VALORISATION OF BAMBARA AND COWPEA HAULMS FOR BIOETHANOL PRODUCTION

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DECLARATION

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

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Manuscripts under review

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Somiame Itseme Okuofu, Suren Singh, Santhosh Pillai (2020) "Deep eutectic solvent pretreatment of bambara haulm for enhanced saccharification and bioethanol production" being prepared for Bioresource Technology

DEDICATION

This thesis is dedicated to my late father Mr. Charles Oshiole Okuofu for his unequivocal love and support for me in his lifetime. He instilled in me the virtue of resilience without which I won't have been able to complete this degree.

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LIST OF ACRONYMS AND SYMBOLS

2, 3- Di-nitro salicylic acid (DNS)

Agricultural Research Council (ARC)

Ammonia fibre/freeze explosion (AFEX)

Ammonia recycle percolation (ARP)

Association of Official Analytical Chemists (AOAC)

Attenuated total reflectance (ATR)

Bambara groundnut haulm (BGH)

Bovine serum albumin (BSA)

Carbon, hydrogen, nitrogen, sulphur and oxygen (CHNS/O)

Central composite design (CCD)

Combined severity factor (CSF)

Consolidated bioprocessing (CBP)

Cowpea haulm (CH)

Deep eutectic solvents (DES)

Dilute acid pretreatment (DAP)

Evaporative light scattering detector (ELSD)

Flame ionization detector (FID)

Fourier transform infrared spectrophotometry (FTIR)

Gas chromatography (GC)

Greenhouse gas (GHG)

High-performance liquid chromatography (HPLC)

Hydrogen bonding acceptors (HBAs)

Hydrogen bonding donors (HBDs)

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

National Renewable Energy Laboratory, Laboratory Analytical Procedure, Technical Reports (NREL/TP)

Polyethylene glycol (PEG)

Response surface methodology (RSM)

Scanning electron microscopy (SEM)

Separate hydrolysis and fermentation (SHF)

Simultaneous saccharification and fermentation (SSF)

Soaking aqueous ammonia (SAA)

Thermogravimetric/differential thermogravimetric (TG/DTG)

Ultra Performance Liquid Chromatography (UPLC)

Water-insoluble solids (WIS)

Yeast peptone dextrose (YPD)

α (alpha)

γ (gamma)

ABSTRACT

Bambara and cowpea are important pulses grown in semi-arid South Africa due to their balanced nutrient profile and drought resilient capacity. The haulm is the lignocellulosic residue obtained after grain harvest and are rich in carbohydrates. However, these haulms are underutilised and under researched. The aim of the study, therefore, was to investigate the potential to valorise bambara haulms (BGH) and cowpea haulms (CH) to bioethanol which is the most promising biofuel with commercial prospects currently.

The structural and chemical composition of BGH and CH was elucidated using techniques such as compositional analysis, XRD, FTIR, ICP-AES, and SEM. Results indicated a volatile matter and fixed carbon mass fraction of 77.70% and 13.15% (w/w) in BGH and 76.16% and 16.26% (w/w) in CH respectively. The polysaccharides make up the largest fraction (51%), followed by extractives (> 20%), while the lignin in BGH (12%) and CH (10%) was low. X-ray diffraction pattern showed a higher percentage of amorphous regions in BGH (78%) than CH (56%). CH was then subjected to dilute acid pretreatment (DAP) to enhance biosugar production for bioethanol fermentation. The effects of operational factors for DAP including temperature, time, and acid concentration on sugar yield and inhibitor formation was investigated and optimised using response surface methodology (RSM). The solid recovered after DAP was subjected to prehydrolysis with simultaneous saccharification and fermentation (PSSF). In addition, the pretreatment hydrolysate was detoxified and fermented to ethanol using cocultures of *Saccharomyces cerevisiae* BY4743 and *Scheffersomyces stipitis* wild type (PsY633). A total ethanol titre of 15.67 g/L was obtained corresponding to 75% conversion efficiency. On the other hand, BGH was subjected to deep eutectic solvent (DES) pretreatment. Five deep eutectic solvents were prepared and screened for their effectiveness in improving enzymatic sugar yield. This was achieved by pretreating BGH with each DES followed by a 48 h enzymatic saccharification. Choline chloride – lactic acid (ChCl-LA) treatment provided the most promising result and was further optimised by investigating the effect of different temperatures and time on cellulose loss and enzymatic sugar yield. ChCl-LA pretreatment at 100°C for 1 h was observed to be the best condition for maximum sugar recovery. The hydrolysate thus obtained was concentrated and fermented for 72 h with *S. cerevisiae* BY4743. A maximum ethanol yield of 11.57 g/L was obtained. From the results, it is evident that bambara and cowpea haulm are promising

substrates for bioethanol production. Dilute acid hydrolysis was shown to be effective in the pretreatment of CH with over 85% of the theoretical sugar recoverable for conversion to bioethanol. In addition, deep eutectic solvents are effective media for breaking the recalcitrance in BGH to achieve high sugar yield for conversion to bioethanol. However, further studies are required to reduce cellulose loss during pretreatment to improve bioethanol yield.

1 CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Pulses have a history as staple crops and are often cited as vital components for sustainable agricultural development in sub-Saharan Africa. They provide food and nutritional security, income generation for smallholder farmers and maintain or improve soil health (Snapp, Cox and Peter 2019). Pulses such as bambara groundnut (*Vigna subterranea* L. Verdc) and cowpea (*Vigna unguiculata*) have been identified as promising crops for the Southern African region due to their ability to thrive in semi-arid to arid conditions (Chivenge *et al.* 2015; Mayes *et al.* 2019). Particularly in South Africa, the need to promote the utilization of bambara groundnut and cowpea by improving their value chain has been reviewed (Chibarabada, Modi and Mabhaudhi 2017). The lignocellulosic residues of bambara groundnut and cowpea are the second most important output of the farming process and input into the value chain. These residues, which are often dumped or burnt, offer a promising yet underutilized resource for valorisation. Two categories of lignocellulosic residues can be obtained from bambara and cowpea production. They include (1) field residues obtained after pod harvest (stalk, stem, leaves collectively called haulm) and processing residues obtained after pod processing (shell/pod). It has been demonstrated that high-quality fuel and other valuable materials could be obtained from bambara groundnut shells *via* pyrolysis (Mohammed *et al.* 2016; Mohammed *et al.* 2017).

Bioethanol or simply ethanol, produced by the biochemical conversion of sugar-rich feedstock is a prominent biofuel (Dehghani Madvar *et al.* 2019). Sugar rich feedstocks applicable for bioethanol production are classified into three generations: first generation (e.g., sugar or starch-rich food crops), second-generation (e.g., lignocellulosic biomass) and third-generation (e.g., algae) (Zabed *et al.* 2017). The utilization of lignocellulosic biomass for bioethanol production eliminates the food versus fuel concerns associated with first-generation feedstock. Lignocellulosic biomass applicable for bioethanol production includes dedicated whole plants, agricultural residues and waste, wood, municipal solid and industrial waste (Zabed *et al.* 2016). The utilization of agricultural residues for bioethanol production has greater advantages including waste beneficiation and reduced pressure on wood

resources (Tye *et al.* 2016). Several agricultural residues have been studied as feedstock for bioethanol including residues from corn, sugarcane, rice, cassava, wheat, etc. (Soccol *et al.* 2019). However, studies utilizing legume crop residues as feedstock for bioethanol are limited. It is, therefore, necessary to harness indigenous crop residues including legume residues in order to diversify the potential cellulosic feedstock portfolio and strengthen agricultural production systems (Ebert 2014).

One key challenge for lignocellulosic bioethanol production is the recovery of monomeric sugars from the biomass for bioconversion to ethanol. In the native form, lignocellulosic biomass sugars are bound by complex structural and chemical associations that hinder the sugar recovery (Bichot *et al.* 2018). Typically, cellulosic ethanol production is comprised of four critical process steps such as pretreatment, hydrolysis, fermentation and recovery (Aditiya *et al.* 2016). The pretreatment process is the first critical control point and is required to deconstruct the biomass cell wall linkages for easy access to enzymes. An array of physical, chemical, physicochemical, biological or combinatory pretreatment methods have been studied in most agricultural lignocellulosic feedstock used for bioethanol (Kumari and Singh 2018; Kumar *et al.* 2019). Previous studies have revealed that the success of the pretreatment process is feedstock dependent, and directly linked to their chemical composition and cell wall structure. Factors such as biomass crystallinity, lignin and hemicellulose composition, biomass particle size and even moisture content are to be considered when choosing a pretreatment method (Bichot *et al.* 2018). Hence, compositional and structural elucidation of the feedstock is vital for process design. In addition, the pretreatment stage is frequently limited by the formation of by-products such as furfural, 5-HMF, formic acid etc. during the process (Kim 2018). These by-products reduce the sugar yield, inhibit the hydrolysis and fermentation phase, consequently lowering ethanol yield. Hence, careful selection and optimization of the pretreatment method is required. Thus far, studies on the characterization, pretreatment and cellulosic ethanol production from BGH and CH are limited. Considering the current global trend of sustainable use of agricultural residues and the development of processes for efficient recovery and conversion of lignocellulosic sugars to bioethanol, the purpose of this study was to evaluate the potential of bambara and cowpea residues as feedstock for cellulosic ethanol production.

1.2 Status of bambara and cowpea production and use in South Africa

1.2.1 Bambara biomass

Bambara nut (*Vigna subterranea* [L] Verdc*)*, is a grain legume indigenous to sub-Saharan Africa where it is widely cultivated by subsistence farmers for its subterranean pods which contain edible seeds. Popularly termed a "complete food", bambara seeds contain on average 63% carbohydrates, 19% proteins and 6.5% fats (Halimi *et al.* 2018). In South Africa, bambara which is locally called izindlubu (Zulu) or Jugo bean is grown majorly in Limpopo, Mpumalanga and KwaZulu-Natal regions (Figure 1.1), mainly for household consumption. Reliable figures for bambara production in South Africa are difficult to obtain because the crop is mainly grown for subsistence purposes. The underutilized status of bambara is attributed to inadequate promotion in local and international markets and the absence of functional value chains to warrant widespread propagation of the plant (Hillocks, Bennett and Mponda 2012). However, there has been a renewed interest in bambara utilisation because bambara is: (1) drought-resilient, (2) able to grow with considerable yield on poor quality soils or low agricultural inputs system, (3) able to replenish soil nutrients through its nitrogen-fixing roots and (4) produces high biomass output even under low water conditions (Mayes *et al.* 2019). To engender a sustainable increase in bambara production in sub-Saharan Africa, market research and development and market-led crop improvement have been suggested (Hillocks, Bennett and Mponda 2012). Although several attempts have been made to valorise bambara seeds (Awobusuyi and Siwela 2019; Hardy and Jideani 2019), the lignocellulosic biomass remains highly understudied.

1.2.2 Cowpea biomass

Cowpea (*Vigna unguiculata* (L.) Walpers) is an annual herbaceous pulse mostly grown in the semi-arid tropics in Africa, Asia, Central and South America, Europe, and the United States. In South Africa, it is grown majorly in Limpopo, Mpumalanga, North-West and KwaZulu-Natal province (Figure 1.1). The sub-Saharan African region accounts for about 84% of cowpea production where its grain and haulm are highly valued for food and forage (Shafique *et al.* 2014). Cowpea is a major source of dietary protein that nutritionally complements staple low-protein cereal and tuber crops and produces income for farmers and traders. Furthermore, it is a valuable component of the farming systems in many areas

because it is drought tolerant and able to restore soil fertility for succeeding cereal crops grown in rotation with it (Timko, Ehlers and Roberts 2007). Despite this, to a large extent, cowpea is an underexploited crop where most research has focused primarily on cowpea breeding and improvement programs. There exists a need to provide new opportunities for the development of cowpea as an economic resource (Timko and Singh 2008).

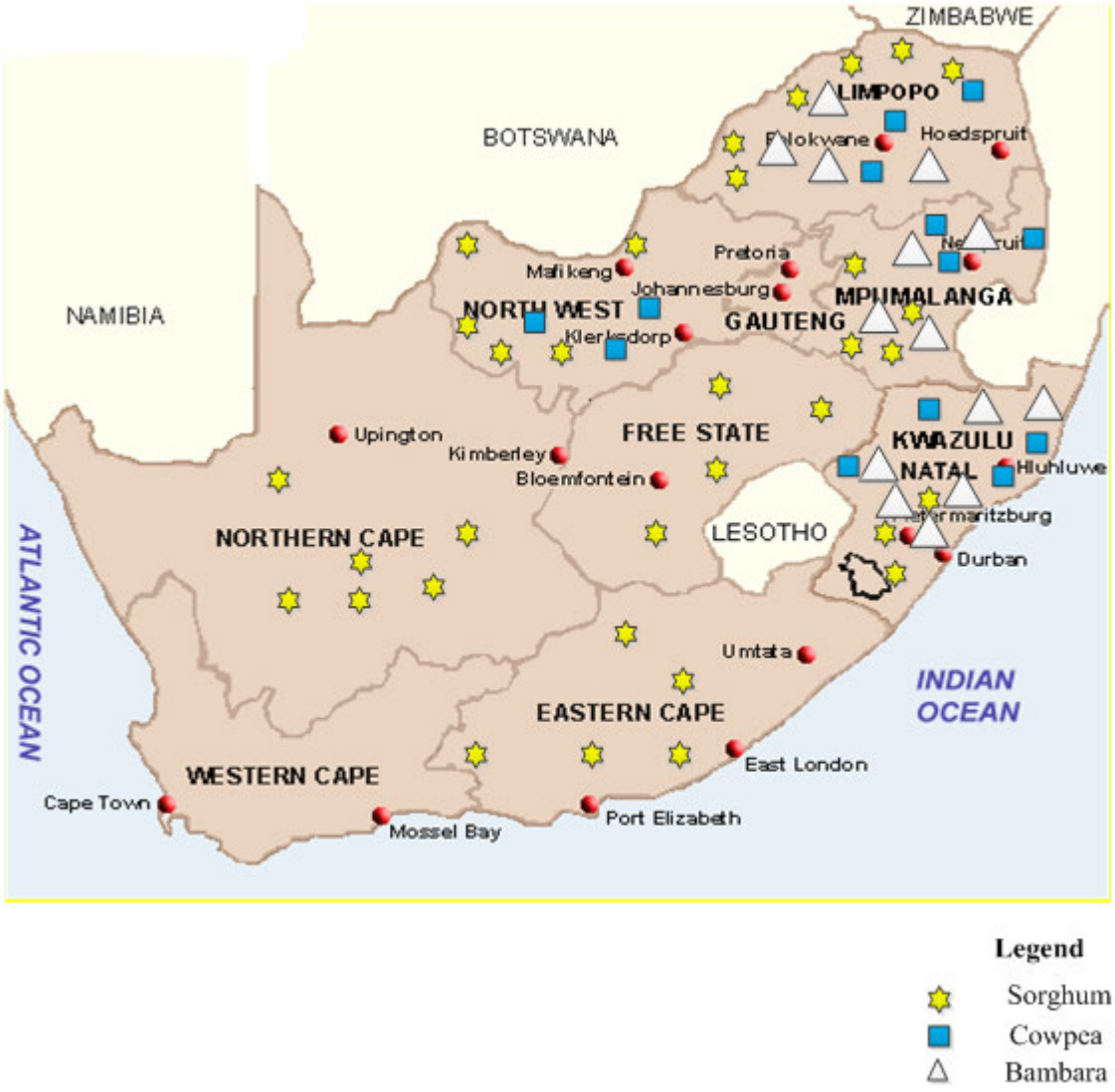

Figure 1.1: Bambara and cowpea producing areas in South Africa (Source: (Department of Agriculture 2016).

1.3 Bioethanol: A globally important commodity

Bioethanol (EtOH or C_2H_5OH) is ethyl alcohol produced by fermentation of sugar-rich feedstock. Bioethanol is a key renewable chemical used primarily as fuel, fuel additive and as feedstock for bio-based production of important industrial chemicals (Bozell and Petersen 2010; Thangavelu, Ahmed and Ani 2016; Rossetti, Tripodi and Ramis 2019). Bioethanol production doubled in the last decade from 13,123 billion gallons in 2007 to 27,050 billion gallons in 2017 (RFA 2018) (Figure 1.2). To date, several national and regional governments have developed policies, strategies and programs to stimulate bioethanol production using locally available resources (Mofijur *et al.* 2015; Solomon *et al.* 2015; Su, Zhang and Su 2015; Sekoai and Yoro 2016; Henley and Fundira 2019).

