60. https://doi.org/10.1016/j.biortech.2013.10.099

Zhang, S., Wang, H., Shi, P., Xu, B., Bai, Y., Luo, H., Yao, B., 2014b. Cloning, expression, and characterization of a thermostable β-xylosidase from thermoacidophilic *Alicyclobacillus* sp. A4. Process Biochem. 49, 1422–1428. https://doi.org/10.1016/j.procbio.2014.05.020

Zhang, Y., Chen, X., Luo, J., Qi, B., Wan, Y., 2014c. An efficient process for lactic acid production from wheat straw by a newly isolated *Bacillus coagulans* strain IPE22. Bioresour. Technol. 158, 396–399. https://doi.org/10.1016/j.biortech.2014.02.128

Zhang, Y., Yu, G., Li, B., Mu, X., Peng, H., Wang, H., 2016. Hemicellulose isolation, characterization, and the production of xylo-oligosaccharides from the wastewater of a

viscose fiber mill. Carbohydr. Polym. 141, 238–243. https://doi.org/10.1016/j.carbpol.2016.01.022

Zhao, C., Wu, Y., Liu, X., Liu, B., Cao, H., Yu, H., Sarker, S.D., Nahar, L., Xiao, J., 2017. Functional properties, structural studies and chemo-enzymatic synthesis of oligosaccharides. Trends Food Sci. Technol. 66, 135–145. https://doi.org/10.1016/J.TIFS.2017.06.008

Zhao, Y., Lu, W.-J., Wang, H.-T., 2009. Supercritical hydrolysis of cellulose for oligosaccharide production in combined technology. Chem. Eng. J. 150, 411–417. https://doi.org/10.1016/j.cej.2009.01.026

Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. Int. J. Agric. Biol. Eng. 2, 51–68. https://doi.org/10.3965/j.issn.1934-6344.2009.03.051-068

Zhou, X., Zhao, J., Zhang, X., Xu, Y., 2019. An eco-friendly biorefinery strategy for xylooligosaccharides production from sugarcane bagasse using cellulosic derived gluconic acid as efficient catalyst. Bioresour. Technol. 289, 121755. https://doi.org/10.1016/J.BIORTECH.2019.121755

Zhu, J., Zhang, D., Tang, H., Zhao, G., 2018. Structure relationship of non–covalent interactions between phenolic acids and arabinan–rich pectic polysaccharides from rapeseed meal. Int. J. Biol. Macromol. 120, 2597–2603. https://doi.org/10.1016/J.IJBIOMAC.2018.09.036

Zhu, J.Y., Wang, G.S., Pan, X.J., Gleisner, R., 2009. Specific surface to evaluate the efficiencies of milling and pretreatment of wood for enzymatic saccharification. Chem. Eng. Sci. 64, 474–485. https://doi.org/10.1016/J.CES.2008.09.026

Zhu, S., Wu, Y., Yu, Z., Zhang, X., Li, H., Gao, M., 2006. The effect of microwave irradiation on enzymatic hydrolysis of rice straw. Bioresour. Technol. 97, 1964–1968. https://doi.org/10.1016/J.BIORTECH.2005.08.008

Zhu, Y., Lee, Y.Y., Elander, R.T., 2005. Optimization of dilute-acid pretreatment of corn stover

using a high-solids percolation reactor. Appl. Biochem. Biotechnol. 124, 1045–1054. https://doi.org/10.1385/ABAB:124:1-3:1045

International Journal of Biological Macromolecules 132 (2019) 987–993

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Xylan from bambara and cowpea biomass and their structural elucidation

Nanthakumar Arumugam [a,b], Peter Biely [b], Vladimír Puchart [b], Abe Shegro Gerrano [c], Koel De Mukherjee [a], Suren Singh [a], Santhosh Pillai [a,*]

[a] Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, PO BOX 1334, Durban 4000, South Africa
[b] Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-845 38 Bratislava, Slovak Republic
[c] Agricultural Research Council-Vegetable and Ornamental Plant Institute, Pretoria, South Africa

ARTICLE INFO

Article history:
Received 31 January 2019
Received in revised form 3 April 2019
Accepted 4 April 2019
Available online 05 April 2019

Keywords:
Xylan
Enzymatic hydrolysis
Xylooligosaccharides
NMR spectroscopy
MALDI-ToF MS
Docking

ABSTRACT

This work is the first report on the isolation and structural elucidation of xylan from bambara and cowpea biomass. The xylans, isolated using acidic delignification followed by NaOH extraction method gave 12.3% and 13.6% yield, respectively. ^{1}H NMR analyses revealed that both the xylans were glucuronoxylan. The presence of xylose and glucuronic acid was confirmed by monosaccharide analysis and uronic acid assay. Further, xylooligosaccharide production from bambara and cowpea xylans was carried out using xylanase from three different glycoside hydrolase families, and the products were analyzed by TLC and MALDI ToF MS. The hydrolysis products of both xylans resembled hardwood glucuronoxylan fragments, generated under similar conditions. The most common oligosaccharides observed in the hydrolysates were Xyl_2, Xyl_3, $MeGlcA^3Xyl_3$, $MeGlcAXyl_4$ and $MeGlcAXyl_5$. A series of computational approaches were also used to study the interactions of the three different xylanases with xylan. Thus, untapped biomass such as bambara and cowpea could serve as a potential source for xylan which could further be converted to xylooligosaccharides and many other value added chemicals.

1. Introduction

Global agricultural waste production exceeds 220 billion tons annually [1]. Lignocellulosic biomass, a principal byproduct of the agricultural sector, has great potential as a source for valuable product generation. Africa has various indigenous agricultural crops generating an enormous amount of biomass as waste, which could be exploited in numerous ways. Bambara (*Vigna subterranea*) and cowpea (*Vigna unguiculata*) plants are few examples of the unexploited biomass that could serve as a source of polysaccharides. However, each crop may have a distinctive polysaccharide structure which depends mainly on their phylogenetic position, varietal origin, tissue or cell type, and the developmental stage of the cells [2]. This warrants the need to understand their structure to develop suitable pretreatment strategies that could improve the effective utilization of lignocellulose and reduce costs [3,4].

The major structural polysaccharide of lignocellulose is cellulose, which is a primary source for the production of many value added chemicals. However, cellulose is naturally recalcitrant to enzymatic and microbial degradation due to its rigid and compact structure. In addition to cellulose, lignin creates a matrix framework which makes the accessibility more critical. On the other hand, hemicellulose covers 25–35% of total lignocellulose and has the potential to act as a precursor for the synthesis of several biochemical side products [5]. Furthermore, the heterogenicity, amorphous and non-crystalline nature of hemicellulose makes it amenable for rapid hydrolysis by chemical or enzymatic treatments [6].

Xylan, the predominant hemicellulose constituent, is a non-crystalline complex polysaccharide consisting of a backbone of 1,4-linked β D-xylopyranosyl units substituted with acetyl, glucuronosyl, and arabinosyl side chains [7]. Based on the components present in the xylan molecule, it can be categorized as linear homoxylan or heteroxylan such as arabinoxylan, glucuronoxylan, and arabinoglucuronoxylan [8]. The foremost products of xylan hydrolysis are xylooligosaccharides (XOS) which include both linear and substituted oligosaccharides comprising the xylose backbone and having distinctive biological effects [9]. Xylan hydrolysis is catalysed by

Abbreviations: Xyl*p*, β-D-xylopyranosyl residue; MeGlcA, 4-*O*-methyl-D-glucuronic acid; GH, glycoside hydrolase; Xyl_n, linear xylooligosaccharide composed of n D-xylopyranosyl residues linked by β-1,4-linkages; $MeGlcA^iXyl_n$, aldouronic acid containing one residue of MeGlcA linked to Xyl*p* residue marked in Xyl_n with 'i' which is the number of Xyl*p* residue counted from the reducing end ('i' would be equal to 1 if MeGlcA would be linked to the reducing end Xyl, 2 if MeGlcA would be linked to the second Xyl*p* residue from the reducing end, *etc*).

* Corresponding author.
E-mail address: santhoshk@dut.ac.za (S. Pillai).

https://doi.org/10.1016/j.ijbiomac.2019.04.030
0141-8130/

endo β 1,4 xylanase (EC 3.2.1.8) which interacts with the xylan back bone and breaks down β 1,4 linkages between the adjacent xylose units. Based on their catalytic mechanism and amino acid sequence in formation, xylanases are grouped into different glycoside hydrolase (GH) families. Among them, GH10 and GH11 are the most widely used xylanases [10]. In addition, GH30 xylanases also drew attention due to its distinct substrate specificity [11].

XOS are reported to have applications in many sectors including food, feed, pharmaceutical or chemical industries. [12,13]. They are available naturally in fruits, shoots, vegetables, honey and milk but XOS content in these foods is very low [14,15]. This offers an opportu nity to explore biomass rich in xylan for XOS production. Furthermore, lignocellulosic biomass could be a good source for the production of XOS rich food and nutraceuticals, which provide health benefits such as increased immunity and prevention of diseases [16].

Till date, there are no reports on the chemical properties of hemicel lulose or XOS derived from bambara and cowpea. Hence, this study has primarily focused on elucidating the potential of bambara and cowpea as a source of xylan. Biochemical characterization of xylan and its con version to XOS was also performed. Lastly, an *in silico* analysis was con ducted to validate the productive binding of xylanase to xylan.

2. Experimental section

2.1. Sources of plant materials and polysaccharide extraction

Bambara and cowpea biomass were collected from the Agricultural Research Council Vegetable and Ornamental Plant Institute, Pretoria. The sun dried biomass was ground with a ball mill (United Scientific, South Africa), sieved into a fine powder (500 μm particle size) and stored in airtight containers. Xylan from these biomass was extracted by acidic delignification and sodium hydroxide dissolution [17]. To re duce the presence of starch in the extracted materials, a commercial *Ba cillus subtilis* α amylase was used (Sigma, St. Louis, MO, USA).

2.2. Characterization of extracted xylan

Total carbohydrate content was estimated by phenol sulphuric acid method [18] using D xylose as a standard. Neutral sugars were quanti fied as alditol acetates after hydrolysis with 2 M trifluoroacetic acid (TFA) for 1 h at 120 °C [19]. Total uronic acid content was determined by the 3 hydroxydiphenyl assay [20] using D glucuronic acid as a stan dard. NMR analysis was carried out by AVANCE III HD 400 MHz equipped with a broad band BB (H F) D 05 Z liquid N_2 Prodigy probe (all from Bruker BioSpin, Rheinstetten, Germany) in D_2O at 25 °C using automatic chemical shift calibration. The following parameters were used for recording 1 D ^{1}H spectra: pre saturation *zgpr* sequence, pre saturation delay 2.5 s, a r.f. 90° pulse and acquisition time 2.5 s.

2.3. Enzymatic hydrolysis of xylan and product analysis

Purified GH11 xylanase from the thermophilic fungus *Thermomyces lanuginosus* SSBP (*Tl*GH11) was prepared as described earlier [21]. *Cellvibrio mixtus* GH10 xylanase (*Cm*GH10) was purchased from Megazyme Int. (Bray, Ireland). GH30 xylanase from *Erwinia chrysanthemi* (*Ec*GH30) was kindly provided by Prof. James F. Preston, University of Florida (Gainesville, FL, USA).

Xylan (2%; w/v) extracted from bambara and cowpea was dissolved in 50 mM sodium citrate buffer (pH 6.5) and incubated with 10 U/g *Tl*GH11 xylanase at 50 °C for 24 h in a water bath. For *Cm*GH10 and *Ec*GH30 xylanases, the incubation was carried out in sodium acetate buffer (50 mM; pH 5.5) at 35 °C. Hydrolysis products were analyzed by TLC on silica gel coated aluminium plates (Merck, Darmstadt, Germany) developed in 1 butanol/ethanol/water (10:8:5, v/v) and de tected with orcinol reagent [22]. MS spectra of XOS were obtained using an Ultraflex MALDI ToF/ToF instrument (Bruker Daltonics GmbH, Bremen, Germany) equipped with a nitrogen laser emitting at 337 nm which was operated in a reflectron positive acquisition mode [17].

2.4. Structure analysis and docking studies

The amino acid sequences of xylanases from *T. lanuginosus* SSBP, *C. mixtus and E. chrysanthemi* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) database with the GenBank IDs AAB94633.1, AAD09439.3 and AAB53151.1, respectively. Primary sequence analysis of the xylanases was performed using ScanProsite [23,24] and InterProScan server. In order to gain information on the active sites, CASTp (http://sts.bioe.uic.edu/castp/index.html?2was) analysis was performed. The docking studies were further executed using AutoDock Vina (http://autodock.scripps.edu/) package. The PDB file of aldopentaouronic acid was transformed as AutoDock suitable coordi nate file (PDBQT) using AutoDock Tools (ADT) [25]. Gasteiger charges were added, and the nonpolar hydrogen atoms were merged to carbon atoms in the ligand structure. A grid box was created with dimension 30 × 35 × 40 Å along the XYZ direction with a grid spacing of 1 Å, using the AutoGrid module for *T. lanuginosus* SSBP xylanase structure. Similar steps were implemented for the other two xylanase modelled struc tures, with slightly modified grid box sizes, 28 × 38 × 36 Å and 32 × 44 × 40 Å for *C. mixtus* and *E. chrysanthemi* respectively. Six conforma tions were generated for each complex, and the best pose with the low est docked binding energy was selected for further analysis. The docking results (pose and their energy values) were saved in CSV format.

3. Results and discussion

3.1. Xylan isolation from bambara and cowpea

The sodium chlorite delignification method was more effective than sodium hypochlorite in removing most of the lignin from both bambara and cowpea biomass. The addition of 1% acetic acid and incubation at 70 °C, apparently induced the relaxation of lignocellulosic structure. The xylan yield (w/w) from bambara and cowpea biomass was 12.3 and 13.6%, respectively.

3.2. Carbohydrate composition of extracted xylan

Total carbohydrate analysis of sodium chlorite delignified xylan showed that bambara and cowpea xylan contained 65.8% and 66.6% (w/w) of total carbohydrates, respectively. Xylose was the major mono saccharide observed in both bambara and cowpea xylan, however, they contained a notable amount of glucose which was an unexpected find ing after xylan extraction (Table 1). The high content of glucose could be partially due to the presence of starch co extracted with xylan or may be a result of the biomass being contaminated by starch containing ma terials [26]. To reduce the glucose contamination of starch origin, the ex tracted xylan was treated with a commercial α amylase which led to the liberation of significant amounts of glucose and short maltooligosaccharides (Fig. 1). It is noteworthy that starch removal by the amylase treatment resulted in a markedly reduced abundance of glucose in amylase treated xylans (Table 1). The NMR spectroscopy also confirmed the removal of starch by the reduction in signals at 5.38 ppm (Fig. 2).

Arabinose and galactose were also detected in xylan from both bam bara and cowpea (Table 1). This might be due to the presence of arabinogalactan coextracted with the main polysaccharide. Both xylans contained 4 *O* methylglucuronic acid in the concentration of 164 mg/g (bambara) and 197 mg/g (cowpea). These results are comparable with a previous study where the presence of glucuronic acid in xylan from the leaves of Algerian *Argania spinosa* was reported [2].

Table 1
Molar ratio of monosaccharides in bambara and cowpea xylan.

Sample	Xylose	Rhamnose	Fucose	Arabinose	Mannose	Galactose	Glucose	Glucose (after amylase treatment)
Bambara xylan	1.00	0.048	0.013	0.310	0.047	0.223	0.39	0.14
Cowpea xylan	1.00	0.040	0.009	0.232	0.031	0.190	0.91	0.35

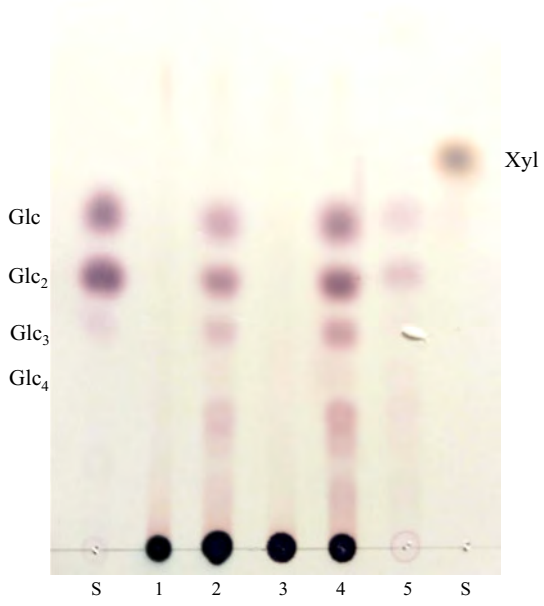

Fig. 1. TLC analysis of products after α-amylase treatment of xylans from bambara and cowpea; S, standards (Glucose; maltooligosaccharides - maltose to maltotetraose; Xyl, xylose); 1, control bambara xylan; 2, products released by α-amylase from bambara xylan; 3, control cowpea xylan; 4, products released by α-amylase from cowpea xylan; 5, products released by α-amylase from commercial starch.

3.3. NMR analysis of the isolated polysaccharides

The ^{1}H NMR spectra of both bambara and cowpea xylan did not show a significant H 1α signal of the reducing end xylopyranosyl residue (at 5.17 ppm) which corresponds to the polymeric nature of the isolated materials. The signals for acetyl and feruloyl groups were absent in both the xylans due to the fact that alkali extraction process destroyed their ester linkages [11].

The NMR spectra of bambara and cowpea xylan were interesting as they contained peaks of MeGlcA both at 5.27 ppm (H 1) and 3.46 ppm (4 *O* methyl ether group) (Fig. 2). In ^{1}H ^{13}C HSQC spectra of the amy lase treated xylans (Figs. S1 and S2) these signals gave cross peaks at 5.27/97.7 ppm and 3.46/60.1 ppm which are unambiguously assigned to glucuronoxylan, providing the evidence for chemical nature of the isolated polysaccharides. The intensity of the side chain signals showed that cowpea and bambara xylan have a similar content of MeGlcA (this finding is in line with the determination of uronic acids). However, in comparison to hardwood, the extracted xylans contained significantly smaller amounts of 2 O α linked MeGlcA side residues.

3.4. Enzymatic breakdown of bambara and cowpea xylan

Enzymatic hydrolysis on the extracted xylans was performed with xylanases from three different GH families and the hydrolysis products were analyzed by TLC (Fig. 3) and MALDI ToF MS (Fig. 4). Xylanases from distinct GH families exhibit different modes of action [11], resulting in a heterogenicity of their hydrolysis products, which might be helpful in the identification of the type of xylan present in the bio mass. TLC analysis showed that *Tl*GH11 xylanase released mainly Xyl_2 and Xyl_3 as neutral oligosaccharides from bambara and cowpea xylan, in addition to some aldouronic acids such as MeGlcA^{3}Xyl_4, MeGlcAXyl_5 and MeGlcAXyl_6. All these fragments are typical hydrolysis products of hardwood glucuronoxylan generated by GH11 xylanases [27,28].

The mode of action of *Cm*GH10 xylanase on both xylans was similar and resulted in similar hydrolysis pattern. The main hydrolysis products

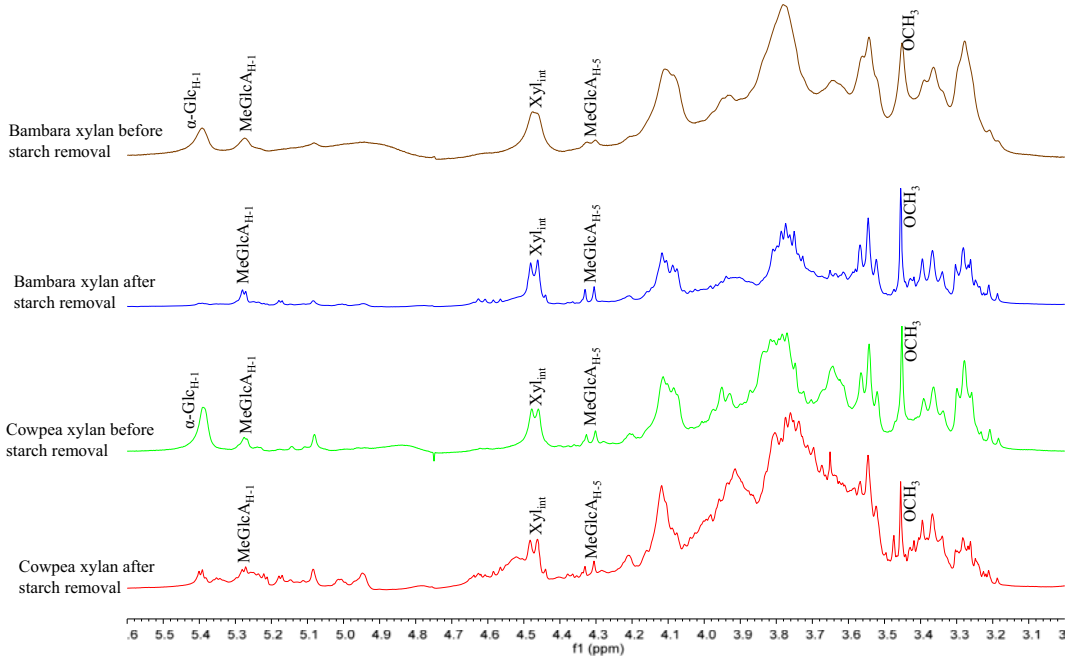

Fig. 2. ^{1}H NMR spectra of the bambara and cowpea xylan before and after starch removal. Description of signals: OCH_3, 4-*O*-methyl ether group of MeGlcA; $MeGlcA_{H\ 1}$ and $MeGlcA_{H\ 5}$, H-1 and H-5 signals of MeGlcA, respectively; Xyl_{int}, H-1 signal of internal xylose; α-$Glc_{H\ 1}$, H-1 of α-1,4-linked glucopyranosyl residues originating from starch.

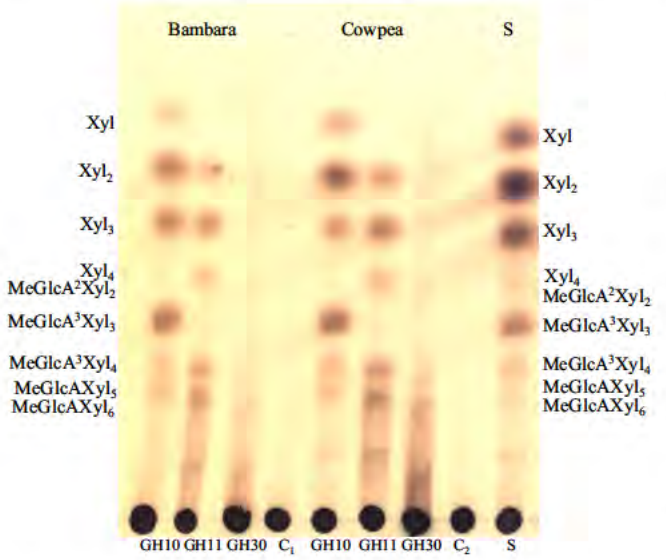

Fig. 3. TLC analysis of XOS produced from bambara and cowpea xylans by GH10, GH11 and GH30 xylanases; C_1 and C_2, Controls (xylan incubated without enzyme); S, oligosaccharide standards; Xyl, xylose; Xyl_2, xylobiose; Xyl_3, xylotriose; Xyl_4, xylotetraose; $MeGlcA^2Xyl_2$, Aldotriouronic acid; $MeGlcA^3Xyl_3$, Aldotetraouronic acid; $MeGlcA^3Xyl_4$, Aldopentaouronic acid; $MeGlcAXyl_5$, Aldohexaouronic acid; $MeGlcAXyl_6$, Aldoheptaouronic acid.

were Xyl, Xyl_2, Xyl_3, $MeGlcA^3Xyl_3$, $MeGlcAXyl_4$ and $MeGlcAXyl_5$. In agreement with the literature [11,17,29], *Cm*GH10 xylanase produced shorter oligosaccharides than *Tl*GH11 xylanase. Conversely, *Ec*GH30 xylanase only acts on uronic acid containing xylan, generating a series of aldouronic acids which are essentially free of linear XOS. It hydrolyzed a smaller portion of both xylans by releasing few acidic oligosaccharides, indicating low uronic acid content in both hemicelluloses. These results correspond to the abundance of MeGlcA deduced from the spectral analyses. It is noteworthy that qualitatively the degradation products generated by all three xylanases from bambara and cowpea xylans resemble those of typical hardwood glucuronoxylan.

The MALDI ToF MS data established the presence of oligosaccharides made up of substituted and unsubstituted pentose sugars in both the xylans (Fig. 4). Since the molecular mass of both xylose and arabinose is the same (150 g/mol), they cannot be distinguished. However, from the length of the hydrolysis products from cowpea, we can assume that *Cm*GH10 xylanase released primarily Xyl_2, Xyl_3 and $MeGlcAXyl_3$. In the case of *Tl*GH11, we detected two longer neutral oligosaccharides having mass to charge ratio values of 569 and 701; the former being presumably xylotetraose, as well as aldouronic acids composed of one MeGlcA and 4 or 5 pentose residues. In *Ec*GH30 hydrolysate, only weak signals for aldouronic acids were observed, particularly in the case of bambara xylan hydrolysate, which is in line with a slightly lower content of MeGlcA. A similar trend was also observed during TLC analysis (Fig. 3).