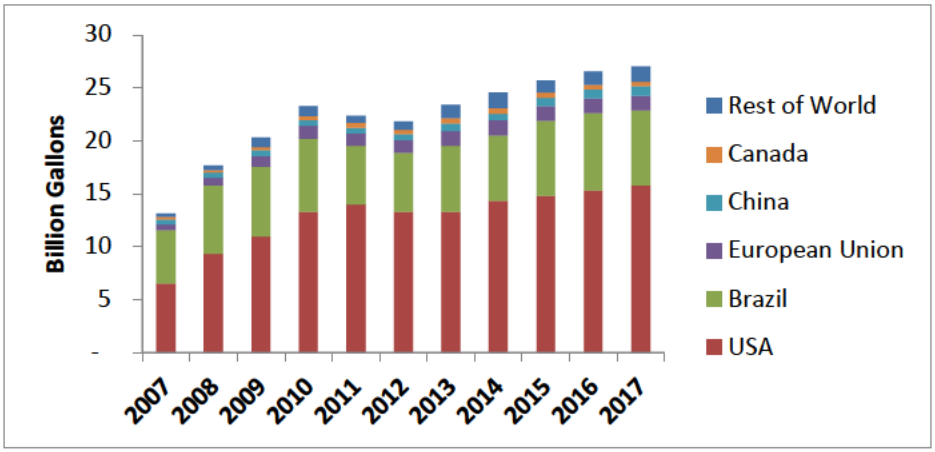

Figure 1.2: Global ethanol production by country and year. Source: www.afdc.energy.gov/data

The growing interest in the use of ethanol as fuel is due to several strategic, environmental and socioeconomic advantages. Ethanol has the potential to reduce particulate emissions in compression–ignition engines due to its oxygen content (34.7%). The use of ethanol fuel and fuel blends reportedly resulted in a decrease in particulate emission by 10-85% depending on the degree of substitution, the feedstock used, ethanol production process and engine type (Morales *et al.* 2015). Ethanol has broader flammability limits, higher flame speeds, higher heats of vaporization and higher-octane number than gasoline (Toor *et al.* 2020). These properties allow for a higher compression ratio with a shorter burning time, resulting in theoretical efficiency advantages over gasoline in an internal combustion engine (Thangavelu, Ahmed and Ani 2016). Furthermore, ethanol is compatible with existing liquid transport fuels and internal combustion engines enabling its immediate use as a blend with

gasoline, without engine modification. However, for blends containing up to 85% ethanol or higher, light-duty ethanol flex-fuel vehicles have been designed (Delavarrafiee and Frey 2018). In addition, ethanol is a carbon-neutral fuel as the carbon dioxide released during combustion is recycled into the plant during photosynthesis, thus, no net carbon dioxide is added to the environment (Saini, Saini and Tewari 2015).

The rapid development of the bioethanol market has been accompanied by a growing interest in its use as a renewable feedstock for the manufacture of bio-based chemicals (Bozell and Petersen 2010). Ethanol can be converted *via* different chemical reactions to various derivatives (Figure. 1.3). Bioethanol is currently used for commercial production of several chemicals (Table 1.1).

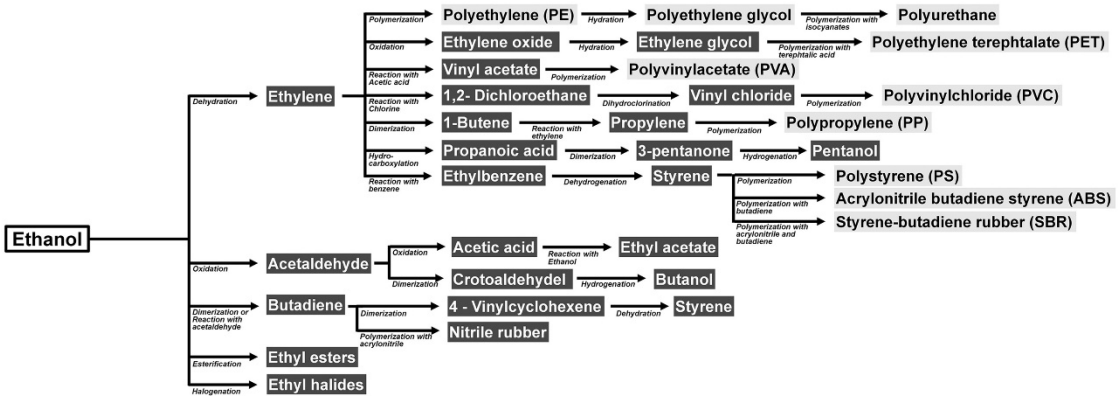

Figure 1.3: Derivatives of ethanol and their production routes (Choi *et al.* 2015).

Table 1.1: Companies producing bioethanol derivatives commercially.

Bioethanol derivatives	Company	Country	Reference
Bioethylene and polyethylene	Braskem Inc.	Brazil	http://plasticoverde.braskem.com.br/site.aspx/Im-greenTM-Polyethylene
Ethylene oxide, its derivatives and various ethylene glycol derivatives	India glycol	India	https://www.indiaglycols.com/product_groups/monoethylene_glycol.htm
Ethyl lactate	Vertec BioSolvents	USA	http://www.vertecbiosolvents.com/vertecbio-el
Acetic acid, ethylene and the polymer vinyl acetate-ethylene (VAE)	Wacker Chemie AG	Germany	https://www.wacker.com/cms/en/wacker_group/divisions/biosolutions/biosolutions.jsp

The top 5 producers in the global market *viz*., USA (56%), Brazil (28%), European Union (5%), China (4%) and Canada (2%) use a variety of food crops (1st generation (1G) feedstock rich in sugar and starch) as feedstock including sugarcane, sugar beets, corn, wheat, sorghum, barley, cassava and rice (Gupta and Verma 2015). 1G bioethanol is a mature technology and cost competitive with petrol (Gupta and Verma 2015). However, 1G bioethanol seems unsustainable for expanding bioethanol markets as they may create unhealthy competition with food production systems and prices, and have minimal green house gas (GHG) reduction benefit (Popp *et al.* 2014; Maga *et al.* 2019). This has driven the emergence of second generation or 2G feedstock called cellulosic biomass. Cellulosic biomass describes all plant matter that contains as part of their cell wall structure, polysaccharides (primarily cellulose and hemicellulose) held in a lignin matrix and associated with proteins, minerals, and other non-structural organic compounds generally called extractives (Singh, Mahanta and Bora 2017). Major categories of cellulosic biomass include agricultural and forest residues, aquatic plants, energy crops and organic fractions of municipal waste (Zabed *et al.* 2017). 2G feedstock obliterates the food versus fuel debate and provides better GHG mitigation benefits (Binod *et al.* 2018). However, the fermentable sugars in cellulosic biomass are organized in a polymeric form and protected by a complex network of structural and chemical associations, creating technical complexities for efficient cellulosic ethanol production and uncertainty in cost (Taha *et al.* 2016).

1.4 Agricultural residues as feedstock for cellulosic ethanol

Agricultural residues are broadly divided into two types such as field residues and process residues. Field residues are the materials left in agricultural land or plantation areas after harvesting the crop, and process residues are leftover materials after the crop is processed into a usable resource (Ali *et al.* 2019). These residues include but are not limited to stem, leaves, straw, stover, peelings, cobs, stalks, bagasse, empty fruit bunches, husks, seeds, roots, and molasses (Hernández *et al.* 2019). The use of agricultural residues for cellulosic bioethanol production presents several comparative advantages over other cellulosic feedstock. These include: (1) a reduction in deforestation by reducing the reliance on forest woody biomass; (2) the development of an eco-friendly disposal measure for these waste resources which are usually left on the field or burnt thereby contributing to GHG emission; (3) adequate feedstock supply all year round (4) lower feedstock cost compared to wood (5) reduction in land use and cost of growing dedicated energy crops and (6) easier amenable structure compared to wood (Saini, Saini and Tewari 2015; Tye *et al.* 2016). Moreover, agricultural wastes represent an abundant, cheap lignocellulosic resource for bioethanol production. Global annual production of crop residues estimated at 2802–3758 million ton/year has a potential ethanol yield of 235–450 L/ton (Zabed *et al.* 2016). Furthermore, the purchase price in the range of USD 12.9 – 80/dry metric ton is competitive with petroleum on an energy basis even at low crude prices (Lee and Lavoie 2013; Lynd *et al.* 2017; Liu and Bao 2019).

1.4.1 Composition of agricultural residues

All plant matter is made up of individual cells which are surrounded by a cell wall that serves to define the morphology of the plant, provide its structural support, and control the passage of water and nutrients (De Lorenzo *et al.* 2019). The plant cell wall consists of three layers, *viz*., middle lamella, primary cell wall and secondary cell wall, where each layer has its own unique structure and function. The chemical composition of these layers vary intra and interspecies depending on the plant species, varieties, age, climate and agronomic conditions (Doblin, Pettolino and Bacic 2010). The middle lamella is made up of pectic substances that cement the cell walls of two adjoining cells together. The primary cell wall is the polysaccharide-rich, thin and flexible layer that surrounds the plant cells (Chen *et al.* 2018). The secondary cell wall is formed within the primary cell wall when the cell is fully

grown and is highly lignified, giving the plant more rigidity (Kumar, Campbell and Turner 2015; Sorieul *et al.* 2016). The cell wall polysaccharides include cellulose, hemicellulose and pectin. However, cellulose and hemicellulose are the main polysaccharides of interest for bioethanol production. The summative chemical composition of cellulosic biomass usually comprises cellulose, hemicellulose, lignin, ash and extractive composition and is reported on a dry basis. Typically, most of the agricultural residues are comprised of about 30–60% cellulose, 20–30% hemicellulose and 10–25% lignin (Table 1.2).

Table 1.2: General composition of agricultural residues.

Agricultural residue	Cellulose	Hemicellulose	Lignin	Ash	Extractives	Reference
Corn cobs	30	34	18	2	1 16	(Xu *et al.* 2017b)
Corn stover	30	26	11	5.2	28	(Xu *et al.* 2017b)
Wheat straw	34	20	20	[1]ND	ND	(Zheng *et al.* 2018)
Rice straw	32	36	24	19	10	(Lim *et al.* 2012)
Rice Husk	29	29	22	20	18	(Lim *et al.* 2012)
Sorghum stalk	38	3	10	3	16	(Xu *et al.* 2017b)
Sweet sorghum bagasse	37	18	20	ND	ND	(Umagiliyage *et al.* 2015)
Sorghum stalk	42	23	18	ND	ND	(Theerarattananoon *et al.* 2012)
Barley straw	33	25	16	8	14	(García-Aparicio *et al.* 2006)
Cassava peels	14	27	11	ND	ND	(Adekunle, Orsat and Raghavan 2016)
Potato peels	8	7	33	7.5	ND	(Hijosa-Valsero, Paniagua-García and Díez-Antolínez 2018)
Spent ground coffee	9	37	ND	2	ND	(Janissen and Huynh 2018)
Cowpea haulm	10 – 26	17 – 19	10.7 – 21	3 – 9	ND	(Anele *et al.* 2012)
Soybean hull	29	20	13	0.2	ND	(Qing *et al.* 2017)
Soybean straw	42	17	22	0.2	ND	(Qing *et al.* 2017)
Sugar cane bagasse	39	28	18	9	3	(Guilherme *et al.* 2015)
Sugarcane tops	38	26	13	ND	ND	(Sherpa, Ghangrekar and Banerjee 2018)
Empty fruit bunch	37	25	22	1	14	(Yang *et al.* 2017)
Cotton gin waste	40	30	25	11	8	(Hamawand *et al.* 2016)

[1] ND – Not determined

1.4.1.1 Cellulose

Cellulose $(C_6H_{10}O_5)n$ is the most abundant polysaccharide on earth with approximately 10^{10} – 10^{11} tons produced annually (Suhas *et al.* 2016; Li *et al.* 2018c). It is formed in the primary and secondary cell walls of plants as the main structural component (Li, Logan Bashline and Gu 2014). Cellulose is a linear, syndiotactic, homopolymer of repeating cellobiose units (a dimer of D-anhydro glucopyranose units (AGU), combined together by β-(l-4)-glycosidic bonds (Coseri 2017). Cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose) chains aggregate by numerous strong intermolecular hydrogen bonds between hydroxyl groups of adjacent macromolecules, forming cellulose microfibrils (García *et al.* 2016). The total number of the repeating structural units of cellobiose gives the degree of polymerization (DP). The DP of cellulose varies from 200 to 44,000, depending on the cellulose source and the isolation/purification method (Nechyporchuk, Belgacem and Bras 2016). Each glucopyranose unit has six carbon atoms with three hydroxyl groups (at C_2, C_3, and C_6 atoms). There are three types of glucopyranose units: (i) a reducing end with free hemiacetal or aldehyde group at C1 atom, (ii) a non-reducing end with free alcohol group at C4 atom, and (iii) internal ring (Nechyporchuk, Belgacem and Bras 2016) (Figure 1.4). The ability of the hydroxyl groups to form strong hydrogen bonds is the main reason for the chirality, and the semi-crystalline hierarchical organization (a highly ordered crystalline domain with strong molecular bonding separated by amorphous, less ordered ones) within the cellulose microfibril (Coseri 2017). Crystalline cellulose domains are insoluble in water, most common organic solvents, and confers recalcitrance to hydrolysis with acids or other chemicals even at higher temperatures, whereas amorphous regions are the potential points for chemical and biochemical attacks (García *et al.* 2016). The degree of crystallinity of cellulose depends on its origin and pretreatment, usually ranging between 40 and 60% (Alemdar and Sain 2008).

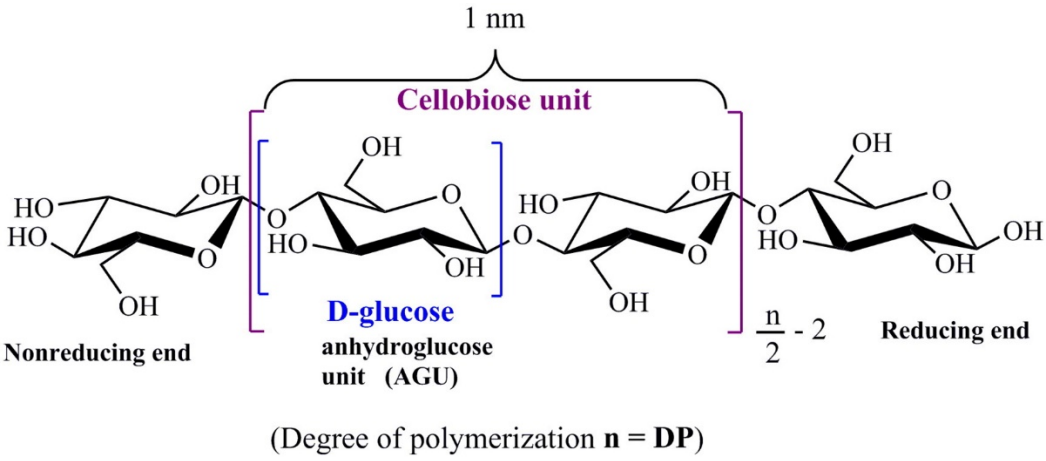

Figure 1.4: Structure of cellulose (Coseri 2017)

1.4.1.2 Hemicellulose

Hemicellulose is a branched hetero-polymer consisting of xylan, mannan, galactan and arabinan polymers which can be further broken down to hexoses (D-mannose, D-glucose, and D-galactose), pentoses (D-xylose and L-arabinose), and sugar acids (D-glucuronic acid) (Naidu, Hlangothi and John 2018). They have lower molecular weights compared to cellulose and branches with short lateral chains that are easily hydrolysed. Xylan is the dominant hemicellulose found in agricultural residues (Hendriks and Zeeman 2009). In many plants, xylans are heteropolysaccharides with backbone chains of 1,4-linked β -D-xylopyranose units. In addition to xylose, xylan may contain arabinose, glucuronic acid, or its 4-*O*-methyl ether, acetic acid, ferulic and p-coumaric acids (Pauly and Keegstra 2016).

1.4.1.3 Lignin

Lignin is an amorphous, highly branched, complex polyphenolic polymer composed of guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units primarily synthesized by the polymerization of phenylpropanoid units such as coniferyl, sinapyl, and *p*-coumaryl alcohol (Pu *et al.* 2013b) (Figure 1.5). During the lignification process, these monolignols produce a complex three-dimensional amorphous lignin polymer *via* carbon-carbon and carbon-oxygen (β-O-4, α-O-4, β-5, β-1, 5-5, 4-O-5 and β–β) linkages which lack the regular and ordered repeating units found in other polymers such as cellulose and proteins (Buranov and Mazza 2008). This biosynthesis process consists of mainly radical coupling and creates a

unique lignin polymer in each plant species (Liu, Luo and Zheng 2018). Lignin in herbaceous crops typically contains all the three types of monolignol units, with peripheral groups (i.e., hydroxycinnamic acids) incorporating into its core structure (Monteil-Rivera *et al.* 2013). On the other hand, softwood lignin consists solely of coniferyl alcohol while hardwood lignin consists mainly of coniferyl alcohol and sinapyl alcohol (Mu *et al.* 2018).

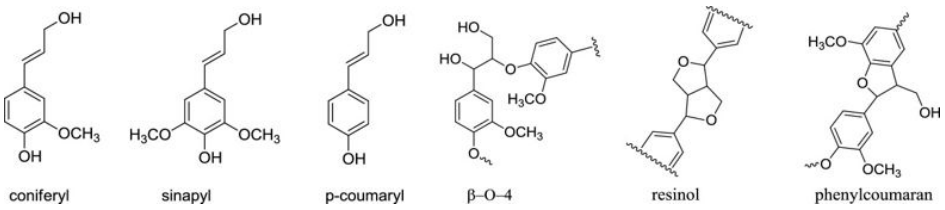

Figure 1.5: Typical phenylpropanoid precursors employed in the biosynthesis of lignin in plant biomass and some primary interunit linkages in lignin macromolecules (Pu *et al.* 2013a).

1.4.2 Bioethanol potential of crop residues

The potential quantity of residues generated each year for a given crop is mainly dependent on three critical factors: (1) quantity of the crop produced, (2) dry residue to crop ratio, and (3) cultivar and/or variety. Among the three factors, the quantity of the crop produced has the greatest influence on the total quantity of residues generated (Go *et al.* 2019). Globally, rice straw, wheat straw, corn straw, and sugarcane bagasse are the major agricultural wastes used in terms of the quantity of available biomass (Saini, Saini and Tewari 2015) (Table 1.3). In addition, the mass of the cell wall polysaccharides (cellulose and hemicellulose) also influence the bioethanol potential of a crop. This is because cell wall polysaccharides are the exclusive substrate for cellulosic ethanol and together with the quantity of available biomass, determine the potential bioethanol yield from a feedstock.

Table 1.3: Global crop production, their respective residues and theoretical ethanol yield (Pandiyan *et al.* 2019).

Crops	**Residue/crop ratio**	**Annual production (MT)**	**Total available residue (MT)**	**Ethanol yield* (L/Kg dry biomass)**	**Ethanol yield (gal/MT dry biomass)**
Rice (straw)	1–1.5	738	738–1107	0.48	109.9
Wheat (straw)	1.3	711	924	0.40	104.5
Maize (stover)	1.0	1017	1017	0.46	113.0
Sugarcane (bagasse)	0.6	1898	1138.8	0.50	111.5
Millets (stalk)	1.2	24.5	29.5	0.44	114.5
Cotton (stalk)	3.5	70	245	ND	–
Pigeon pea (stalk)	3.5	4.5	15.75	ND	–

1.5 The cellulosic ethanol production process

Although cellulosic biomass is a good source of fermentable sugars, it is a highly recalcitrant feedstock, with complex characteristics to protect its carbohydrates from degradation by micro-organisms or enzymes (Yao *et al.* 2018). Several factors have been implicated in the recalcitrance of cellulosic biomass including cellulose crystallinity, cellulose degree of polymerization, complex carbohydrate – carbohydrate and carbohydrate – lignin linkages, the degree of lignification and composition of lignin, cell wall specific surface area, acetylation of hemicellulose etc. (Buranov and Mazza 2008; Zhao, Zhang and Liu 2012; McCann and Carpita 2015; Li *et al.* 2018b). Hence, cellulosic biomasses are first subjected to a pretreatment stage prior to saccharification and fermentation. Bioethanol production from agricultural residues consists of four major unit operations: pretreatment, hydrolysis, fermentation, and recovery.

1.5.1 Pretreatment

Pretreatment of lignocellulosic biomass is the first critical control step for cellulosic ethanol production. It is required to alter the biomass size and structure thereby improving access to hydrolytic enzymes (Mosier *et al.* 2005). It is considered critical because it impacts cellulose digestibility and down-stream process costs from the removal of inhibitory products, enzyme loading, water use, waste treatment demands, amongst others and accounts for up to 40% of the total processing costs (Sindhu, Binod and Pandey 2016). Depending on the pretreatment regime, the hydrolysis of pretreated agricultural residues can yield up to 90% of the total sugars compared to untreated feedstock which yields no more than 30% (Gaur *et al.* 2016). With every different feedstock used for bioethanol production, the selection of pretreatment methods vary depending on their distinct chemical composition and physical structure (DeMartini *et al.* 2013). Factors such as cellulose crystallinity, lignin content, cell wall porosity, hemicellulose side chain branching and crosslinking are critical in choosing the pretreatment method. For instance, enhanced digestibility of steam pretreated agricultural residues was strongly correlated to hemicellulose solubilization (Chandra, Arantes and Saddler 2015). On the other hand, the efficiency of alkali pretreatment was correlated with its action on lignin (Yan *et al.* 2015). The mode of action, reaction conditions and outcomes of the pretreatment processes vary significantly from each other, each presenting some advantages and disadvantages with respect to their techno-economic and process conditions (Hassan, Williams and Jaiswal 2018). Hence, no "best" option exists as research and development efforts target continuous improvement in performance and reduce cost. Zabed *et al.* (2016) highlighted some general criteria, an effective pretreatment method should meet. They include: (1) Effective alteration of the lignocellulosic complex, enabling maximum polysaccharides recovery with minimum damage and high digestibility to enzymes or other treatments applied in the subsequent saccharification step; (2) minimal loss of sugars and degradation to sugar derived inhibitors; (3) minimum size-reduction requirements prior to pretreatment of the biomass; (4) low capital and operational cost requirements; (5) low operational energy requirements; (6) high lignin recovery for valorization; (7) high recovery of digestible and fermentable solids; (8) capability to operate in reasonable batch size and moderate cost reactors.

Many pretreatment methods have been investigated on a wide range of agricultural residues and are classified as physical (mechanical pretreatment, extrusion, irradiation, ultrasound

hydrodynamic cavitation and shock pretreatment), thermochemical (acids, bases, oxidants, organosolv, ozonolysis and ionic liquids), physicochemical methods (ammonia-based methods, steam explosion, wet oxidation, liquid hot water, hydrothermal, CO_2 explosion) and biological (use of microbes) (Chen *et al.* 2017; Kumari and Singh 2018). Extensive reviews on the various pretreatment methods are available in literature (Agbor *et al.* 2011; Gu 2013; Duque *et al.* 2016; Kim, Lee and Kim 2016; Loow *et al.* 2016; Sindhu, Binod and Pandey 2016; Travaini *et al.* 2016; Zhang, Pei and Wang 2016; Zhuang *et al.* 2016; Loow *et al.* 2017; Halder *et al.* 2019; Sharma, Xu and Qin 2019). Brief description of pretreatment methods employed in this study including comminution, dilute acid and deep eutectic solvent pretreatment are further provided.

1.5.1.1 Comminution

Comminution involves applying the forces of compression, impact, shearing and abrasion/attrition on the biomass (Mayer-Laigle *et al.* 2018a). The associated effects of comminution on the lignocellulose structure include; (a) increase in biomass surface area due to particle size reduction; (b) the degradation of lignin-carbohydrate complex (LCC) linkages; (c) cleavage of β-O-4′ linkages in lignin and β-1,4 glycosidic bonds in cellulose; (d) reduction in cellulose crystallinity and degree of polymerization; (e) decomposition of arabinoxylans (Liu *et al.* 2018a; Liu *et al.* 2019b). This leads to an improvement in the yield of subsequent processes (Ji *et al.* 2017; Dahunsi 2019). Common methods of comminution include milling and grinding (Mayer-Laigle *et al.* 2018a). Size reduction is an essential requirement for the effectiveness of other pretreatment methods (Kapoor *et al.* 2019), hence, comminution is commonly used prior to or in conjunction with other pretreatments (Araújo, Vilarinho and Machado 2019; Xie *et al.* 2020). The main advantages of comminution methods include improvement in biomass handling, hydrolysis yield and low production of inhibitors. However, comminution is energy-intensive and has low operational effectiveness in relation to energy input and cost (Mayer-Laigle *et al.* 2018a; Mayer-Laigle *et al.* 2018b).

1.5.1.2 Acid pretreatment

Dilute acid pretreatment (DAP) is one of the most popular methods used to obtain high sugar yields from lignocellulosic residues. It is commonly used prior to enzymatic hydrolysis of cellulose to glucose (Slathia *et al.* 2019). Several advantages associated with dilute acid pretreatment include low acid consumption, low enzyme requirements (Deshavath *et al.*

2017) and lower cost compared to other pretreatment methods due to higher sugar yield (Kazi *et al.* 2010; Baral and Shah 2017). Also, low acid concentration produces vital nutrients (P and S) that increase downstream fermentation (Mussatto and Roberto 2005; Haykiri-Acma and Yaman 2019). Typically, this pretreatment process is carried out by exposing the agricultural residues to low acid concentration (< 4 % w/w) at elevated temperatures (100 – 215°C) and pressure (2 – 10 atm) for specific residence time (5 – 150 min) in a reactor (Solarte-Toro *et al.* 2019). Dilute sulfuric acid has been the predominant choice for the established processes due to its effectiveness, low cost, and availability (Li *et al.* 2016a). However, other mineral acids including hydrochloric acid (Li *et al.* 2016b), phosphoric acid (Wu *et al.* 2018a), nitric acid (Skiba *et al.* 2017), and organic acids such as acetic, citric, formic, oxalic and maleic acid have been investigated (Amnuaycheewa *et al.* 2016; Saha *et al.* 2016; Rattanaporn *et al.* 2018). The use of mixed acids has also been reported (Zhang *et al.* 2012).

Dilute acids induce selective solubilization of hemicelluloses and lignin redistribution within tissues, that together, facilitates the loosening of cell wall structure and increases cell porosity and cellulose accessibility (Donohoe *et al.* 2008; Pingali *et al.* 2010; Ji *et al.* 2015; Ji *et al.* 2016; Pingali *et al.* 2016). This results in a highly digestible, cellulose enriched biomass solids in addition to a liquid fraction containing most of the hemicellulose sugars in monomeric or oligomeric forms (Díaz-Blanco et al., 2018). The hydrolysis of polysaccharide in a dilute-acid medium is complex because the substrate is in a solid phase while the acid catalyst is in a liquid phase. Nonetheless, the hydrolysis mechanism involves several steps (Aguilar *et al.* 2002). These include: (1) dissociation of acid to form protons (H^+) and diffusion into the lignocellulosic matrix; (2) protonation of the oxygen of a heterocyclic ether bond between the sugar monomers; (3) cleavage of the ether bond; (4) breakdown of the conjugate acid to a carbocation as intermediate; (5) solvation of the carbocation with water; (6) liberation of the proton and free sugar monomer, oligomer or polymer depending on the position of the ether bond; (7) diffusion of the reaction products in the liquid phase depending on the form and size. Due to its heterogeneous nature, the solubilization of hemicellulose results in the formation of a variety of pentoses (xylose, arabinose, mannose) and hexoses (glucose and galactose). However, since xylan is the main hemicellulose in softwoods and grasses, xylose is the predominant sugar obtained in the acid hydrolysate (Gaur *et al.* 2016). Amorphous portions of cellulose are also cleaved into glucose. As the

hydrolysis continues, the free sugars released can be dehydrated to other compounds such as furans and aliphatic acids in addition to phenolic compounds released from partial lignin solubilization. The individual and synergistic interaction of these degradation products have inhibitory effects on enzymatic hydrolysis and fermentation process (Zhai, Hu and Saddler 2018). This leads to higher costs associated with sugar loss and detoxification strategies (Jönsson and Martín 2016).

1.5.1.3 Deep eutectic solvents pretreatment

Deep eutectic solvents (DESs) are nascent green solvents that have physical and chemical properties comparable to ionic liquids (Xu *et al.* 2017a). However, they are preferred over conventional ionic liquids because they are easy to synthesize, cheaper, recoverable, environmental-friendly, recyclable, highly tuneable and have high solubility. It is estimated that DES costs only 20% of an ionic liquid (Satlewal *et al.* 2018). DES are eutectic mixtures prepared by complexating hydrogen bonding donors (HBDs, usually a quaternary ammonium salt) and hydrogen bonding acceptors (HBAs, usually a metal salt) at specific molar ratio (Zdanowicz, Wilpiszewska and Spychaj 2018). DESs contain large, nonsymmetric ions that have low lattice energy and hence much lower melting point than its individual components (HBD and HBA) due to charge delocalization occurring through strong hydrogen-bonding interaction between HBD and HBA (Smith, Abbott and Ryder 2014). The versatility of DES has fuelled research into a wide range of industrial application including biomass pretreatment and biocatalysis (Liu *et al.* 2018b; Pätzold *et al.* 2019)

Several DES have been evaluated as solvents for biomass pretreatment. ChCl is the most studied HBA, although others including betaine, proline, etc., have also been studied for DES pretreatment of biomass. On the other hand, several HBDs have been investigated including acidic (lactic, formic, acetic, citric, malonic etc.), basic (ethylene glycol, imidazole, urea,) and neutral (glycerol) HBDs. Studies indicate that DES is effective in solubilisation of lignin and hemicellulose leading to a reduction in recalcitrance, crystallinity and improvement in enzymatic hydrolysis (Satlewal *et al.* 2018; Shen *et al.* 2019). The solubilized lignin was also recovered by the addition of an antisolvent enabling full utilization of the biomass (Chen, Reznicek and Wan 2018). One key disadvantage in DES pretreatment of biomass is the long pretreatment time, which is uneconomical for industrial applications (Procentese *et al.* 2015a; Zhang, Xia and Ma 2016; Procentese and Rehmann

2018). The use of alternative heating such as microwaves could reduce the pretreatment time as compared to conventional heating (Chen and Wan 2018).

1.5.2 Hydrolysis

The microbes involved in the bioconversion of sugars to ethanol are only able to utilize simple sugars, hence a saccharification step is required (Kumari and Singhal 2019). Saccharification is the second critical step for bioethanol production and involves further depolymerisation of cellulose and hemicelluloses in the pretreated solid into hexose and pentose sugars respectively, for subsequent ethanol fermentation. This stage is considered critical because the quality of hydrolysate will affect the subsequent fermentation process and consequently the quality of the ethanol produced. Although hydrolysis can be done using acids (Khawla *et al.* 2014; Kumar *et al.* 2015; Łukajtis *et al.* 2018) or enzymes (Guilherme *et al.* 2015; Kouteu Nanssou, Jiokap Nono and Kapseu 2016; Qing *et al.* 2017; Yang *et al.* 2017; Amini, Haritos and Tanksale 2018; Meinita *et al.* 2019), the latter is the dominant technology. Compared to acid hydrolysis, enzymatic hydrolysis requires less energy, milder operational conditions, simpler and less expensive reactor configuration (Binod *et al.* 2018). Furthermore, no inhibitors are produced during the saccharification process. However, enzyme systems are highly substrate-specific and contribute to the major cost of cellulosic ethanol production (Klein-Marcuschamer *et al.* 2012; Liu, Zhang and Bao 2016). Due to the complex nature of lignocellulosic biomass and the pretreatment employed, the complete deconstruction of lignocellulose to fermentable sugars by enzymes requires the concerted activities of cellulases, hemicellulases, lignases as well as various accessory enzymes and proteins (Binod *et al.* 2018). Hence, most of the multinational companies producing enzymes are now focused on the production of multi-enzyme complexes. Commercially available enzyme complex and their constituents have been described (Van Dyk and Pletschke 2012). Two main suppliers of commercial enzymes are Novozyme (Celluclast, Novozyme 188 etc.) and Genencor (Spezyme and Accellerase 1500 etc.).

1.5.2.1 Cellulases and Hemicellulases

Two major enzymes from the glycoside hydrolase (GH) family *viz*., cellulases and hemicellulases are involved in the saccharification of plant polysaccharides. The cellulases are enzymes which hydrolyse cellulose to glucose. Complete degradation of cellulose takes place by the synergistic action of three cellulases such as

endoglucanases, cellobiohydrolases and β-glucosidases (Sharma *et al.* 2016). Endoglucanases (EG, endo 1,4-D glucanohydrolase or endocellulases, EC 3.2.1.4), hydrolyse internal β-1, 4-glucosidic linkages randomly at amorphous sites in the cellulose chain while cellobiohydrolases (CBH, 1,4-β-D glucan cellobiohydrolase or exocellulases or exoglucanases, EC 3.2.1.91), cleaves the free ends of long chain oligosaccharides produced by the action of endoglucanases to cellobiase units, and finally cellobiose units are hydrolysed to glucose by β - glucosidases (BG, also known as β–glucoside glucohydrolases or cellobiases, EC 3.2.1.21) (Prasad *et al.* 2019).

Compared to cellulases, hemicellulase enzyme systems are more complex due to the heterogeneous nature of hemicellulose (Brigham, Adney and Himmel 2018). The complete breakdown takes place by the synergistic action of several hydrolytic enzymes including endo-1,4-β-D-xylanases, α-L-arabinofuranosidases, endo-1,4-β-D mannanases, β-mannosidases, acetyl xylan esterases, α-glucoronidases and α-galactosidases (Gupta and Verma 2015). The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyse glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyse ester linkages of acetate or ferulic acid side groups (Chadha, Rai and Mahajan 2019). Microorganisms with glycoside hydrolytic potential are distributed throughout the entire microbial kingdoms and include several bacterial and fungal species (Sharma *et al.* 2016). Among the various cellulolytic microbial strains, *Trichoderma* is one of the most well-studied cellulase and hemicellulase producing fungal strains. On the other hand, *Aspergillus* is a very efficient β-glucosidase producer (Passos, Pereira and Castro 2018). Nonetheless, high β-glucosidase production has been reported in other organisms including *Penicillium oxalicum* (Yao et al. 2016)*, Lichtheimia ramose* (Li *et al.* 2019a)*, Aspergillus saccharolyticus* (Lübeck and Lübeck 2018) etc.

1.5.2.2 Lignin degrading enzymes: Peroxidases and Laccases

Generally, oxidoreductases including lignin peroxidase (LiP, E.C. 1.11.1.7), manganese peroxidase (MnP, E.C. 1.11.1.7), versatile peroxidase (VP), dye-decolorizing peroxidase (DyP) and laccase are known to depolymerize lignin (Kamimura *et al.* 2019). Oxidoreductases generally utilize low-molecular-weight oxidants, such as veratryl alcohol, Mn^{2+}, and unsaturated lipids as diffusible redox mediators for the delignification of lignocellulosics (Chen and Wan 2017). Lignolytic microbes include white-rot, brown-rot

and soft-rot fungi as well as strains of actinomycetes, α-proteobacteria, and γ-proteobacteria (Agarwal, Rana and Park 2018). Fungal pretreatment involving white-rot and brown-rot fungus on corn cob enhanced lignin extraction efficiency (62.3%) (You *et al.* 2019). The recovered lignin had less phenolic hydroxyl, methoxyl, tricin, ester-linked *p*-coumaric acid and more ratio of syringyl to guaiacyl units, carboxylic acid, β-O-4′ linkage and molecular weight. This led to an improvement in the thermal stability and an increase in the phenol and alkyl-phenol contents in pyrolysis products. A sequential 10-day inoculation of lignin degrading white-rot fungus *Ganoderma lobatum* and brown-rot fungus *Gloeophyllum trabeum* improved the enzymatic hydrolysis of wheat 2.8-fold higher than untreated wheat straw (Hermosilla *et al.* 2018).