3.5. Structure elucidation and docking analysis

To circumvent expensive, time and labour consuming wet lab experiments, computational modelling and docking analysis are widely applied to understand the nature of biomolecular interactions. In this study, the focus was placed on the analysis of three xylanase structures

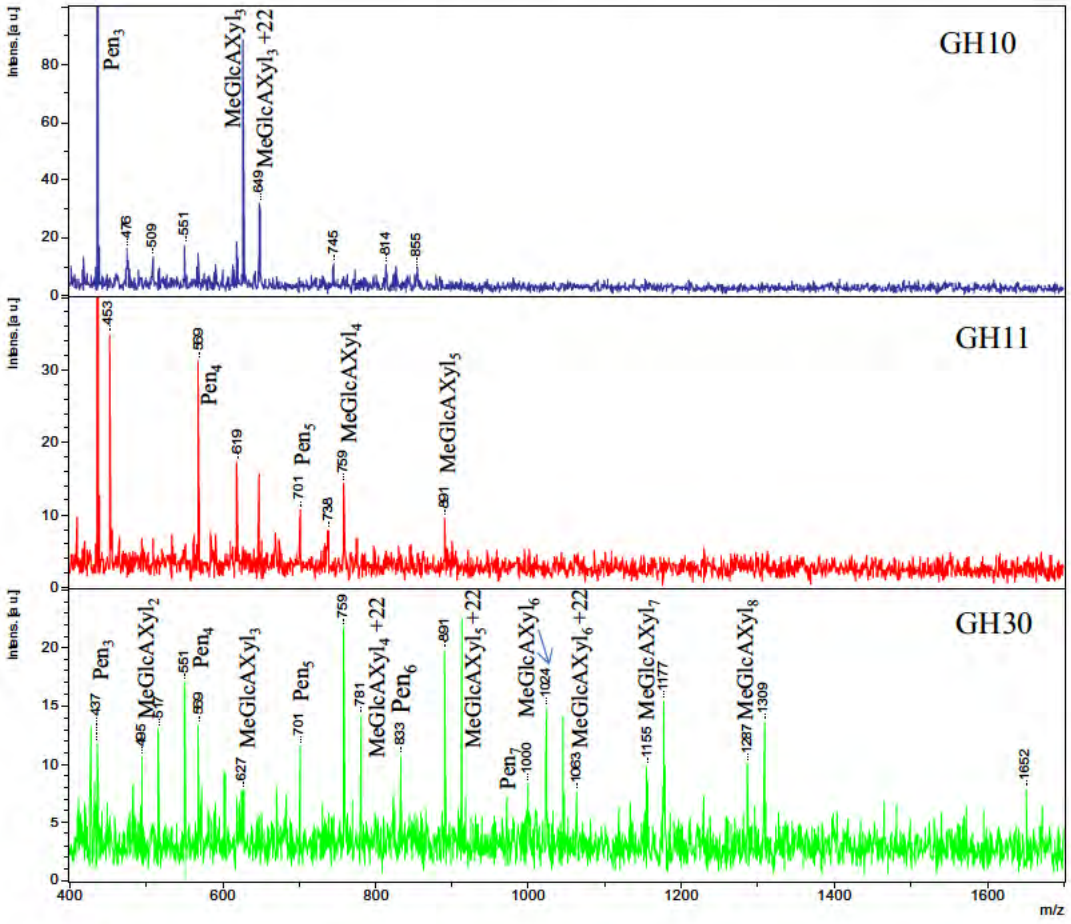

Fig. 4. MALDI-ToF MS spectrum of xylooligosaccharides produced from cowpea glucuronoxylan by *C. mixtus* GH10 xylanase, *T. lanuginosus* GH11 xylanase and *E. chrysanthemi* GH30 xylanase. Abbreviations: Pen_x, ions of neutral xylooligosaccharides; $MeGlcAXyl_x$, ions of acidic oligosaccharides; $MeGlcAXyl_x + 22$, ions of sodium salts of acidic oligosaccharides.

and docking of xylan to them. Primary sequence analysis revealed some characteristics of all the xylanase proteins. InterProScan results showed that all the three xylanases possessed a hydrolase domain. The results also confirmed that the sequences of *C. mixtus*, *T. lanuginosus*, and *E. chrysanthemi* belong to the GH10, GH11 and GH30 family, respec tively. The InterProScan results revealed the domain positions and their sequence length, indicating that more than half of the sequence lengths comprise hydrolase domain. For *Tl*GH11, two amino acids near 150 and 255 residue position were predicted within the hydrolase do main. It is reported that normally catalytic residues can form a charged network that activates nucleophile, which helps in releasing product with the help of substrate [30]. The location of the active site within the hydrolase domain was also predicted for the other two xylanases studied (Fig. 5).

Further, molecular docking studies using AutoDock Vina suite was performed to explore the best docked pose of the complex structures. The results obtained from the CASTp server suggested that the pockets stretching from amino acid residues 147 157 and 239 250, form an ac tive site of *T. lanuginosus* SSBP xylanase. Similar results were also ob served for *C. mixtus* amino acid residues 165 200 and 309 319, and *E. chrysanthemi* regions 145 168 and 330 350. Docking the ligand was successfully performed for these predicted active sites with all the three xylanase structures. Each structure generated six poses of interac tion mode with different scoring values (Table 2). Multiple binding poses and the scoring values of their complexes aim to identify the key residues and predict the best binding mode inside the cleft of given xylanase. The lowest score (binding affinity) was identified as −10.24 kcal/mol for complex structure (Pose 2) of *T. lanuginosus* SSBP. Further, interaction study was focused on pose 2 to identify the interac tions of *Tl*GH11 with the ligand. They were also visualized using PyMOL plugin (Fig. 6).

Table 2
Interaction data of xylanase-ligand complexes obtained by AutoDock Vina suite.

Enzyme	Receptor	Ligand	Active sites[a]	Pose	Scoring values[b] (kcal/mol)
*Tl*GH11	Xylanase-model1. pdbqt (GH11)	Xylan. pdbqt	**147–157**, 239–250	1	9.0
				2	10.24
				3	7.97
				4	8.49
				5	8.89
				6	9.30
*Cm*GH10	Xylanase-model2. pdbqt (GH10)	Xylan. pdbqt	165–200, **309–319**	1	9.40
				2	9.53
				3	9.38
				4	9.09
				5	8.80
				6	8.44
*Ec*GH30	Xylanase-model1. pdbqt (GH30)	Xylan. pdbqt	**145–168**, 330–350	1	9.93
				2	8.85
				3	8.56
				4	7.24
				5	8.44
				6	8.01

[a] Bold letter highlights the best final hit.
[b] Scoring values shows the binding energy of each pose of the predicted complexes.

The modelled structure of *Tl*GH11 holds a β barrel type of super secondary structure that acts as a nice cleft for the aldopentaouronic acid to be bound inside (Fig. 6a and b). Tyrosine, tryptophan, histidine, lysine and arginine residues are known as strong binding factors as they comprise aromatic and positive charged side chains [23,31]. In the complex structure, these amino acids (Tyr137, Tyr152, Trp48, Trp49) indeed form non polar stacking interactions between xylanase

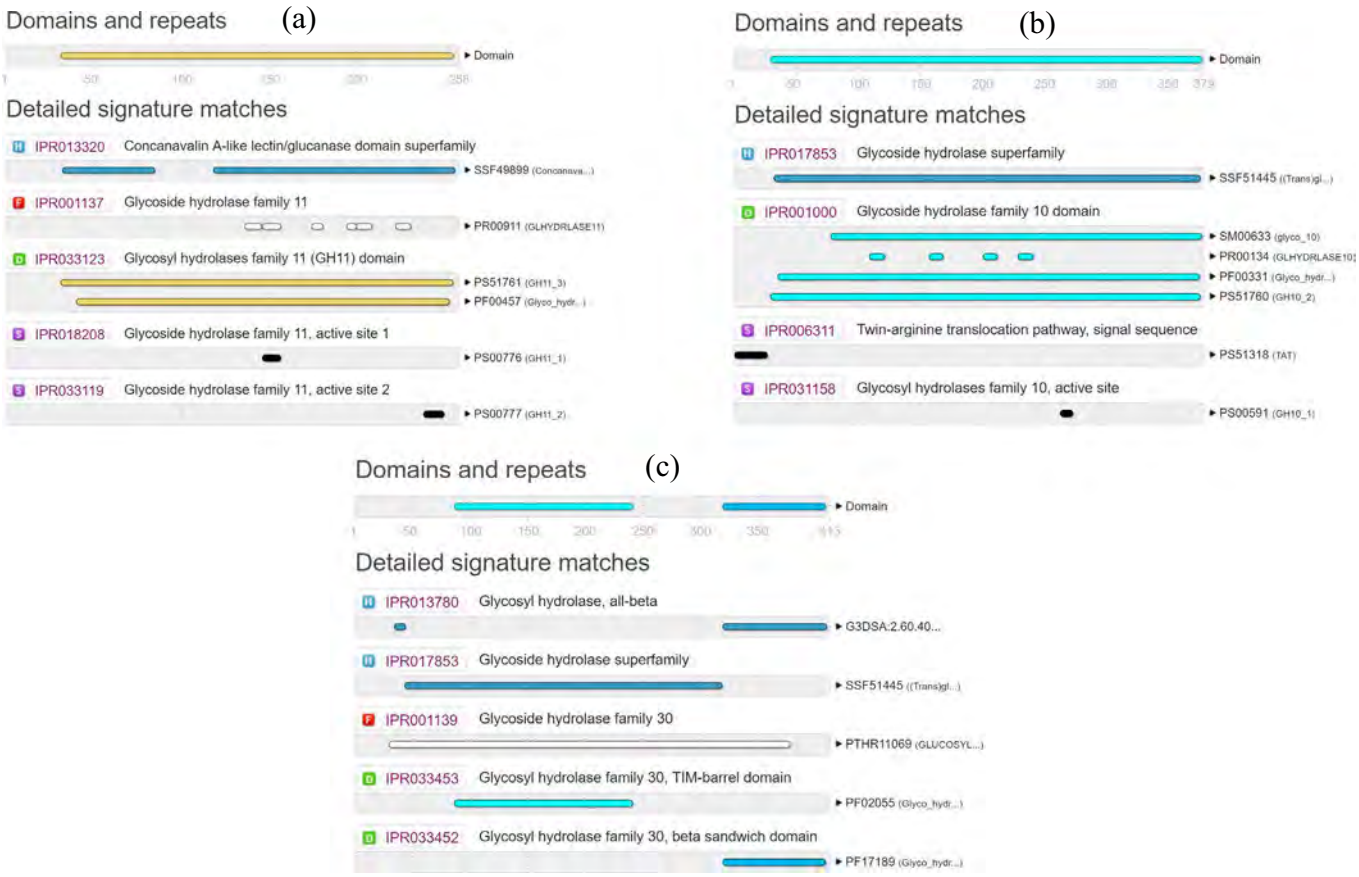

Fig. 5. Sequential insight to xylanase domains identified using InterProScan server. (a) *Tl*GH11, (b) *Cm*GH10, (c) *Ec*GH30.

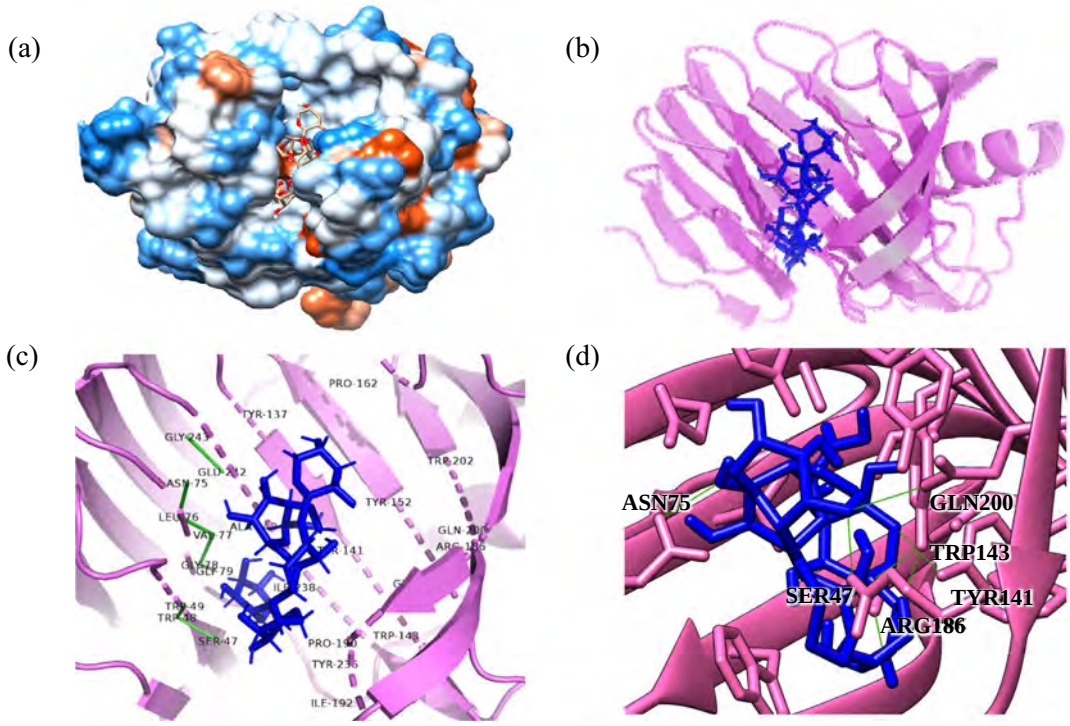

Fig. 6. Schematic illustrations of *Tl*GH11 xylanase (*T. lanuginosus* SSBP) complex with aldopentaouronic acid $MeGlcA^3Xyl_4$. (a) Hydrophobicity surface view of the complex using a colour range from blue (the most positive charge) to red (the most negative charge); (b) Cartoon view of the complex; (c) Three letter code of the residues of the enzyme interacting with the ligand; (d) Inter-molecular H-bonds are represented by green bonds. In parts (b) to (d) the ligand is shown in blue sticks and the enzyme in pink colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the ligand. Proline residues at positions 162 and 190 (Fig. 6c) might have also helped in non bonded interaction. Moreover, receptors (pro tein) are amphoteric in nature, showing the properties of both hydro gen acceptor and donor. Hydrogen bond is one of the main contributors to the ligand affinity to the receptor. In the docked struc ture, seven intermolecular H bonds were visible in the docked pose with the following residues of *Tl*GH11 xylanase: Ser47, Asn75, Tyr141 (two hydrogen bonds), Arg186, Gln200, and Tyr236 (Fig. 6d). The bind ing energy (−10.24 kcal/mol) and the number of H bonds formed in the complex clearly indicate that the enzyme is well suited for accom modating the polymeric xylan.

4. Conclusion

A detailed structural characterization of xylans isolated from bam bara and cowpea biomass which represent unexplored lignocellulose feedstock, was provided. By biochemical and spectroscopic methods, the extracted xylans were identified as glucuronoxylan. In addition, the hydrolysis of these xylans by three different xylanases resulted in the release of linear XOS (2 7 dp) and acidic oligosaccharides differing in the degree of polymerization. To support the interaction between xylanase and xylan, an *in silico* study was performed which suggested that *Tl*GH11 xylanase was more appropriate for binding to xylan. Fur ther, XOS produced from these indigenous crops could be explored for their value addition in the forthcoming research.

Acknowledgments

This study was supported by the grants from the National Research Foundation (Innovation doctoral grant no. 101418; Thuthuka grant no. 93982 and 114227; TWAS post doctoral fellowship no. 110794), Re public of South Africa, and the ARC DUT UFS collaborative consortium. This work was also supported by the Slovak Research and Development Agency under the contract No. APVV 0602 12, and by Scientific Grant Agency VEGA under the contract No. 2/0016/18.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.04.030.

References

[1] J. Wu, S. Upreti, F. Ein-Mozaffari, Ozone pretreatment of wheat straw for enhanced biohydrogen production, Int. J. Hydrog. Energy 38 (2013) 10270–10276, https://doi.org/10.1016/j.ijhydene.2013.06.063.
[2] K. Hachem, C. Faugeron, M. Kaid-Harche, V. Gloaguen, Structural investigation of cell wall xylan polysaccharides from the leaves of Algerian *Argania spinosa*, Molecules 21 (2016) 1–10, https://doi.org/10.3390/molecules21111587.
[3] H. Chen, J. Liu, X. Chang, D. Chen, Y. Xue, P. Liu, H. Lin, S. Han, A review on the pre-treatment of lignocellulose for high-value chemicals, Fuel Process. Technol. 160 (2017) 196–206, https://doi.org/10.1016/j.fuproc.2016.12.007.
[4] R. Chandra, J. Saddler, A "compare-and contrast" of pulping process that have been adapted for biomass pretreatment, J-FOR 3 (2013) 6–14.
[5] S. Sousa, A. Ramos, D.V. Evtuguin, J.A.F. Gamelas, Xylan and xylan derivatives-their performance in bio-based films and effect of glycerol addition, Ind. Crop. Prod. 94 (2016) 682–689, https://doi.org/10.1016/j.indcrop.2016.09.031.
[6] D.O. Otieno, B.K. Ahring, The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: xylooligosaccharides (XOS), arabinooligosaccharides (AOS), and mannooligosaccharides (MOS), Carbohydr. Res. 360 (2012) 84–92, https://doi.org/10.1016/j.carres.2012.07.017.
[7] M.I. Ja'afaru, Screening of fungi isolated from environmental samples for xylanase and cellulase production, ISRN Microbiol. 2013 (2013), 283423. https://doi.org/10.1155/2013/283423.
[8] F.L. Motta, C.C.P. Andrade, M.H.A. Santana, A review of xylanase production by the fermentation of xylan: classification, characterization and applications, in: A.K. Chandel, S.S. da Silva (Eds.), Sustain. Degrad. Lignocellul. Biomass - Tech. Appl. Commer, Intech, Croatia 2013, pp. 251–275, https://doi.org/10.5772/53544.
[9] C. Amorim, S.C. Silvério, R.F.S. Gonçalves, A.C. Pinheiro, S. Silva, E. Coelho, M.A. Coimbra, K.L.J. Prather, L.R. Rodrigues, Downscale fermentation for xylooligosaccharides production by recombinant *Bacillus subtilis* 3610, Carbohydr. Polym. 205 (2019) 176–183, https://doi.org/10.1016/j.carbpol.2018.09.088.

[10] A.M. Sajib, P. Falck, R.R.R. Sardari, S. Mathew, C. Grey, E.N. Karlsson, Valorization of Brewer's spent grain to prebiotic oligosaccharide: production, xylanase catalyzed hydrolysis, in-vitro evaluation with probiotic strains and in a batch human fecal fermentation model, J. Biotechnol. 268 (2018) 61–70, https://doi.org/10.1016/j.jbiotec.2018.01.005.
[11] P. Biely, S. Singh, V. Puchart, Towards enzymatic breakdown of complex plant xylan structures: state of the art, Biotechnol. Adv. 34 (2016) 1260–1274, https://doi.org/10.1016/j.biotechadv.2016.09.001.
[12] A.K. Samanta, N. Jayapal, C. Jayaram, S. Roy, A.P. Kolte, S. Senani, M. Sridhar, Xylooligosaccharides as prebiotics from agricultural by-products: production and applications, Bioact. Carbohydr. Diet. Fibre 5 (2015) 62–71, https://doi.org/10.1016/j.bcdf.2014.12.003.
[13] N.A. Mazlan, K.A. Samad, H. Wan Yussof, S.M. Saufi, J. Jahim, Xylooligosaccharides from potential agricultural waste: characterization and screening on the enzymatic hydrolysis factors, Ind. Crop. Prod. 129 (2019) 575–584, https://doi.org/10.1016/j.indcrop.2018.12.042.
[14] I. Jain, V. Kumar, T. Satyanarayana, Xylooligosaccharides: an economical prebiotic from agroresidues and their health benefits, Indian J. Exp. Biol. 53 (2015) 131–142http://nopr.niscair.res.in/handle/123456789/30744.
[15] A.A. Aachary, S.G. Prapulla, Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties and applications, Compr. Rev. Food Sci. Food Saf. 10 (2011) 2–16, https://doi.org/10.1111/j.1541-4337.2010.00135.x.
[16] F.C. De Figueiredo, F.A.A. Carvalho, M. Brienzo, T.S. Campioni, P. De Oliva-neto, Chemical input reduction in the arabinoxylan and lignocellulose alkaline extraction and xylooligosaccharides production, Bioresour. Technol. 228 (2017) 164–170, https://doi.org/10.1016/j.biortech.2016.12.097.
[17] N. Arumugam, P. Biely, V. Puchart, S. Singh, S. Pillai, Structure of peanut shell xylan and its conversion to oligosaccharides, Process Biochem. 72 (2018) 124–129, https://doi.org/10.1016/j.procbio.2018.06.024.
[18] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356, https://doi.org/10.1021/ac60111a017.
[19] H.N. Englyst, J.H. Cummings, Simplified method for the measurement of total non-starch polysaccharides by gas - liquid chromatography of constituent sugars as alditol acetates, Analyst 109 (1984) 937–942, https://doi.org/10.1039/an9840900937.
[20] N. Blumenkrantz, G. Asboe-Hansen, New method for quantitative determination of uronic acids, Anal. Biochem. 54 (1973) 484–489, https://doi.org/10.1016/0003-2697(73)90377-1.
[21] J. Lin, L.M. Ndlovu, S. Singh, B. Pillay, Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP, Biotechnol. Appl. Biochem. 30 (1999) 73–79, https://doi.org/10.1111/j.1470-8744.1999.tb01162.x.
[22] K.S. Kumar, A. Manimaran, K. Permaul, S. Singh, Production of β-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp, J. Biosci. Bioeng. 107 (2009) 494–498, https://doi.org/10.1016/j.jbiosc.2008.12.020.
[23] B. Dutta, A. Banerjee, P. Chakraborty, R. Bandopadhyay, In silico studies on bacterial xylanase enzyme: structural and functional insight, J. Genet. Eng. Biotechnol. 16 (2018) 749–756, https://doi.org/10.1016/j.jgeb.2018.05.003.
[24] E. de Castro, C.J.A. Sigrist, A. Gattiker, V. Bulliard, P.S. Langendijk-Genevaux, E. Gasteiger, A. Bairoch, N. Hulo, ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins, Nucleic Acids Res. 34 (2006) 362–365, https://doi.org/10.1093/nar/gkl124.
[25] S. Forli, R. Huey, M.E. Pique, M. Sanner, D.S. Goodsell, J. Arthur, Computational protein-ligand docking and virtual drug screening with the AutoDock suite, Nat. Protoc. 11 (2016) 905–919, https://doi.org/10.1038/nprot.2016.051.
[26] A.K. Ghosh, A.K. Naskar, M.L. Jana, S. Khowala, S. Sengupta, Purification and characterization if an amyloglucosidase from *Termitomyces clypeatus* that liberates glucose from xylan, Biotechnol. Prog. 11 (1995) 453–456, https://doi.org/10.1021/bp00034a013.
[27] M. Nieto-Domínguez, L.I. de Eugenio, M.J. York-Durán, B. Rodríguez-Colinas, F.J. Plou, E. Chenoll, E. Pardo, F. Codoñer, M. Jesús Martínez, Prebiotic effect of xylooligosaccharides produced from birchwood xylan by a novel fungal GH11 xylanase, Food Chem. 232 (2017) 105–113, https://doi.org/10.1016/j.foodchem.2017.03.149.
[28] R. Sydenham, Y. Zheng, A. Riemens, A. Tsang, J. Powlowski, R. Storms, Cloning and enzymatic characterization of four thermostable fungal endo-1,4-β-xylanases, Appl. Microbiol. Biotechnol. 98 (2014) 3613–3628, https://doi.org/10.1007/s00253-013-5244-8.
[29] H. Li, Y. Xue, J. Wu, H. Wu, G. Qin, C. Li, J. Ding, J. Liu, L. Gan, M. Long, Enzymatic hydrolysis of hemicelluloses from *Miscanthus* to monosaccharides or xylo-oligosaccharides by recombinant hemicellulases, Ind. Crop. Prod. 79 (2016) 170–179, https://doi.org/10.1016/j.indcrop.2015.11.021.
[30] P.A. Frey, S.A. Whitt, J.B. Tobin, A low-barrier hydrogen bond in the catalytic triad of serine proteases, Science 264 (1994) 1927–1930, https://doi.org/10.1126/science.7661899.
[31] A. Lammerts Van Bueren, S. Otani, E.P. Friis, K.S. Wilson, G.J. Davies, Three-dimensional structure of a thermophilic family GH11 xylanase from *Thermobifida fusca*, Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 68 (2012) 141–144, https://doi.org/10.1107/S1744309111049608.

Process Biochemistry 72 (2018) 124–129

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Structure of peanut shell xylan and its conversion to oligosaccharides

Nanthakumar Arumugam[a,b], Peter Biely[b], Vladimír Puchart[b], Suren Singh[a], Santhosh Pillai[a,*]

[a] Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, PO Box 1334, Durban, 4000, South Africa
[b] Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-845 38 Bratislava, Slovak Republic

ARTICLE INFO

Keywords:
Peanut shell
Glucuronoxylan
Xylanase
Xylooligosaccharides
^{1}H NMR spectroscopy
MALDI-TOF MS

ABSTRACT

We present here that peanut shell, an underutilized agro-residue, is suitable for the isolation of xylan and the production of prebiotic xylooligosaccharides (XOS). Two different alkaline extraction procedures coupled to delignification were applied for the extraction of xylan. A one-step extraction in the presence of sodium hypochlorite produced xylan I (14.8% yield) contaminated with other hemicelluloses, mainly xyloglucan. A two-step extraction of sodium chlorite-delignified material yielded much purer polysaccharide, assigned as xylan II (15.5% yield). This polysaccharide was characterized by NMR, and MALDI ToF MS analysis was performed on xylooligosaccharides generated by various xylanases. The polysaccharide appears to be a glucuronoxylan similar to a hardwood glucuronoxylan, however, with a relatively higher degree of substitution of the main chain with 4-*O*-methyl-D-glucuronic acid (Xyl:MeGlcA ratio ~6–7). Consequently, the peanut shell glucuronoxylan hydrolysates produced by GH10, GH11 and GH30 xylanases contained considerable portion of acidic xylooligosaccharides. A process for the production of peanut shell xylooligosaccharides from alkali extracted peanut shell xylan using a thermophilic *Thermomyces lanuginosus* GH11 xylanase has been proposed.

1. Introduction

Peanut shell represents an abundant agro residue which has not been sufficiently explored. The Food and Agriculture Organizationof the United Nations reports that peanut production worldwide corre sponds to about 46 million tons, of which shells account for 20% of the entire weight [1]. In South Africa alone, approximately 22,000 tons of peanut shells are produced annually [2] and exploitation of this un derutilized biomass offers a great potential for added value, besides waste management. Peanut shells contain ~25% of their total dry weight as hemicellulose [3], which is predominantly xylan. Literature presents several examples of the use of peanut shells, either intact or after mechanical pretreatment. They are primarily used as a carbon source for the production of plant biomass degrading enzymes [4,5], and in powder form, as an adsorbent for sequestering heavy metals from aqueous solutions [6], and in combination with corn stover as a material for the production of briquettes [3]. Peanut shells pretreated by wet oxidation, dilute acid or ionic liquids can serve as a potential source of sugars fermentable to ethanol, following enzymatic hydrolysis [1,7 10]. However, majority of the peanut shells are still either dumped or burnt as waste.

The present work focuses on utilizing the unexplored peanut shell biomass, isolation of its major hemicellulose i.e., xylan, its structural characterization and possibility of its enzymatic conversion to prebiotic xylooligosaccharides (XOS). XOS not only serve as a selective carbon source for health promoting bacteria in the human digestive tract [11] but may also have many other positive effects, such as improvement of mineral absorption and prevention of gut infections [12,13]. XOS have also been used as functional and water binding food additives [14,15].

Thus far, there are no detailed studies dedicated to xylan present in peanut shells. In this work, alkaline extraction of deacetylated peanut shell xylan and its first structural characterization by NMR spectroscopy and enzymatic hydrolysis is reported. This, together with the analysis of XOS generated by three types of endoxylanases suggests that we have isolated a glucuronoxylan with unusually high content of 4 *O* methyl D glucuronic acid side residues. In the final phase of completion of this manuscript, a new report emerges which suggest peanut shells as a source of bioactive oligosaccharides [16], however, the nature of xylan has not been studied. To the best of our knowledge, this is the first report on the characterization of xylan from peanut shells and its en zymatic conversion to XOS.

Abbreviations: Xyl, *p*β-D-xylopyranosyl residue; MeGlcA, 4-*O*-methyl-D-glucuronic acid; GH, glycoside hydrolase; Xyl_n, β-1,4-xylooligosaccharide of 'n' Xyl residues; $MeGlcA^iXyl_n$, aldouronic acid containing one residue of MeGlcA linked to Xyl*p* residue marked in Xyl_n with 'i' which is the number of Xyl*p* residue counted from the reducing end 'i' would be equal to 1 if MeGlcA would be linked to the reducing end Xyl 2 if MeGlcA would be linked to the second Xyl*p* residue from the reducing end etc.); Glc, glucose; Gal, galactose; Man, mannose; Ara, arabinose; Fuc, fucose; Rha, rhamnose

* Corresponding author.
E-mail address: santhoshk@dut.ac.za (S. Pillai).

https://doi.org/10.1016/j.procbio.2018.06.024
Received 29 March 2018; Received in revised form 30 May 2018; Accepted 26 June 2018
Available online 28 June 2018
1359-5113/

2. Materials and methods

2.1. Biomass collection

Peanut shells were obtained from a peanut processing plant in Durban, KwaZulu Natal, South Africa during the autumn season (April). The shells were washed, dried to constant weight at a temperature of 60 °C, ground in a ball mill grinder and sieved. The fraction consisting of particles smaller than 0.35 mm was collected and used for xylan isolation.