1.5.2.3 Accessory enzymes

Accessory enzymes enhance the performance of enzymatic hydrolysis of cellulosic biomass, though they are not directly involved in cellulose hydrolysis. Common accessory enzymes and proteins include the lytic polysaccharide monooxygenases (LPMOs) and swollenins (Andberg, Penttilä and Saloheimo 2015; Forsberg *et al.* 2019). LPMOs are a class of copper enzymes recognized to play major roles in biomass degradation. LPMOs use an active site copper ion to activate oxygen and oxidize polysaccharides (Tandrup et al. 2018). Swollenin is a helper protein that is associated with a "loosening" effect on the cellulosic network within plant cell walls (Binod *et al.* 2019).

1.5.2.4 Process factors affecting enzymatic hydrolysis

Various process conditions have been shown to influence the hydrolysis rate and yield of sugars from cellulosic biomass. These factors include temperature, pH, mixing rate, substrate concentration, enzyme loading, time and surfactant addition (Mussatto *et al.* 2008; Ferreira *et al.* 2009; Ruangmee and Sangwichien 2013; Verardi *et al.* 2016; Kapoor *et al.* 2019). For most studies, enzymatic hydrolysis was conducted under the following conditions: temperature (40 to 55°C), pH (4 to 5), agitation (100 to 200 rpm) and time (24 to 96 h). However, enzyme loadings varied significantly depending on the enzyme type and concentration of substrates (Table 1.4). During enzymatic treatment, antimicrobials such as sodium azide, tetracycline and cycloheximide were added to inhibit microbial growth and consumption of glucose (Resch, Baker and Decker 2015).

Biomass loading is one of the critical factors affecting enzymatic hydrolysis. Although most laboratory-scale investigations were carried out at low solid loading (< 15%), profitable industrial-scale hydrolysis will require high solid loading. Recently, several studies have focused on carrying out enzymatic hydrolysis at high biomass loading because of its attractive economic prospects (Chen and Liu 2017; Chi *et al.* 2019). However, biomass loading affects the mixing of biomass with the enzyme due to viscosity (Fockink *et al.* 2016). Geng *et al.* (2015) presented three strategies to achieve maximum carbohydrate conversion during high solid loading-enzymatic hydrolysis. These strategies include fed-batch, splitting/thickening, and clarifier processes. Moreover, sugar loss and enzyme inhibition due to oligosaccharide accumulation have been reported (Xue *et al.* 2015).

Furthermore, studies reveal that the addition of surfactants improves enzymatic hydrolysis especially with biomass containing high lignin content (Li *et al.* 2016c). Surfactants block the interaction between lignin and enzymes through lignin modification or by blocking the adsorption sites of lignin (Agrawal *et al.* 2017). This prevents the enzymes from unproductive binding with lignin, enhancing the availability of reaction areas, and lowering the enzyme loading requirements. The enzymatic hydrolysis yield of acid pretreated bamboo residue improved from 24.3% to 71.9% by pre-adsorbing with 0.8 g/L amphiphilic surfactant obtained from rosin acid (Lin *et al.* 2019). Amphiphilic surfactants pre-adsorbed on acid pretreated bamboo residue reduced the hydrophobicity, adsorption affinity and adsorption capacity of lignin for cellulase from 0.51 L/g to 0.48–0.32 L/g, from 2.9 mL/mg to 1.8–1.4 mL/mg, and from 122.3 mg/g to 101.9–21.4 mg/g, respectively. Previous research has demonstrated that the addition of non-ionic surfactants such as Tween 20 and polyethylene glycol (PEG) to pretreated biomass significantly improved enzymatic digestibility (Parnthong, Kungsanant and Chavadej 2018; Li *et al.* 2019b; Zhu *et al.* 2019).

Table 1.4: Operational parameters reported for the enzymatic hydrolysis of crop residues.

Biomass type	Pretreatment	Enzyme type and activity	Hydrolysis conditions	Antimicrobial used and dosage	Key result	Reference
Corn cob	Deep eutectic solvent	Cellic CTec 2 enzymes (Novozyme) 166 FPU ml^{-1}	6% (w v^{-1}) dry matter/buffer 55°C, 0.1 M sodium citrate buffer (pH: 4.8), 180 rpm, 80 h	80 µl tetracycline and 60 µl cycloheximide	76% of the maximum theoretical yield	(Procentese *et al.* 2015b)
Soybean hull and straw	Dilute acid	N/A	solid loading of 2% (w/v), 30 FPU/g-DM, 50°C 0.05 M acetate buffer (pH 4.8), 160 rpm for 72 h	800 µg of 20 mg/mL tetracyc line	86.9 and 70.3% total carbohydrate recovery from soybean hull and straw respectively	(Qing *et al.* 2017)
Oil palm trunk	Acid pretreatment	20 FPU of celluclas1.5L (20 FPU) and 100 CBU of cellobiase,	0.25g of sample is mixed with 20 FPU of celluclas1.5L and 100 CBU of cellobiose, 45°C, 20 mL of 50 mM sodium citrate buffer (pH 4.7)	200 ul of 2M sodium azide	144 mg sugar /g pretreated biomass	(Rattanaporn *et al.* 2018)
Agave lechuguilla	Acid	Cellic CTec3, Fungal β-glucosidase	5% (w/v) substrate loading, Fungal β-glucosidase enzyme loading of 15 IU/g pretreated solid, Cellic CTec3, enzyme load of 15 FPU/g pretreated solid. 0.05 M sodium citrate buffer (pH 4.8), 150 rpm for 72 h.	[2]N/A	68 g glucose/100 g glucose in raw agave	(Díaz-Blanco *et al.* 2018)
Empty fruit bunch		Accellerase® 1500 (37 FPU/Ml) or Accellerase® XC (2778 U/Ml)	25.0 mL of sodium citrate buffer (pH 4.8, 50.0 mM), 1250 mg of the sample and 25.0×10^{-2} mL of the enzyme Accellerase® 1500 or Accellerase® XC, 45°C, 100 rpm) for 48 h.[3]	N/A	Sugar yield was nearly 630 mg glucose/g original dry EFB biomass	(Palamae *et al.* 2017)

[2] NA – Not available

1.5.3 Fermentation and recovery of bioethanol

Fermentation is the third critical process in cellulosic ethanol production, where the ethanol is directly produced by the metabolic activity of the fermentation agent. The pretreatment and enzymatic hydrolysates contain sugar which is "food" for the specific fermentation agent (yeast or bacteria) to digest according to its ability, leaving ethanol compound as the product. Theoretically, each kg of glucose or xylose can produce 0.49 kg of carbon dioxide and 0.51 kg of ethanol (Rijal *et al.* 2016). Considering the heterogeneous nature of the cell wall sugars and the effect of the pretreatment and saccharification process, the hydrolysate usually consists of fractions of different monomers and several oligosaccharides with probable inhibitors or indigestible substances (Yang *et al.* 2019). Hence, no single fermentation agent (yeast or bacteria) is considered ideal. For a commercially viable ethanol production method, an ideal microorganism should have broad substrate utilization, high ethanol yield and productivity, should have the ability to withstand high concentrations of ethanol and high temperature, should be tolerant to inhibitors present in hydrolysate and have cellulolytic activity (Liu *et al.* 2019a). There are several wild types and recombinant ethanologens reported in the literature (Radecka *et al.* 2015; Benarji and Ayyanna 2016). Nonetheless, the most known ethanologens include the hexose fermenting yeast, *Saccharomyces cerevisiae* and bacteria *Zymomonas mobilis* as well as the pentose fermenting yeasts *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* (Karagoz, Bill and Ozkan 2019).

Bioethanol fermentation can be performed as a batch, fed-batch, cell recycle batch or continuous process (Mahboubi *et al.* 2017). The batch fermentation process is a simplistic, commonly used model for studying the fermentation activity of microbes. The batch process is a "closed system" in which the substrate and the inoculum are added at the beginning of the fermentation and until the fermentation is complete, nothing is added or removed from the reactor except in cases where pH and /or oxygen is regulated by the addition of acid/base or oxygen respectively (Rudolf *et al.* 2005; Phukoetphim *et al.* 2017). The process starts with a high sugar concentration at the beginning and ends with a high product concentration at the end (Khalseh *et al.* 2019). The reactor configuration for the batch system is less capital intensive, flexible and can be adjusted to suit product specifications and needs (Karagoz, Bill and Ozkan 2019). Labour skills required for the operation of a batch system are basic. Moreover, the production and sterilization errors only affect a batch such that losses can be

minimised. However, the presence of high sugar concentration in the fermentation medium creates an unfavourable osmotic environment for the microbes that lead to substrate inhibition and results in the inhibition of cell growth and low ethanol productivity (Thatipamala, Rohani and Hill 1992; Chang *et al.* 2018). Moreover, there is also a high downtime between batches, which includes charge and discharge of the bioreactor, cleaning, sterilization, and restart of the process (Gavahian *et al.* 2019).

The fed-batch is a hybrid between the continuous and batch process. Fed-batch process starts off as a batch mode with a small amount of biomass and substrate in the fermenter. At the peak of the log phase, the nutrients are fed into the reactor in a specific manner (by scheduled additions or continuously) and the products remain in the bioreactor until the end of the run (Nielsen *et al.* 2017). Fed-batch operation aids in regulating the concentration of compounds that control the key reaction rates which allow the conversion of a sufficient amount of fermentable sugars to ethanol thus providing a unique advantage over a batch or continuous operation (Phukoetphim *et al.* 2017; Chang *et al.* 2018). The advantages of this process include shortened fermentation time, higher productivity associated with a reduction of substrate inhibition and the toxic effects of the medium components, which are present at high concentrations (He *et al.* 2018). The application of cell recycling technology can contribute to a reduction in time and costs associated with inoculum preparation in batch and fed-batch processes.

In the continuous process, a bioreactor containing active microorganisms is continuously fed with the substrates, culture medium and nutrients at the same rate at which the fermentation products are taken continuously from the media, hence, the culture volume is constant (Shokrkar, Abbasabadi and Ebrahimi 2019). The advantages of continuous system over batch and fed-batch system are enhanced product consistency, minimal product loss and higher volumetric productivity. This reduces the reactor volume requirement, investment and operational costs (Kang *et al.* 2015; Zahed *et al.* 2016). At a high dilution rate, ethanol productivity is increased while ethanol yield is decreased due to incomplete substrate consumption by yeasts (Mahboubi *et al.* 2017). However, the possibility for contamination to occur is higher than other types of fermentation (Brexó and Sant'Ana 2017; Zhang *et al.* 2019). Moreover, the ability of yeasts to produce ethanol in a continuous process is reduced due to long cultivation time (Azhar *et al.* 2017).

In the conventional bioethanol fermentation strategy such as separate hydrolysis and fermentation (SHF), enzymatic hydrolysis is performed separately from the fermentation operation. In SHF, liquid streams from the pretreatment and hydrolysis reactors jointly flow into the fermentation reactors and are sequentially fermented (Hemansi *et al.* 2018). The hexoses are first fermented by a hexose fermenting microbe such as *S. cerevisiae* and the ethanol produced is recovered. Then, the remaining pentose rich liquor is fermented using pentose fermenting yeast (e.g., *Pichia stipitis*) and ethanol is also recovered (Balat, Balat and Öz 2008). The major advantage of the SHF strategy is that each process can be operated at optimal conditions. However, the industrial feasibility of the strategy is low due to product inhibition and cost implications. Consequently, several integrated strategies have been adopted to enhance the performance of bioethanol fermentation. They include simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) (Ayodele, Alsaffar and Mustapa 2019). Comparative studies between SHF and SSF indicate that SSF showed higher ethanol titre, yield and productivity compared to SHF (Dahnum *et al.* 2015; Nguyen, Yang and Bae 2017; Guerrero, Ballesteros and Ballesteros 2018). In SSF, the sugar is fermented to ethanol as soon as it is hydrolysed, thus, the concentration is kept low. This addresses the problem of substrate inhibition associated with SHF and reduces the fermentation time and enzyme load (Mithra *et al.* 2018). However, the optimal temperature for cellulose hydrolysis (40 to 50°C) is higher than the optimum for ethanol fermentation (20 to 35°C) (Gonçalves *et al.* 2016b) creating a major drawback to this strategy. The application of a pre-hydrolysis stage tends to improve the SSF performance parameters (Gonçalves *et al.* 2016b). On the other hand, co-fermentation is a beneficial strategy for improving the SHF and SSF performance (Novy, Longus and Nidetzky 2015; Kamoldeen *et al.* 2017). Various factors have been reported to affect the efficiency of hydrolysis and fermentation of the biomass, which are mostly related to substrate, enzyme, microorganism, and process parameters. They include temperature, pH, initial substrate concentration, inoculum size, fermentation time, agitation rate and the accumulation of soluble by-products in the fermentation medium (Mohd Azhar *et al.* 2017; Zabed *et al.* 2017). An array of agricultural residues has been investigated as feedstock for bioethanol production using various ethanologens, operational modes, fermentation strategy, and outcome. The performance of ethanol production was often reported in terms of ethanol concentration, productivity and yield (Fernandes *et al.* 2018).

Popping pretreated mixed residues was subjected to simultaneous saccharification and fermentation (pH 4.8, 37°C, 200 rpm for 96 h) using *S. cerevisiae* KCTC 7906 and resulted in 77.3% ethanol yield (Nguyen, Yang and Bae 2017). Steam pretreated wheat straw was fermented with *S. cerevisiae* TMB3400 in fed-batch mode, using the simultaneous hydrolysis and co-fermentation strategy (SHCF, 32°C, 300 rpm, 120 h, 7.5% WIS) and 53.3 g/L of ethanol was produced (Mohd Azhar *et al.* 2017). From previous reports, it is evident that most of the fermentation process was carried out at 30°C, except for *Kluyveromyces marxianus* performed at 40°C (Wu *et al.* 2016). In addition, the studies were usually carried out for 24 to 72 h at pH 4.5 to 5.5 with an agitation rate of 120 to 200 rpm using various sugar concentrations and inoculum sizes (5 to 10%).

Finally, the ethanol solution resulting from the fermentation process needs to undergo further processing to remove the water content, giving anhydrous ethanol. The removal of water content is generally carried out by distillation which involves utilising the difference of boiling points of the mixtures in a solution. When the mixture is heated to the ethanol boiling point (78.2°C), ethanol in the mixture will be vaporised and separated from the other components in solution (Gavahian *et al.* 2019). Anhydrous ethanol holds a minimum of 99.5% of ethanol by volume, with the water content strictly no more than 0.5% by volume. The existing distillation techniques to produce anhydrous ethanol include (i) adsorption process, (ii) azeotropic distillation, (iii) chemical dehydration, (iv) diffusion distillation, (v) extractive distillation, (vi) membrane process, and (vii) vacuum distillation (Aditiya *et al.* 2016).

In view of the global efforts to produce bioethanol from locally available lignocellulosic residues as an approach to sustainable energy security, bambara groundnut and cowpea haulm were investigated in this study as potential bioethanol feedstock.

1.6 Aim and objectives of the study

To investigate the potential of bambara groundnut and cowpea haulm as feedstock for bioethanol production.

The following objectives were carried out:

1. Compositional and structural characterization of bambara and cowpea haulm to assess their feasibility as a substrate for bioethanol production
2. Dilute acid pretreatment of cowpea haulm for enhanced biosugar recovery and bioethanol fermentation
3. Deep eutectic solvent pretreatment of bambara haulm for fermentable sugar production and bioethanol fermentation

2 CHAPTER TWO
PHYSICOCHEMICAL CHARACTERIZATION OF BAMBARA AND COWPEA HAULM FOR POTENTIAL VALORIZATION

2.1 Introduction

In recent years, globally, efforts are underway to produce industrial raw materials, chemicals and fuel from biomass in a sustainable manner in order to meet the rising demand. Amongst other descriptions, sustainability entails the efficient exploitation of variable feedstock and its components within a region to produce high-value products. In view of this, the "biorefinery concept" is an interesting platform for the sustainable utilization of biomass resources, ensuring the conversion of multiple components of a feedstock into value-added marketable products such as biofuels, industrial chemicals and products (Cherubini 2010). Agricultural residues constitute an important category of feedstock with high application potential in biorefineries. They are abundant, cheap, renewable and include roots, stalks, leaves, bark, straw residues, seeds, wood residues, animal residues etc., produced by agri-food industries in their daily operations (Forster-Carneiro *et al.* 2013). Agricultural residues are primarily composed of cellulose, hemicellulose and lignin, although other constituents such as lipids, starch, proteins, amino acids, minerals, polar and non-polar extracts, non-structural sugars are also present in variable concentration thereby ensuring a viable repository of convertible products for a sustainable biorefinery (Deshavath, Veeranki and Goud 2019). However, wide variation in the type and composition of agro-residues creates the need for constant exploration of locally available residues. Furthermore, the characterization of biomass is also necessary to assess their potential applicability since the characteristics of a feedstock may affect the performance of the process in which it is used (Ramesh *et al.* 2019). Characterization also aids in efficient process design, feedstock preparation and handling. Accurate compositional analysis of lignocellulosic biomass enables the evaluation of conversion yields and process economics, particularly in biorefinery processes.

Numerous studies that evaluate the potential of several agricultural residues as biorefinery feedstocks are available (García, González Alriols and Labidi 2014; Sanchez *et al.* 2017;

Sillero *et al.* 2019). However, except for soybean, very little attention has been given to legume residues as feedstock in a biorefinery (Jensen *et al.* 2012), meanwhile, legume residues could also be potentially viable biorefinery feedstocks. It has been shown previously that the lignocellulosic component of soybean (straw and hulls) was compared favourably with commonly used cereal residues like wheat straw (Alemdar and Sain 2008; Reddy and Yang 2009). Faba beans residual biomass was characterized by a high polysaccharide (48.8 to 56.5%) and low lignin content (13.1 to 14.4%) (Alkhtib *et al.* 2016). Moreover, legume residues could contain constituents other than carbohydrates that may be relevant in a biorefinery concept (Jensen *et al.* 2012). For instance, protein-derived amino acids in legume residues could be used as feedstocks in the production of bio-based chemicals (Lammens *et al.* 2012).

Bambara groundnut and cowpea are important pulses grown in semi-arid tropics of Africa, Asia, Central and South America (Baptista *et al.* 2017). These pulses are highly nutritive, drought-tolerant and contribute to soil fertility through nitrogen fixation which has led to their increased production (Cleasby, Massawe and Symonds 2016). Post-harvest generates large quantities of residues referred to as "haulms", which are under-researched and underutilized. Information on the compositional characteristics of bambara groundnut haulm (BGH) and cowpea haulm (CH) is limited. Hence, this study investigated the compositional characteristics of BGH and CH to evaluate their potential as biorefinery feedstock.

2.2 Materials and methods

2.2.1 Feedstock provenance and preparation

Bambara groundnut (*Vigna subterranea* L. Verdc) and cowpea (*Vigna unguiculata*) of the brown crowder genotype was obtained from the South African Agricultural Research Council (ARC) gene bank and planted at the Roodeplaat research farm of the Vegetable and Ornamental Plants. Agronomic management practices such as land clearing, land preparation, irrigation and weeding were carried out during the cropping season. Weeds were controlled manually. No fertilizer was added; however, the field was irrigated thrice a week, as necessary, to avoid stress. The pods were harvested after 20 weeks and the residues including the leaves, stalks and stem referred to hereafter as haulms were collected, washed several times with tap water followed by distilled water to remove debris, and dried for 48 h

at 30°C in a drier. The dried haulms were milled using a hammer mill followed by a laboratory blender (MRC SM – 450L) and sieved to a particle size of ≤ 0.5 mm (Universal Test Sieve -117547, South Africa). Milled BGH and CH were stored at room temperature in airtight plastic containers.

2.2.2 Characterization of BGH and CH

BGH and CH biomass samples were characterized for their chemical, thermal, nutritional and morphological properties. The compositional results for both BGH and CH biomass were obtained from three independent replicates and mean data was reported. Results are expressed as weight percentages on a dry weight basis (% w/w db).

2.2.3 Calorific value, ultimate and proximate analyses

The proximate composition of BGH and CH including moisture (Sluiter *et al.* 2008a), ash (Sluiter *et al.* 2008b), volatile matter (ASTM 2007) and fixed carbon was determined using standard methods. The fixed carbon (FC) content was calculated with the empirical formula as given below (Eq. 1):

FC = 100 - % (Ash + Volatiles) (Equation 1)

The ultimate analysis was performed to determine the elemental composition of BGH and CH. The proportion of carbon (C), hydrogen (H), nitrogen (N) and sulphur was measured using an elemental analyser (Thermo Scientific Flash 2000 CHNS/O Analyser, USA) and oxygen was determined by difference. The atomic ratios O/C, H/C and N/C was calculated from the empirical formula of the biomasses (Méndez *et al.* 2019).

Higher heating value (HHV) in BGH and CH was determined experimentally following standard protocol using a bomb calorimeter (DryCal modular calorimeter, USA) and theoretically using the modified Dulong's formula (Nanda *et al.* 2013) (Eq. 2).

HHV (MJ/Kg) = (33.5 * wt. % C + 142.3 * wt. % H - 33.5 * wt. % O) / 100 (Equation 2)

2.2.4 Nutritional composition

The crude lipid mass fraction was determined using the AOAC method (Horwitz 2000). Protein mass fraction was determined by multiplying the nitrogen mass fraction (N) with a

conversion factor of 6.25. The total carbohydrate was calculated using the formula below (Felisberto *et al.* 2019).

Total carbohydrate (g/Kg DM) = 1000 – (Mass fraction mg/Kg DM of Moisture + Ash + Crude lipid + Crude protein)

The amino acid composition was determined using a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) fitted with a photodiode array (PDA) detector after derivatization with 6-aminoquinolyl- N-hydroxysuccinimidyl carbamate (AQC). The sample/standard solution (1 µl) was injected into the mobile phase which conveyed the derivatized amino acids onto a Waters UltraTag C 18 column (2.1 x 50 mm x 1.7 µm) held at 60°C. Instrument control and data acquisition were performed by MassLynx software which integrates the peaks at the defined retention times and plots calibration curves for each amino acid based on the peak response (peak area/internal standard peak area) against concentration (Biancarosa *et al.* 2017)

2.2.5 Determination of mineral composition

Inorganic composition in BGH and CH ash was measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Shimadzu ICPE 9000, Japan). BGH and CH ash (1 g) was solubilized in dilute nitric acid and made up to a 100 ml volume. A standard sample containing metals *viz*., Mg, Al, Ca, P, K, Si, Na, Mn, Fe, Cu, Zn and Pb were used for the calibration (signal intensity vs. mass to charge ratio) of ICP-AES. A full scan m/z 40–250 was carried out for the quantification study (Cindrić *et al.* 2011).

2.2.6 Chemical summative composition

The chemical summative analyses of BGH and CH included the determination of polysaccharides, lignin, extractives and ash. The composition of cellulose, hemicellulose, and lignin was determined using the Van Soest and Wine method (Van Soest and Wine 1968). Extractives were obtained after successive Soxhlet extractions with dichloromethane : ethanol (100:100, v/v) and water (Milli-Q) until complete extraction was achieved with each solvent. The solvents were recovered, and the mass of extractives was calculated as the difference between the initial mass (7 g) of dry unextracted BGH and CH samples and the mass of the solid residue obtained after extraction and drying at 105°C, to constant weight.

2.2.7 Phytochemical profiling of extracts

The qualitative preliminary detection of alkaloids, cardiac glycosides, flavonoids, tannins, terpenoids, phlobatannins, saponins and steroids was carried out following the procedure described by Iqbal, Salim and Lim (2015). Tests specific for each class of secondary metabolites were based on the change in colour or formation of precipitates on the addition of specific reagents.

2.2.8 Thermogravimetric analysis

The thermal behaviour of BGH and CH was evaluated by Thermogravimetric/differential thermogravimetric (TG/DTG) analysis performed in a thermogravimetric analyser (TGA/DSC 1 Mettler Toledo, Switzerland) on samples (5 mg) under nitrogen, at a heating rate of 10°C min^{-1} from 25 to 700°C. The weight loss vs temperature data was analysed using the STAR^{e} 11.0x software (Silva *et al.* 2019)

2.2.9 Fourier Transform Infrared (FTIR) Spectroscopy of BGH and CH

FTIR was performed using Perkin Elmer Spectrum 100 (Waltham, MA, USA) with attenuated total reflectance (ATR) sampling accessory. The spectrum was recorded between 380 and 4000 cm^{-1}.

2.3 Results and discussion

2.3.1 Proximate composition

The proximate composition analysis of BGH and CH has shown that the moisture content in BGH (10.07 wt.%) was lower than CH (11.02 wt.%) (Table 7). Generally, moisture in herbaceous and agricultural biomass has been reported to vary from 4.4 to 47.9 wt.% (mean 12 wt.%) (Vassilev *et al.* 2010). The low moisture (≤ 15 wt.%) content of these biomass is a desirable characteristic for long term storage and thermochemical conversion processes. Moisture adds a counter-productive weight to biomass, reducing its thermal efficiency, pre-processing performance and increases susceptibility to microbial attack, consequently increasing process cost (García *et al.* 2014). BGH and CH showed high quantities of volatile matter (around 65 wt.% excluding moisture) and fixed carbon (16 to 17 wt.%) comparable to other biomass fuel (García *et al.* 2014). Chemical energy (which can be released *via* direct

or indirect combustion) is stored mainly in the form of fixed carbon and volatile matter in biomass (Wiselogel, Tyson and Johnson 2018). Fixed carbon (FC) indicates the mass of non-volatile organic matter present in biomass while volatile matter (VM) can be referred to as the fuel fraction of biomass (except moisture) released when it is heated at high temperature in the absence of air (Kumar, Upadhyay and Mishra 2019). VM in biomass is formed by the combustible fraction (light hydrocarbons, CO, H_2) and incombustible fraction (CO_2, SO_2, SO_3 NO_X), influencing the reactivity of the combustion process. Generally, biomass with high fixed carbon produces high biochar quantities. On the other hand, high volatile matter results in the production of high quantities of bio-oil and syngas *via* thermochemical processes (Vassilev *et al.* 2010). This indicates that these feedstocks are suitable for thermal conversion processes such as pyrolysis, combustion, and gasification. A higher yield of syngas and bio-oil compared to biochar may be expected due to the higher volatile matter than fixed carbon in BGH and CH. Ash is the inorganic material remaining after fixed carbon combustion. Ash in BGH (8.83 wt.%) is higher than CH (7.5 wt.%), although, within the range (1 – 20 wt.%) reported for herbaceous and agricultural biomass feedstock (Vassilev *et al.* 2010). Ash (≥ 3 wt.%) can lead to operational issues such as the formation of slag at higher temperatures during thermochemical conversion (Niu, Tan and Hui 2016), reduction in the higher heating value (Hansted *et al.* 2018) or enzyme inhibition during saccharification (Bin and Hongzhang 2010), which reduces the process efficiency and increases cost. Therefore, the ash in BGH and CH could limit their potential use on an "as-is" basis. This could be resolved by processes such as sieving, screening, thorough washing to remove water-soluble and loosely adhered inorganic materials, or a customized pretreatment method (e.g. use of chelating agent) prior to use (Edmunds *et al.* 2017). Nevertheless, it is well known that biomass ash contains significant amounts of water-soluble components of major and minor elements which are bioavailable. ICP-AES analysis showed a considerable amount of macro and microelements in the following order of relative abundance Ca>K>Mg>S>Si>P>Al>Fe>Sr>Na>Mn in BGH and K>Ca>P>Mg>S>P>Si>Na>Fe>Al>Sr>Mn in CH. Thus, the ash could be used in applications such as soil amelioration, construction, bio-absorbent production and for mineral recovery from the ash itself (Vassilev *et al.* 2013; Prasara-A and Gheewala 2017).

2.3.2 Ultimate composition and calorific value

The higher heating value (HHV) of a biomass is equivalent to the maximum energy contained in a biomass and is independent of the conversion process used (such as thermochemical, biochemical or mechanical processes); although, the form and amount of energy obtained will vary depending on the conversion process (Nanda *et al.* 2013). The HHV results showed consistency for both experimental and theoretical data. BGH had a higher HHV (18.68 MJ/Kg) than CH (17.15 MJ/Kg) (Table 2.1). The HHV in BGH and CH compares favourably with other biomass residues commonly used as biofuel feedstock such as wheat straw (17.8 MJ/Kg), sugarcane bagasse (17.98 MJ/Kg) (Kuan *et al.* 2013) and cornstalk (16.55 MJ/Kg) (Özyuğuran and Yaman 2017). Hence, BGH and CH could be used as substrates for biofuel production (Oyebanji *et al.* 2018).

Carbon, hydrogen, nitrogen, sulphur and oxygen (CHNS/O) are the major elements in a biomass material. CHNS/O composition influences the higher heating value, gas emission and ash composition of biomass fuels (Mishra and Mohanty 2018). While N and S are precursors of SO_x and NO_x gaseous emissions which are environmentally non-friendly; oxygen reduces the HHV of biomass fuels, increases the polarity and reduces the stability of pyrolysis products (Kumar, Upadhyay and Mishra 2019). The elemental mass fractions for both BGH and CH were similar, however, BGH exhibited slightly higher carbon with lower hydrogen, nitrogen and oxygen mass fractions (Table 2.1). Carbon was the most abundant element in BGH and CH, followed by oxygen, hydrogen and nitrogen, whereas, sulphur was below the detection limit. The absence of sulphur in BGH and CH implies that there will not be any SO_x emission during the conversion processes. The high nitrogen content in BGH (1.97 wt.%) and CH (2.08 wt.%) may be attributed to the high content of protein. The presence of nitrogen can result in NOx emissions during thermochemical processes occurring above 500ºC. Nonetheless, the elementary composition (wt.%) in BGH and CH is comparable to the classical values for herbaceous and agricultural biomass which are typically in the range of 42 –58 for C, 3 – 9 for H, 34 – 49 for O and 0.1 – 3.4 for N (Vassilev *et al.* 2010). The high oxygen content in BGH and CH indicates the need for a deoxygenation step to make BGH and CH better suited as solid fuels. The influence of the elemental composition on the HHV can be seen by correlating the calculated molar ratio with the HHV (Table 2.1). CH with a higher H/C, O/C ratio had lower HHV both experimentally and theoretically. This could be attributed to the fact that the H/C, O/C ratio are indices of the

degree of carbonation and aromaticity (Wang *et al.* 2019). The chemical energy in the various bonds are in the order, C – C bonds > C – H bonds > C – O bonds. Therefore, the lower the H/C, O/C ratio, the higher the HHV, as seen in BGH (Table 7). This correlation confirms the need for deoxygenation of BGH and CH prior to their utilization, to render them more suitable fuels. The N/C ratio of BGH (1:30) and CH (1:25) indicate the decomposability of these feedstocks which could be useful in composting and fermentative bioprocesses. Studies by Mao *et al.* (2019) reveal that the complicated microbial ecology system and microbial community changes of archaea and bacterial taxa were significantly correlated with the N:C ratio. An N/C ratio between 1:20 – 1:30 is required for microbial growth and degradation of organic substrates (Nakasaki et al. 1992).

Table 2.1: Proximate and ultimate composition of BGH and CH.

	Proximate analysis (% dry weight, w/w)				
	Moisture	**Ash**	**Volatile matter**	**Fixed carbon**	
BGH	10.04	9.15	77.70	13.15	
CH	11.22	7.58	76.16	16.26	
	Ultimate analysis (% dry weight, w/w)				
	Carbon	**Hydrogen**	**Nitrogen**	**Oxygen**	**Empirical formula**
BGH	50.50	6.27	1.97	41.26	$C_{30.17}O_{18.5}H_{44.65}N$
CH	49.48	6.36	2.08	42.08	$C_{28}O_{17.88}H_{43}N$
	Molar ratios and HHV(MJ/Kg)				
	H/C	O/C	N/C	HHV experimental	HHV calculated
BGH	1.48	0.61	0.03	18.68	17.97
CH	1.53	0.64	0.04	17.15	17.39

2.3.4 Chemical composition of BGH and CH

Cellulose, hemicellulose, lignin and extractive composition were 36%, 15%, 12% and 28% of dry matter, respectively in BGH and 35%, 16%, 10% and 31% of dry matter, respectively in CH. Compared to cereal straws such as rice, wheat, corn and oat (Tian, Zhao and Chen 2018), BGH and CH have comparable cellulose content and lower hemicellulose and lignin content. This is indicative that BGH and CH may be less recalcitrant than cereal straws. Cellulose and hemicellulose are complex sugar polymers that can be hydrolysed into monomers such as glucose, xylose and arabinose for saccharification bioprocesses. On the other hand, lignin acts as a protective covering thereby acting as a hindrance to enzymatic saccharification of cellulose and hemicellulose (Bichot *et al.* 2018). The polysaccharides make up the largest fraction in BGH and CH (51%) which is an indicator of their high potential as feedstocks for fermentative bioprocesses. In addition, the relatively low lignin content in BGH (12%) and CH (10%) indicate that these feedstocks might be more readily deconstructed into fermentable sugars than biomass from traditional herbaceous or woody feedstocks. Furthermore, both haulms had a high extractive composition (>20%) which can be utilized for the production of high-value biochemicals including food flavours, feeds, pharmaceuticals, cosmeceuticals, and nutraceutical, etc., using integrated processing techniques (Wang *et al.* 2011; Peng, Xue and Ohkoshi 2014; Tao *et al.* 2019). Aqueous extracts of BGH and CH were positive for reducing sugar and starch while the phytochemical screening of crude dichloromethane – ethanol extracts of BGH and CH revealed the presence of some secondary metabolites such as tannins, steroids, terpenoids, triterpenoids and cardiac glycosides (data not shown). The phytochemical compounds detected are known to have medicinal and industrial importance. For instance, tannins are the second most abundant source of natural aromatic biomolecules after lignin and can be used as an alternative feedstock for the expansion of chemical building blocks to develop bio-based polymers and materials (Arbenz and Avérous 2015). Tannins have also been reported to have antifungal, antioxidant and antidiarrheal effects (Bonelli *et al.* 2018; de Freitas *et al.* 2018; Molino *et al.* 2018; Olchowik-Grabarek *et al.* 2018). Similarly, other phytochemicals detected including steroids, cardiac glycosides and terpenoids have known therapeutic uses (Wadood *et al.* 2013). The carbohydrate fraction in the form of starch and reducing sugars in the aqueous extract could be converted to industrially important chemicals through fermentation (Smit and Huijgen 2017).