2.2. Extraction of xylan from peanut shells

Two different alkali extractions were employed for xylan isolation. For the first procedure, i.e., one step NaClO/NaOH extraction, 50 g of powdered shells were stirred with 125 ml of 1% NaClO solution for 1 h at room temperature. The wet material was washed several times with distilled water and soaked in 15% NaOH for 24 h at room temperature, as described by Chapla et al. [17]. The mixture was filtered through a muslin cloth followed by neutralizing with glacial acetic acid, and the filtrate was precipitated by 3 volumes of ice cold 95% ethanol. The neutralized precipitate was centrifuged at a speed of 8800 × *g* for 10 min at 4 °C, washed with 75% ethanol and dried at 60 °C till constant weight was reached.

Xylan II was extracted according to the procedure of Ebringerová et al. [18]. Briefly, powdered peanut shells (50 g) were soaked in 500 ml of distilled water and heated to a temperature of 50 °C. Acidification with glacial acetic acid (7 ml) was followed by the gradual addition of sodium chlorite (35 g). The mixture was heated at 70 °C for 1 h in a protected fume hood to liberate lignin and coloured materials. This was followed by extensive washing with water. The neutral wet cake was suspended in 350 ml of 1% NH_4OH at room temperature for 2 h. The extract was then filtered off and discarded. The remaining solid material was extracted with 5% NaOH at room temperature for 2 h under occasional stirring. The mixture was then filtered through a muslin cloth; the solid material extraction was repeated, and the two filtrates were combined and mixed with 3 volumes of 95% ethanol to precipitate the polysaccharide. The precipitate thus formed was collected by filtration, followed by washing with 75 80% ethanol containing 2% acetic acid to neutralize the residual alkali and subsequently washed several times with 80% ethanol. Finally, the precipitated polysaccharide, assigned as xylan II, was suspended in water and freeze dried.

2.3. Monosaccharide composition of isolated polysaccharides

Neutral sugars were quantified by GLC in the form of alditol acetates after hydrolysis of the samples with 2 M trifluoroacetic acid (TFA) for 1 h at 120 °C [19]. The uronic acid content of the extracted xylan was determined using H_2SO_4/3 hydroxybiphenyl reagent [20].

2.4. Enzymes used in the study

GH11 family xylanase from the thermophilic fungus *T. lanuginosus* SSBP was purified as already described [21]. *Cellvibrio mixtus* GH10 xylanase, *Clostridium thermocellum* GH16 endo 1,3(4) β glucanase and *Paenibacillus* sp. GH5 endo xyloglucanase were obtained from Megazyme (Bray, Ireland). GH30 xylanase from *Erwinia chrysanthemi* was supplied by Prof. James F. Preston (University of Florida, Gainesville, FL, USA). *Bacillus subtilis* α amylase was procured from Sigma Aldrich (St. Luis, MO, USA). Xylanase activity was determined on beechwood glucuronoxylan (Sigma Aldrich, USA) following the formation of reducing groups quantified by Somogyi Nelson procedure [22]. One unit of xylanase activity is defined as the amount of μmol equivalents of xylose generated in 1 min. Other enzymes were applied in units reported by the supplier.

Table 1
Molar ratio of monosaccharides in peanut shell xylan.

Xylan preparation	Xyl	MeGlcA	Glc	Gal	Man	Ara	Fuc	Rha
Xylan I (NaClO/NaOH one-step extraction)	1.00	0.13	0.14	0.09	0.02	0.12	0.02	0.03
Xylan II (NaOH extraction after $NaClO_2$ delignification and NH_4OH treatment)	1.00	0.14	0.009	0.02	0.003	0.04	0.001	0.01

2.5. Enzymatic degradation of peanut shell xylan and beechwood glucuronoxylan

Isolated peanut shell xylan II and commercial beechwood glucuronoxylan were dissolved (1%, w/v) in 50 mM sodium citrate buffer (pH 6.5) and incubated in a water bath at 50 °C for 24 h with *T. lanuginosus* GH11 xylanase (10 U/g). The citrate buffer was replaced with sodium acetate buffer (pH 5.5) for reaction mixtures of GH10 and GH30 xylanases (10 U/g) and was incubated at 35 °C for 24 h. In the search for co extracted polysaccharides, both xylan I and xylan II were treated with other pure glycoside hydrolases under similar conditions. The hydrolysis products of xylan II were analysed by thin layer chromatography (TLC) and MALDI TOF MS as described below. The GH11 hydrolysate of xylan II was also subjected to ^{1}H NMR spectroscopy.

2.6. TLC analysis of enzymatically released carbohydrates

TLC analysis was carried out on silica gel coated aluminium sheets (Merck, Darmstadt, Germany) and developed in a solvent system of n butanol/ethanol/water (10:8:5, v/v). Appropriately diluted samples and standards were spotted (3 μl) and detected using orcinol reagent (1% in 10% H_2SO_4 in ethanol). Standards of linear XOS were procured from Megazyme Int. (Bray, Ireland). Shorter aldouronic acids in the peanut shell xylan hydrolysate were identified using hydrolysates of beechwood glucuronoxylan by GH10 and GH11 xylanases containing $MeGlcA^3Xyl_3$ and $MeGlcA^3Xyl_4$ as the predominant acidic products, respectively [23]. Supporting information on the nature of acidic oligosaccharides was obtained by NMR spectroscopy and MALDI TOF mass spectrometry.

2.7. NMR spectroscopy and MALDI TOF mass spectrometry

For NMR analysis, all samples were freeze dried twice from D_2O. ^{1}H NMR spectra were measured at 25 °C using automatic chemical shift calibration in D_2O on either AVANCE III HD X 600 MHz equipped with a Triple inverse TCI H C/N D 05 Z cryo probe, or AVANCE III HD 400 MHz with a broad band BB (H F) D 05 Z liquid N_2 Prodigy probe (all from Bruker BioSpin, Rheinstetten, Germany), using a pre saturation *zgpr* sequence, with a pre saturation delay of 2 s, a r.f. 90° pulse and an acquisition time of 2.5 s.

Analysis of oligosaccharides was done by MALDI ToF MS as previously described [24]. An Ultraflex MALDI TOF/TOF instrument (Bruker Daltonics GmbH, Bremen, Germany) equipped with a nitrogen 337 nm laser beam was operated in reflectron positive acquisition mode and controlled using the Flex Control 3.3 software package. Ions of sodium adducts of xylan fragments were marked using monosaccharide codes while ions of fragments of xyloglucan were marked using the one letter nomenclature for xyloglucan derived oligosaccharides [25].

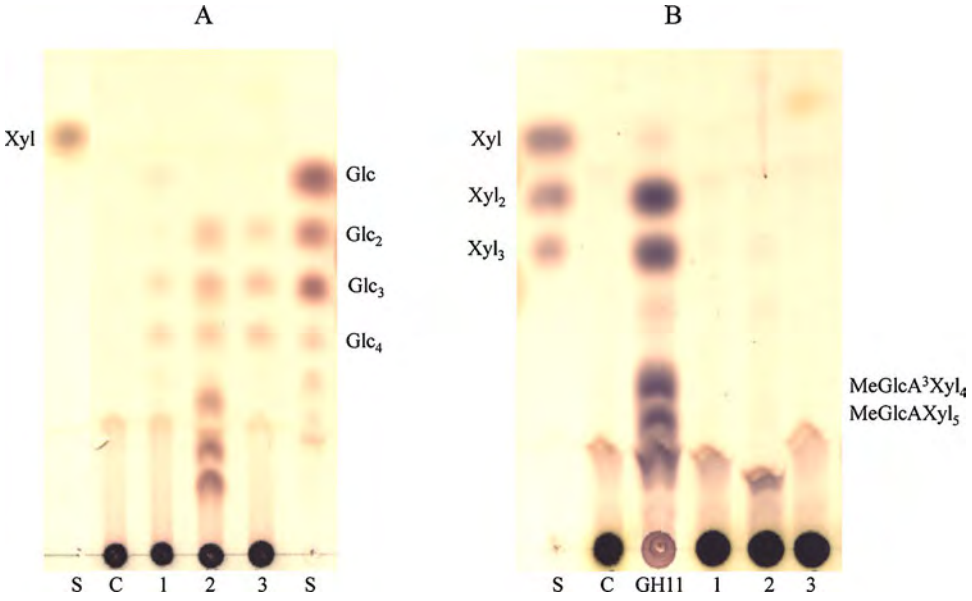

Fig. 1. TLC analysis of products released by α-amylase (1), xyloglucanase (2), β-1,4-β-1,3-glucanase (lichenase) (3) and GH11 xylanase (GH11) from one-step-extracted peanut shell xylan I (A) and from xylan II, extracted from $NaClO_2$-delignified and NH_4OH pretreated peanut shells (B). C, control, enzyme untreated samples; S, standards (Glc_2-Glc_4, maltooligosaccharides; Xyl, xylose; Xyl_2, xylobiose; Xyl_3, xylotriose); $MeGlcA^3Xyl_4$, Aldopentaouronic acid; $MeGlcAXyl_5$, Aldohexaouronic acid.

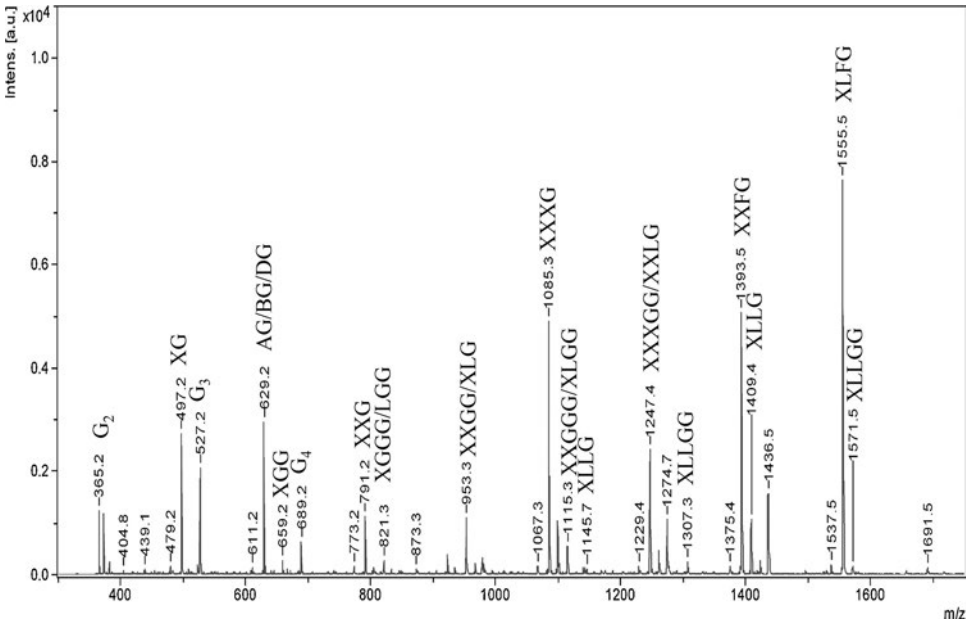

Fig. 2. MALDI ToF MS spectrum of hydrolysate of one-step-extracted xylan I by endo-xyloglucanase. The numbers correspond to *m/z* values of xyloglucan fragments. The one-letter abbreviations of oligosaccharides follow the nomenclature introduced by Fry et al. [25]: X = Glc-Xyl (backbone Glc substituted with Xyl), L = Glc-Xyl-Gal, F = Glc-Xyl-Gal-Fuc, A,B,D = backbone Glc disubstituted by two pentoses or by side chain Xyl substituted with another pentose. Abbreviations: G_n, n = 2–4, ions of non-substituted β-1,4-glucosaccharides (cellooligosaccharides).

3. Results and discussion

3.1. Extraction of xylan and its monosaccharide composition

Both extraction procedures produced a xylan yield range of 14 15.5% from peanut shells. Monosaccharide analysis (Table 1) has revealed that the product isolated with one step procedure, xylan I (yield, 14.8%), was contaminated with other hemicelluloses, particu larly, Glc containing polysaccharide(s). Hydrolysis of this material with pure α amylase, β 1,3 β 1,4 glucanase (lichenase) and xyloglucanase, followed by TLC analysis (Fig. 1A), has shown that the major source of Glc in xylan I is xyloglucan. Of the three endoglycanases, only xy loglucanase generated significant amount of oligosaccharides and MALDI ToF MS analysis (Fig. 2) confirmed their identity as typical xylosylated glucooligosaccharides with the following three types of side chains: 1) Xyl (X); 2) Gal Xyl (L) and Fuc Gal Xyl (F). The presence of Fuc is evidence that some of the oligosaccharides also contain Gal*p* residues which cannot be differentiated from Glc*p* residues by MS. Fuc occurs in the xyloglucan side chains as the non reducing sugar linked to Gal attached to the side chain Xyl*p* residues [26]. The presence of Xyl free hexooligosaccharides in xyloglucanase hydrolysate suggests that peanut shell xyloglucan may have unsubstituted or less substituted re gions of the main β 1,4 glucan chain. The identification of xyloglucan as an admixture also implies that a small portion of the overall Xyl is a constituent of this minor polysaccharide. The molar ratios of sugars in xylan I suggest that xyloglucan admixture could account for 5 10%.

The second extraction procedure that involved acid delignification with $NaClO_2$ and pre extraction with ammonium hydroxide [18] af forded much purer peanut shell xylan, assigned as xylan II (yield, 15.5%) and composed mainly of two sugars, neutral Xyl and uronic acid (Table 1). Obviously, the polysaccharides contaminating the one step extracted xylan I were eliminated during the two step treatment pre ceding the NaOH extraction. This is apparent from the comparison of chromatograms (Fig. 1A and B) showing products released with α amylase and certain hemicellulases. In contrast to xylan I, xylan II was essentially free of other polysaccharide admixtures. It should be noted here that the method used for uronic acid analysis [20] does not dis criminate between D glucuronic acid and 4 *O* methyl D glucuronic acid,

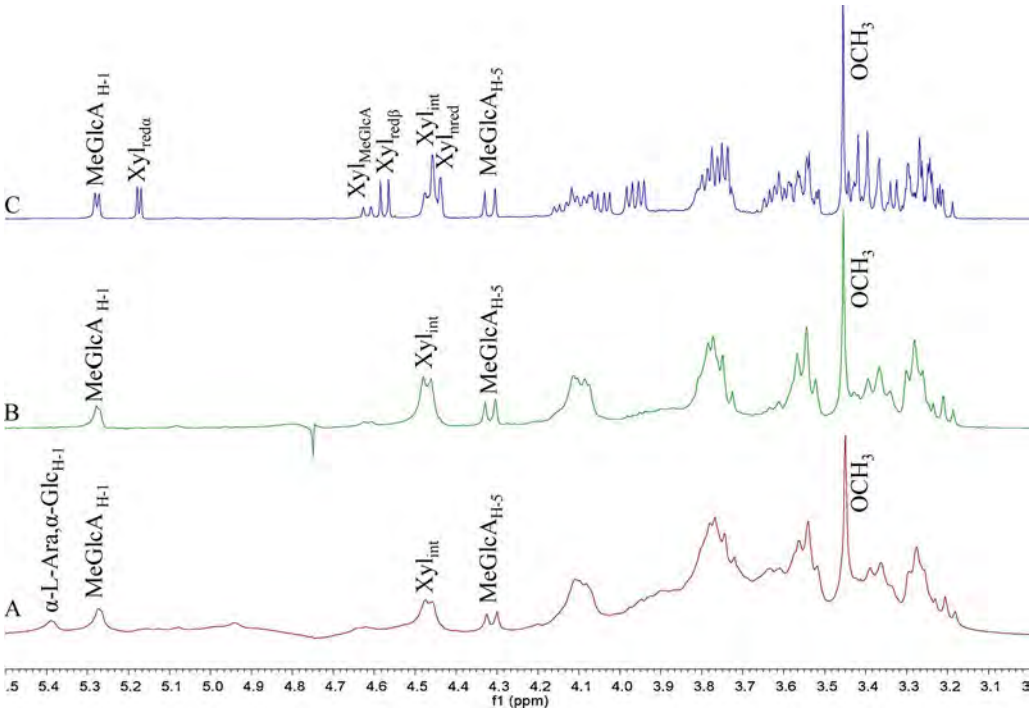

Fig. 3. ^{1}H NMR spectrum of peanut shell xylan I isolated by one-step NaClO/NaOH extraction procedure (A) and xylan II isolated by NaOH extraction of $NaClO_2$-delignified and NH_4OH pretreated peanut shells (B), and ^{1}H NMR spectrum of peanut shell xylan II hydrolysate by *T. lanuginosus* GH11 xylanase (C). In contrast to spectra B and C, spectrum A is less sharp and contains a signal at 5.38 ppm that may correspond to H-1 of α-1,4-linked Glc*p* or α-1,3-L-Ara*f* residues. The spectrum of GH11-generated XOS (C) contains signals of reducing-end ($Xyl_{red\alpha}$, and $Xyl_{red\beta}$) and non-reducing-end Xyl residues (Xyl_{nred}).

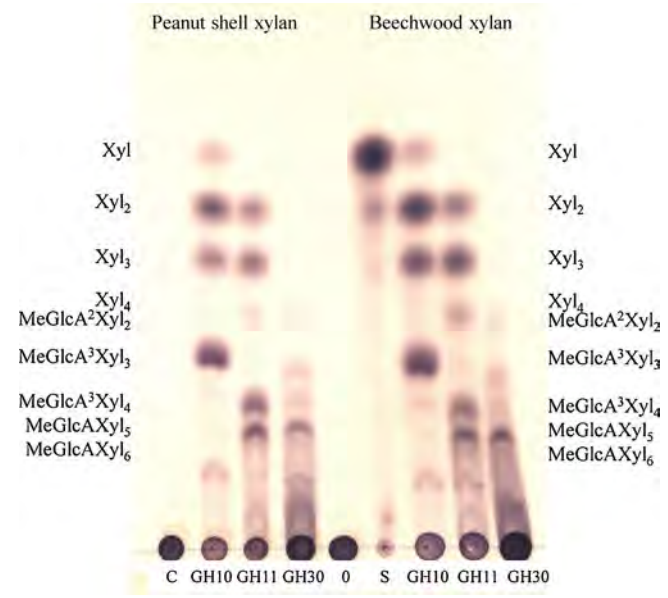

Fig. 4. TLC analysis of XOS produced from peanut shell xylan II and beechwood xylan by GH10, GH11 and GH30 xylanases; C, control (xylan incubated without enzyme); S, standards. Xyl, Xylose; Xyl_2, Xylobiose; Xyl_3, Xylotriose; Xyl_4, Xylotetraose; $MeGlcA^2Xyl_2$, aldotriouronic acid; $MeGlcA^3Xyl_3$, aldotetraouronic acid; $MeGlcA^3Xyl_4$, aldopentaouronic acid; $MeGlcAXyl_5$ - aldohexaouronic acid; $MeGlcAXyl_6$ - aldoheptaouronic acid.

however, it has displayed a higher degree of branching with uronic acid than in hardwood xylan [27,28]. The identification of the uronic acid as a 4 *O* methyl etherified derivative and as the sole uronic acid present in peanut shell xylan was confirmed by spectral methods (see below). The molar ratio of Xyl:MeGlcA in peanut shell glucuronoxylan thus appear to be around 7 (Table 1). Purity of xylan II is evident from ^{1}H NMR spectroscopy results. The spectrum (Fig. 3, spectrum B) of xylan II ap pears to be sharper than that of xylan I (Fig. 3, spectrum A) and does not contain a signal at 5.38 ppm which could correspond to H 1 of α 1,4 linked Glc*p* or α 1,3 L Ara*f*. Consequently, the xylan II extracted from $NaClO_2$ delignified material was used in all further experiments.

3.2. Characterization of peanut shell xylan by NMR spectroscopy

^{1}H NMR spectrum of peanut shell xylans extracted by both proce dures (Fig. 3, spectra A and B) showed unusually high signals of H 1 for the MeGlcA at 5.28 ppm and for the 4 *O* methyl ether group of MeGlcA at 3.45 ppm, which corresponds to a higher uronic acid content than that observed in hardwood xylans [29]. Both xylan preparations did not show any significant H 1α signal for the reducing end Xyl (at 5.17 ppm) which confirms polymeric nature of the isolated material. The sharper NMR spectrum of xylan II extracted from $NaClO_2$ delignified peanut shells (spectrum B) also confirms its higher purity.

3.3. Enzymatic evidence for the nature of isolated peanut shell xylan

A striking similarity exists between the products generated by the three types of xylanases from peanut shell xylan II and beechwood glucuronoxylan (Fig. 4). From both polysaccharides, GH10 xylanase generated some Xyl, but mainly Xyl_2, Xyl_3 and $MeGlcA^3Xyl_3$. The *T. lanuginosus* GH11 xylanase generated mainly Xyl_2, Xyl_3, $MeGlcA^3Xyl_4$ and $MeGlcAXyl_5$. A slightly higher proportion of aldouronic acids in the peanut shell xylan hydrolysate than in the beechwood xylan hydro lysate is in accordance with previous information on higher MeGlcA content.

GH30 xylanase hydrolysed peanut shell xylan II to a series of al douronic acids with different number of Xyl*p* residues, as reported earlier for hardwood xylan [30,31]. The action of this enzyme depends on the presence of MeGlcA side residues, and the primary products of its action on glucuronoxylan are aldouronic acids of the structure $MeGlcA^2Xyl_n$ [27,32].

Final evidence for the glucuronoxylan nature of the peanut shell xylan was provided by MALDI TOF MS analysis of the XOS generated from xylan II using the three xylanases (Fig. 5). Principal ions in the hydrolysate of GH10 xylanase correspond to Xyl_3 and aldouronic acids $MeGlcAXyl_3$ and $MeGlcAXyl_4$ (the ion of the main neutral product, Xyl_2, is not shown). The GH11 xylanase hydrolysate showed mainly ions of Xyl_3, Xyl_4 and aldouronic acids $MeGlcAXyl_4$, $MeGlcAXyl_5$ and

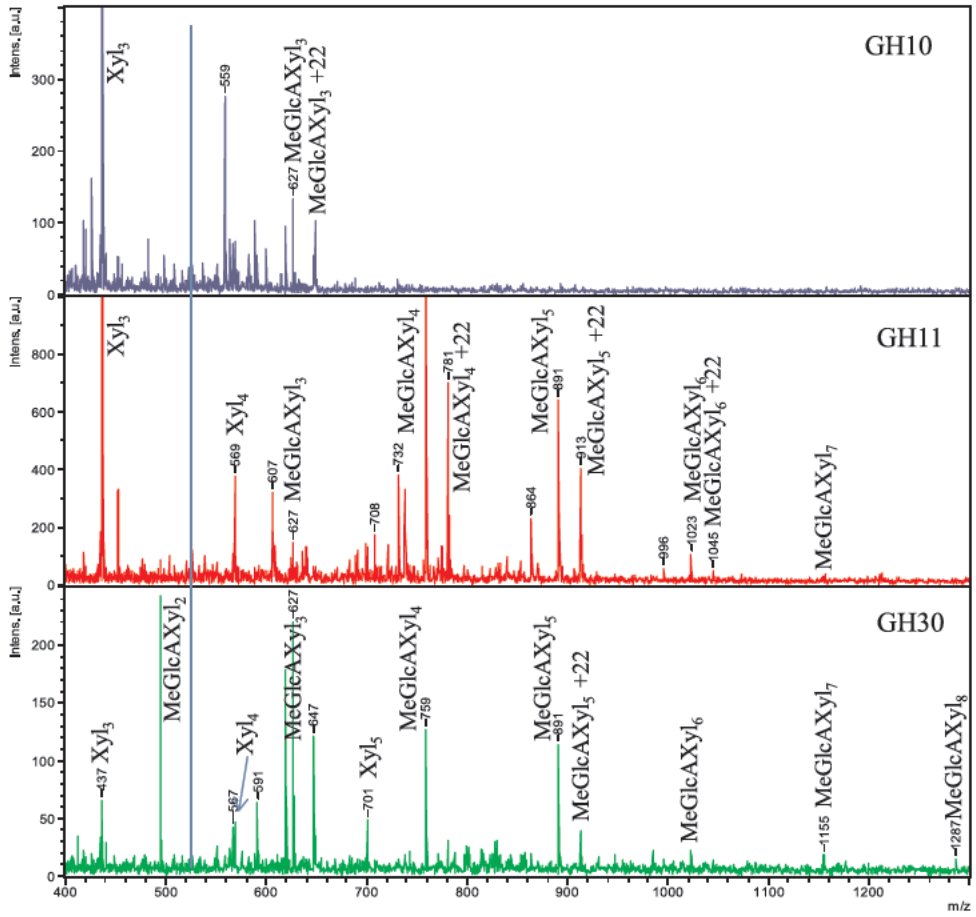

Fig. 5. MALDI ToF MS spectra of hydrolysates of xylan II by GH10, GH11 and GH30 xylanases. The numbers correspond to *m/z* values of generated oligosaccharides. Xyl_n, Xylooligosaccharides; $MeGlcA^1Xyl_n$- Aldouronic acid; + 22 – Disodium ions.

$MeGlcAXyl_6$. Since such ions were not observed in the GH10 xylanase hydrolysate, the larger aldouronic acids must have been hydrolysed by this xylanase. GH10 xylanases require just two unsubstituted residues between two substituted ones for the cleavage of the main xylan chain [33,34]. However, GH11 xylanase requires three unsubstituted residues [30,35]. Under the experimental conditions used, all aldouronic acids also afforded ions of their sodium salts, that means by *m/z* 22 larger than the ions of their sodium adducts. The absence of ions containing hexoses supports the view that hexoses are not constituents of the peanut shell xylan.

4. Conclusion

This work provides the first insight into structural features of xylan isolated from peanut shells, which represent abundant lignocellulose feedstock. The alkali extracted polysaccharide from delignified peanut shell is very similar to hardwood glucuronoxylan but contains a much higher degree of substitution with MeGlcA. Consequently, the portion of acidic oligosaccharides will be higher, but they could be separated easily from neutral ones on anion exchangers and used as a separate group of biologically active compounds. The area of neutral xylooligosaccharides applied as prebiotics [36] is not as new as the area of application of acidic xylooligosaccharides [37]. One Japanese patent is dedicated to the isolation of acidic xylooligosaccharides [38]. Aldouronic acids derived from hardwood xylan have been shown to have potential to be applied in medicine. On experimental animals (mouse and rat), it has been demonstrated that the aldouronic acids promote recovery from iron deficiency anaemia by enhancing serum ion levels [37] and prevent the development of atopic dermatitis like skin lesions [39]. The GH11 xylanase from *T. lanuginosus* SSBP was found to be very efficient in hydrolysing peanut shell glucuronoxylan into XOS with essentially no release of free xylose. Suitability of peanut shell XOS as prebiotics and nutraceuticals in foods and beverages is under current investigation.