2.3.5 Thermogravimetric analysis

The thermal degradation profile of BGH and CH between 25°C and 700°C at a heating rate of 10°C/min showed a multi-stage decomposition followed by slow and continuous weight loss (Figure 2.1). The first significant weight loss, which corresponded to 16% in BGH and 12% in CH respectively, occurred at temperatures below 100°C, while the main devolatilization stage (49%) spanned from 209 – 379°C in BGH and 180 – 359°C in CH. The maximum rate of weight loss occurred between 330 – 350°C. Smaller peaks showing decomposition above 400°C could be attributed to lignin degradation. These results agree well with those obtained for other biomass such as pinewood, hazelnut and olive stone (Biagini, Barontini and Tognotti 2006; Pasangulapati *et al.* 2012). The first stage of devolatilization could be attributed to moisture evaporation, loss of adsorbed water and possibly degradation of light organic compounds (Aghamohammadi, Nik Sulaiman and Aroua 2011). In the second stage, a wide spectrum of volatile species was liberated due to the degradation of the essential polymeric fractions that make up lignocellulosic biomass (cellulose, hemicellulose, lignin and extractives). Generally, herbaceous annuals with low lignin content such as BGH and CH are more reactive considering that hemicellulose and cellulose decompose more rapidly. Yang *et al.* (2007) studied the pyrolysis characteristics of cellulose, hemicellulose and lignin. They reported weight loss of hemicellulose at 220 – 315°C and cellulose at 315 – 400°C, while lignin exhibited a more thermostable decomposition over a wide range of temperatures (160 – 900°C). The main devolatilization stage in CH started at a temperature of 29°C lower than BGH. This is due to a higher content of extractives in CH than BGH. Extractives, having a low molecular mass, promote the ignitability of biomass at lower temperatures due to their higher volatility, thereby, accelerating the degradation process (Poletto 2016).

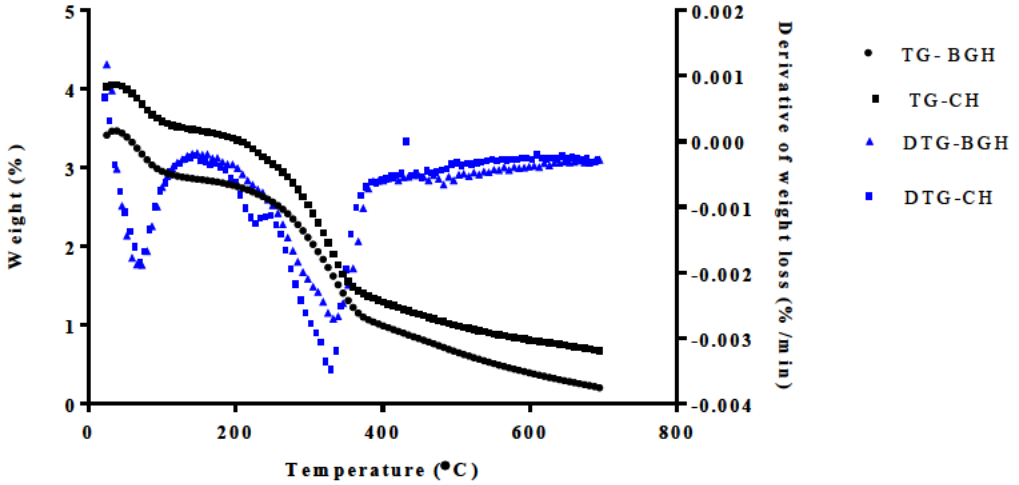

Figure 2.1: Thermogravimetric and Differential thermogravimetric (DTG) analysis of BGH and CH

2.3.6 Nutritional and amino acid composition

About 60% and 8% (w/w) of the dry mass of BGH and CH is made up of carbohydrates and crude lipids respectively which is indicative of an energy-dense feedstock. The crude protein yield of BGH and CH was 12.3% and 13% respectively. Similar values were reported for the CH of South African origin (Mohatla *et al.* 2016). Some authors reported higher crude protein values for several varieties of BGH (14.32 - 16.5%) (Anderson 2016) and CH (131 – 212 g/kg of DM) (Anele *et al.* 2010; Anele *et al.* 2011). The variability in crude protein content may be attributed to varietal differences, plant age, growth conditions etc., and is typical of lignocellulosic biomass (Vassilev *et al.* 2010). The carbohydrate, lipid and protein mass fraction of BGH and CH highlight their potential as a nutritionally balanced feedstock.

Furthermore, aspartic acid, glutamic acid and proline were the most predominant amino acids found in both the biomasses, whereas, methionine was the least. BGH and CH contained all essential amino acids (Table 2.2). Overall, BGH had a higher concentration of essential amino acids than CH, except for lysine. The high lysine content of cowpea and its residues has been previously reported (Gonçalves *et al.* 2016a). The amino acid concentration in BGH and CH makes these biomasses an attractive feedstock for the biorefining of nitrogen-containing bulk chemicals as well as feed and nutraceuticals. For instance, glutamic acid isolated from biomass can be used to produce bio-based acrylonitrile, the polyamide precursor; succinonitrile (CAS 110-61-2), and pyrrolidone-derivatives such

as N-methylpyrrolidone (NMP) and N-vinylpyrrolidone (NVP, CAS 88-12-0) (Lammens *et al.* 2012).

Table 2.2: Amino acid composition in BGH and CH.

Amino acid concentration (mg/g dry solid)	**BGH**	**CH**
His	1.88	1.56
Ser	3.82	4.27
Arg	6.29	3.76
Gly	5.33	3.75
Glu	9.00	8.51
Asp	6.44	10.28
Thr	4.06	3.53
Ala	3.83	4.49
Pro	15.38	7.32
Lys	2.81	4.94
Tyr	4.70	2.80
Met	0.43	0.21
Val	4.70	4.17
Ile	3.25	3.12
Leu	6.82	5.75
Phe	6.14	3.92
Sum	84.87	72.38

2.3.7 X-ray diffraction

The X-ray diffraction patterns showed that both BGH and CH contained amorphous and crystalline regions with the amorphous region being the major portion. BGH had a higher amorphous region (78%) than CH (56%). A larger percentage of the amorphous region makes BGH and CH easily amenable to pretreatment and enzymatic hydrolysis. The intense X-ray diffraction peaks were detected at 26.25° (2Θ) for CH and 26.38° (2Θ) for BGH (Figure 2.2). The crystallinity index was 0.22 and 0.44 for BGH and CH respectively.

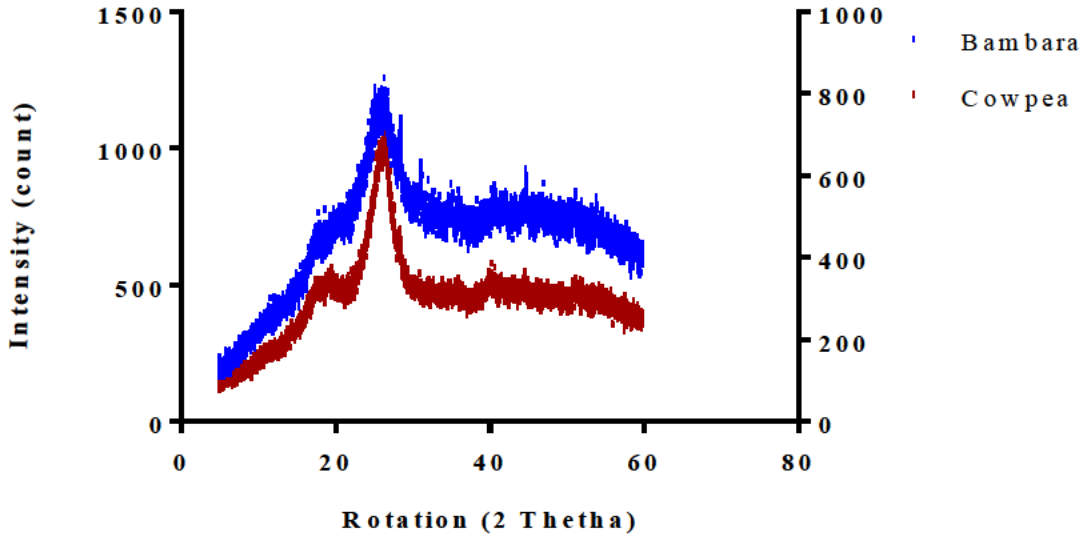

Figure 2.2: X-ray diffractogram of BGH and CH

2.3.8 Fourier transform infra-red spectroscopy (FTIR)

The FTIR studies revealed that the most prominent peaks in the spectrum for both BGH and CH originated from a C–N stretching vibration at 1025 cm^{-1} and 1022 cm^{-1} respectively (Figure 2.3). This peak is characteristic of primary amines (Coates 2000) and is expected from leguminous residues such as BGH and CH. The broad peaks at (3282 – 3296 cm^{-1}) were assigned to O–H stretch, which was attributed to water molecules in carbohydrates and aromatic rings. The bands around (2850 – 2925 cm^{-1}) were assigned to asymmetric and symmetric methylene stretching in a saturated aliphatic (alkane/alkyl) group from cellulose, lignin, and hemicellulose, without providing specific identification of any of these components (Chen *et al.* 2019). The bands at ~1732 cm^{-1} corresponded to acetyl groups present in hemicellulose (Pandey 1999). Sharp peaks at (1625 – 1627 cm^{-1}) were assigned to the aromatic skeleton vibrations involving both C–Ph and C=C, respectively and are generally found in the lignin structure (Pereira *et al.* 2016). The bands around 1373 – 1420 cm^{-1} was associated with the crystalline cellulose (Biswas *et al.* 2017).

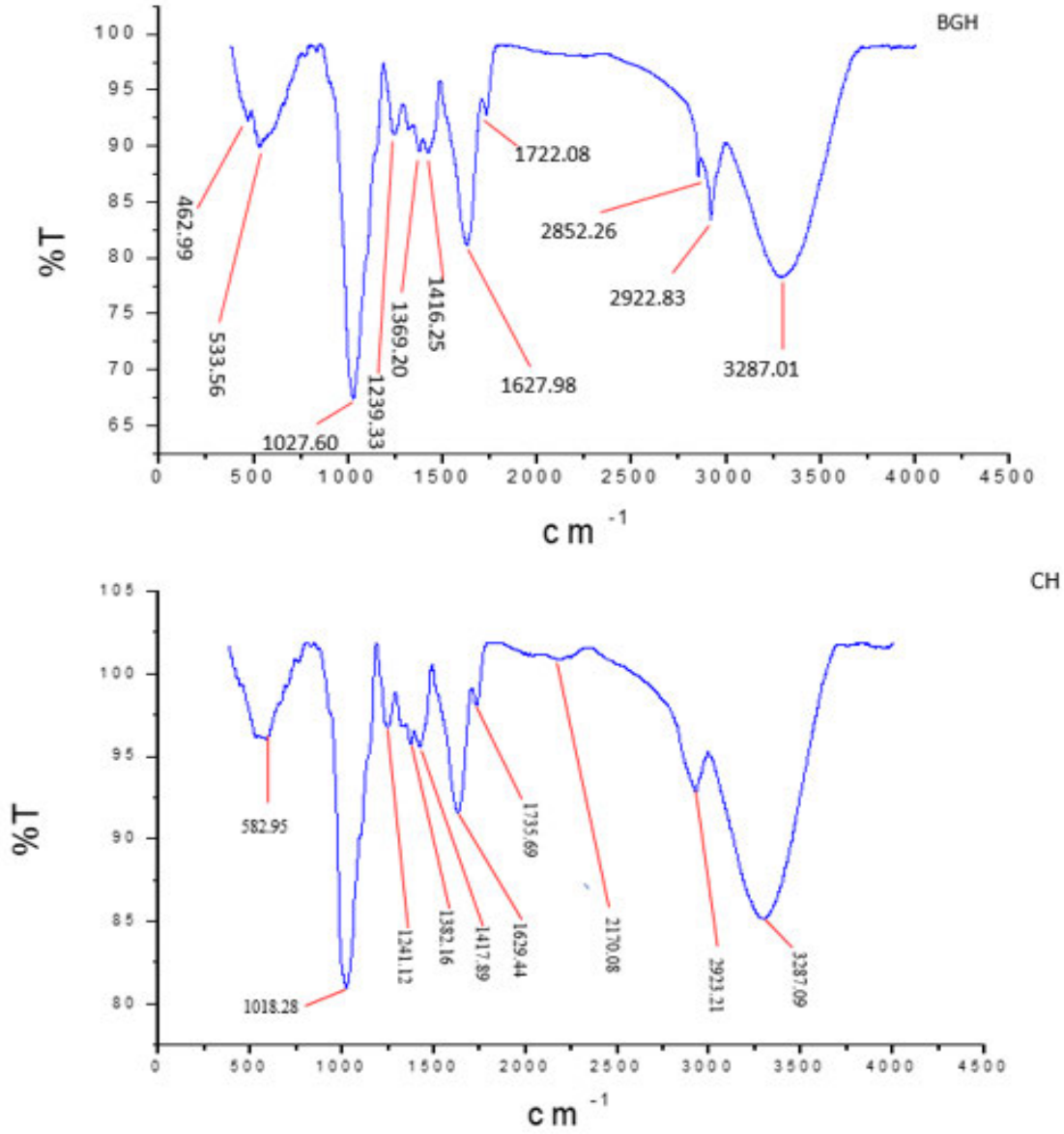

Figure 2.3: Fourier transform infrared spectroscopy of BGH and CH

2.4 Conclusions

So far, efforts at promoting the utilization of bambara groundnut and cowpea especially in water-stressed regions have stimulated research into the value addition of the seed, whereas, the haulms are discarded off as waste. However, this study, for the first time, demonstrates that BGH and CH have characteristics that compare well with other commonly used biorefinery feedstocks. This makes BGH and CH promising alternatives for a multi-product biorefinery, if they are integrated as single and or co-substrates in various biorefinery processes. The results obtained in this study indicate that BGH and CH can be converted to a range of biorefinery products using both thermochemical and biochemical conversion technologies. However, appropriate technologies for the deoxygenation and depolymerization of BGH and CH for thermochemical and biochemical processes as well as isolation and purification of bioactive compounds is key to their suitable utilization.

3 CHAPTER THREE
DILUTE ACID PRETREATMENT OF COWPEA HAULM AS POTENTIAL SUBSTRATE FOR BIOETHANOL PRODUCTION

3.1 Introduction

Bioethanol is a prominent biofuel and chemical feedstock that can be produced from lignocellulosic agricultural residues. Cowpea haulm is the above-ground lignocellulosic biomass residue remaining after the cowpea pod harvest. It is an underutilised yet promising candidate for bioethanol due to its high sugar and low lignin content. Typically, lignocellulosic biomass is a highly recalcitrant feedstock and therefore requires a pretreatment step prior to utilization for bioethanol production (Toor *et al.* 2020). The pretreatment step helps to disrupt the recalcitrant matrix, solubilizing hemicellulose, and lignin for increased enzyme access and digestibility of cellulose. Various pretreatments including steam, hydrothermal, extrusion, and ammonia-based methods have proven effective in increasing biomass digestibility (Sun *et al.* 2016).

Dilute acid pretreatment (DAP) is the most frequently studied chemical pretreatment method for agriculture biomass because it is low cost and can achieve up to 70 – 90% sugar recovery (Díaz-Blanco *et al.* 2018). DAP is influenced by several process parameters including temperature, pH, pretreatment time, solid loading etc. (Loow *et al.* 2016). The interaction between these factors influences the severity of the pretreatment. Under strong pretreatment conditions (high temperature or acid concentration), the hydrolysed sugars are degraded into by-products that are inhibitory to the saccharification and fermentation process (Kim 2018). It is, therefore, necessary to optimally combine process parameters for maximum sugar recovery. Response surface methodology (RSM) is a multivariate statistical method based on the design of experiments that analyse the influence of the independent variables (factors) on the dependent variables (responses) and predict optimal process conditions by maximizing the dependent variables (Myers, Montgomery and Anderson-Cook 2016). It has been used severally to optimise the effectiveness of pretreatment of lignocellulosic biomass (Jiang, Ding and Tang 2019; Ramaraj and Unpaprom 2019).

DAP usually results in a hydrolysate that contains a mix of pentose and hexose sugars which can be fermented sequentially using co-cultures of *S. cerevisiae* and *S. stipitis* (Sahu and Pramanik 2018). In addition, the solid fraction recovered from DAP is usually hydrolysed using enzymes and fermented into bioethanol. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are the two commonly reported bioethanol fermentation strategies (Chen and Fu 2016). Additionally, reports on SSF processes have indicated that a pre-hydrolysis step could significantly enhance the fermentation process, ethanol titer and conversion efficiency (Sewsynker-Sukai and Gueguim Kana 2018). The aim of this work was to evaluate the potential of CH as feedstock for bioethanol production. To this end, response surface methodology was used to optimize dilute acid pretreatment for optimum sugar recovery and the recovered solid fraction and liquid fractions were fermented to bioethanol by prehydrolysis with simultaneous saccharification and fermentation (SSF) and simultaneous hydrolysis and fermentation (SHF) respectively (Figure 3.1).

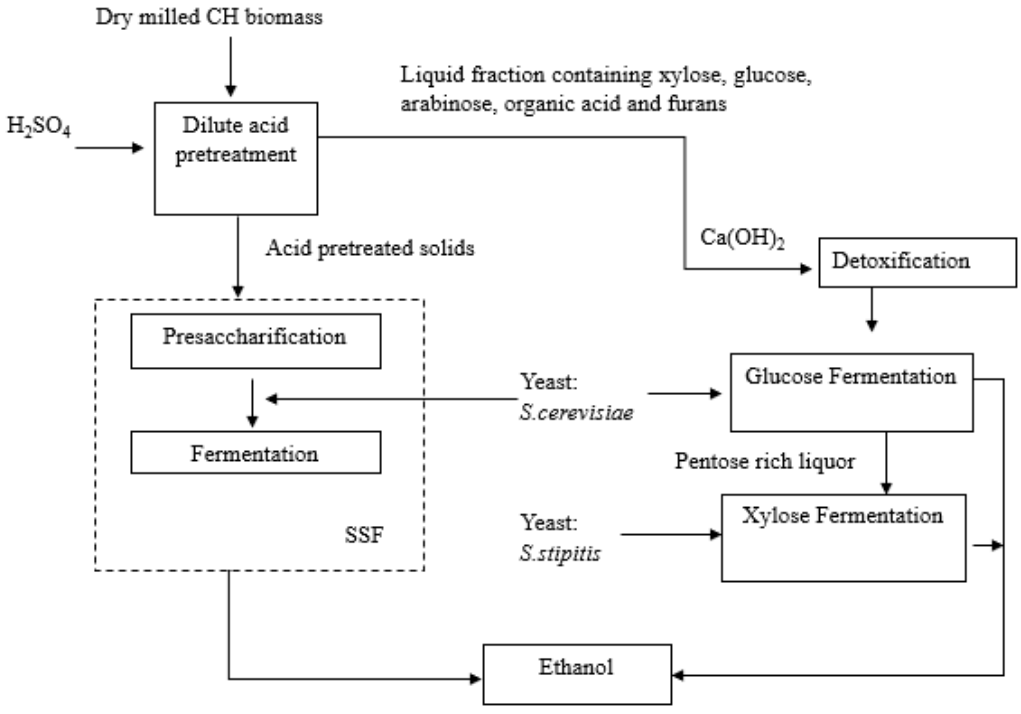

Figure 3.1: Flow chart demonstrating the experimental process used for bioethanol production

3.2 Materials and methods

3.2.1 Feedstock

Cowpea (*Vigna unguiculata*) haulms were obtained from the Agricultural Research Council (ARC), VOPI, Pretoria, South Africa. The haulms were air-dried, milled using a hammer mill, followed by a laboratory blender (MRC SM – 450L) and sieved to a particle size of ≤ 0.5 mm (Universal Test Sieve -117547, South Africa). Milled CH was stored at room temperature in airtight plastic containers.

3.2.2 Microorganisms and inoculum development

The pentose rich hydrolysate was fermented using S. *stipitis* wild type (PsY633) (CSIR, South Africa). *S. stipitis* culture was activated from frozen glycerol stock by two successive sub-culturing on malt extract agar and incubation at 30°C. Malt extract agar contained (g/L) malt extract (20), dextrose (20), peptone (6) and agar (15) (Chandel *et al.* 2011). Glucose hydrolysate was fermented using *S. cerevisiae* BY4743 (Discipline of Microbiology, University of KwaZulu-Natal, South Africa) previously maintained on a yeast peptone dextrose (YPD) agar containing (g/L) yeast extract (10), bacteriological peptone (20), dextrose (20) and agar (15). *S. cerevisiae* BY4743 and *S. stipitis* wild type (PsY633) inoculum were grown on YPD and malt extract broth respectively at 30°C for 18 h in an orbital shaker (120 rpm) to the early log phase (OD 0.8) prior to use. Cell growth was checked by measuring the optical density at 600 nm, using a spectrophotometer (Genesys 150 UV-Vis, Thermo Fischer Scientific, USA).

3.2.3 Experimental design

A three-variable central composite design (CCD) was used to optimize the pretreatment conditions of CH with dilute H_2SO_4. The input variables selected based on preliminary experimentation were temperature (100 – 120°C), acid concentration (1 – 4%) and pretreatment time (30 – 90 min), while the output variables were xylose yield (%), glucose yield (%) and total inhibitor concentration (g/L) in the pre-hydrolysate (Table 3.1). The CCD was constructed using 8 axial points, 6-star points and 6 replicates at the central point giving a total of 20 experimental runs (Table 3.2). The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

Table 3.1: Range and levels of independent process variables used in experimental design.

Variable		Range and levels				
		−α	−1	0	1	+α
Temperature (°C)	A	93.18207	100	110	120	126.8179
Time (min)	B	9.546215	30	60	90	110.4538
Acid concentration (%)	C	-0.02269	1	2.5	4	5.022689

Table 3.2: Central composite experimental design during the assessment of dilute acid pretreatment of cowpea haulm.

Std	Run	A: Temperature (°C)	B:Pretreatment time (min)	C:Acid concentration (%)	CSF
7	1	120	90	1	1.833116
14	2	126.8179	60	2.5	2.18777
10	3	110	60	5.022689	1.992588
18	4	110	60	2.5	1.692588
15	5	110	60	2.5	1.692588
8	6	120	90	4	2.403116
9	7	110	60	-0.02269	-3.61741
2	8	100	30	4	1.337121
12	9	110	110.4538	2.5	1.957618
20	10	110	60	2.5	1.692588
5	11	120	30	1	1.355995
6	12	120	30	4	1.925995
3	13	100	90	1	1.244243
11	14	110	9.546215	2.5	0.894268
19	15	110	60	2.5	1.692588
13	16	93.18207	60	2.5	1.197406
1	17	100	30	1	0.767121
16	18	110	60	2.5	1.692588
4	19	100	90	4	1.814243
17	20	110	60	2.5	1.692588

The intensity of the pretreatment conditions was expressed in a combined severity factor (CSF). The CSF expresses the severity of the pretreatment by combining the effect of reaction temperature, pH and reaction time. The CSF is generally defined as shown in Eq. 3

$$\log R'_o = \log\left(t.e^{\left(\frac{T-100}{14.75}\right)} - pH\right) \quad \text{(Equation 3)}$$

Where 'T' is the hydrolysis temperature in °C and 't' is the reaction time in minutes.

An optimization criterion that maximizes xylose and glucose yield in the prehydrolysate with minimal inhibitor production was set. Regression analysis was performed for each response in order to estimate the effect of combined independent variables on the responses using the statistical software package Design-Expert 11.1 (Stat Ease, Inc., Minneapolis, USA). Each response was tested for possible linear, quadratic and cubic models to find out the best fitting model. The quadratic polynomial model (Eq. 4) was used to calculate regression coefficients. The significance of each model term was determined with analysis of variance (ANOVA). The fit of the models was evaluated by comparing R^2 and adjusted-R^2. The statistical significance was checked by *F*-test. Three-dimensional surface plots were drawn based on the final equation determined for each response to demonstrate the effects of independent variables on the responses (Anita *et al.* 2019).

$$Y=\beta_0+\sum_{i=1}^{3}\beta_I X_i \sum_{i=1}^{3}\beta_{ii} X_i^2+\sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_I X_J \quad \text{(Equation 4)}$$

where *Y* is the response variable (xylose yield, glucose yield, and total inhibitors) and β_0, β_i, β_{ii}, and β_{ij} are the intercept, linear, quadratic, and interaction model coefficients, respectively. Additional experiments were subsequently conducted to verify the validity of the statistical experimental design under specific conditions.

3.2.4 Dilute acid pretreatment

CH biomass (5 g) was mixed with sulphuric acid (10% (w/v) substrate loading) in 250 ml Erlenmeyer flasks and pretreated in an autoclave according to the experimental design (Table 8). The pretreated slurry was cooled and filtered through a muslin cloth to separate the solid and liquid fraction. The recovered solid was washed with Milli-Q water. The pre-hydrolysate and wash water were centrifuged at 3000 g for 10 min and assayed for

monomeric sugars and inhibitors. The solid residue was further washed and dried at 30°C for gravimetric and compositional analysis (Frederick *et al.* 2014).

3.2.5 Detoxification and co-culture fermentation of prehydrolysate

The prehydrolysate obtained under optimal DAP conditions was detoxified by treating with $Ca(OH)_2$ to reach a pH of 11 ± 0.1, kept at 30°C for 1 h and then adjusted to pH 6 ± 0.1 with 72% H_2SO_4, followed by centrifugation. The detoxified hydrolysate was supplemented with fermentation medium (composed of yeast extract 5 g/L, peptone 5 g/L, $Mg_2SO_4 \cdot 7H_2O$ 1 g/L, $(NH_4)2SO_4$ 1 g/L, and KH_2PO_4 2 g/L, at pH 6). The medium was sterilized by autoclaving at 121°C for 15 min. Once the media attained room temperature, fermentation was initiated by inoculating first with *S. cerevisiae* BY4743 and *S. stipitis* was added after 24 h. Both the inocula were added in 1:1 ratio at 10% (v/v) level to the production medium (50 ml, pH 6) and incubated at 30°C in an orbital shaker (150 rpm). Samples were routinely taken for the analysis of glucose, xylose and ethanol.

3.2.6 Pre-hydrolysis with simultaneous saccharification and fermentation (PSSF) of dilute acid pretreated CH

The solid fraction recovered from DAP was washed to remove inhibitors and characterised for the glucan and pentosan content using the NREL method. This was further subjected to pre-hydrolysis with simultaneous saccharification and fermentation (PSSF) in 250 ml Erlenmeyer flasks with 50 mL working volume. The prehydrolysis step was performed at 50°C, 120 rpm for 24 h using the commercial enzyme complex Accellerase 1500 (20 mg/mL total protein content) and the accessory enzyme Accellerase XY (10 mg/mL total protein content), from Genencor (Netherlands). The hydrolysis solution was composed of the washed pretreated CH residue at 10% solid loading, sterile citrate buffer (pH 4.8, 0.05 M) supplemented with fermentation nutrients (g/L) (yeast extract 5 , peptone 5 , $Mg_2SO_4 \cdot 7H_2O$ 1 , $(NH_4)_2SO_4$ 1 and KH_2PO_4 2 ; pH 6), an enzyme load of 2 mg protein per gram of glucan and pentosan in the pretreated CH for Accellerase 1500 and Accellerase XC respectively. The pre-hydrolysed CH residue was subjected to simultaneous saccharification and fermentation process **(**SSF) for ethanol production in sterile 250 mL Erlenmeyer flasks. *S. cerevisiae* BY4743 grown on YPD broth for 18 h was used as the inoculum. The flask was seeded aseptically with 10% (w/v) inoculum (OD 0.8) to make a final volume of 50 mL. The

culture was incubated at 35°C and 120 rpm for 48 h and routinely sampled for sugars and ethanol (Carrillo-Nieves *et al.* 2017).

3.2.7 Analytical methods

3.2.7.1 Compositional analysis

Characterization of the raw materials was accomplished using the National Renewable Energy Laboratory, Laboratory Analytical Procedure, Technical Reports (NREL/TP). It included: total solids and moisture (NREL/TP 510-42621), ash (NREL/TP 510-42622), extractable substances in water and ethanol (NREL/TP 510-42619), structural carbohydrates: glucans, xylans and arabinans, acetyl groups and lignin: acid-insoluble lignin, and acid-soluble lignin (NREL/TP 510-42618). The same analyses, except for the determination of extractable substances in water and ethanol, were carried out on the resulting solids from all stages.

3.2.7.2 Determination of sugars and inhibitors

The pre-hydrolysate and wash water were analysed for monomeric sugars (glucose, xylose, and arabinose) and total inhibitors, quantified as the sum of furfural, 5-HMF, formic acid, acetic acid, and levulinic acid using high-performance liquid chromatography (HPLC, Shimadzu LC-20AD, Japan). Monomeric sugars were quantified using HPLC equipped with an ELSD detector and Biorad Aminex HPX-87H column with the following conditions: oven temperature (40°C), detector temperature (50°C), Nitrogen as carrier gas (350 kPa), Gain (6) and Milli-Q water as mobile phase (0.5 mL/min). Furfural and 5-HMF were determined using HPLC equipped with photodiode array detector (Shimadzu, SPD-M20AV) and a Kinetex EVO C18 reversed-phase column (30 × 2.1 mm) at 50°C column temperature. The mobile phase was a mixture of buffer solution (1.25 g/L each of monobasic sodium phosphate and dibasic sodium phosphate, pH 7± 0.2) and methanol (90: 10 v/v) at a flow rate of 1.0 mL/ min, with UV detection at 280 nm (Oliva *et al.* 2003). Formic acid, acetic acid and levulinic acid were quantified using HPLC equipped with photodiode array detector with UV detection at 210 nm and Biorad Aminex HPX-87H column at 50°C and 50 mM sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min (Oliva *et al.* 2003). The concentration of each sugar and inhibitor were quantified based on the calibration curves constructed using respective standards. The ethanol resulting from the fermentation trials

was analysed by gas chromatography (GC) using a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (FID) and a Zebron ZB-FFAP (30 m x 0.25 mm x 0.25 μm) capillary column. The chromatographic conditions used were as follows. Nitrogen was used as the carrier gas with an oven temperature of 200°C, injector temperature of 200°C and detector temperature of 250°C respectively. The ethanol standards were prepared using HPLC grade ethanol and methyl butyl ketone was used as the internal standard (Dogan *et al.* 2009).

The crystalline nature of raw and pretreated CH was determined by X-ray diffraction using a PANalytical Empyrean Diffractometer equipped with an X'Celerator detector and a Cobalt X-Ray tube. The scanning was performed over the angle range of $3^{o}<2\Theta>60^{o}$. A peak deconvolution method was used to analyse the XRD data and the built-in Gaussian function in Origin 8 was used to fit the peaks (Park *et al.* 2010).

Modification in the chemical moieties in CH was observed by Fourier transform infrared (FTIR) spectrophotometry. FTIR was performed using Perkin Elmer Spectrum 100 (Waltham, MA, USA) with attenuated total reflectance (ATR) sampling accessory. The spectrum was recorded between 380 and 4000 cm^{-1} (Thi and Lee 2019).

The morphological difference of the raw and pretreated BGH was examined using scanning electron microscopy (SEM, ZEISS EVO LS 15, Carl Zeiss, Germany). The samples were air-dried, mounted on aluminium specimen mounts and gold sputter-coated (Eiko IB-3 Ion Coater) before the examination.

3.2.8 Calculation

The yields of the solid fraction obtained in the pretreatment of CH was determined gravimetrically in relation to the dry weight of the biomass initially submitted to the process Eq. (5).

$$\mathbf{M} = \frac{\textbf{weight (g) of biomass after pretreatment (dry basis)}}{\textbf{weight (g) of biomass before pretreatment (dry basis).}} \times \mathbf{100} \qquad \text{(Equation 5)}$$

where M = yield of solid fraction (%)

$$\textbf{Glucose yield} = \frac{\textbf{weight (g) of glucose in prehydrolysate} * \mathbf{0.9}}{\textbf{weight (g) of glucan in raw biomass}} \times 100 \qquad \text{(Equation 6)}$$

$$\textbf{Xylose yield} = \frac{\textbf{weight (g) of xylose in prehydrolysate} * \mathbf{0.88}}{\textbf{weight (g) of xylan in raw biomass}} \times \mathbf{100} \qquad \text{(Equation 7)}$$

$$\textbf{Component Recovery} = \frac{\textbf{weight (g) of cellulose/hemicellulose/lignin in recovered solids}}{\textbf{weight (g) of cellulose/hemicellulose/lignin in raw biomass}} \times \mathbf{100}$$
(Equation 8)

The glucose utilisation (%), ethanol yield, productivity and fermentation efficiency were calculated according to Equation (9 – 12) respectively

$$\text{Glucose utilisation (\%)} = \frac{\textbf{(Initial glucose concentration final glucose concentration)}}{\textbf{Initial glucose concentration}} \times \mathbf{100}$$
(Equation 9)

$$\text{Ethanol yield (g-ethanol/g-glucose)} = \frac{\textbf{Maximum ethanol concentration } (\frac{\mathbf{g}}{\mathbf{L}})}{\textbf{Utilized glucose } (\frac{\mathbf{g}}{\mathbf{L}})}$$ (Equation 10)

$$\text{Ethanol productivity (g/L/h)} = \frac{\textbf{Highest ethanol produced } (\frac{\mathbf{g}}{\mathbf{L}})}{\textbf{Fermentation time (h)}}$$ (Equation 11)

$$\text{Fermentation efficiency (\%)} = \frac{\textbf{Ethano yield (g/l)}}{\textbf{Theoritical ethanol yield (g/l)}} \times \mathbf{100}$$ (Equation 12)

3.3 Results and discussion

3.3.1 Feedstock composition

About 53.9% of the dry mass of CH was composed of carbohydrates, mainly glucans (43%), xylans (7.2%) and arabinans (3.1%) while lignin content (4.5%) was low (Table 3.3). Hemicellulosic sugars represented 10.3% of the raw material with xylose as the main sugar (70%). The lignin content of CH used in this study was much lower than values reported for feedstock such as corn cob (14 – 15%), wheat straw (17 – 19%), sugar cane bagasse (20 – 42%) and rice husk (26 – 31%) (Zabed *et al.* 2016). Anele *et al.* (2010) have reported 12.6 – 22.1% lignin in various varieties of cowpea haulm. However, low lignin values similar to those observed in this study have been reported for other lignocellulosic biomasses including water hyacinth (3.5%), orchard grass (4.7%), pineapple leaf fibre (1-5%) and bermuda grass (6.4%). The variation in lignin content could be attributed to the differences in variety, agronomic and physiological conditions of the plant and the method of lignin measurement (Vavilala, Ghag and D'Souza 2019). The particularly low lignin content (4.5%) and high sugar content (53.9%) observed in this study make CH a promising feedstock for bioethanol. This is because cellulose and hemicellulose are the main sugar precursors for bioethanol production while lignin provides a protective sheath over hemicellulose and cellulose by complex physical and chemical association (De Bhowmick, Sarmah and Sen 2018). Therefore, a low lignin content could ease the solubilization of polysaccharide fractions

(Studer *et al.* 2011). Moreover, the degradation of lignin during acid pretreatment leads to the formation of phenolic monomers which have individual and synergistic inhibitory effects on bioethanol fermentation (Jönsson and Martín 2016). CH is also composed of a high mass fraction of extractives (33%, Table 3.3). High extractive components have been reported for other agricultural residues including olive tree (Martínez-Patiño *et al.* 2017) and *Agave lechuguilla* (Díaz-Blanco *et al.* 2018). Biomass extractives encompass a wide variety of high-value chemical compounds with reported therapeutic and industrial potentials that could be used to boost the economic viability of bioethanol production in a biorefinery process (Golets, Ajaikumar and Mikkola 2015; Pan *et al.* 2018; Tao *et al.* 2019).