Acknowledgments

This study was supported by the grants from the National Research Foundation (Innovation doctoral grant no. 101418; Thuthuka grant no. 93982), Republic of South Africa and the ARC DUT UFS collaborative consortium. This work was also supported by the Slovak Research and Development Agency under the contract No. APVV 0602 12, and by Scientific Grant Agency VEGA under the contract No. 2/0016/18.

References

[1] A.P. Carneiro, O. Rodríguez, E.A. Macedo, Dissolution and fractionation of nut shells in ionic liquids, Bioresour. Technol. 227 (2017) 188–196, http://dx.doi.org/10.1016/j.biortech.2016.11.112.
[2] F. Meyer, G. van der Burgh, Evaluating the Sustainability of the South African Groundnut Industry, (2012).
[3] C. Gong, D. Lu, G. Wang, L. Tabil, D. Wang, Compression characteristics and energy requirement of briquettes made from a mixture of corn stover and peanut shells, BioResources 10 (2015) 5515–5531, http://dx.doi.org/10.15376/biores.10.3.5515-5531.
[4] N.H. Abd El-Nasser, S.M. Helmy, A.A. El-Gammal, Formation of enzymes by biodegradation of agricultural wastes with white rot fungi, Polym. Degrad. Stab. 55 (1997) 249–255, http://dx.doi.org/10.1016/S0141-3910(96)00117-6.
[5] C.-H. Yang, S.-F. Yang, W.-H. Liu, Production of xylooligosaccharides from xylans by extracellular xylanases from Thermobifida fusca, J. Agric. Food Chem. 55 (2007) 3955–3959, http://dx.doi.org/10.1021/jf0635964.
[6] D. Sud, G. Mahajan, M.P. Kaur, Agricultural waste material as potential adsorbent

for sequestering heavy metal ions from aqueous solutions - a review, Bioresour. Technol. 99 (2008) 6017–6027, http://dx.doi.org/10.1016/j.biortech.2007.11.064.
[7] C. Martín, A.B. Thomsen, Wet oxidation pretreatment of lignocellulosic residues of sugarcane, rice, cassava and peanuts for ethanol production, J. Chem. Technol. Biotechnol. 181 (2007) 174–181, http://dx.doi.org/10.1002/jctb.1648.
[8] C. Martin, B. Alriksson, A. Sjöde, N.O. Nilvebrant, L.J. Jönsson, Dilute sulfuric acid pretreatment of agricultural and agro-industrial residues for ethanol production, Appl. Biochem. Biotechnol. 137–140 (2007) 339–352, http://dx.doi.org/10.1007/s12010-007-9063-1.
[9] P. Chandrakant, V.S. Bisaria, Application of a compatible xylose isomerase in simultaneous bioconversion of glucose and xylose to ethanol, Biotechnol. Bioprocess Eng. 5 (2000) 32–39, http://dx.doi.org/10.1007/s002530050025.
[10] Z.F. Fang, K.L. Liu, F.S. Chen, L.F. Zhang, Z. Guo, Cationic surfactant-assisted microwave-NaOH pretreatment for enhancing enzymatic hydrolysis and fermentable sugar yield from peanut shells, BioResources (9) (2014) 1290–1302, http://dx.doi.org/10.15376/biores.9.1.1290-1302.
[11] A.M. Sajib, P. Falck, R.R.R. Sardari, S. Mathew, C. Grey, E.N. Karlsson, Valorization of Brewer's spent grain to prebiotic oligosaccharide: production, xylanase catalyzed hydrolysis, in-vitro evaluation with probiotic strains and in a batch human fecal fermentation model, J. Biotechnol. 268 (2018) 61–70, http://dx.doi.org/10.1016/j.jbiotec.2018.01.005.
[12] A.K. Samanta, N. Jayapal, C. Jayaram, S. Roy, A.P. Kolte, S. Senani, M. Sridhar, Xylooligosaccharides as prebiotics from agricultural by-products: production and applications, Bioact. Carbohydr. Diet. Fibre 5 (2015) 62–71, http://dx.doi.org/10.1016/j.bcdf.2014.12.003.
[13] M.C.R. Mano, I.A. Neri-Numa, J.B. da Silva, B.N. Paulino, M.G. Pessoa, G.M. Pastore, Oligosaccharide biotechnology: an approach of prebiotic revolution on the industry, Appl. Microbiol. Biotechnol. 102 (2018) 17–37, http://dx.doi.org/10.1007/s00253-017-8564-2.
[14] W.F. Broekaert, C.M. Courtin, K. Verbeke, T. Van de Wiele, W. Verstraete, J.A. Delcour, Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides, Crit. Rev. Food Sci. Nutr. 51 (2011) 178–194, http://dx.doi.org/10.1080/10408390903044768.
[15] T.M. Cantu-Jungles, M. Iacomini, T.R. Cipriani, L.M.C. Cordeiro, Isolation and characterization of a xylan with industrial and biomedical applications from edible açaí berries (*Euterpe oleraceae*), Food Chem. 221 (2017) 1595–1597, http://dx.doi.org/10.1016/j.foodchem.2016.10.133.
[16] X. Rico, B. Gullón, J.L. Alonso, J.C. Parajó, R. Yáñez, Valorization of peanut shells: manufacture of bioactive oligosaccharides, Carbohydr. Polym. 183 (2018) 21–28, http://dx.doi.org/10.1016/j.carbpol.2017.11.009.
[17] D. Chapla, P. Pandit, A. Shah, Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics, Bioresour. Technol. 115 (2012) 215–221, http://dx.doi.org/10.1016/j.biortech.2011.10.083.
[18] A. Ebringerová, A. Kramár, F. Rendoš, R. Domansky, Die stufenextraktion der hemicellulosen ausdemholz derhagebuche (*Carpinus betulus* L.), Holzforschung 21 (1967) 74–77, http://dx.doi.org/10.1515/hfsg.1967.21.3.74.
[19] H.N. Englyst, J.H. Cummings, Simplified method for the measurement of total non-starch polysaccharides by gas - liquid chromatography of constituent sugars as alditol acetates, Analyst 109 (1984) 937–942, http://dx.doi.org/10.1039/an9840900937.
[20] N. Blumenkrantz, G. Asboe-Hansen, New method for quantitative determination of uronic acids, Anal. Biochem. 54 (1973) 484–489, http://dx.doi.org/10.1016/0003-2697(73)90377-1.
[21] J. Lin, L.M. Ndlovu, S. Singh, B. Pillay, Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP, Biotechnol. Appl. Biochem. 30 (1999) 73–79, http://dx.doi.org/10.1111/j.1470-8744.1999.tb01162.x.
[22] L.G. Paleg, Citric acid interference in estimation of reducing sugars with alkaline copper reagents, Anal. Chem. 31 (1959) 1902–1904, http://dx.doi.org/10.1021/ac60155a072.
[23] P. Biely, M. Vršanská, M. Tenkanen, D. Kluepfel, Endo-β-1,4-xylanase families: differences in catalytic properties, J. Biotechnol. 57 (1997) 151–166, http://dx.doi.org/10.1016/S0168-1656(97)00096-5.
[24] P. Biely, M. Cziszárová, I. Uhliariková, J.W. Agger, X.-L. Li, V.G.H. Eijsink, B. Westereng, Mode of action of acetylxylan esterases on acetyl glucuronoxylan and acetylated oligosaccharides generated by a GH10 endoxylanase, Biochim. Biophys. Acta 1830 (2013) 5075–5086, http://dx.doi.org/10.1016/j.bbagen.2013.07.018.
[25] S.C. Fry, W.S. York, P. Albersheim, A. Darvill, T. Hayashi, J.-P. Joseleau, Y. Kato, E.P. Lorences, G.A. Maclachlan, M. McNeil, A.J. Mort, J.S. Grant Reid, H.U. Seitz, R.R. Selvendran, A.G.J. Voragen, A.R. White, An unambiguous nomenclature for xyloglucan derived oligosaccharides, Physiol. Plant. 89 (1993) 1–3, http://dx.doi.org/10.1111/j.1399-3054.1993.tb01778.x.
[26] A. Mishra, A.V. Malhotra, Tamarind xyloglucan: a polysaccharide with versatile application potential, J. Mater. Chem. 19 (2009) 8528–8536, http://dx.doi.org/10.1039/b911150f.
[27] F.J.St. John, J.D. Rice, J.F. Preston, Characterization of XynC from *Bacillus subtilis* subsp. *subtilis* strain 168 and analysis of its role in depolymerization of glucuronoxylan, J. Bacteriol. 188 (2006) 8617–8626, http://dx.doi.org/10.1128/JB.01283-06.
[28] J. Li, K. Kisara, S. Danielsson, M.E. Lindström, G. Gellerstedt, An improved methodology for the quantification of uronic acid units in xylans and other polysaccharides, Carbohydr. Res. 342 (2007) 1442–1449, http://dx.doi.org/10.1016/j.carres.2007.03.031.
[29] A.K. Verma, A. Goyal, A novel member of family 30 glycoside hydrolase subfamily 8 glucuronoxylan endo-β-1,4-xylanase (CtXynGH30) from *Clostridium thermocellum* orchestrates catalysis on arabinose decorated xylans, J. Mol. Catal. B Enzym. 129 (2016) 6–14, http://dx.doi.org/10.1016/j.molcatb.2016.04.001.
[30] P. Biely, S. Singh, V. Puchart, Towards enzymatic breakdown of complex plant xylan structures: State of the art, Biotechnol. Adv. 34 (2016) 1260–1274, http://dx.doi.org/10.1016/j.biotechadv.2016.09.001.
[31] M.S. Rhee, L. Wei, N. Sawhney, J.D. Rice, J.S. John, J.C. Hurlbert, F. Preston, Engineering the xylan utilization system in *Bacillus subtilis* for production of acidic xylooligosaccharides, Appl. Environ. Microbiol. 80 (2014) 917–927, http://dx.doi.org/10.1128/AEM.03246-13.
[32] M. Vršanská, K. Kolenová, V. Puchart, P. Biely, Mode of action of glycoside hydrolase family 5 glucuronoxylan xylanohydrolase from Erwinia chrysanthemi, FEBS J. 274 (2007) 1666–1677, http://dx.doi.org/10.1111/j.1742-4658.2007.05710.x.
[33] G. Pell, E.J. Taylor, T.M. Gloster, J.P. Turkenburg, C.M.G.A. Fontes, L.M.A. Ferreira, T. Nagy, S.J. Clark, G.J. Davies, H.J. Gilbert, The mechanisms by which family 10 glycoside hydrolases bind decorated substrates, J. Biol. Chem. 279 (2004) 9597–9605, http://dx.doi.org/10.1074/jbc.M312278200.
[34] Z. Fujimoto, S. Kaneko, A. Kuno, H. Kobayashi, I. Kusakabe, H. Mizuno, Crystal structures of decorated xylooligosaccharides bound to a family 10 xylanase from *Streptomyces olivaceoviridis* E-86, J. Biol. Chem. 279 (2004) 9606–9614, http://dx.doi.org/10.1074/jbc.M312293200.
[35] Q. Wan, Q. Zhang, S. Hamilton-Brehm, K. Weiss, M. Mustyakimov, L. Coates, P. Langan, D. Graham, A. Kovalevsky, X-ray crystallographic studies of family 11 xylanase Michaelis and product complexes: implications for the catalytic mechanism, Acta Crystallogr. D Biol. Crystallogr. 70 (2014) 11–23, http://dx.doi.org/10.1107/S1399004713023626.
[36] K. Koga, S. Fujikawa, Xylo-oligosaccharides, in: T. Nakakuki (Ed.), Oligosaccharides Prod. Prop. Appl. Gordon and Breach, Phildelphia, 1993, pp. 130–143.
[37] Y. Kobayashi, E. Wakasugi, T. Ohbuchi, M. Yokoyama, R. Yasui, M. Kuwahata, Y. Nakabou, Y. Kido, Acidic oligosaccharide promotes recovery from iron deficiency anemia by enhancing serum iron level in rats, Biomed. Res. 22 (2011) 417–423.
[38] Y. Izumi, N. Azumi, A. Furujyo, T. Kimura, N. Uotsu, R. Sa-kamoto, Acidic oligosaccharide composition and the process of manufacture, (2003), p. JP183303.
[39] T. Ohbuchi, M. Sakaino, T. Takahashi, N. Azumi, K. Ishikawa, S. Kawazoe, Y. Kobayashi, Y. Kido, Oral administration of acidic xylooligosaccharides prevents the development of atopic dermatitis-like skin lesions in NC/Nga mice, J. Nutr. Sci. Vitaminol. 56 (2010) 54–59, http://dx.doi.org/10.3177/jnsv.56.54.

CHAPTER 5

Thermostable Enzymes and Their Industrial Applications

Santhosh Kumar, Nanthakumar Arumugam, Kugenthiren Permaul, and Suren Singh

CONTENTS

Abstract....117
Introduction....117
Xylanases....118
Enzyme Sources....118
Enzyme Production Level....119
Applications....120
Cellulases....121
Enzyme Sources....122
Enzyme Production Level....122
Applications....123
Amylase....123
Enzyme Sources....124
Enzyme Production Level....124
Applications....125
Pullulanase....125
Enzyme Sources....126
Enzyme Production Level....126
Applications....126
Xylosidase....127
Enzyme Sources....127

Enzyme Production Level 127
Applications 128
Lipase 128
Enzyme Sources 129
Enzyme Production Level 129
Applications 130
Phospholipase 130
Enzyme Sources 131
Enzyme Production Level 131
Applications 132
Chitinase 132
Enzyme Sources 132
Enzyme Production Level 133
Applications 133
Laccases 134
Enzyme Sources 134
Enzyme Production Level 134
Applications 135
Protease 135
Enzyme Sources 136
Enzyme Production Level 136
Applications 136
Peroxidases 137
Enzyme Sources 137
Enzyme Production Level 137
Applications 138
Ligases 138
Enzyme Sources 139
Applications 139
DNA Polymerase 140
Enzyme Sources 140
Applications 140
Reverse Transcriptase 141
Enzyme Sources 141
Applications 142
Conclusions 142
References 142

ABSTRACT

Thermophilic organisms provide a reservoir for a plethora of enzymes that are generally thermostable and many of them can also withstand denaturants of extremely acidic or alkaline nature, which is ideal for many industrial applications. Furthermore, thermostable enzymes have better substrate solubility, high mass transfer rate, and lowered risk of contamination. Enzyme production from thermophilic microorganisms can be achieved either by using optimized fermentation conditions or cloning and expressing the functional genes in fast-growing bacteria or yeast by recombinant DNA technology. Latest developments in the field of genetic and protein engineering provide a platform for the advancement of enzymes with superior properties. In this chapter, the commonly used industrial enzymes, their source microorganisms, properties, and applications are discussed.

INTRODUCTION

Environmental and economic concerns over the application of conventional chemical processes in industries have led scientists to explore alternative technologies that are safer and that do not negatively impact the environment. Biocatalysis that exploits the catalytic potential of enzymes through enantioselectivity and regioselectivity under appropriate conditions has emerged as a promising approach to the chemical synthesis of novel and industrially significant compounds (Schmid et al. 2001; Shoemaker et al. 2003). Enzymes have been used in several manufacturing processes since ancient times, in the production of food products, such as cheese, sourdough, beer, wine and vinegar, and in the manufacture of commodities such as leather, indigo, and linen. The development of fermentation processes during the latter part of the last century has resulted in the production of enzymes on a large scale. The continuously expanding applications of enzymes for the chemical, pharmaceutical, and food industries are creating a growing demand for biocatalysts that exhibit improved or new properties. Advancements in biotechnology, especially in the area of genetics and protein engineering have made it possible to provide tailor-made enzymes displaying new activities and adapted to new process conditions, enabling a further expansion of their industrial use. Based on such favorable properties, enzymes are widely used as catalysts and processing aids in many industrial processes.

Despite the fact that more than 3000 diverse enzymes have been identified and many of these have found their way into biotechnological and industrial applications, the current enzyme pool is still insufficient to meet all demands. A major reason for this is the fact that many available enzymes are incapable of withstanding harsh industrial reaction conditions. This has resulted in the screening and characterization of thermophilic microorganisms that are able to thrive in extreme environments as a potential enzyme source. Such enzymes generally exhibit good temperature, pH and enzymatic stability, and faster reaction rates, which are important parameters for the industrial application of any enzyme. Some commonly used industrial enzymes and their application are described in this chapter.

XYLANASES

Among the various hydrolases, hemicellulases are a diverse group of enzymes that are widely used in industry. Xylanases are glycosidases (O-glycoside hydrolases) that catalyzes the endohydrolysis of 1, 4-β-D-xylosidic linkages in the xylan backbone resulting in its conversion into xylooligosaccharides and xylose (Verma and Satyanarayana 2012; Rakotoarivonina et al. 2015). However, the extend of xylanase action depends on the type, solubility, degree of polymerism, and degree of substitution of the polysaccharide. Endoxylanases have also been reported to catalyze intermolecular transglycosylation in the presence of high concentrations of xylooligomers (Masui et al. 2012). Furthermore, there are also reports regarding the existence of multiple forms of xylanases produced by microorganisms (Turner et al. 2007). Elegir et al. (1994) reported that *Streptomyces* sp. B-12-2 produced five endoxylanases when grown on oat spelt xylan. Around 15 xylanases have been reported from the culture filtrates of *Aspergillus niger* and 13 xylanases from *Trichoderma viride* (Biely 1985). From *Phanerochaete chrysosporium*, more than 30 different xylanases have been reported when grown on Avicel (Dobozi et al. 1992). Heteroxylans having a complex structure require the action of multiple xylanases with overlapping but different specificities as all of the xylosidic linkages in the substrates are not equally accessible to xylan-degrading enzymes (Elleuche et al. 2015).

Enzyme Sources

Xylanases are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods, and arthropods (Knob and Carmona 2010) and some members of higher animals, including freshwater mollusks

(Yamura et al. 1997). Most of the microbial xylanases have been reported from bacteria and fungi (Kulkarni et al. 1999; Chavez et al. 2006); however, many of them are mesophilic in nature. A number of thermophilic (optimal growth at 50–80°C) and hyperthermophilic (optimal growth at >80°C) xylanase-producing microorganisms have been isolated from a variety of sources, including terrestrial and marine solfataric fields, thermal springs, hot pools, volcanic islands, composts, and self-heating decaying organic debris (Haki and Rakshit 2003; Singh et al. 2003; Cannio et al. 2004; Bouacem et al. 2014; Elleuche et al. 2015; Palavesam 2015).

Xylanases with high thermostability are better candidates for industrial applications, particularly for enzymatic hydrolysis at elevated temperatures (e.g., biopulping), where mesophilic xylanases fail to meet the desired results. Several thermophilic strains have been screened for the production of thermostable enzymes and they are found more appropriate for industrial applications, as compared with their mesophilic counterparts (Uday et al. 2016). Among the reported fungal xylanase producers, strains of *Thermomyces* have been found to produce high titers of thermostable xylanolytic enzymes (Kumar et al. 2009) with *Thermomyces lanuginosus* SSBP being the highest (Singh et al. 2000a,b).

Enzyme Production Level

Enzyme production level varies with different microorganisms depending on the class, genus and species as well as the type of media/substrate used for enzyme production. *Thermobacillus xylanilyticus* has been reported to produce xylanase with a specific activity of 480.66 U/mg (Rakotoarivonina et al. 2015) and *Stenotrophomonas maltophilia* strain X6 with 313.38 U/mg (Raj et al. 2013). Xylanase produced by *Halomonas meridiana* APCMST-KS4 has 26.13 U/mg specific activity (Palavesam 2015). *Clostridium thermocellum* has been reported to produce a family 10 xylanase with a specific activity of 93 U/mg and *Streptomyces lividans* produced family 11 xylanase with 119.5 U/mg specific activity (Gonçalves et al. 2015). Xylanase produced by *Streptomyces* sp. CS428 has 926,103 U/mg of specific activity (Pradeep et al. 2013). *Scytalidium thermophilum* has been observed to produce xylanase with a specific activity of 841.1 U/mg (Kocabaş et al. 2015). Among the different thermophilic fungal strains, *T. lanuginosus* SSBP has recorded the highest endoxylanase (family 11) activity of 3575.28 U/mg (Singh et al. 2000a,b) when grown on corn cob media. *Penicillium janczewskii* also produced xylanase with a specific activity of 179.1 U/mg (Terrasan et al. 2013).

There are several reports on the cloning and expression of xylanase on prokaryotic and eukaryotic hosts. A xylanase gene from the extremely thermophilic bacterium *Geobacillus thermoleovorans* was cloned and expressed in *Escherichia coli* BL21 (DE3) with a specific activity of 270 U/mg (Verma and Satyanarayana 2012). Similarly family 10 endoxylanase gene from *Geobacillus* sp. WSUCF1 was cloned and expressed in *E. coli* with a specific activity of 461 U/mg (Bhalla et al. 2014). The genes encoding for endoxylanase of *B. subtilis* M015 was also expressed in *E. coli* JE5505 (Banka et al. 2014). A highly thermostable xylanase from *Thermotoga thermarum* was cloned and expressed in *E. coli* BL21 (DE3) with a specific activity of 145.8 U/mg (Shi et al. 2013). Recombinant xylanases have also been expressed in *E. coli* and *Pichia pastoris* from *Neocallimastix patriciarum* with a specific activity of 5778.3 U/mg and 7995.3 U/mg, respectively (Cheng et al. 2014). An overexpression of xylanase from *Penicillium occitanis* Pol6 in *P. pastoris* was also observed with a specific activity of 8549.85 U/mg (Uday et al. 2016).

Applications

Among the different hemicellulase enzymes, xylanases represent the major commercial proportion; however, they only constitute a small percentage of the total enzyme market. Microbial xylanases have attracted a great deal of attention owing to their biotechnological potential in various industrial processes such as food, feed, and pulp and paper industries (Chavez et al. 2006; Patel and Savanth 2015). Other potential applications include the conversion of xylan in biomass from food and agricultural industry into xylose and xylooligosaccharides (Bhalla et al. 2013), and in the bioconversion of lignocellulosic materials to fuels and chemical feedstocks (Banka et al. 2014; Thomas et al. 2014; Palavesam 2015). Other less well documented putative applications include: brewing, to increase wort filterability and reduce haze in the final product (Tikhomirov et al. 2003; Raj et al. 2013; Elleuche et al. 2015); in coffee extraction and in the preparation of soluble coffee (Wong et al. 1988); in detergents (Kamal Kumar et al. 2004); in the protoplastation of plant cells (Kulkarni et al. 1999); in the production of pharmacologically active polysaccharides for use as antimicrobial agents (Christakopoulos et al. 2003) or antioxidants (Katapodis et al. 2003); in the production of alkyl glycosides for use as surfactants (Matsumura et al. 1999); and in the washing of precision devices and semiconductors (Imanaka and Sakurai 1992).

Xylanases are of significant importance to the pulp and paper industries because hydrolysis of xylan by xylanase facilitates the release of lignin from pulp and aid in reducing the level of chlorine usage as a bleaching agent (Moraïs et al. 2011; Ellis and Magnuson 2012). In the food industry, xylanases are used in a number of applications including baking, juice preparation, and starch processing (MacCabe et al. 2002). Xylanases can also be used in bread-making, together with α-amylase, malting amylase, glucose oxidase, and proteases. Synergistic action of xylanase and cellulase mixtures could result in the efficient release of sugars from lignocelluloses (Harris et al. 2014; Gonçalves et al. 2015). Xylanases break down the hemicellulose in wheat-flour thereby helping in the redistribution of water and leaving the dough softer and easier to knead (Polizeli et al. 2005; Javier et al. 2007; Butt et al. 2008).

In juice making processes, xylanases, in conjunction with cellulases, amylases, and pectinases, help to improve the yield of juice by means of liquefaction of fruit; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments, etc. It also helps in the reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate. Xylanases are also used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytases, galactosidases, and lipases. These enzymes break down arabinoxylans in the ingredients of the feed, reducing the viscosity of the raw material (Polizeli et al. 2005; Knob et al. 2014).

CELLULASES

Cellulases, a general term used for cellulolytic enzymes, are composed of three classes of enzymes and are recognized on the basis of their mode of action and substrate specificities: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74 and EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Várnai et al. 2014; Haq et al. 2015). Endo-β-glucanase acts randomly on the cellulose polysaccharide and produces cello-oligosaccharides, while exo-β-glucanase acts on the exposed chain ends by splitting off cellobiose (Raghuwanshi et al. 2014). The release of glucose is as a result of the synergetic action of these enzymes. Action of cellulases results in either exo- or endo-cleavage of the substrate and mostly all the cellulases target specifically on the β 1,4-glycosidic bonds (Juturu and Wu 2014), whereas β-glucosidases or cellobiases cleave the products of exocellulase into monosaccharides (Jabbour et al. 2012; Lee et al. 2015). Most of the

reported cellulases are from mesophilic organisms that cannot fulfill the requirements for industrial application, where the physical factors, such as pH, temperature, ionic strength, acidity, and alkalinity are at their extremes (Gunny et al. 2014; Khelila and Cheba 2014; Bhalla et al. 2015). The performance of cellulase mixtures in biomass conversion processes relies on several of their properties including stability, product inhibition, specificity, synergism between the different enzymes, productive binding to the cellulose, physical characteristics as well as the composition of cellulosic biomass (Heinzelman et al. 2009).

Enzyme Sources

Many microorganisms have been reported to produce cellulases, which include bacteria, and fungi, aerobes and anaerobes, mesophiles and thermophiles. Organisms generally adopt two strategies for utilizing their cellulases: (i) distinct noncomplexed cellulases that are typically secreted by aerobic bacteria and fungi and (ii) complexed cellulases (cellulosome) that are typically expressed on the surface of anaerobic bacteria and fungi. Cellulose degradation mechanism by aerobic bacteria and fungi are similar; however, it differs with anaerobic fungi and bacteria (Kuhad et al. 2016).

A plethora of bacteria and archea belonging to diverse genera such as *Bacillus, Clostridia, Fervidobacterium, Rhodothermus, Thermoplasma, Thermotoga, Pyrococcus, Sulfolobus, Thermococcus,* and *Desulfurococcus* have been reported to produce thermostable cellulases with different properties (Kuhad et al. 2016). Most of the fungal cellulases are produced by the genus *Trichoderma*, however, of mesophilic nature. On the other hand, a thermo-alkalistable cellulase has been produced by extremophilic fungus *Penicillium citrinum* with multiple pH optima (Dutta et al. 2008).

Enzyme Production Level

Clostridium thermocellum has been reported to produce cellulase with a specific activity of 2.4 U/mg (Thomas et al. 2014). A recombinant cellulase from *Thermococcus* sp. AM4 was expressed in *E. coli* BL21 (DE3) which has a specific activity of 700.4 U/mg (Leis et al. 2015). A marine *Bacillus* VITRKHB has been reported to produce cellulase with a specific activity of 1.92 U/mg (Singh et al. 2013) and cellulase from *Bacillus* sp. BCCS A3 has been reported with a high level of enzyme production of 50.3 U/mL (Kazemi et al. 2014).

Xanthomonas sp. EC102 has shown to produce endoglucanase with a specific activity of 1.97 U/mg (Woo et al. 2014) and a recombinant

endoglucanase from *C. thermocellum* ATCC 27405 has been reported with a specific activity of 30 U/mg (Haq et al. 2015).

Endocellulase gene from *Ciboria shiraiana* has been cloned into pPIC9K and expressed in *P. pastoris* has an activity of 17.44 U/mL (Lu et al. 2015). A gene encoding for cellobiohydrolase from *Chaetomium thermophilum* was cloned into pMD 18 T and expressed in *P. pastoris* (Li et al. 2009). Similarly, cellobiohydrolase gene from *Penicillium funiculosum* NCL1 was cloned into pPICZαA4 and expressed in *P. pastoris* with a specific activity of 0.8 U/mg (Chinnathambi et al. 2015).

Carboxy methyl cellulase (CMCase) gene from *C. thermocellum* was cloned into pET21a which was expressed in *E. coli* with a specific activity of 3.5 U/mg (Mutreja et al. 2011). Similarly, CMCase gene from *Neocallimastix* sp was cloned into pCT and expressed in *E. coli* EC100 with a specific activity of 2.06 U/mg (Comlekcioglu et al. 2010). *Bacillus* sp. BSS3 has produced CMCase which showed a maximum specific activity of 104.06 U/mL (Sreedevi et al. 2013). The mutant strain *T. asperellum* SR1-7 has produced CMCase (13.2 U/g) and β-glucosidase (9.2 U/g) simultaneously under controlled conditions (Raghuwanshi et al. 2014).

Applications

Cellulases have been commercially available for more than 3 decades and have also demonstrated their potential in industries such as food, animal feed, brewing and wine, agriculture, pulp and paper, textile, and laundry (Kuhad et al. 2011; Ferreira et al. 2014). However, the type of cellulases required is completely different with respect to different industries. The predominant application of cellulolytic enzymes is in biomass processing specifically for biofuel and bioenergy generation. The biofuel industry prefers thermostable cellulases that are resistant to acidic conditions, while the detergent industry prefers enzymes operating at higher pH with good thermostability (Kumar et al. 2011). Cellulolytic enzymes are also used in detergent industries for softening and color brightening, stoning of jeans, and in the pre-treatment of industrial wastes (Salahuddin et al. 2012).

AMYLASE

Amylases are mostly a group of extracellular enzymes that break down the complex polysaccharide starch to yield assorted products such as dextrins, maltose, glucose, and maltooligosaccharides (Jyoti et al. 2011; Janeček et al. 2014). The complete hydrolysis of starch requires a combination of enzymes which include α-amylases (EC 3.2.1.1), glucoamylases or

β-amylases (EC 3.2.1.3), and pullulanases (Oziengbe and Onilude 2012). α-Amylase (endo-1,4-alpha-D-glucan glucohydrolase) randomly cleaves the 1,4-α-D-glucosidic linkages between the adjacent glucose units in the linear amylose chain. These endoacting enzymes cleave the substrate in the interior of the molecules and are categorized based on their properties and mode of action. Amylases that liberate free sugars are termed as "saccharogenic" and those that liquefy starch without generating free sugars are known as "starch-liquefying." Amylolytic enzymes are categorized by the similarities in their amino acid sequences and three-dimensional structures, reaction mechanisms, and catalytic machineries which reflect their evolutionary relatedness than specificity (Janeček et al. 2014).

Enzyme Sources

Amylases can be obtained from several sources such as plants, animals, and microorganisms. However, the enzymes from microbial sources particularly obtained from extreme environments, proved to be useful for industrial processes (Joshi 2011; Jyoti et al. 2011; Ibrahim et al. 2013; Zafar et al. 2015). Furthermore, microorganisms offer easy manipulation for obtaining α-amylases of desired characteristics with good expression levels (Abdel-fattah et al. 2013). Some of the bacteria that produce amylases are *Bacillus licheniformis* (Oziengbe and Onilude 2012), *B. licheniformis* BT5.9 (Ibrahim et al. 2013), *B. circulans* (Joshi 2011), *B. subtilis* JS-2004, *B. megaterium, Bacillus* sp. Strain PM1, *Pyrococcus furiosus* (Cuong et al. 2015), *Amphibacillus* sp. NM-Ra2 (Mesbah and Wiegel 2014), and *Halobacillus* sp. LY9 (Sharma et al. 2014). Some of the fungal species that produce amylases are *Rhizomucor pusillus* (He et al. 2014), *Penicillium fellutanum*, (Sharma et al. 2014), *Penicillium camemberti*, *Pestalotiopsis microspore, Aspergillus oryzae*, and *Acremonium sporosulcatum* (Rana et al. 2013).

Enzyme Production Level

Bacillus licheniformis JAR-26 was reported to produce α-amylase with a specific activity of 317.9 U/mg (Jyoti et al. 2011) and *B. licheniformis* AI20 produced amylase with a specific activity of 748.98 U/mg (Abdelfattah et al. 2013). α-Amylase gene from *B. licheniformis* was cloned into a Gateway shuttle vector pMMC and expressed in *E. coli* and *B. megaterium* (Atanassov et al. 2013). Specific activity of amylase produced from *Bacillus* sp. strain EF_TYK1-5 was 132.44 U/mg (Pathak and Rekadwad 2013). *B. circulans* PN5 produced amylase which had 2625 U/mg of specific activity

(Joshi 2011). α-Amylase from *Amphibacillus* sp. NM-Ra2 has 250 U/mg of specific activity (Mesbah and Wiegel 2014). An α-amylase gene from the thermophilic bacterium *B. subtilis* was cloned and expressed in *E. coli* DH5α which has 15950 U/mg specific activity (Park et al. 2013). *B. subtilis* A28 α-amylase gene was also cloned and expressed *in E. coli* with a specific activity of 2814 U/mg (Ozturk et al. 2013). A gene encoded for α-amylase from acidophilic bacterium *B. acidicola* was cloned into pET28a(+) vector and expressed in *E. coli* BL21 (DE3) with a specific activity of 1166 U/mg (Sharma and Satyanarayana 2012). Similarly *Thermotoga petrophila* was cloned into pET-21a(+) and expressed in *E. coli* BL21 (DE3) and has a specific activity of 126.31 U/mg (Zafar et al. 2015). Amylase produced from *Thermoactinomyces thalpophilus* KSV 17 has a specific activity of 145.8 U/mg (Rao et al. 2012). α-Amylase gene of a fungal glucoamylase and the α-amylase genes from *Rhizomucor pusillus* were cloned, and expressed in *P. pastoris* which have a specific activity of 1953 U/mg and 20732 U/mg, respectively (He et al. 2014).

Applications

Amylases are the important digestive enzymes of starch which have various applications in several industries such as food, clinical, medical, and agriculture (Pathak and Rekadwad 2013; Sharma et al. 2014). In food industry, amylases aid in starch processing which includes starch liquefaction and saccharification and also in brewing and sugar production (Nigam 2013). Amylases are also used for baking (to delay the staling of bread), in textile industries for sizing of textile fibers and in detergent manufacturing processes (Joshi 2011; Jyoti et al. 2011; Mesbah and Wiegel 2014). Amylases are also used for the production of fructose and glucose by the enzymatic conversion of starch (Van Der Maarel et al. 2002). Amylases also play an important role in removing stains from fabrics when added to detergents (Sundarram and Murthy 2014).

PULLULANASE

Pullulanase (3.2.1.41) belongs to the α-amylase family and hydrolyses the glycosidic linkages in pullulan, amylopectin, starch, and glycogen (Li et al. 2015). Pullulanase is generally used as a debranching enzyme during starch saccharification. Pullulanases are grouped into four categories: pullulan hydrolase type I (neopullulanase), pullulan hydrolase type II (isopullulanase), pullulanase type I, and pullulanase type II (amylopullulanase)

(Ramanathan 2011). Microbial pullulanases are gaining more interest due to their specific action on α-1,6 linkages in pullulan, a linear α-glucan made of maltotriosyl units (Hii et al. 2012).

Enzyme Sources

Pullulanases are mainly produced by bacteria compared with fungi or other organisms. Pullulanase has been reported from *Anaerobranca gottschalkii*, *Fervidobacterium pennavorans*, *Thermotoga neapolitana*, *Bacillus acidopullulyticus*, and *Bacillus* sp. CICIM 263 (Kang et al. 2011; Li et al. 2012, 2015). Pullulanase has also been produced by *Bacillus flavocaldarius*, *B. acidopullulyticus*, *B. deramificans* (Duan and Wu 2015), *B. thermoleovorans* US 105, *Aspergillus niger* (Hii et al. 2012), *Pyrococcus furiosus, Pyrococcus woesei, Thermococcus aggregans, T. hydrothermalis, T. celer, T. hydrothermalis, Sulfolobus solfataricus, Thermus caldophilus, Thermoanaerobacter ethanolicus, Clostridium thermosulfurogenes,* and *Desulfurococcus mucosus* (Bertoldo and Antranikian 2002; Kang et al. 2004; Chiang et al. 2005; Mrudula et al. 2011; Ramanathan 2011). Pullulanase is also reported from *Staphylothermus marinus* (Li et al. 2013b), *Streptomyces* sp. No. 27, and *Geobacillus* sp. LM14-3 (Sun et al. 2011).

Enzyme Production Level

Bacillus halodurans has been reported to produce pullulanase with a specific activity of 87.64 U/mg (Asha et al. 2013). A high level of amylopullulanase was noticed with *Geobacillus thermoleovorans* NP33 with a specific activity of 1260 U/mg (Nisha and Satyanarayana 2013). *Staphylothermus marinus* produced amylopullulanase with a specific activity of 42.1 U/mg (Li et al. 2013b) and *S. erumpens* had a specific activity of 98.84 U/mg (Kar et al. 2012). *Thermus thermophilus* HB27 has been reported with 280 U/mg specific activity (Wu et al. 2014). A recombinant pullulanase from *Thermococcus kodakarensis* KOD1 was reported with a specific activity of 118 U/mg (Han et al. 2013) and pullulanase from *Geobacillus* sp. was expressed in *E. coli* BL21 (DE3) with a specific activity of 134.3 U/mg (Jasilionis et al. 2014). There are reports on the cloning of pullulanase from *Anaerobranca gottaschalkii* and expression in *E. coli* BL21 (DE3) with a specific activity of 56 U/mg.

Applications

Pullulanase is used as a principal enzyme for the industrial production of high-glucose and high-maltose syrups and has major applications in

starch processing (Malakar et al. 2010; Li et al. 2015). Pullulanases also have applications in other industries. This includes production of cyclodextrins, liquefaction and saccharification of starch, making of low-calorie beer, and as an antistaling agent to improve texture, volume, and flavor of bakery products and also as a dental plaque control agent (Sun et al. 2011; Hii et al. 2012; Asha et al. 2013; Wu et al. 2014).

XYLOSIDASE

β-xylosidases (EC 3.2.1.37) are exo-type glycosidases that catalyze the hydrolysis of 1,4-β-D-xylooligosaccharides by removing successive xylose residues from the nonreducing termini. It also releases xylose from branched or substituted xylo-oligosaccharides produced by the action of endo-1,4-β-xylanases (Subramaniyan and Prema 2002; Biely 2003; Terrasan et al. 2013). The systematic name is 1,4-β-D-xylan xylohydrolase; however, the commonly used name is β-xylosidase and is found in families 3, 39, 43, 52, and 54 (Shallom and Shoham 2003). For many industrial applications such as improving bread dough, production of xylitol, and deinking of recycled paper, β-xylosidases are used in combination with xylanases (Jordan and Wagschal 2010).

Enzyme Sources

Various microorganisms including bacteria and fungi are reported to produce β-xylosidase; however, very few yeast strains are known to produce the enzyme (Basaran and Ozcan 2008). *Bacillus subtilis* M015 was reported to produce intracellular β-xylosidase (Banka et al. 2014). β-Xylosidase production has been documented from fungal species such as *Thermomyces lanuginosus* (Singh et al. 2000a), *Aspergillus awamori* (Paredes et al. 2015), *A. terricola* and *A. ochraceus* (Michelin et al. 2012a,b), *Neocallimastix frontalis* (Hebraud and Fevre 1990), *Neocallimastix* sp. M2 (Comlekcioglu et al. 2011), *Aspergillus japonicas* (Wakiyama et al. 2008), *Penicillium janthinellum* (Kundu and Ray 2013), *Fusarium verticillioides* (Saha 2001), *F. proliferatum* (Saha 2003), *Trichoderma reesei* RUT C-30 (Herrmann et al. 1997), and *Penicillium janczewskii* (Terrasan et al. 2013).

Enzyme Production Level

Bacillus thermantarcticus has been reported to produce β-xylosidase with a specific activity of 160 U/mg (Lama et al. 2004). β-Xylosidase with a specific activity of 261.1 U/mg was produced by *Alicyclobacillus* sp. A4 (Zhang et al. 2014) and 41.43 U/mg by *A. ochraceus* (Michelin, Peixoto-Nogueira,

et al. 2012). *Paecilomyces thermophila* has been reported to produce β-xylosidase with 45.4 U/mg specific activity (Teng et al. 2011). A thermotolerant β-xylosidase of *Aspergillus* sp. BCC125 was cloned and expressed as a secreted protein using the *P. pastoris* KM71 expression system with a specific activity of 156 U/mg (Wongwisansri et al. 2013).

The genes (Xyn A, GH Family 11 and Xyn B, GH Family 43) encoding for β-xylosidase from *B. subtilis* M015 was isolated and expressed in *E. coli* JE5505 which has an activity of 2.75 ± 0.30 U/mL and 0.41 ± 0.02 U/mL, respectively (Banka et al. 2014). A β-xylosidase gene (Tlxyn1) from the thermophilic fungus *T. lanuginosus* SSBP was cloned and expressed in *P. pastoris* GS115 with a specific activity of 2.29 U/mg (Gramany et al. 2015). A gene (designated TlXyl43) encoding β-xylosidase was cloned from *T. lanuginosus* CAU44 and expressed in *E. coli* with a specific activity of 45.4 U/mg (Chen et al. 2012). Kirikyali et al. (2014) implemented the heterologous expression of *Aspergillus oryzae* β-xylosidase (XylA) in *P. pastoris* under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter which has a specific activity of 150 U/mg.

Applications

β-xylosidase has immense biotechnological potential especially in food, pharmaceutical animal feed, paper, and pulp industries. It is also used in the bioconversion of lignocellulosic wastes into value-added chemicals (Beg et al. 2001; Chapla et al. 2010). β-Xylosidase in combination with xylanase cocktails is also used in the bleaching of pulp liquor (Marques et al. 2003; Kumar et al. 2009) and processing of wood pulp (Tsujibo et al. 2001). In food industry, it is used in the extraction of juice by hydrolyzing the bitter xylosylated compounds and liberates aroma from grapes during wine making (Jordan and Wagschal 2010). β-Xylosidase also plays a role in improving bread dough baking and nutritional quality (Dornez et al. 2007) and in the release of D-xylose residues from xylan for subsequent reduction to xylitol, a sweetener used in food industry (Polizeli et al. 2005; Jordan and Wagschal 2010).

LIPASE

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are lipolytic enzymes that catalyze the hydrolysis of long-chain triglycerides by forming diacylglycerides, monoglycerides, glycerol, and free fatty acids at the interface between the insoluble substrate and water (Masomian et al. 2013). Thermostable microbial lipases can be used for a variety of applications

including many bioconversion processes. Hydrolysis, interesterification, alcoholysis, aminolysis, esterification, and acidolysis are some of the bioconversion processes that have been performed effectively by microbial lipases (Deive et al. 2012; Borrelli and Trono 2015). Their industrial significance is due to their distinctive characteristics such as substrate specificity, stereospecificity, regioselectivity, and the ability to catalyze heterogeneous reactions at the interface of water soluble and water insoluble systems (Haki and Rakshit 2003; Saxena et al. 2003; Li and Zhang 2005; Ebrahimpour et al. 2011; Puchart et al. 2015).

Enzyme Sources

Lipases are available in most of the flora and fauna and also in microbial sources such as bacteria, fungi, and yeasts. One of the major sources for lipases are *Bacillus* sp. such as *B. acidocaldarius, B. thermocatenletus, B. thermoleovorans,* and *Bacillus* sp. RSJ-1 (Nawani and Kaur 2000; Haki and Rakshit 2003; Dror et al. 2014; Espinosa-Luna et al. 2015). *Bacillus stearothermophilus* MC 7 has expressed lipase activity even at an elevated temperature of 75°C to 80°C (Kambourova et al. 2003). Lipase QL, an extracellular enzyme produced by *Alcaligenes* sp., has optimum activity at pH and temperature of 7.0 and 50°C, respectively (Wilson et al. 2006). An organic solvent-tolerant lipase was reported from *Aneurinibacillus thermoaerophilus* HZ with an optimal temperature and pH of 65°C and 7.0, respectively (Masomian et al. 2013). Lipase from *Aspergillus carneus* is reported to be active at alkaline pH and has extreme temperature tolerance (Saxena et al. 2003). Similarly, a lipase produced by *Thermomyces lanuginosus* is optimally active at 80°C and at a pH of 10 (Ávila-Cisneros et al. 2014). Some other fungi that are reported to produce lipases are *Rhizopus homothallicus, Candida rugosa, Penicillium simplicissimum, Humicola lanuginosa, A. niger,* etc.

Enzyme Production Level

Bacillus coagulans BTS-3 produced lipase with a specific activity of 4.8 U/mg (Kumar et al. 2005). *Bacillus thermoamylovorans* CH6B produced significant levels (0.45 U/mL) of extracellular lipase (Deive et al. 2012). Lipase gene from *Geobacillus* strain T1 has been cloned and expressed in *E. coli* with a specific activity of 30.19 U/mg (Leow et al. 2004). *Alcaligenes* sp. has produced a thermostable lipase with 0.049 U/mg specific activity (Wilson et al. 2006). Gutarra et al. (2009) have reported a lipase from *Penicillium simplicissimum* with a specific activity of 4.5 U/mg. A high level production of lipase was reported from *Aspergillus carneus* with a specific activity

of 502 U/mg (Saxena et al. 2003). *Thermomyces lanuginosus* produced lipase with a specific activity of 0.12 U/mg (Ávila-Cisneros et al. 2014).

Applications

Thermostable lipases have a wide range of applications in various sectors including bakery food dressing, beverages, oleochemical, agrochemical, polymer synthesis, pulp and paper, leather, synthesis of surfactants, and pharmaceutical industries (Haki and Rakshit 2003; Sharma et al. 2011; Espinosa-Luna et al. 2015). In detergents, lipase is used to remove oil stains from fabric and in cleaning products, it aids in fat removal. Lipases are also used to treat oily wastewaters and for biodiesel production from vegetable oil (Cammarota and Freire 2006; Gutarra et al. 2009). Lipases are also used to enhance the flavor and aroma in dairy products (milk, cheese, and butter) and beverages. Lipases help the interesterification of fats and oils and they catalyze the hydrolysis of lipids in butter, fats, and cream. Lipases also serve as an emulsifier in food, cosmetics, and pharmaceuticals and they prolong the shelf life of bakery foods and improve their flavor (Sharma et al. 2011).

PHOSPHOLIPASE

Phospholipases, are a group of lipolytic enzymes that cleave the ester bonds of phospholipids, are classified into A, B, C, and D classes, based on the type of reaction they catalyze. Phospholipases A1 (PLA1, 3.1.1.32) and A2 (PLA2, 3.1.1.4) catalyze the hydrolysis of the ester bond at *sn*-1 and *sn*-2 positions, respectively, of the phospholipids, thus producing a free fatty acid and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively (Borrelli and Trono 2015). Phospholipases A1, in general, constitute a large group of 1-acyl hydrolases, some of which also degrade neutral lipids (Istivan and Coloe 2006). Phospholipases B (PLB, 3.1.1.5) can hydrolyze fatty acids esterified at both the *sn*-1 or *sn*-2 position of the phospholipid. Phospholipase C (PLC, 3.1.4.3) breaks down the glycerophosphate bond, thus releasing diacylglycerol and the phosphorylated head group, while phospholipase D (PLD, 3.1.4.4) cleaves the terminal phosphodiesteric bond, thus releasing phosphatidic acid (PA) along with the head group (Borrelli and Trono 2015). Phospholipase D is abundant in nature and is secreted by a plethora of organisms ranging from viruses to bacteria, yeast, plants, and animals (Simkhada et al. 2009). Phospholipases have various functions, ranging from nutrient digestion to bioactive molecule formation, making them a vital enzyme in life (Istivan and Coloe

2006). Substrate specificity, stability to organic solvents, tolerance to high and low temperatures, and tolerance to acidic and alkaline pHs, tolerance to proteases are some of the outstanding properties of phospholipases (Wei et al. 2015). The amphipathic character of phospholipids restricts the enzymes by forming bilayers or micelles making them very rare to have as a single soluble substrate. Since all phospholipases target phospholipid as the substrate, variations are observed with their specific active site, mode of action and regulation (Istivan and Coloe 2006).

Enzyme Sources

Commercial phospholipases are mostly produced by yeasts and fungi, followed by bacteria. The most important genera of yeasts and fungi that are exploited for the production of phospholipases include *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans, Thermomyces lanuginosus, Tuber borchii, Gibberella zeae, Magnaporthe grisea, Aspergillus oryzae, A. fumigatus, A. nidulans,* and *Neurospora crassa* (Istivan and Coloe 2006; Borrelli and Trono 2015). The most important bacteria that have been investigated for the production of phospholipases are *Serratia liquefaciens, Yersinia enterocolitica, E. coli, Streptomyces alboflavus, S. coelicolor, S. olivochromogenes, S. violaceoruber, Ochrobactrum* sp., *Bacillus subtilis, B. cereus, Clostridium perfringens, Listeria monocytogenes, L. monocytogenes, Pseudomonas fluorescens, P. aeruginosa, P. cepacia, Thermotoga lettingae, Burkholderia pseudomallei*, and *Legionella pneumophila* (Hu et al. 2013; Borrelli and Trono 2015).

Enzyme Production Level

PLA_1 has been produced from *A. oryzae* with a specific activity of 2000 U/mg which was the higher level reported from filamentous fungi (Shiba et al. 2001). PLA_2 gene from a hyperthermophilic archaeon *Aeropyrum pernix* K1, which comprised 474 bases was cloned and expressed in *E. coli* BL21 (DE3) which has a specific activity of 120 U/mg (Wang et al. 2004). Phospholipase B from *Thermotoga lettingae* TMO has been cloned, and functionally overexpressed in *E. coli* with a specific activity of 158 U/mg (Wei et al. 2015). A lysophospholipase/PLB gene from the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1 (LysoPL-tk) was cloned and expressed in *E. coli* which has a specific activity of 95.5 U/mg (Cui et al. 2012). Phospholipase D was produced from *Streptomyces* sp. CS684 with a specific activity of 37.5 U/mg (Simkhada et al. 2009) and from *Ochrobactrum* sp. ASAG-PL1 with 83.5 U/mg specific activity (Hu et al. 2013).

Applications

Phospholipases have various functions, ranging from the breakdown of nutrients to the formation of bioactive molecules (Borrelli and Trono 2015). Among the fungal phospholipases, PLA1s and PLA2s from *Fusarium oxysporum, T. lanuginosus, A. niger,* and *Trichoderma reesei* have been commercialized and are used for the degumming of vegetable oils, while PLA1s, PLA2s, and PLBs from *A. oryzae* and *A. niger* have been used primarily in food industry (Maria et al. 2007; Casado et al. 2012). Furthermore, PLA2 proteins are of great interest to the pharmaceutical industry. They are responsible for the release of arachidonic acid from membranes, and the successive conversion of fatty acids to leukotrienes and prostaglandins (Istivan and Coloe 2006). PLDs from *Actinomycetes* strains are also commercially available and are used in many industrial processes, owing to their high transphosphatidylation and hydrolytic activities (Casado et al. 2012; Borrelli and Trono 2015). Phospholipases play essential roles in a number of different physiological processes, including phospholipid metabolism, signal transduction, cell cycle progression, cytoskeletal organization, and inflammatory responses (Cockcroft 2001; Cherif et al. 2010; Wei et al. 2015).

CHITINASE

Chitinases (EC 3.2.1.14) are essential enzymes that hydrolyze the β (1, 4) linkages of chitin and convert the polysaccharide to its monomeric or oligomeric components (low-molecular-weight products). The breakdown of chitin happens in two steps. First chitinase cleaves the chitin polymer into chitin oligosaccharides and further release *N*-acetylglucosamine, and monosaccharides catalyzed by chitobiases (Suginta et al. 2000; Hamid et al. 2013). Chitinases have been found in a wide range of organisms, including bacteria, plants, viruses, fungi, animals, insects, and crustaceans (Dahiya et al. 2006). The chitin-binding domain of bacterial chitinases can either be located in the amino terminal or in the carboxyl terminal domains of the enzyme. Fungal chitinases play an important role in the nutrition, morphogenesis, and fungal development processes (Hamid et al. 2013).

Enzyme Sources

The thermophilic organisms such as *Bacillus licheniformis* X-7u (Takayanagi et al. 1991), *Bacillus* sp. BG-11 (Bharat and Hoondal 1998), and *Streptomyces thermoviolaceus* OPC-520 (Tsujibo et al. 1995) were

reported to be the chief sources of chitinases. Thermostable exochitinases were also isolated from *B. stearothermophilus* CH-4, isolated from a compost of organic solid wastes (Haki and Rakshit 2003). *Bacillus thuringiensis* subsp. kurstaki strain HBK-51 (Kuzu et al. 2012) and *B. cereus* (Liang et al. 2014) also produced thermostable and alkaline chitinase. *Brevibacillus laterosporus* also can produce chitinase which is stable at 70°C and 6–8 pH (Prasanna et al. 2013). Chitinase is also produced from the extreme thermophilic anaerobic arachaeon *Thermococcus chitinophagus* (Huber et al. 1995).

Enzyme Production Level

Chitinase has been reported from *B. cereus* with a specific activity of 16598 U/mg (Liang et al. 2014). *Bacillus* sp. Hu1 that was isolated from hot springs produced a chitinase with a specific activity of 11.1 U/mg (Dai et al. 2011). Another chitinase enzyme with a specific activity of 494.5 U/mg has been reported from *B. licheniformis* strain LHH100 and was cloned and expressed in *E. coli* (Laribi-Habchi et al. 2015). Chitinase gene from *Aeromonas veronii* was cloned into *P. pastoris* GS115 using pPIC9 vector with a specific activity of 553.8 U/mg (Y. Zhang et al. 2014). *Aeromonas hydrophila* SBK1 has been known to produce chitinase with 71.6 U/mg of specific activity (Halder et al. 2012). *Paenibacillus barengoltzii* has secreted chitinase (30.1 U/mg) after recombination into *E. coli* (Yang et al. 2016). Chitinase II was produced from *T. lanuginosus* with 150 ± 3.48 U/mg specific activity (Zhang et al. 2015).

Applications

Chitinolytic enzymes have a wide range of applications in diverse fields. This includes preparation of pharmaceutically important chitooligosaccharides with antimicrobial, anticholesterol, and antitumor activities. Chitinases are also used for the production of *N*-acetyl D-glucosamine, preparation of single-cell protein, isolation of protoplasts from fungi and yeast, treatment of chitin wastes, etc. (Haki and Rakshit 2003; Dahiya et al. 2006; Hamid et al. 2013). Chitinases have also been implicated in plant resistance against fungal pathogens and has shown considerable antifungal activities *in vitro* (Cho et al. 2011). Chitinases are also used as mosquitocides (Halder et al. 2012) and as biocontrol agents in agricultural applications against worms and insects that cause crop damage (Hamid et al. 2013; Liang et al. 2014).

LACCASES

Laccases (1.10.3.2) are copper-containing enzymes that belong to the group of blue oxidase which is produced by many bacteria, fungi, and yeasts (Lu et al. 2013). These enzymes are characterized by their unusual substrate specificity and a wide range of oxidizable substrates that depend on the source organism (Madhavi and Lele 2009). Laccases mostly have the structure as monomeric, dimeric, and tetrameric glycoprotein. The presence of glycosylation is important for copper retention, thermal stability, and susceptibility to proteolytic degradation. After purification, laccase enzymes show considerable heterogeneity. Variation in laccase glycosylation and composition of glycoprotein is a result of various growth medium composition (Shraddha et al. 2011). Substrates that are oxidized by laccases include mono-, di-, and polyphenols, methoxyphenols, aminophenols, aromatic amines, and ascorbate, with the associated four-electron reduction of oxygen to water (Madhavi and Lele 2009; Giardina et al. 2010).

Enzyme Sources

Majority of the laccases are reported from fungi, whereas few reports are from bacteria. Fungi belonging to the class *Ascomycetes, Basidiomycetes,* and *Deuteromycetes* have been implicated for the production of most laccases (Kiiskinen et al. 2004; Gochev and Krastanov 2007). Fugal species that are known to produce laccases are *Stereum ostrea, S. hirsutam, Fomitella fraxinea, Lentinus tigrinus, Trametes versicolor, T. hirsuta, T. ochracea, T. villosa, T. gallica, Ganoderma* sp. MK05, *Cerrena unicolor, C. byrsiana, C. maxima*, *H. cylindrosporum, Pycnoporus sanguineus, Trichoderma harzianum, Coriolopsis polyzona, Pleurotus eryngii,* and *P. ostreatus*. Bacterial species such as *B. subtilis, Azospirillum lipoferum, Streptomyces lavendulae, S. coelicolor,* and *Stenotrophomonas maltophilia* AAP56 are also reported for the production of laccases (Morozova et al. 2007; Madhavi and Lele 2009; Desai and Nityanand 2011).

Enzyme Production Level

A thermostable laccase from *Pleurotus* sp. MAK-II has been reported with a specific activity of 1613 U/mg (Manavalan et al. 2015). Laccase produced from *Neurospora crassa* has a specific activity of 333 U/mg (Grotewold et al. 1998). A laccase produced by the basidiomycete *Marasmius quercophilus* C30 has a specific activity of 934 U/mg (Klonowska et al. 2002). The white-rot fungus *Trametes pubescens* MB 89 has been reported to produce laccase with an activity of 743 U/mL (Galhaup et al. 2002). The

laccase gene *lac48424-1* from *Trametes* sp. 48424 was cloned and expressed in *P. pastoris* and the enzyme has a specific activity of 49.32 U/mg (Fan et al. 2011). Similarly a thermo-alkali-stable laccase gene from *Bacillus licheniformis* was cloned and expressed in *P. pastoris* with a maximum activity of 227.9 U/L (Lu et al. 2013). *Pleurotus ostreatus* strain 32 has been induced with ABTS and produced laccase with 410 U/mL activity (Hou et al. 2004). Phenol has been used as an inducer for laccase production by *T. versicolor* with an activity level of 2.575 U/mL (Pazarlioğlu et al. 2005).

Applications

Laccases are used for a variety of applications including textile, food, wood processing, chemical, and pharmaceutical industries (Kunamneni et al. 2007). Laccases are also used for coupling reactions during organic synthesis (Kudanga and Le Roes-Hill 2014). Laccases also catalyze some of the processes such as dye decolorization, degradation of xenobiotics, and effluent treatment. They also play a vital role in the oxidation of toxins and contaminants from industries. Laccases also have potential for biological delignification of pulp. In food industry they are used to eliminate the phenolics, haze formation, and turbidity development in fruit juice, wine, and beer. Laccases are also used in hair dyes, which are less irritant and are easier to handle (Kunamneni et al. 2007; Roriz et al. 2009; Desai and Nityanand 2011).

PROTEASE

Proteases are lytic enzymes that cleave other proteins by hydrolyzing the peptide linkages (Li et al. 2013a). Cleavage of peptide bonds result in the degradation of protein substrates into their principal amino acids, or it can be specific, leading to selective protein chopping for post-translational modification and processing. Proteases are classified as peptide hydrolases or peptidases (EC 3.4) and are commonly grouped into two categories (exopeptidases—that slice off amino acids from the ends of the protein chain and endopeptidases—which cleave peptide bonds within the protein) (de Souza et al. 2015). Proteases have wide applications in the food, pharmaceutical, leather, and textile industries (Fan et al. 2001; Mozersky et al. 2002).

Proteases play a critical role in many physiological and pathological functions such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in cell line development, tumor growth and metastasis, activation of zymogens, release of

hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes (Li et al. 2013a; de Souza et al. 2015). Extracellular proteases convert the proteins into smaller peptides and amino acids for subsequent absorption into cells, thereby playing a vital role in nitrogen metabolism (Sabotic and Kos 2012).

Enzyme Sources

The dominant producers of proteases are microorganisms of the genera *Pyrococcus, Thermococcus,* and *Staphylothermus.* Few examples are *Pyrococcus* sp. KODI, *S. marinus, T. aggreganes, T. celer, T. litoralis, Thermoacidophiles* (archeal and bacterial origin), and *Thermotoga maritima*. Few thermophilic protease-producing *Bacillus* spp. have been identified, such as *B. brevis, B. licheniformis*, *B. stearothermophilus, B. stearothermophilus* TP26, *Bacillus* sp. JB-99, and *B. thermoruber* (Haki and Rakshit 2003). A huge number of fungal strains have also been known to produce proteases such as *Aspergillus, Penicillium, Rhizopus, Mucor, Humicola, Thermoascus, Thermomyces* sp. (de Souza et al. 2015), *Trichoderma asperellum* (Yang et al. 2013), and *Aureobasidium pullulans* (Banani et al. 2014).

Enzyme Production Level

Bacillus megaterium has been reported to produce protease with 41.09 U/mg specific activity (Asker et al. 2013) and *Anoxybacillus* sp. KP1 with 16.39 U/mg (Bekler et al. 2015) activity. *Bacillus subtilis* has shown a good protease production level of 205.87 U/mg (Pant et al. 2015). Protease with 1052 U/mg of specific activity was produced by *B. pumilus* (Jayakumar et al. 2012). A nattokinase/subtilisin (serine proteases family) from *B. subtilis* VTCC-DVN-12-01 was expressed in *B. subtilis* WB800 with a specific activity of 12.7 U/mg (Nguyen et al. 2013). Haloalkaliphilic bacteria isolated from saline habitats have been reported to produce protease with 6765.76 U/mg specific activity (Purohit and Singh 2013). A protease gene APL5 of *Aureobasidium pullulans* strain PL5 was cloned and expressed in *E. coli* BL21 with a specific activity of 129 U/mg (Zhang et al. 2012).

Applications

A vast array of commercial proteases is available which has potential applications in various industrial processes such as textile, food, dairy, and pharmaceutical preparations (Nguyen et al. 2013; de Souza et al. 2015). Proteases such as alkaline protease, pancreatic protease, rennin,

and papain have major applications in detergent formulations (to enhance the ability to remove tough stains and making the detergent environmentally safe), baking (altering the viscoelastic properties of dough), meat tenderization, and leather industries (dehairing of animal hides and skin) (Zambare et al. 2011; Kumari et al. 2012; de Souza et al. 2015).

PEROXIDASES

Peroxidases (1.11.1.x) are pervasive enzymes that oxidize a wide range of reducing substrates with the help of H_2O_2 or other peroxides (Fodil et al. 2012). This extensively utilized group of heme-containing peroxidases is produced from fungal or plant source and has advantages due to its wide range of substrates (Loncar and Fraaije 2015). Catalase has been transformed into either peroxidase or oxidase and is resistant to inactivation by hydrogen peroxide, this being the drawback of many peroxidases. Bacterial enzymes are preferred for industrial application because of their thermostability as fungal/plant is normally quite labile at higher temperatures. Thermostable peroxidases may be used *in situ* for the treatment of process water enabling fast recycling and with low energy usage (Loncar and Fraaije 2015).

Enzyme Sources

A catalase which has peroxidase activity has been reported from *Thermobifida fusca* (Loncar and Fraaije 2015). An extracellular thermostable humic acid peroxidase (HaP3) was isolated from a *Streptomyces* sp. strain AH4 (Fodil et al. 2012). Dye-decolorizing peroxidases were isolated from *B. subtilis*, *P. putida* MET94 (Santos et al. 2014), and *Kocuria rosea* MTCC 1532 (Parshetti et al. 2012). Microorganisms belonging to the genera *Agaricales, Corticiales, Polyporales,* and *Hymenochaetales* are reported for manganese peroxidase production (Tello et al. 2000; Hilden et al. 2008; Morgenstern et al. 2010; Janusz et al. 2013). Fungal species such as *Phlebia* sp. MG60, *P. radiata* 79, *Dichomitus squalens , Lentinula edodes*, *Phanerochaete chrysosporium, P. sordida, Ganoderma lucidum, Ceriporiopsis subvermispora,* and *Trametes versicolor* 9522-1 have genes encoded for Mn peroxidase (Janusz et al. 2013). A novel peroxidase (SviDyP) was isolated, purified, and characterized from *Saccharomonospora viridis* DSM 43017, a pentachlorophenol-degrading thermophilic *actinomycete* (Webb et al. 2001).

Enzyme Production Level

Kocuria rosea MTCC 1532 produced lignin peroxidase which was having 168.33 U/mg specific activity (Parshetti et al. 2012). Humic acid

peroxidase (HaP3) was produced from a *Streptomyces* sp. strain AH4 which has a specific activity of 9.45 U/mg (Fodil et al. 2012). Peroxidase from *Saccharomonospora viridis* DSM 43017 was reported with 17.8 U/mg specific activity (Yu et al. 2014). *Thermobifida fusca* also produced peroxidase with a specific activity of 67 U/mg (Loncar and Fraaije 2015). Peroxidase genes from *B. subtilis* and *P. putida* MET94 were cloned into plasmid pET-21a(+) to yield plasmids pRC-1 and pRC-2, respectively. These were introduced into the host expression strains *E. coli* BL21 and *E. coli* BL21 star, respectively, in which the target genes were expressed under the control of the T7lac promoter with a production level of 40 U/mg and 15 U/mg, respectively (Santos et al. 2014).

Applications

Peroxidases have applications in the selective delignification of lignocellulosic materials for the production of cellulose or conversion into feed and biofuels. They also have applications in the treatment of toxic industrial effluents, such as those containing synthetic dyes, generated in numerous industrial practices as they have also potential to be used as biological decolorizing agents (Santos et al. 2014). Peroxidase has potential applications in biopulping, biobleaching (Yu et al. 2014), biodegradation, and bioremediation (Fujii et al. 2013; Janusz et al. 2013). Peroxidase is also used in analytical chemistry, immune chemistry, biosensor construction, food processing, and food storage (Mall et al. 2013).

LIGASES

Ligases are enzymes that join the DNA fragments together by catalyzing bond formation between neighboring nucleotides. DNA and RNA ligases are ubiquitous enzymes that catalyze the formation of phosphodiester bonds between opposing 5′-phosphate and 3′-hydroxyl termini in nucleic acids (Wang et al. 2013). They belong to the nucleotidyl transferase superfamily together with the RNA capping enzymes and tRNA ligases. All of the enzymes in this superfamily catalyze phosphodiester bond formation in a conserved, three-step mechanism that utilizes ATP, GTP, or NAD+ as a high-energy cofactor (Wang and Shuman 2005; Shuman 2009; Chambers and Patrick 2015). Ligases are the most important catalysts in the central biological processes, including DNA replication, recombination, and rearrangement of immunoglobulin genes. Their activities *in vitro* have also been exploited in numerous molecular biology protocols, making them crucial tools for modern biotechnology (Chambers and Patrick 2015). For

decades, DNA ligases have been used to create recombinant DNA molecules (i.e., cloning) and for genetic disease detection using the ligation chain reactions (Gibson et al. 2009).

In vivo, DNA ligases catalyze the formation of phosphodiester bonds at single-stranded nicks in double-stranded DNA. This activity is critical for maintaining genomic integrity during DNA replication, DNA recombination, and DNA excision repair (Wang et al. 2013). They are essential in all organisms and they are conventionally categorized into two families according to their cofactor specificity (Doherty and Suh 2000; Wang et al. 2013). ATP-dependent ligases (EC 6.5.1.1) are typically found in Eukarya, Archaea, and viruses (including bacteriophages), while the NAD+-dependent DNA ligases (EC 6.5.1.2) are typically found in bacteria and some eukaryotic viruses. Most notably, the archaeal species *Haloferax volcanii* holds two active DNA ligases: one ATP-dependent (LigA) and the other NAD+-dependent (LigN) (Zhao et al. 2006). However, there is some protein sequence uniformity between bacterial and eukaryotic DNA ligases. Further, bacterial DNA ligase requires NAD+ as a cofactor, while eukaryotic and most viral DNA ligases utilize ATP (Stokes et al. 2011).

Enzyme Sources

Several bacteria are capable of producing the ligase enzymes. This include *Thermus aquaticus*, *Aeropyrum pernix*, *Desulfurolobus ambivalens*, *Staphylothermus marinus*, *Sulfolobus acidocaldarius*, *S. shibatae*, *S. solfataricus*, *Sulfophobococcus zilligii*, *Archaeoglobus fulgidus*, *Methanothermobacter thermautotrophicus*, *Pyrococcus horikoshii*, *Thermococcus* sp. 1519, *T. fumicolans*, *T. kodakaraensis*, *T. onnurineus*, *T. sibiricus*, *Methanocaldococcus jannaschii*, *Thermotoga maritima*, and *Schizosaccharomyces pombe* (Lai et al. 2002; Lohman et al. 2011; Stewart et al. 2011; Le et al. 2013; Wang et al. 2013; Chambers and Patrick 2015).

Applications

DNA ligases are most importantly having a role in DNA repair and most archeal DNA ligases have the ability to seal single-stranded nicks in double-stranded DNA (Kotani et al. 2012; Le et al. 2013). Their ability to ligate the double-stranded, cohesive-, or blunt-end fragments made these enzymes receive great attention in biotechnological applications. Ligations of cohesive-ended fragments have been performed by the enzymes from *Aeropyrum pernix*, *Staphylothermus marinus*, *Thermococcus* sp. 1519, and *T. fumicolans*. In addition, the *S. marinus* (Seo et al. 2007) and *T. fumicolans*

DNA ligases could also ligate blunt-ended fragments (Rolland et al. 2004; Chambers and Patrick 2015). A number of next-generation sequencing methods also depend on DNA ligases (Quail et al. 2008; Lohman et al. 2011) either for adapter ligation during sample preparation (e.g., Illumina and 454 sequencing) or for the sequencing reaction itself (SOLiD sequencing) (Chambers and Patrick 2015).

DNA POLYMERASE

The polymerase chain reaction (PCR) process has directed a huge advancement in genetic engineering due to its capacity to amplify DNA. The three sequential steps in this process include denaturation or melting of the DNA strand (separation) carried out at 90–95°C, renaturation, or primer annealing at 55°C followed by synthesis or primer extension at around 75°C. Development in this process has been to a large extent facilitated by the availability of thermostable DNA polymerases, which catalyze the elongation of primer DNA strand (Haki and Rakshit 2003). Many microbial sources have been used for the production of DNA polymerases.

Enzyme Sources

DNA polymerase has been reported from *E. coli,* however, did not retain the activity at higher temperatures. *Taq* polymerase from the bacterium *Thermus aquaticus* was the first thermostable DNA polymerase identified and characterized biochemically (Chien et al. 1976; Kaledin et al. 1980). Some commercially used DNA polymerases are produced from bacteria which includes *Thermus caldophilus, T. filiformis, T. flavis, T. thermophiles, Pyrococcus species* GB-D, *P. abyssi, P. furiosus, Thermococcus kodakaraensis, T. brokianus, T. fumiculans, T. gorgonarius, T. litoralis, T. peptonophilus, T. zilligii, Thermotoga maritima,* and *T. neopolitana* (Terpe 2013). The DNA polymerase I (Taq Pol I) gene from *T. aquaticus* was cloned into a plasmid expression vector that utilizes the strong bacteriophage PL promoter. It was transferred to *E. coli* for expression with a specific activity of 292,000 U/mg (Lawyer et al. 1993). *Escherichia coli* BL21 was transformed with p*Taq* gene and expressed the DNA polymerase which has a specific activity of 5263.16 U/mg (Engelke et al. 1990).

Applications

DNA polymerases have applications in forensic science, nucleic acid sequencing industries, molecular characterization of plants, animals, and

microorganisms. The enzyme is involved in molecular processes such as the construction of gene cloning (Ikehara et al. 2004; Herrin et al. 2005), genomic DNA cloning (Nisole et al. 2004), synthesis of second-strand cDNA (Sasaki et al. 2004), knockout targeting vector (Kim et al. 2005), and synthetic gene manufacture (Wu et al. 2006).

REVERSE TRANSCRIPTASE

Reverse transcriptase (RTase) is the enzyme that catalyzes DNA polymerization using RNA as a template (RNA-dependent DNA polymerase) (Baranauskas et al. 2012). The enzyme is used in various genetic experiments, such as in microarray analysis by synthesizing cDNA or the start-site mapping of transcript mRNA. RTases play central roles in these genetic experiments, and the enzymes used are derived from retroviruses, such as the Moloney murine leukemia virus (MMLV) or the avian myeloblastosis virus which are not thermostable (AMV) (Arezi and Hogrefe 2007; Sano et al. 2012). To develop thermostable RTase, several strategies have been attempted (Yasukawa et al. 2008; Arezi and Hogrefe 2009; Kranaster et al. 2010; Mizuno et al. 2010; Jozwiakowski and Connolly 2011), and some genetically engineered enzymes are commercially available. RTase possesses three enzymatic activities: the RNA-dependent DNA polymerase, the DNA-dependent DNA polymerase, and RNase H, which degrade RNA strand in the RNA–DNA hybrid (Sambrook and Russell 2001). The synthesis of cDNA is probably the second most important technique in present molecular biology after the PCR and its modifications (Baranauskas et al. 2012).

Enzyme Sources

Thermus thermophiles has been reported for RTase activity in the presence of Mn^{2+} (Mohr et al. 2013). A DNA polymerase from *T. aquaticus* has been mutated to have RTase activity (Kranaster et al. 2010). RTase can also be produced from Moloney murine leukemia virus RTase (M-MuLV RTase) variants (Baranauskas et al. 2012). A mutant RTase has been produced from *Thermotoga petrophila* K4 (Sano et al. 2012). Some other organisms that produce RTase are avian myeloblastosis virus (Arezi and Hogrefe 2009), human immunodeficiency virus type 1 (HIV-1) (Sarafianos et al. 2009), and *Carboxydothermus hydrogenoformans* (Vieille and Zeikus 2001). Thermostable DNA-dependent DNA polymerase of *Bacillus stearothermophilus* has been reported which have RTase activity (Jestin et al. 2015).

Applications

RTases are extensively used to generate cDNA libraries for cloning, end-point and quantitative RT-polymerase chain reaction (RT-PCR), RACE technique, microarray analysis, RNA amplification (Sambrook and Russell 2001; Baranauskas et al. 2012), transcriptome and miRNA profiling, next-generation RNA sequencing (RNA-seq), RNA structure mapping, and the analysis of protein- or ribosome-bound RNA fragments (Wang et al. 2009; Mayer et al. 2011; Ozsolak and Milos 2011).

CONCLUSIONS

The application of thermostable enzymes as effective catalysts in industry would result in substantial savings of resources, mainly energy and water. With the fast increasing global population and shrinking natural resources, enzyme technology offers a great perspective for many industries to help meet the future challenges. Furthermore, with a paradigm shift in industry moving from natural to renewable resource utilization, the need for thermostable microbial catalysts is predicted to increase in the future. This warrants for further research in identifying novel thermostable enzymes with superior properties that address specific industrial needs.

REFERENCES

Abdel-fattah, Y.R. et al., 2013. Production, purification, and characterization of thermostable-amylase produced by *Bacillus licheniformis* isolate AI20. *Journal of Chemistry*, 2013, pp.1–11. http://dx.doi.org/10.1155/2013/673173.

Arezi, B. and Hogrefe, H., 2009. Novel mutations in Moloney Murine Leukemia Virus reverse transcriptase increase thermostability through tighter binding to template-primer. *Nucleic Acids Research*, 37, pp. 473–481.

Arezi, B. and Hogrefe, H.H., 2007. *Escherichia coli* DNA polymerase III epsilon subunit increases Moloney murine leukemia virus reverse transcriptase fidelity and accuracy of RT-PCR procedures. *Analytical Biochemistry*, 360, pp. 84–91.

Asha, R., Niyonzima, F.N., and Sunil, S.M., 2013. Purification and properties of pullulanase from *Bacillus halodurans*. *International Research Journal of Biological Sciences*, 2, pp. 35–43.

Asker, M.M.S. et al., 2013. Purification and characterization of two thermostable protease fractions from *Bacillus megaterium*. *Journal of Genetic Engineering and Biotechnology*, 11, pp. 103–109. Available at: http://dx.doi.org/10.1016/j.jgeb.2013.08.001.

Atanassov, I. et al., 2013. Seamless GFP and GFP-Amylase cloning in gateway shuttle vector, expression of the recombinant proteins in *E. coli* and *Bacillus megaterium* and assessment of the GFP-amylase thermostability. *Biotechnology and Biotechnological Equipment*, 27, pp. 4172–4180. Available at: http://www.tandfonline.com/doi/abs/10.5504/BBEQ.2013.0079.

Ávila-Cisneros, N. et al., 2014. Production of thermostable lipase by Thermomyces lanuginosus on solid-state fermentation: Selective hydrolysis of sardine oil. *Applied Biochemistry and Biotechnology*, 174, pp. 1859–1872. Available at: http://dx.doi.org/10.1007/s12010-014-1159-9.

Banani, H. et al., 2014. Biocontrol activity of an alkaline serine protease from *Aureobasidium pullulans* expressed in Pichia pastoris against four postharvest pathogens on apple. *International Journal of Food Microbiology*, 182–183, pp. 1–8. Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2014.05.001.

Banka, A.L., Albayrak Guralp, S., and Gulari, E., 2014. Secretory expression and characterization of two hemicellulases, xylanase, and β-xylosidase, isolated from Bacillus subtilis M015. *Applied Biochemistry and Biotechnology*, 174, pp. 2702–2710. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4237932&tool=pmcentrez&rendertype=abstract.

Baranauskas, A. et al., 2012. Generation and characterization of new highly thermostable and processive M-MuLV reverse transcriptase variants. *Protein Engineering Design and Selection*, 25, pp. 657–668. Available at: http://peds.oxfordjournals.org/cgi/doi/10.1093/protein/gzs034.

Basaran, P. and Ozcan, M., 2008. Characterization of b-xylosidase enzyme from a *Pichia stipitis* mutant. *Bioresource Technology*, 99, pp. 38–43.

Beg, Q.K. et al., 2001. Microbial xylanases and their industrial applications: A review. *Applied Microbiology and Biotechnology*, 56(3–4), pp. 326–338.

Bekler, F.M., Acer, O., and Guven, K., 2015. Production and purification of novel thermostable alkaline protease from *Anoxybacillus* sp. KP1. *Cellular and Molecular Biology*, 61, pp. 113–120.

Bertoldo, C. and Antranikian, G., 2002. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. *Current Opinion in Chemical Biology*, 6, pp. 151–160.

Bhalla, A. et al., 2013. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, 128, pp. 751–759. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23246299 (Accessed September 29, 2014).

Bhalla, A. et al., 2014. Novel thermostable endo-xylanase cloned and expressed from bacterium *Geobacillus* sp. WSUCF1. *Bioresource Technology*, 165, pp. 314–318. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0960852414004179.

Bhalla, A. et al., 2015. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, 128, pp. 751–759.

Bharat, B. and Hoondal, G., 1998. Isolation, purification and properties of thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. *Biotechnology Letters*, 20, pp. 157–159.

Biely, P., 1985. Microbial xylanolytic systems. *Trends in Biotechnology*, 3, pp. 286–290.

Biely, P., 2003. Xylanolytic enzymes. In J.R. Whitaker, A.G.J. Voragen, and D.W.S. Wong, eds. *Handbook of Food Enzymology*. Marcel Dekker, Inc., New York, NY, pp. 879–915.

Borrelli, G. and Trono, D., 2015. Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *International Journal of Molecular Sciences*, 16, pp. 20774–20840. Available at: http://www.mdpi.com/1422-0067/16/9/20774/.

Bouacem, K. et al., 2014. Partial characterization of xylanase produced by *Caldicoprobacter algeriensis*, a new thermophilic anaerobic bacterium isolated from an Algerian hot spring. *Applied Biochemistry and Biotechnology*, 174, pp. 1969–1981. doi:10.1007/s12010-014-1153-2.

Butt, M.S. et al., 2008. Xylanases and their application in baking industry. *Food Technology and Biotechnology*, 46, pp. 22–31.

Cammarota, M.C. and Freire, D.M.G., 2006. A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. *Bioresource Technology*, 97, pp. 2195–2210.

Cannio, R., Di Prizito, N., and Rossi Alessandra Morana, M., 2004. A xylan-degrading strain of *Sulfolobus solfataricus*: Isolation and characterization of the xylanase activity. *Extremophiles*, 8, pp. 117–124.

Casado, V. et al., 2012. Phospholipases in food industry: A review. In G. Sandoval, ed. *Lipases and Phospholipases: Methods and Protocols*. Springer, New York, NY, pp. 495–523.

Chambers, C.R. and Patrick, W.M., 2015. *Archaeal* nucleic acid ligases and their potential in biotechnology. Archaea, 2015, pp.1–10. http://dx.doi.org/10.1155/2015/170571.

Chapla, D. et al., 2010. Utilization of agro-industrial waste for xylanase production by *Aspergillus foetidus* MTCC 4898 under solid state fermentation and its application in saccharification. *Biochemical Engineering Journal*, 49, pp. 361–369.

Chavez, R., Bull, P., and Eyzaguirre, J., 2006. The xylanolytic enzyme system from the genus Penicillium. *Journal of Biotechnology*, 123, pp. 413–433.

Chen, Z. et al., 2012. Secretory expression of a β-xylosidase gene from *Thermomyces lanuginosus* in *Escherichia coli* and characterization of its recombinant enzyme. *Letters in Applied Microbiology*, 55, pp. 330–337.

Cheng, Y.-S. et al., 2014. Structural analysis of a glycoside hydrolase family 11 xylanase from neocallimastix patriciarum: Insight into the molecular basis of a thermophilic enzyme. *Journal of Biological Chemistry*, 289, pp. 11020–11028. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.M114.550905.

Cherif, S. et al., 2010. Crab digestive phospholipase: A new invertebrate member. *Bioresource Technology*, 101, pp. 366–371.

Chiang, C.M. et al., 2005. Expression of a bi-functional and thermostable amylopullulanase in transgenic rice seeds leads to autohydrolysis and altered composition of starch. *Molecular Breeding*, 15, pp. 125–143.

Chien, A., Edgar, D.B., and Trela, J.M., 1976. Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *Journal of Bacteriology*, 127, pp. 1550–1557.

Chinnathambi, V. et al., 2015. Molecular cloning and expression of a family 6 Cellobiohydrolase gene cbhII from *Penicillium funiculosum* NCL1. *Advances in Bioscience and Biotechnology*, 6, pp. 213–222.

Cho, E.K., Choi, I.S., and Choi, Y.J., 2011. Overexpression and characterization of thermostable chitinase from *Bacillus atrophaeus* SC081 in *Escherichia coli*. *BMB Reports*, 44(3), pp. 193–198.

Christakopoulos, P. et al., 2003. Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. *International Journal of Biological Macromolecules*, 31, pp. 171–175.

Cockcroft, S., 2001. Signalling roles of mammalian phospholipase D1 and D2. *Cellular and Molecular Life Science*, 58, pp. 1674–1687.

Comlekcioglu, U. et al., 2010. Cloning and characterization of cellulase and xylanase coding genes from anaerobic fungus *Neocallimastix* sp. GMLF1. *International Journal of Agriculture and Biology*, 12(5), pp. 691–696.

Comlekcioglu, U. et al., 2011. Effects of various agro-wastes on xylanase and b-xylosidase production of anaerobic ruminal fungi. *Journal of Scientific and Industrial Research*, 70(4), pp. 293–299.

Cui, Z. et al., 2012. High level expression and characterization of a thermostable lysophospholipase from *Thermococcus kodakarensis* KOD1. *Extremophiles*, 16, pp. 619–625.

Cuong, N.P. et al., 2015. Continuous production of pure maltodextrin from cyclodextrin using immobilized Pyrococcus furiosus thermostable amylase. *Process Biochemistry*. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1359511315301318.

Dahiya, N. et al., 2006. Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. *World Journal of Microbiology and Biotechnology*, 21, pp. 1611–1616.

Dai, D. et al., 2011. Purification and characterization of a novel extracellular chitinase from thermophilic *Bacillus* sp. Hu1. *African Journal of Biotechnology*, 10, pp. 2476–2484.

Deive, F. et al., 2012. A process for extracellular thermostable lipase production by a novel Bacillus thermoamylovorans strain. *Bioprocess and Biosystems Engineering*, 35, pp. 931–941. Available at: http://dx.doi.org/10.1007/s00449-011-0678-9.

Desai, S.S. and Nityanand, C., 2011. Microbial laccases and their applications: A review. *Asian Journal of Biotechnology*, 3, pp. 98–124.

de Souza, P.M. et al., 2015. A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology*, 46, pp. 337–346. Available at: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822015000200337&lng=en&nrm=iso&tlng=en.

Dobozi, M.S., Szakács, G., and Bruschi, C.V., 1992. Xylanase activity of *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*, 58, pp. 3466–3471.

Doherty, A.J. and Suh, S.W., 2000. Structural and mechanistic conservation in DNA ligases. *Nucleic Acids Research*, 28, pp. 4051–4058.

Dornez, E. et al., 2007. Impact of wheat flour-associated endoxylanases on arabinoxylan in dough after mixing and resting. *Journal of Agricultural and Food Chemistry*, 55, pp. 7149–7155.

Dror, A. et al., 2014. Protein engineering by random mutagenesis and structure-guided consensus of *Geobacillus stearothermophilus* Lipase T6 for enhanced stability in methanol. *Applied and Environmental Microbiology*, 80, pp. 1515–1527. Available at: http://aem.asm.org/cgi/doi/10.1128/AEM.03371-13.

Duan, X. and Wu, J., 2015. Enhancing the secretion efficiency and thermostability of a *Bacillus deramificans* pullulanase mutant (D437H/D503Y) by N-terminal domain truncation. *Applied and Environmental Microbiology*, 81, pp. 1926–1931. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25556190.

Dutta, T. et al., 2008. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: Production and characterization. *Journal of Industrial Microbiology and Biotechnology*, 35, pp. 275–282.

Ebrahimpour, A. et al., 2011. High level expression and characterization of a novel thermostable, organic solvent tolerant, 1,3-regioselective lipase from *Geobacillus* sp. strain ARM. *Bioresource Technology*, 102, pp. 6972–6981. Available at: http://www.sciencedirect.com/science/article/pii/S0960852411004408 (Accessed October 29, 2015).

Elegir, G., Szakács, G., and Jeffries, T.W., 1994. Purification, characterization, and substrate specificities of multiple xylanases from *Streptomyces sp.* Strain B-12-2. *Applied and Environmental Microbiology*, 60, pp. 2609–2615. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=201691&tool=pmcentrez&rendertype=abstract.

Elleuche, S. et al., 2015. Exploration of extremophiles for high temperature biotechnological processes. *Current Opinion in Microbiology*, 25, pp. 113–119. Available at: http://www.sciencedirect.com/science/article/pii/S1369527415000624.

Ellis, J.T. and Magnuson, T.S., 2012. Thermostable and alkalistable xylanases produced by the thermophilic bacterium *Anoxybacillus flavithermus* TWXYL3. ISRN Microbiol, 2012, pp.1–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23762752.

Engelke, D.R. et al., 1990. Purification of *Thermus aquaticus* DNA polymerase expressed in *Escherichia coli*. *Analytical Biochemistry*, 191, pp. 396–400. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2085185\n, http://www.sciencedirect.com/science/article/pii/0003269790902385.

Espinosa-Luna, G. et al., 2015. Gene cloning and characterization of the *Geobacillus thermoleovorans* CCR11 Carboxylesterase CaesCCR11, a New Member of Family XV. *Molecular Biotechnology*. Available at: http://link.springer.com/10.1007/s12033-015-9901-2.

Fan, F. et al., 2011. Cloning and functional analysis of a new laccase gene from Trametes sp. 48424 which had the high yield of laccase and strong ability for decolorizing different dyes. *Bioresource Technology*, 102, pp. 3126–3137. Available at: http://dx.doi.org/10.1016/j.biortech.2010.10.079.

Fan, Z., Zhu, Q., and Dai, J., 2001. Enzymatic treatment of wool. *Journal of Dong Hua University*, 18 (2), pp. 112–115. https://www.scopus.com/record/display.uri?eid=2-s2.0-0035382286&origin=inward&txGid=0

Ferreira, N.L. et al., 2014. Use of cellulases from *Trichoderma reesei* in the twenty-first century—Part I: Current industrial uses and future applications in the production of second ethanol generation. In: Gupta, V. K. et al. (eds.), *Biotechnology and Biology of Trichoderma*. The Netherlands: Elsevier Ltd, pp. 245–261. http://dx.doi.org/10.1016/B978-0-444-59576-8.00017-5.

Fodil, D. et al., 2012. A thermostable humic acid peroxidase from *Streptomyces* sp. strain AH4: Purification and biochemical characterization. *Bioresource Technology*, 111, pp. 383–390. Available at: http://dx.doi.org/10.1016/j.biortech.2012.01.153.

Fujii, K. et al., 2013. Environmental control of lignin peroxidase, manganese peroxidase, and laccase activities in forest floor layers in humid Asia. *Soil Biology and Biochemistry*, 57, pp. 109–115. Available at: http://dx.doi.org/10.1016/j.soilbio.2012.07.007.

Galhaup, C. et al., 2002. Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme and Microbial Technology*, 30, pp. 529–536.

Giardina, P. et al., 2010. Laccases: A never-ending story. *Cellular and Molecular Life Sciences*, 67, pp. 369–385.

Gibson, D.G. et al., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6, pp. 343–345.

Gochev, V.K. and Krastanov, A.I., 2007. Isolation of laccases producing *Trichoderma* pp. *Bulgarina*. *Journal of Agricultural Science*, 13, pp. 171–176.

Gonçalves, G.A.L. et al., 2015. Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. *Enzyme and Microbial Technology*, 72, pp. 16–24.

Gramany, V. et al., 2015. Cloning, expression, and molecular dynamics simulations of a xylosidase obtained from *Thermomyces lanuginosus*. *Journal of Biomolecular Structure and Dynamics*, 1102(11), pp. 1–12. Available at: http://www.tandfonline.com/doi/full/10.1080/07391102.2015.1089186.

Grotewold, E. et al., 1998. Purification of an extracellular fungal laccase. *Mircen Journal of Applied Microbiology and Biotechnology*, 4, pp. 357–363.

Gunny, A.A.S. et al., 2014. Potential halophilic cellulases for *in situ* enzymatic saccharification of ionic liquids pretreated lignocelluloses. *Bioresource Technology*, 155, pp. 177–181.

Gutarra, M.L.E. et al., 2009. Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. *Bioresource Technology*, 100, pp. 5249–5254. Available at: http://dx.doi.org/10.1016/j.biortech.2008.08.050.

Haki, G.D. and Rakshit, S.K., 2003. Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, 89, pp. 17–34.

Halder, S.K. et al., 2012. Chitinolytic enzymes from the newly isolated *Aeromonas hydrophila* SBK1: Study of the mosquitocidal activity. *BioControl*, 57, pp. 441–449.

Hamid, R. et al., 2013. Chitinases: An update. *Journal of Pharmacy and Bioallied Sciences*, 5, pp. 21–29. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3612335/.

Han, T. et al., 2013. Biochemical characterization of a recombinant pullulanase from *Thermococcus kodakarensis* KOD1. *Letters in Applied Microbiology*, 57, pp. 336–343.

Haq, I. ul et al., 2015. CenC, a multidomain thermostable GH9 processive endoglucanase from *Clostridium thermocellum*: Cloning, characterization and saccharification studies. *World Journal of Microbiology and Biotechnology*, 31, pp. 1699–1710. Available at: http://link.springer.com/10.1007/s11274-015-1920-4.

Harris, P. V. et al., 2014. New enzyme insights drive advances in commercial ethanol production. *Current Opinion in Chemical Biology*, 19, pp. 162–170. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1367593114000271.

He, Z. et al., 2014. Cloning of a novel thermostable glucoamylase from thermophilic fungus *Rhizomucor pusillus* and high-level co-expression with α-amylase in *Pichia pastoris*. *BMC Biotechnology*, 14, pp. 1–10. Available at: http://www.biomedcentral.com/1472-6750/14/114.

Hebraud, M. and Fevre, M., 1990. Purification and characterization of an extracellular beta-xylosidase from the rumen anaerobic fungus *Neocallimastix frontalis*. *FEMS Microbiology Letters*, 60(1–2), pp. 11–16. Available at: http://search.ebscohost.com/login.aspx?direct=true&db=mnh&AN=2126511&site=ehost-live.

Heinzelman, P. et al., 2009. A family of thermostable fungal cellulases created by structure-guided recombination. *Proceedings of the National Academy of Sciences of the United States of America*, 106, pp. 5610–5615. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667002/.

Herrin, B.R., Groeger, A.L., and Justement, L.B., 2005. The adaptor proteinHSH2 attenuates apoptosis in response to ligation of the B cell antigen receptor complex on the B lymphoma cell line, WEHI-231. *Journal of Biological Chemistry*, 280, pp. 3507–3515.

Herrmann, M.C. et al., 1997. The b-D-xylosidase of *Trichoderma ressei* is a multifunctional b-D-xylan xylohydrolase. *Biochemistry Journal*, 321, pp. 375–381.

Hii, S.L. et al., 2012. Pullulanase: Role in starch hydrolysis and potential industrial applications. *Enzyme Research*, 2012, pp. 1–14. Available at: http://www.hindawi.com/journals/er/2012/921362/.

Hilden, K.S. et al., 2008. Molecular characterization of the basidiomycete isolate *Nematoloma frowardii* b19 and its manganese peroxidase places the fungus in the corticioid genus Phlebia. *Microbiology*, 154, pp. 2371–9.

Hou, H. et al., 2004. Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochemistry*, 39, pp. 1415–1419.

Hu, F. et al., 2013. A novel phospholipase D constitutively secreted *by Ochrobactrum* sp. ASAG-PL1 capable of enzymatic synthesis of phosphatidylserine. *Biotechnology Letters*, 35, pp. 1317–1321. Available at: http://dx.doi.org/10.1007/s10529-013-1207-5.

Huber, R. et al., 1995. *Thermococcus chitonophagus* sp. Nov., a novel chitin degrading, hyperthermophilic archeum from the deep sea hydrothermal vent environment. *Archives Microbiology*, 164, pp. 255–264.

Ibrahim, D. et al., 2013. *Bacillius licheniformis* BT5.9 isolated from Changar hop spring, Malang, Indonesia as a potential producer of Thermostable a-amylase. *Tropical Life Sciences*, 24, pp. 72–84.

Ikehara, Y., Ikehara, S.K., and Paulson, J.C., 2004. Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *Journal of Biological Chemistry*, 279, pp. 43117–43125.

Imanaka, T. and Sakurai, S., 1992. Method of washing super precision devices,semiconductors, with enzymes. https://www.google.com/patents/US5078802.

Istivan, T.S. and Coloe, P.J., 2006. Phospholipase A in Gram-negative bacteria and its role in pathogenesis. *Microbiology*, 152, pp. 1263–1274.

Jabbour, D., Klippel, B., and Antranikian, G., 2012. A novel thermostable and glucose-tolerant β-glucosidase from *Fervidobacterium islandicum*. *Applied Microbiology and Biotechnology*, 93, pp. 1947–1956. Available at: http://link.springer.com/10.1007/s00253-011-3406-0.

Janeček, Š., Svensson, B., and MacGregor, E.A., 2014. α-Amylase: An enzyme specificity found in various families of glycoside hydrolases. *Cellular and Molecular Life Sciences*, 71, pp. 1149–1170.

Janusz, G. et al., 2013. Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation. *Enzyme and Microbial Technology*, 52, pp. 1–12. Available at: http://dx.doi.org/10.1016/j.enzmictec.2012.10.003.

Jasilionis, A., Petkauskaite, R., and Kuisiene, N., 2014. A novel type I thermostable pullulanase isolated from a thermophilic starch enrichment culture. *Microbiology*, 83, pp. 227–234. Available at: http://link.springer.com/10.1134/S0026261714030084.

Javier, P.I. et al., 2007. Xylanases: Molecular properties and applications. In J. Polaina and A. MacCabe, eds. *Industrial Enzymes*. Springer, Dordrecht, the Netherlands, pp. 65–82.

Jayakumar, R. et al., 2012. Characterization of thermostable serine alkaline protease from an alkaliphilic strain *Bacillus pumilus* MCAS8 and its applications. *Applied Biochemistry and Biotechnology*, 168, pp. 1849–1866.

Jestin, J.-L., Vichier-Guerre, S., and Ferries, S., 2015. Methods for obtaining thermostable enzymes, DNA polymerase I variants from Thermus aquaticus having new catalytic activities, methods for obtaining the same, and applications to the same. https://www.google.com/patents/US8927699

Jordan, D.B. and Wagschal, K., 2010. Properties and applications of microbial β-D-xylosidases featuring the catalytically efficient enzyme from *Selenomonas ruminantium*. *Applied Microbiology and Biotechnology*, 86, pp. 1647–1658.

Joshi, B.H., 2011. A novel thermostable alkaline α-amylase from Bacillus circulans PN5: Biochemical characterization and production. *Asian Journal of Biotechnology*, 3, pp. 58–67.

Jozwiakowski, S.K. and Connolly, B.A., 2011. A modified family-B archaeal DNA polymerase with reverse transcriptase activity. *Chem Bio Chem*, 12, pp. 35–37.

Juturu, V. and Wu, J.C., 2014. Microbial cellulases: Engineering, production and applications. *Renewable and Sustainable Energy Reviews*, 33, pp. 188–203.

Jyoti, J. et al., 2011. Partial purification and characterization of an acidophilic extracellular A—Amylase from *Bacillus Licheniformis* Jar-26 abstract: *International Journal of Advanced Biotechnology and Research*, 2, pp. 315–320.

Kaledin, A.S., Sliusarenko, A.G., and Gorodetskii, S.I., 1980. Isolation and properties of DNA polymerase from extremely thermophilic bacterium *Thermus aquaticus* YT1. *Biokhimiia*, 45, pp. 644–651.

Kamal Kumar, B., Balakrishnan, H., and Rele, M.V., 2004. Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87-6-10) with commercial detergents and proteases. *Journal of Industrial Microbiology and Biotechnology*, 31, pp. 83–87.

Kambourova, M. et al., 2003. Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7. *Journal of Molecular Catalysis B: Enzymatic*, 22(5–6), pp. 307–313.

Kang, J. et al., 2011. Molecular cloning and biochemical characterization of a heat-stable type I pullulanase from *Thermotoga neapolitana*. *Enzyme and Microbial Technology*, 48, pp. 260–266.

Kang, S., Vieille, C., and Zeikus, J.G., 2004. Identification of *Pyrococcus furiosus* amylopullulanase catalytic residues. *Applied Microbiology and Biotechnology*, 66, pp. 408–413.

Kar, S., Ray, R.C., and Mohapatra, U.B., 2012. Purification, characterization and application of thermostable amylopullulanase from *Streptomyces erumpens* MTCC 7317 under submerged fermentation. *Annals of Microbiology*, 62, pp. 931–937. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84871613270&partnerID=40&md5=d95672b70ab4fa6dbcd309cea022589e.

Katapodis, P. et al., 2003. Enzymatic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan. *European Journal of Nutrition*, 42, pp. 55–60.

Kazemi, A. et al., 2014. Isolation, identification and media optimization of high level cellulase production by Bacillus sp. BCCS A3, in a fermentation system using response surface methodology. *Preparative Biochemistry and Biotechnology*, 44, pp. 107–118.

Khelila, O. and Cheba, B., 2014. Thermophilic cellulolytic microorganisms from western Algerian sources: Promising isolates for cellulosic biomass recycling. *Procedia Technology*, 12, pp. 519–528.

Kiiskinen, L.L. et al., 2004. Expression of *Melanospora albamyces* laccase in Trichoderma reesei and characterization of the purified enzyme. *Microbiology*, 150, pp. 3065–3074.

Kim, T.S. et al., 2005. Delayed dark adaptation in 11-cis-retinol dehydrogenase-deficient mice: A role of RDH11 in visual processes *in vivo*. *Journal of Biological Chemistry*, 280, pp. 8694–8704.

Kirikyali, N., Wood, J., and Connerton, I.F., 2014. Characterisation of a recombinant β-xylosidase (xylA) from *Aspergillus oryzae* expressed in *Pichia pastoris*. *AMB Express*, 4, p.68. Available at: http://www.amb-express.com/content/4/1/68.

Klonowska, A. et al., 2002. Characterization of a low redox potential laccase from the basidiomycete c30. *European Journal of Biochemistry*, 269, pp. 6119–6125.

Knob, A. and Carmona, E.C., 2010. Purification and characterization of two extracellular xylanases from *Penicillium sclerotiorum*: A novel acidophilic xylanase. *Applied Biochemistry and Biotechnology*, 162, pp. 429–443. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19680819.

Knob, A. et al., 2014. Agro-residues as alternative for xylanase production by filamentous fungi. *BioResources*, 9, pp. 5738–5773.

Kocabaş, D.S., Güder, S., and Özben, N., 2015. Purification strategies and properties of a low-molecular weight xylanase and its application in agricultural waste biomass hydrolysis. *Journal of Molecular Catalysis B: Enzymatic*, 115, pp. 66–75. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1381117715000296.

Kotani, A. et al., 2012. EndoV/DNA ligase mutation scanning assay using microchip capillary electrophoresis and dual-color laser-induced fluorescence detection. *Analytical Methods*, 4, p.58.

Kranaster, R. et al., 2010. One-step RNA pathogen detection with reverse transcriptase activity of a mutated thermostable *Thermus aquaticus* DNA polymerase. *Biotechnology Journal*, 5, pp. 224–231.

Kudanga, T. and Le Roes-Hill, M., 2014. Laccase applications in biofuels production: Current status and future prospects. *Applied Microbiology and Biotechnology*, 98, pp. 6525–6542.

Kuhad, R.C., Gupta, R., and Singh, A., 2011. Microbial cellulases and their industrial applications. *Enzyme Research*, 2011, pp. 1–10. Available at: http://www.hindawi.com/journals/er/2011/280696/.

Kuhad, R.C. et al., 2016. Revisiting cellulase production and redefining current strategies based on major challenges. *Renewable and Sustainable Energy Reviews*, 55, pp. 249–272. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1364032115012113.

Kulkarni, N., Shendye, A., and Rao, M., 1999. Molecular and biotechnological aspects of xylanases. *FEMS Microbiology Reviews*, 23, pp. 411–456.

Kumar, K.S. et al., 2009. Production of beta-xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. *Journal of Bioscience and Bioengineering*, 107, pp. 494–498. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19393546 (Accessed November 14, 2014).

Kumar, L., Awasthi, G., and Singh, B., 2011. Extremophiles: A novel source of industrially important enzymes. *Biotechnology*, 10, pp. 121–135.

Kumar, S. et al., 2005. Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expression and Purification*, 41, pp. 38–44.

Kumari, M., Sharma, A., and Jagannadham, M.V., 2012. Religiosin B, a milk-clotting serine protease from *Ficus religiosa*. *Food Chem*, 131, pp. 1295–1303.

Kunamneni, A. et al., 2007. Fungal laccase—a versatile enzyme for biotechnological applications. In A. Mendez-Vias, ed. *Communication Current Research and Educational Topics and Trends in Applied Microbiology*. Formex, Badajoz, pp. 233–245.

Kundu, A. and Ray, R.R., 2013. Production of intracellular b-xylosidase from the submerged fermentation of citrus wastes by *Penicillium janthinellum* MTCC. *3 Biotech*, 3, pp. 241–246.

Kuzu, S.B., Güvenmez, H.K., and Denizci, A.A., 2012. Production of a thermostable and alkaline chitinase by *Bacillus thuringiensis* subsp. kurstaki Strain HBK-51. *Biotechnology Research International*, 2012, p. 135498. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3532916&tool=pmcentrez&rendertype=abstract.

Lai, X. et al., 2002. Biochemical characterization of an ATP-dependent DNA ligase from the hyperthermophilic crenarchaeon *Sulfolobus shibatae*. *Extremophiles*, 6, pp. 469–477.

Lama, L. et al., 2004. Purification and characterization of thermostable xylanase and beta-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus*. *Research in Microbiology*, 155, pp. 283–289. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15142626.

Laribi-Habchi, H. et al., 2015. Purification, characterization, and molecular cloning of an extracellular chitinase from *Bacillus licheniformis* stain LHH100 isolated from wastewater samples in Algeria. *International Journal of Biological Macromolecules*, 72, pp. 1117–1128. Available at: http://dx.doi.org/10.1016/j.ijbiomac.2014.10.035.

Lawyer, F.C. et al., 1993. High-level expression, purification, and enzymatic characterization of full-length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5′ to 3′ Exonuclease activity. *Research—PCR Methods and Applications*, 2, pp. 275–287.

Le, Y. et al., 2013. Thermostable DNA ligase-mediated PCR production of circular plasmid (PPCP) and its application in directed evolution via *in situ* error-prone PCR. *DNA Research*, 20, pp. 375–382.

Lee, I., Evans, B.R., and Woodward, J., 2015. The mechanism of cellulase action on cotton fibres: Evidence from atomic force microscopy. *Ultramicroscopy*, 82, pp. 213–221.

Leis, B. et al., 2015. Functional screening of hydrolytic activities reveals an extremely thermostable cellulase from a deep-sea archaeon. *Frontiers in Bioengineering and Biotechnology*, 3(7), p. 95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26191525.

Leow, T.C. et al., 2004. High level expression of thermostable lipase from *Geobacillus* sp. strain T1. *Bioscience Biotechnology and Biochemistry*, 68, pp. 96–103.

Li, H. and Zhang, X., 2005. Characterization of thermostable lipase from thermophilic *Geobacillus* sp. TW1. *Protein Expression and Purification*, 42, pp. 153–159.

Li, Q. et al., 2013a. Commercial proteases: Present and future. *FEBS Letters*, 587, pp. 1155–1163.

Li, S. et al., 2015. Structure and sequence analysis-based engineering of pullulanase from *Anoxybacillus* sp. LM18-11 for improved thermostability. *Journal of Biotechnology*, 210, pp. 8–14. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168165615300328.

Li, X., Li, D., and Park, K.-H., 2013b. An extremely thermostable amylopullulanase from *Staphylothermus marinus* displays both pullulan- and cyclodextrin-degrading activities. *Applied Microbiology and Biotechnology*, 97, pp. 5359–5369. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23001056.

Li, Y. et al., 2012. Cloning, expression, characterization, and biocatalytic investigation of a novel *Bacilli thermostable* type i pullulanase from *Bacillus* sp. CICIM 263. *Journal of Agricultural and Food Chemistry*, 60, pp. 11164–11172.

Li, Y.-T. et al., 2009. Preparation of homogenous oligosaccharide chains from glycosphingolipids. *Glycoconjugate Journal*, 26, pp. 929–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18415015.

Liang, T.-W., Hsieh, T.-Y., and Wang, S.-L., 2014. Purification of a thermostable chitinase from *Bacillus cereus* by chitin affinity and its application in microbial community changes in soil. *Bioprocess and Biosystems Engineering*, 37, pp. 1201–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24342954.

Lohman, G.J., Tabor, S., and Nichols, N.M., 2011. DNA ligases. In: Ausubel, F.M. et al. (eds.), *Current Protocols in Molecular Biology*. 94:III:3.14:3.14.1–3.14.7. DOI:10.1002/0471142727.mb0314s94.

Loncar, N. and Fraaije, M.W., 2015. Not so monofunctional—A case of thermostable *Thermobifida fusca* catalase with peroxidase activity. *Applied Microbiology and Biotechnology*, 99, pp. 2225–2232. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25227535\n, http://download.springer.com/static/pdf/175/art%253A10.1007%252Fs00253-014-6060-5.pdf?auth66=1427795261_56f36dc55730aafb5ce2f7d5f3cf4665&ext=.pdf.

Lu, L. et al., 2013. Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization. *Bioresource Technology*, 134, pp. 81–86. Available at: http://dx.doi.org/10.1016/j.biortech.2013.02.015.

Lu, R. et al., 2015. Screening cloning and expression analysis of a cellulase derived from the causative agent of hypertrophy sorosis sclerotenosis *Ciboria shiraiana*. *Gene*, 565, pp. 221–227.

MacCabe, A.P. et al., 2002. Improving extracellular production of food-use enzymes from *Aspergillus nidulans*. *Journal of Biotechnology*, 96, pp. 43–54.

Madhavi, V. and Lele, S.S., 2009. Laccase: Properties and applications. *Bio Resources*, 4, pp. 1694–717.

Malakar, R., Tiwari, D.A., and Malviya, S.N., 2010. Pullulanase: A potential enzyme for industrial application. *International Journal of Biomedical Research*, 1, pp. 10–20.

Mall, R. et al., 2013. Purification and characterization of a thermostable soluble peroxidase from *Citrus medica* leaf. *Preparative Biochemistry and Biotechnology*, 43, pp. 137–151. Available at: http://www.tandfonline.com/doi/abs/10.1080/10826068.2012.711793.

Manavalan, A. et al., 2015. Characterization of a solvent, surfactant and temperature-tolerant laccase from *Pleurotus* sp. MAK-II and its dye decolorizing property. *Biotechnology Letters*, 37, pp. 2403–2409. Available at: http://dx.doi.org/10.1007/s10529-015-1937-7.

Maria, L. De et al., 2007. Phospholipases and their industrial applications. *Applied Microbiology and Biotechnology*, 74, pp. 290–300.

Marques, S. et al., 2003. Characterisation and application of glycanases secreted by *Aspergillus terreus* CCMI 498 and *Trichoderma viride* CCMI 84 for enzymatic deinking of mixed office wastepaper. *Journal of Biotechnology*, 100, pp. 209–219.

Masomian, M. et al., 2013. A new thermostable and organic solvent-tolerant lipase from *Aneurinibacillus thermoaerophilus* strain HZ. *Process Biochemistry*, 48, pp. 169–175. Available at: http://www.sciencedirect.com/science/article/pii/S135951131200387X (Accessed October 12, 2015).

Masui, D.C. et al., 2012. Production of a xylose-stimulated β-glucosidase and a cellulase-free thermostable xylanase by the thermophilic fungus *Humicola brevis* var. *thermoidea* under solid state fermentation. *World Journal of Microbiology and Biotechnology*, 28, pp. 2689–2701.

Matsumura, S., Sakiyama, K., and Toshima, K., 1999. Preparation of octyl β-D-xylobioside and xyloside by xylanase catalyzed direct transglycosylation reaction of xylan and octanol. *Biotechnology Letters*, 21, pp. 17–22.

Mayer, G., Muller, J., and Lunse, C.E., 2011. RNA diagnostics: Real-time RT-PCR strategies and promising novel target RNAs. *Wiley Interdisciplinary Reviews RNA*, 2, pp. 32–41.

Mesbah, N.M. and Wiegel, J., 2014. Halophilic alkali- and thermostable amylase from a novel polyextremophilic *Amphibacillus sp.* NM-Ra2. *International Journal of Biological Macromolecules*, 70C, pp. 222–229. Available at: http://www.sciencedirect.com/science/article/pii/S014181301400436X.

Michelin, M. et al., 2012a. A novel xylan degrading β-d-xylosidase: Purification and biochemical characterization. *World Journal of Microbiology and Biotechnology*, 28, pp. 3179–3186.

Michelin, M. et al., 2012b. Production of xylanase and β-xylosidase from autohydrolysis liquor of corncob using two fungal strains. *Bioprocess and Biosystems Engineering*, 35, pp. 1185–1192. Available at: http://link.springer.com/10.1007/s00449-012-0705-5.

Mizuno, M., Yasukawa, K., and Inouye, K., 2010. Insight into the mechanism of the stabilization of moloney murine leukaemia virus reverse transcriptase by eliminating RNase H activity. *Bioscience, Biotechnology and Biochemistry*, 74, pp. 440–442.

Mohr, S. et al., 2013. Thermostable group II intron reverse transcriptase fusion proteins and their use in cDNA synthesis and next-generation RNA sequencing. *RNA*, 19, pp. 958–970. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3683930&tool=pmcentrez&rendertype=abstract.

Moraïs, S. et al., 2011. Assembly of xylanases into designer cellulosomes promotes efficient hydrolysis of the xylan component of a natural recalcitrant cellulosic substrate. *MBio*, 2(6), pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22086489.

Morgenstern, I., Robertson, D.L., and Hibbett, D.S., 2010. Characterization of three mnp genes of *Fomitiporia mediterranea* and report of additional class II peroxidases in the order Hymenochaetales. *Applied and Environment Microbiology*, 76, pp. 6431–6440.

Morozova, O. V et al., 2007. "Blue" laccases. *Biochemistry (Mosc)*, 72, pp. 1136–1150.

Mozersky, S., Marmer, W., and Dale, A.O., 2002. Vigorous proteolysis: Relining in the presence of an alkaline protease and bating (Post-Liming) with an extremophile protease. *JALCA*, 97, pp. 150–155.

Mrudula, S., Gopal, R., and Seenayya, G., 2011. Effect of substrate and culture conditions on the production of amylase and pullulanase by thermophilic *Clostridium thermosulforegenes* SVM17 in solid state fermentation. *Malaysian Journal of Microbiology*, 7, pp. 19–25.

Mutreja, R. et al., 2011. Bioconversion of agricultural waste to ethanol by SSF using recombinant cellulase from *Clostridium thermocellum*. *Enzyme Research*, 2011, pp. 1–6. Available at: http://www.hindawi.com/journals/er/2011/340279/.

Nawani, N. and Kaur, J., 2000. Purification, characterization and thermostability of lipase from a thermophilic *Bacillus* sp. J33. *Molecular and Cellular Biochemistry*, 206, pp. 91–96.

Nguyen, T.T., Quyen, T.D., and Le, H.T., 2013. Cloning and enhancing production of a nattokinase from *Bacillus subtilis* VTCC-DVN-12-01 by using an eight-protease-gene-deficient *Bacillus subtilis* WB800. *Microbial Cell Factories*, 12, pp. 1–11. Available at: Microbial Cell Factories.

Nigam, P.S., 2013. Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules*, 3, pp. 597–611.

Nisha, M. and Satyanarayana, T., 2013. Characterization of recombinant amylopullulanase (gt-apu) and truncated amylopullulanase (gt-apuT) of the extreme thermophile *Geobacillus thermoleovorans* NP33 and their action in starch saccharification. *Applied Microbiology and Biotechnology*, 97, pp. 6279–6292.

Nisole, S. et al., 2004. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proceedings of National Academy of Sciences*, 101, pp. 13324–13328.

Oziengbe and Onilude, 2012. Production of a thermostable a-amylase and its assay using *Bacillus licheniformis* isolated from excavated land sites in Ibadan, Nigeria. *Bayero Journal of Pure Applied Sciences*, 5, pp. 132–138.

Ozsolak, F. and Milos, P.M., 2011. RNA sequencing: Advances, challenges and opportunities. *Nature Review Genetics*, 12, pp. 87–98.

Ozturk, M.T. et al., 2013. Ligase-independent cloning of amylase gene from a local *Bacillus subtilis* isolate and biochemical characterization of the purified enzyme. *Applied Biochemistry and Biotechnology*, 171, pp. 263–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23832859.

Palavesam, A., 2015. Investigation on lignocellulosic saccharification and characterization of haloalkaline solvent tolerant endo-1,4 β-d-xylanase from *Halomonas meridiana* APCMST-KS4. *Biocatalysis and Agricultural Biotechnology*, 4, pp.761–766. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1878818115001279.

Pant, G. et al., 2015. Production, optimization and partial purification of protease from *Bacillus subtilis*. *Journal of Taibah University for Sciences*, 9, pp. 50–55. Available at: http://dx.doi.org/10.1016/j.jtusci.2014.04.010.

Paredes, R. de S. et al., 2015. Production of xylanase, α-l-arabinofuranosidase, β-xylosidase, and β-glucosidase by *Aspergillus awamori* using the liquid stream from hot-compressed water treatment of sugarcane bagasse. *Biomass Conversion and Biorefinery*, pp. 3–11. Available at: http://link.springer.com/10.1007/s13399-015-0159-5.

Park, J.-T. et al., 2013. Molecular cloning and characterization of a thermostable α-amylase exhibiting an unusually high activity. *Food Science and Biotechnology*, 23, pp. 125–132. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84894434916&partnerID=tZOtx3y1.

Parshetti, G.K. et al., 2012. Industrial dye decolorizing lignin peroxidase from *Kocuria rosea* MTCC 1532. *Annals of Microbiology*, 62(1), pp.217–223. doi:10.1007/s13213-011-0249-y.

Patel, S.J. and Savanth, V.D., 2015. Review on fungal xylanases and their applications xylan xylanase fungal xylanases xylanase production. *International Journal of Advanced Research*, 3, pp. 311–315.

Pathak, A.P. and Rekadwad, B.N., 2013. Isolation of thermophilic *Bacillus* sp. strain EF _TYK1-5 and production of industrially important thermostable a-amylase using suspended solids for fermentation. *Journal of Scientific and Industrial Research*, 72, pp. 685–689.

Pazarlioğlu, N.K., Sariişik, M., and Telefoncu, A., 2005. Laccase: Production by *Trametes versicolor* and application to denim washing. *Process Biochemistry*, 40, pp. 1673–1678.

Polizeli, M.L.T.M. et al., 2005. Xylanases from fungi: Properties and industrial applications. *Applied Microbiology and Biotechnology*, 67, pp.577–591.

Pradeep, G.C. et al., 2013. A novel thermostable cellulase free xylanase stable in broad range of pH from *Streptomyces* sp. CS428. *Process Biochemistry*, 48, pp. 1188–1196. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1359511313002766.

Prasanna, L. et al., 2013. A novel strain of *Brevibacillus laterosporus* produces chitinases that contribute to its biocontrol potential. *Applied Microbiology and Biotechnology*, 97, pp. 1601–1611.

Puchart, V. et al., 2015. A unique CE16 acetyl esterase from *Podospora anserina* active on polymeric xylan. *Applied Microbiology and Biotechnology*, pp. 10515–10526. Available at: http://link.springer.com/10.1007/s00253-015-6934-1.

Purohit, M.K. and Singh, S.P., 2013. A metagenomic alkaline protease from saline habitat: Cloning, over-expression and functional attributes. *International Journal of Biological Macromolecules*, 53, pp. 138–143. Available at: http://dx.doi.org/10.1016/j.ijbiomac.2012.10.032.

Quail, M.A. et al., 2008. A large genome center's improvements to the Illumina sequencing system. *Nature Methods*, 12, pp. 1005–1010.

Raghuwanshi, S. et al., 2014. Bioprocessing of enhanced cellulase production from a mutant of *Trichoderma asperellum* RCK2011 and its application in hydrolysis of cellulose. *Fuel*, 124(2014), pp. 183–189. Available at: http://dx.doi.org/10.1016/j.fuel.2014.01.107.

Raj, A., Kumar, S., and Singh, S.K., 2013. A highly thermostable xylanase from *Stenotrophomonas maltophilia*: Purification and partial characterization. *Enzyme Research*, 2013, p. 429305. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24416589.

Rakotoarivonina, H. et al., 2015. Engineering the hydrophobic residues of a GH11 xylanase impacts its adsorption onto lignin and its thermostability. *Enzyme and Microbial Technology*, 81, pp. 47–55. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0141022915300351.

Ramanathan, S., 2011. *Production of Thermostable Pullulanase from Bacillus flavothermus KWF-1 in Fed-Batch Culture*. Universiti Teknologi Malaysia.

Rana, N., Walia, A., and Gaur, A., 2013. α-Amylases from microbial sources and its potential applications in various industries. *National Academy Science Letters*, 36, pp. 9–17.

Rao, S., Ellaiah, P., and Biradar, K.V., 2012. Purification and characterization of thermostable amylase from a strain of thermoactinomyces thalpophilus KSV 17. *RGUHS Journal of Pharmaceutical Sciences*, 2, pp. 83–89. Available at: http://www.rjps.in/article/65.

Rolland, J.-L. et al., 2004. Characterization of a thermophilic DNA ligase from the archaeon *Thermococcus fumicolans*. *FEMS Microbiology Letters*, 236, pp. 267–273.

Roriz, M.S. et al., 2009. Application of response surface methodological approach to optimise Reactive black 5 decolouration by crude laccase from *Trametes pubescens*. *Journal of Hazardous Materials*, 169, pp. 691–696.

Sabotic, J. and Kos, J., 2012. Microbial and fungal protease inhibitors—Current and potential applications. *Applied Microbiology and Biotechnology*, 93, pp. 1351–1375.

Saha, B.C., 2001. Purification and characterization of an extracellular Fusarium verticillioides. *Journal of Industrial Microbial Biotechnology*, 27, pp. 241–245.

Saha, B.C., 2003. Purification and properties of an extracellular beta-xylosidase from a newly isolated *Fusarium proliferatum*. *Bioresource Technology*, 90, pp. 33–38.

Salahuddin, K. et al., 2012. Biochemical characterization of thermostable cellulase enzyme from mesophilic strains of actinomycete. *African Journal of Biotechnology*, 11, pp. 10125–10134. Available at: http://www.academicjournals.org/ajb/abstracts/abs2012/29May/Salahuddin et al.htm.

Sambrook, J. and Russell, D.W., 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sano, S. et al., 2012. Mutations to create thermostable reverse transcriptase with bacterial family A DNA polymerase from *Thermotoga petrophila* K4. *Journal of Bioscience and Bioengineering*, 113, pp. 315–21. Available at: http://dx.doi.org/10.1016/j.jbiosc.2011.11.001.

Santos, A. et al., 2014. New dye-decolorizing peroxidases from *Bacillus subtilis* and *Pseudomonas putida* MET94: Towards biotechnological applications. *Applied Microbiology and Biotechnology*, 98, pp. 2053–2065.

Sarafianos, S.G. et al., 2009. Structure and function of HIV-1 reverse transcriptase: Molecular mechanisms of polymerization and inhibition. *Journal of Molecular Biology*, 385, pp. 693–713. Available at: http://dx.doi.org/10.1016/j.jmb.2008.10.071.

Sasaki, Y. et al., 2004. TNF family member B cell-activating factor (BAFF) receptor dependent and -independent roles for BAFF in B cell physiology. *Journal of Immunology*, 173, pp. 2245–2252.

Saxena, R.K. et al., 2003. Purification and characterization of an alkaline thermostable lipase from *Aspergillus carneus*. *Process Biochemistry*, 39, pp. 239–247.

Schmid, A. et al., 2001. Industrial biocatalysis today and tomorrow. *Nature*, 409, pp. 258–268.

Seo, M.S. et al., 2007. Cloning and expression of a DNA ligase from the hyperthermophilic archaeon *Staphylothermus marinus* and properties of the enzyme. *Journal of Biotechnology*, 128, pp. 519–530.

Shallom, D. and Shoham, Y., 2003. Microbial hemicellulases. *Current Opinion in Microbiology*, 6, pp. 219–228.

Sharma, A. and Satyanarayana, T., 2012. Cloning and expression of acid-stable, high maltose-forming, Ca2+-independent-amylase from an acidophile *Bacillus acidicola* and its applicability in starch hydrolysis. *Extremophiles*, 16, pp. 515–522.

Sharma, D., Sharma, B., and Shukla, A.K., 2011. Biotechnological approach of microbial lipase: A review. *Biotechnology*, 10, pp. 23–40.

Sharma, K. et al., 2014. Isolation, identification and optimization of culture conditions of *Bacillus* sp. strain PM1 for alkalo-thermostable amylase production. *British Microbiology Research Journal*, 4, pp. 369–380.

Shi, H. et al., 2013. A novel highly thermostable xylanase stimulated by Ca2+ from *Thermotoga thermarum*: Cloning, expression and characterization. *Biotechnology for Biofuels*, 6, p. 26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23418789\n, http://www.biotechnologyforbiofuels.com/content/6/1/26.

Shiba, Y. et al., 2001. High-level secretory production of phospholipase A1 by *Saccharomyces cerevisiae* and *Aspergillus oryzae*. *Bioscience, Biotechnology, and Biochemistry*, 65(2), pp. 94–101.

Shoemaker, G.K., Juers, D.H., and Coombs, J.M.L., 2003. Crystallization of β-galactosidase does not reduce the range of activity of individual molecules. *Biochemistry*, 42, pp. 1707–1710.

Shraddha et al., 2011. Laccase: Microbial sources, production, purification, and potential biotechnological applications. *Enzyme Research*, p. 217861.

Shuman, S., 2009. DNA ligases: Progress and prospects. *The Journal of Biological Chemistry*, 284, pp. 17365–17369.

Simkhada, J.R. et al., 2009. A novel low molecular weight phospholipase D from *Streptomyces* sp. CS684. *Bioresource Technology*, 100, pp. 1388–93. Available at: http://www.sciencedirect.com/science/article/pii/S0960852408007748.

Singh, K. et al., 2013. Statistical media optimization and cellulase production from marine *Bacillus* VITRKHB. *3 Biotech*, 4, pp. 591–598. Available at: http://link.springer.com/10.1007/s13205-013-0173-x (Accessed November 17, 2014).

Singh, S. et al., 2000a. Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. *Journal of Applied Microbiology*, 88, pp. 975–982.

Singh, S. et al., 2000b. Relatedness of *Thermomyces lanuginosus* strains producing a thermostable xylanase. *Journal of Biotechnology*, 81(2–3), pp. 119–128.

Singh, S., Madlala, A.M., and Prior, B.A., 2003. *Thermomyces lanuginosus*: Properties of strains and their hemicellulases. *FEMS Microbiology Reviews*, 27(1), pp. 3–16.

Sreedevi, S., Sajith, S., and Benjamin, S., 2013. Cellulase producing bacteria from the wood-yards on Kallai river bank. *Advances in Microbiology*, 3(8), pp. 326–332. Available at: http://afrjournal.org/index.php/afr/article/view/80 (Accessed November 17, 2014).

Stewart, E.V. et al., 2011. Yeast SREBP cleavage activation requires the Golgi Dsc E3 ligase complex. *Molecular Cell*, 42, pp. 160–171. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1097276511002528.

Stokes, S.S. et al., 2011. Discovery of bacterial NAD+-dependent DNA ligase inhibitors: Optimization of antibacterial activity. *Bioorganic and Medicinal Chemistry Letters*, 21, pp. 4556–4560. Available at: http://www.sciencedirect.com/science/article/pii/S0960894X11007694.

Subramaniyan, S. and Prema, P., 2002. Biotechnology of microbial xylanases: Enzymology, molecular biology, and application. *Critical Reviews in Biotechnology*, 22, pp. 33–64.

Suginta, W. et al., 2000. Chitinases from vibrio: Activity screening and purification of chiA from *V. carchariae*. *Journal of Applied Microbiology*, 89, pp. 76–84.

Sun, S. et al., 2011. Cloning, expression and characterization of a thermostable pullulanase from newly isolated thermophilic Geobacillus sp. LM14-3. In *4th International Conference on Biochemical Engineering and Informatics*. Shanghai, China, pp. 1567–1570. DOI: 10.1109/BMEI.2011.6098577.

Sundarram, A. and Murthy, T.P.K., 2014. α-Amylase production and applications: A review. *Journal of Applied and Environmental Microbiology*, 2, pp. 166–175.

Takayanagi, T. et al., 1991. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X_7u. *Biochemica et Biophysica Acta*, 1078, pp. 404–410.

Tello, M. et al., 2000. Characterization of three new manganese peroxidase genes from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. *Biochimica et Biophysica Acta*, 1490, pp. 137–44.

Teng, C. et al., 2011. High-level expression of extracellular secretion of a β-xylosidase gene from *Paecilomyces thermophila* in *Escherichia coli*. *Bioresource Technology*, 102, pp. 1822–1830. Available at: http://dx.doi.org/10.1016/j.biortech.2010.09.055.

Terpe, K., 2013. Overview of thermostable DNA polymerases for classical PCR applications: From molecular and biochemical fundamentals to commercial systems. *Applied Microbial Biotechnology*, 97, pp. 10243–10254.

Terrasan, C.R.F. et al., 2013. Xylanase and β-xylosidase from *Penicillium janczewskii*: Production, physico-chemical properties, and application of the crude extract to pulp biobleaching. *BioResources*, 8, pp. 1292–1305.

Thomas, L., Joseph, A., and Gottumukkala, L.D., 2014. Xylanase and cellulase systems of *Clostridium* sp.: An insight on molecular approaches for strain improvement. *Bioresource Technology*, 158, pp. 343–350. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24581864 (Accessed October 19, 2014).

Tikhomirov, D.F. et al., 2003. Non-starch polysaccharide hydrolysing microbial enzymes in grain processing. In C.M. Courtin, W.S. Veraverbeke, and J.A. Delcour, eds. *Recent Advances in Enzymes in Grain Processing*. Katholieke University, Leuven, pp. 423–418.

Tsujibo, H. et al., 1995. Expression in *Escherichia coli* of a gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Bioscience, Biotechnology and Biochemistry*, 59, pp. 145–146.

Tsujibo, H. et al., 2001. Cloning, sequencing, and expression of the gene encoding an intracellular β-D-xylosidase from *Streptomyces thermoviolaceus* OPC-520. *Bioscience, Biotechnology, Biochemistry*, 65, pp. 1824–1831.

Turner, P., Mamo, G., and Karlsson, E.N., 2007. Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbial Cell Factories*, 6, p. 9.

Uday, U.S.P. et al., 2016. Classification, mode of action and production strategy of xylanase and its application for biofuel production from water hyacinth. *International Journal of Biological Macromolecules*, 82, pp. 1041–1054. Available at: http://linkinghub.elsevier.com/retrieve/pii/S014181301530088X.

Van Der Maarel, M.J. et al., 2002. Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology*, 94, pp. 137–155.

Várnai, A. et al., 2014. Expression of endoglucanases in *Pichia pastoris* under control of the GAP promoter. *Microbial Cell Factories*, 13, p. 57. Available at: http://www.microbialcellfactories.com/content/13/1/57.

Verma, D. and Satyanarayana, T., 2012. Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. *Bioresource Technology*, 107, pp. 333–338. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22212694.

Vieille, C. and Zeikus, G.J., 2001. Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews*, 65, pp. 1–43.

Wakiyama, M. et al., 2008. Purification and properties of an extracellular b-xylosidase from *Aspergillus japonicus* and sequence analysis of the encoding gene. *Journal of Bioscience and Bioengineering*, 106, pp. 398–404.

Wang, B. et al., 2004. A novel phospholipase A2/esterase from hyperthermophilic archaeon *Aeropyrum pernix* K1. *Protein Expression and Purification*, 35, pp. 199–205. Available at: http://www.sciencedirect.com/science/article/pii/S1046592804000440 (Accessed October 30, 2015).

Wang, L.K. and Shuman, S., 2005. Structure-function analysis of yeast tRNA ligase. *RNA*, 11(6), pp. 966–975.

Wang, Y. et al., 2013. Expression, purification and biochemical characterization of *Methanocaldococcus jannaschii* DNA ligase. *Protein Expression and Purification*, 87(2), pp. 79–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23147204.

Wang, Z., Gerstein, M., and Snyder, M., 2009. RNA-Seq: A revolutionary tool for transcriptomics. *Nature Review Genetics*, 10, pp. 57–63.

Webb, M.D. et al., 2001. Metabolism of pentachlorophenol by *Saccharomonospora viridis* strains isolated from mushroom compost. *Soil Biology and Biochemistry*, 33, pp. 1903–1914.

Wei, T. et al., 2015. Characterization of a novel thermophilic phospholipase B from *Thermotoga lettingae* TMO: Applicability in enzymatic degumming of vegetable oils. *Journal of Industrial Microbiology and Biotechnology*, 42, pp. 515–522. Available at: http://link.springer.com/10.1007/s10295-014-1580-7.

Wilson, L. et al., 2006. Improvement of the functional properties of a thermostable lipase from *Alcaligenes* sp. via strong adsorption on hydrophobic supports. *Enzyme and Microbial Technology*, 38, pp. 975–980.

Wong, K.K.Y., Tan, L.U.L., and Saddler, J.N., 1988. Multiplicity of β-1, 4-xylanase in microorganisms: Functions and applications. *Microbiology Reviews*, 52, pp. 305–317.

Wongwisansri, S. et al., 2013. High-level production of thermotolerant β-xylosidase of *Aspergillus* sp. BCC125 in *Pichia pastoris*: Characterization and its application in ethanol production. *Bioresource Technology*, 132, pp. 410–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23265813.

Woo, M.H. et al., 2014. First thermostable endo-β-1,4-glucanase from newly isolated *Xanthomonas* sp. EC102. Protein Journal, 33, pp. 110–117.

Wu, G. et al., 2006. Simplified gene synthesis: A one-step approach to PCR-based gene construction. *Journal of Biotechnology*, 124, pp. 496–503.

Wu, H. et al., 2014. Cloning, overexpression and characterization of a thermostable pullulanase from *Thermus thermophilus* HB27. *Protein Expression and Purification*, 95, pp. 22–27. Available at: http://www.sciencedirect.com/science/article/pii/S1046592813002544.

Yamura, I., Koga, T., and Matsumoto, T., 1997. Purification and some properties of endo-1,4-β xylanase from a fresh water mollusk Pomacea insularus (de Oringny). *Bioscience Biotechnology and Biochemistry*, 61, pp. 615–620.

Yang, S. et al., 2016. Cloning, expression, purification and application of a novel chitinase from a thermophilic marine bacterium *Paenibacillus barengoltzii*. *Food Chemistry*, 192, pp. 1041–1048. Available at: http://dx.doi.org/10.1016/j.foodchem.2015.07.092.

Yang, X., Cong, H., and Song, J., 2013. Heterologous expression of an aspartic protease gene from biocontrol fungus Trichoderma asperellum in *Pichia pastoris*. *World Journal of Microbiology and Biotechnology*, 29, pp. 2087–2094.

Yasukawa, K., Nemoto, D., and Inouye, K., 2008. Comparison of the thermal stabilities of reverse transcriptases from avian myeloblastosis virus and Moloney murine leukaemia virus. *Journal of Biochemistry*, 143, pp. 261–268.

Yu, W. et al., 2014. Application of a novel alkali-tolerant thermostable DyP-type peroxidase from *Saccharomonospora viridis* DSM 43017 in biobleaching of Eucalyptus Kraft Pulp. *PLoS ONE*, 9, p. e110319. Available at: http://dx.plos.org/10.1371/journal.pone.0110319.

Zafar, A. et al., 2015. Cloning, purification and characterization of a highly thermostable amylase gene of *Thermotoga petrophila* into *Escherichia coli*. *Applied Biochemistry and Biotechnology*. Available at: http://link.springer.com/10.1007/s12010-015-1912-8.

Zambare, V., Nilegaonkar, S., and Kanekar, P., 2011. A novel extracellular protease from *Pseudomonas aeruginosa* MCMB-327: Enzyme production and its partial characterization. *New Biotechnology*, 28, pp. 173–181.

Zhang, D. et al., 2012. Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens. *International Journal of Food Microbiology*, 153, pp. 453–464. Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2011.12.016.

Zhang, M. et al., 2015. The multi-chitinolytic enzyme system of the compost-dwelling thermophilic fungus *Thermomyces lanuginosus*. *Process Biochemistry*, 50, pp. 237–244. Available at: http://dx.doi.org/10.1016/j.procbio.2014.11.008.

Zhang, S. et al., 2014. Cloning, expression, and characterization of a thermostable β-xylosidase from thermoacidophilic *Alicyclobacillus* sp. A4. *Process Biochemistry*, 49, pp. 1422–1428. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1359511314003225.

Zhang, Y. et al., 2014. High-yield production of a chitinase from *Aeromonas veronii* B565 as a potential feed supplement for warm-water aquaculture. *Applied Microbiology and Biotechnology*, 98, pp. 1651–1662.

Zhao, A., Gray, F.C., and MacNeill, S.A., 2006. ATP- and NAD+-dependent DNA ligases share an essential function in the halophilic archaeon *Haloferax volcanii*. *Molecular Microbiology*, 59, pp. 743–752.