Table 3.3: Composition of native cowpea haulm (g/ 100 g) on oven-dry basis.

Components	Mass fraction (% w/w)
Cellulose (as glucan)	43.9 ± 2.1
Hemicellulose as	10.3 ± 0.6
Xylan	7.2 ± 0.5
Arabian	3.1 ± 0.1
Lignin	4.5 ± 0.4
ASL	1.4 ± 0.1
AIL	3.1 ± 0.3
Ash	6.7 ± 0.4
Extractives	33.8 ± 0.5
Acetyl group	0.1 ± 0.2

3.3.2 Yield and composition of solid and liquid fractions of pretreated CH

The yield and composition of the solid and liquid fraction are highly dependent on the pretreatment conditions. Generally, with increasing process severity (i.e., increase in acid concentration, temperature and pretreatment time), the hydrolysis of cellulose and hemicelluloses is accelerated, leading to a loss in solid and an increase in the amount of monomeric sugars that can be recovered in solution. The combined severity factors for the dilute acid pretreatment in this study ranged from 2.40 to 3.62 . Solid recovery exhibited a decline with increasing combined severity factor (CSF) and ranged between 26 to 63% corresponding to the highest (CSF = 2.4, run 6) and the lowest CSF (CSF = −3.62, run 7) respectively (Table 3.4). The experiments carried out in the centre of the domain (runs 4, 5, 10, 15, 18, 20) with a combined severity average of 1.69 resulted in a solid recovery of around 33.5%. The solubilisation of cellulose and hemicellulose was confirmed by examining the composition of the recovered solid fraction (Table 3.4). As expected, the hemicelluloses were most susceptible to dilute acid pretreatment. Under mild pretreatment conditions (CSF = -3.62), only 4.79% solubilization occurred while in the most severe runs (CS above 2), over 90% xylan solubilization was observed. The cellulose content in the pretreated solids ranged between 22 and 42%, amounting to a recovery of 52.14 – 96.5% of the initial cellulose content for the mildest and most severe runs respectively.

Consequently, monomeric sugars in the hydrolysate increased up to a certain point and began to decrease. Glucose was the most abundant sugar in the filtrates (0.2 to 15.6 g/L), followed by xylose (0.2 to 6.79 g/L), while arabinose was found in relatively minor concentration (Table 3.5). The glucose obtained was attributed to the solubilization of amorphous portions of cellulose and even hemicellulose (Loow *et al.* 2016). Some agricultural residues are known to be composed of glucuronoarabinoxylan as their hemicelluloses (Brigham, Adney and Himmel 2018). Xylose and arabinose could be released during hemicellulose hydrolysis. The decrease in the sugar yield at higher CSF is attributed to sugar degradation to secondary products. In addition to the sugars, furfural, 5-HMF, acetic, formic, and levulinic acids, were detected in the prehydrolysate (Table 3.6). During acid hydrolysis, the pentoses and uronic acids released from hemicellulose undergo dehydration into furfural while the hexose sugars are dehydrated into 5-HMF (Jönsson and Martín 2016). With increasing severity in pretreatment conditions, such as long pretreatment time, acid concentration or temperature, furfural is further degraded into formic acid while 5-HMF is degraded into formic and

levulinic acids. Acetic acid is obtained from the acetyl groups during hemicellulose solubilization (Hofmann 2019). The production of inhibitors in various concentration was also observed following the pretreatment of cotton stalk (Gaur *et al.* 2016), wheat straw (Barisik *et al.* 2016) and pine needles (Slathia *et al.* 2019).

Table 3.4: Yield and composition of solid fraction after dilute acid pretreatment.

		Composition of solids % (w/w)						
Run	CSF	Solid recovery	Glucan	Xylan	Arabian	Lignin	Cellulose recovery	Xylan solubilization
7	-3.62	62.8	42.34	7.8	2.254	4.28	96.46	4.79
17	0.77	50.5	32.25	6.05	1.333	4.55	73.47	26.15
14	0.89	41.4	32.6	3.15	1.02	3.64	74.27	61.51
16	1.2	42.4	30.81	2.42	1.082	3.95	70.19	70.49
13	1.24	43.6	33.99	4.09	1.15	4.49	77.44	50.09
8	1.34	42.3	33.94	2.00	1.65	3.4	77.32	75.53
11	1.36	40.5	31.59	3.57	1.054	2.96	71.96	56.42
15	1.69	38.6	34.63	1.75	0.88	3.01	78.89	78.57
4	1.69	37.6	33.52	3.18	0.874	3.13	76.37	61.13
10	1.69	37.2	33.28	1.75	0.962	4.24	75.81	78.58
5	1.69	36.3	32.65	2.25	0.941	2.42	74.39	72.52
18	1.69	34.6	33.71	1.68	0.875	3.48	76.78	79.52
20	1.69	34	32.96	1.6	0.827	3.58	75.08	80.49
19	1.81	37	33.01	2.17	0.913	3.12	75.21	73.55
1	1.83	35.1	31.97	2.48	0.934	2.73	72.82	69.72
12	1.93	33.3	32.52	1.51	0.759	4.04	74.08	81.55
9	1.96	33.3	33.11	1.12	0.818	2.53	75.42	86.31
3	1.99	35	34.83	1.1	0.882	2.5	79.34	86.61
2	2.19	31.1	33.22	0.77	0.738	2.45	75.67	90.63
6	2.4	26	22.89	0.65	0.62	2.91	52.14	92.06

Table 3.5: Analysis of sugar composition in the hydrolysate after pretreatment.

Run	Acid conc. (%)	Time (min)	Temp (°C)	pH	CSF	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Glucose yield (%)	Xylose yield (%)
7	-0.02	60	110	5.69	-3.62	1.28	0.85	0.10	2.63	10.38
17	1	30	100	0.71	0.77	0.23	0.21	0.18	0.48	2.531
14	2.5	9.55	110	0.38	0.89	5.89	4.22	1.41	12.08	51.51
16	2.5	60	93.2	0.38	1.2	5.36	4.20	1.35	11.00	51.26
13	1	90	100	0.71	1.24	3.54	2.50	1.27	7.26	30.51
8	4	30	100	0.14	1.34	6.11	4.85	1.19	12.54	59.25
11	1	30	120	0.71	1.36	5.10	3.39	1.31	10.46	41.45
4	2.5	60	110	0.38	1.69	15.6	4.80	2.49	31.98	58.65
5	2.5	60	110	0.38	1.69	14.0	5.75	2.35	28.76	70.22
10	2.5	60	110	0.38	1.69	13.3	6.17	2.27	27.34	75.38
15	2.5	60	110	0.38	1.69	13.8	6.11	2.18	28.39	74.63
18	2.5	60	110	0.38	1.69	13.8	6.26	2.09	28.39	76.4
20	2.5	60	110	0.38	1.69	13.6	6.26	2.16	27.79	76.5
19	4	90	100	0.14	1.81	7.21	5.99	1.25	14.78	73.12
1	1	90	120	0.89	1.83	6.74	5.28	1.52	13.92	64.46
12	4	30	120	0.14	1.93	7.52	6.61	1.2	15.41	80.74
9	2.5	110	110	0.38	1.96	7.61	6.79	1.31	15.61	82.96
3	5.02	60	110	0.08	1.99	7.08	6.01	1.11	14.52	73.41
2	2.5	60	127	0.38	2.19	7.42	5.98	1.08	15.22	73.10
6	4	90	120	0.14	2.4	7.75	5.96	1.27	15.89	72.79

Table 3.6: Analysis of inhibitor composition (g/L) of the hydrolysate after pretreatment.

Run	CSF	Acetic	Formic	Levulinic	Furfural	5-HMF	Total inhibitor
7	-3.62	ND	2.38	ND	ND	ND	2.38
17	0.77	ND	2.28	0.12	0.42	ND	2.82
14	0.89	0.03	1.09	0.23	0.63	ND	1.98
16	1.20	0.03	1.4	0.12	0.6	0.02	2.17
13	1.24	0.02	1.02	0.11	0.52	ND	1.68
8	1.34	0.04	1.38	0.23	0.39	0.04	2.08
11	1.36	0.02	0.96	ND	0.63	ND	1.62
4	1.69	0.05	1.34	0.26	0.77	0.08	2.50
5	1.69	0.05	1.48	ND	0.73	0.06	2.33
10	1.69	0.07	1.73	0.47	0.85	0.11	3.23
15	1.69	0.03	0.84	0.24	0.81	0.14	2.07
18	1.69	0.04	1.05	0.14	0.57	0.19	2.00
20	1.69	0.03	0.79	0.24	0.71	ND	1.91
19	1.81	0.04	1.08	0.24	0.75	0.18	2.29
1	1.83	0.05	1.18	0.4	0.91	0.18	2.71
12	1.93	0.05	0.62	0.24	0.79	0.41	2.11
9	1.96	0.04	1.47	0.31	0.7	0.39	3.04
3	1.99	0.07	1.09	0.45	0.54	0.49	2.64
2	2.19	0.08	1.6	0.65	0.63	0.73	3.70
6	2.40	0.1	2.39	1.20	0.45	0.79	4.92

ND – Not detected

3.3.3 Modelling and optimisation of dilute acid pretreatment conditions

3.3.3.1 Analysis of variance and model fitting

To define the optimum conditions of the independent variables, the experimental results obtained (Table 3.4 – 3.6) were fitted to a second-order polynomial regression equation (Equation 4). The values of the regression coefficients were calculated, and the predicted model for each response was described in terms of coded values by equations 13 – 15. The three model equations were derived using statistically significant regression coefficients ($p < 0.05$).

Xylose yield = 72.0636 + 18.5229 * A + 8.04102 * B + 9.57308 * C + -5.63569 * AB + -6.46357 * AC + -3.34878 * BC + -11.2849 * A^2 + -2.32457 * B^2 + -4.11168 * C^2 (Equation 13)

Glucose yield = 28.7573 + 3.40559 * A + 1.38401 * B + 2.03142 * C + -0.939293 * AB + -1.58358 * AC + -0.635929 * BC + -7.03096 * A^2 + -5.16809 * B^2 + -5.42675 * C^2 (Equation 14)

Total inhibitor concentration = 2.34244 + 0.2193 * A + 0.347797 * B + 0.370734 * C + 0.385377 * AB + 0.353631 * AC + 0.602266 * BC + 0.0312669 * A^2 + 0.0306822 * B^2 + 0.180194 * C^2 (Equation 15)

Where *A* is acid concentration, *B* is pretreatment time and *C* is temperature.

The adequacy of the regression equation and the significance of each coefficient was checked by the analysis of variance (ANOVA) (Tables 3.7 – 3.9). The significance of the models and each coefficient was determined by *F*- and *P*-values. The model F-values of 25.42, 78.81 and 6.96 with low probability value ($P < 0.0001$), ($P < 0.0001$), ($P = 0.0028$) for xylose yield, glucose yield and total inhibitor concentration respectively imply a high significance of the model. Furthermore, the lack of fit was also insignificant for all the models. The high R^2-values of 0.9581, 0.9861, and 0.8424 indicated a satisfactory agreement of the quadratic model to the experimental data such that 95.8%, 98.6% and 84.2% of the variations in the responses could be explained by the models for xylose yield, glucose yield and total inhibitor concentrations respectively. Further, the adjusted-R^2 was computed to determine the percentage of variation explained by the independent variables that affected the model output. It was found that the adjusted-R^2 for xylose yield (0.9204), glucose yield (0.9736), and inhibitor concentration (0.7386) was close to the R^2-value, indicating that the independent variables were important, and no data overfitting occurred. The response surface quadratic model reveals that in descending order, the model terms A,

A^2, C, B, AC, AB, C^2 and A^2, C^2, B^2, A, C, B, AC, as well as C, B, BC, AB, AC, have a statistically significant effect on xylose yield, glucose yield, and total inhibitor concentration respectively. The linear terms have a positive effect on xylose and glucose yield with acid concentration exerting the most significant positive influence followed by temperature and time, whereas, the interactive and quadratic terms all had a negative effect (Eq. 10 and 11). On the other hand, both linear and interaction terms had significant positive effects on total inhibitor concentration (Eq. 12). Gonzales, Kim and Kim (2019) also observed that temperature, reaction time, and dilute acid concentration all exerted significant individual influences on the sugar concentration during the dilute acid hydrolysis of oil palm empty fruit bunch.

Table 3.7: ANOVA for response surface quadratic model for xylose yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9476.5	9	1052.94	25.42	< 0.0001	significant
A-Acid concentration	4685.61	1	4685.61	113.11	< 0.0001	
B-Pretreatment time	883.02	1	883.02	21.32	0.001	
C-Temperature	1251.57	1	1251.57	30.21	0.0003	
AB	254.09	1	254.09	6.13	0.0327	
AC	334.22	1	334.22	8.07	0.0175	
BC	89.71	1	89.71	2.17	0.1719	
A^2	1835.25	1	1835.25	44.3	< 0.0001	
B^2	77.87	1	77.87	1.88	0.2003	
C^2	243.64	1	243.64	5.88	0.0357	
Residual	414.26	10	41.43			
Lack of Fit	174.8	5	34.96	0.73	0.6309	not significant
Pure Error	239.46	5	47.89			
Cor Total	9890.75	19				
Model	9476.5	9	1052.94	25.42	< 0.0001	significant

CV = 10.73%; R^2 = 0.9581; Predicted R^2 =0.8254; Adj R^2 = 0.9204; Adeq. Precision = 16.5551

Table 3.8: ANOVA for response surface quadratic model for glucose yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1548.97	9	172.1	78.81	< 0.0001	significant
A-Acid concentration	158.39	1	158.4	72.53	< 0.0001	
B-Pretreatment time	26.16	1	26.16	11.98	0.0061	
C-Temperature	56.36	1	56.36	25.81	0.0005	
AB	7.06	1	7.06	3.23	0.1024	
AC	20.06	1	20.06	9.19	0.0127	
BC	3.24	1	3.24	1.48	0.2515	
A^2	712.41	1	712.4	326.2	< 0.0001	
B^2	384.91	1	384.9	176.25	< 0.0001	
C^2	424.41	1	424.4	194.33	< 0.0001	
Residual	21.84	10	2.18			
Lack of Fit	8.23	5	1.65	0.6053	0.7025	not significant
Pure Error	13.6	5	2.72			
Cor Total	1570.81	19				

CV = 8.84%; R^2 = 0.9861; Predicted R^2 = 0.9474; Adj R^2 = 0.9736; Adeq. Precision = 26.4174.

Table 3.9: ANOVA for response surface quadratic model for inhibitor concentration

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9.75	9	1.08	6.96	0.0028	significant
A-Acid concentration	0.6568	1	0.6568	4.22	0.0669	
B-Pretreatment time	1.65	1	1.65	10.62	0.0086	
C-Temperature	1.88	1	1.88	12.07	0.006	
AB	1.19	1	1.19	7.64	0.02	
AC	1	1	1	6.43	0.0296	
BC	2.9	1	2.9	18.66	0.0015	
A^2	0.0141	1	0.0141	0.0906	0.7696	
B^2	0.0136	1	0.0136	0.0872	0.7738	
C^2	0.4679	1	0.4679	3.01	0.1135	
Residual	1.56	10	0.1555			
Lack of Fit	0.3623	5	0.0725	0.3037	0.8915	not significant
Pure Error	1.19	5	0.2386			
Cor Total	11.3	19				

CV = 15.73%; R^2 = 0.8424; Predicted R^2 = 0.6024; Adj R^2 = 0.7386; Adeq. Precision = 11.3152

3.3.3.2 Diagnostics and adequacy of the model

The model adequacy was checked by analysing the generated diagnostic plots. The main aim of a diagnostic test is to graphically analyse the model. The first diagnostic tool is the normal plot of the residuals. This plot indicates whether the residuals follow a normal distribution and in such a case, the points will lie along a straight line. For all the responses in this study, the normal probability plot of the studentized residuals are linear (Figure 3.2 a, b, c). This linearity indicates that the error terms follow a normal distribution, the models underlying assumption are adequate and would not be improved by a change in the transformation (Nayak and Vyas 2019) Model suitability was also confirmed from the random scatter trend obtained when residuals were plotted against the predicted responses (Figure 3.3 a, b, c). The plot of the predicted versus experimental values (Figure 3.4 a, b, c) showed point clustering around the diagonal which indicates the satisfactory correlation between the experimental and predicted.

Usually, in the case of abnormal distribution of data, the cause of abnormality should be ascertained, and adequate remedial action taken. Transformation of the response is essential when the error (residual) is a function of the response magnitude (predicted values). Power law transformation is the transformation described by the power function. The Box-Cox power transformation is a remedial diagnostic tool available on design expert that provides guidelines for selecting the correct power law transformation if required (Anderson and Whitcomb 2016). A recommended transformation is listed based on the lambda value which is found at the minimum point of the curve generated by the natural log of the sum of squares of the residuals (Myers, Montgomery and Anderson-Cook 2016). If the 95% confidence interval around this lambda includes 1, then the software does not recommend a specific transformation. In this study, no transformation was recommended, hence the current transformation of one (1) was selected as the best possible transformation (Figure. 3.5 a, b, c).

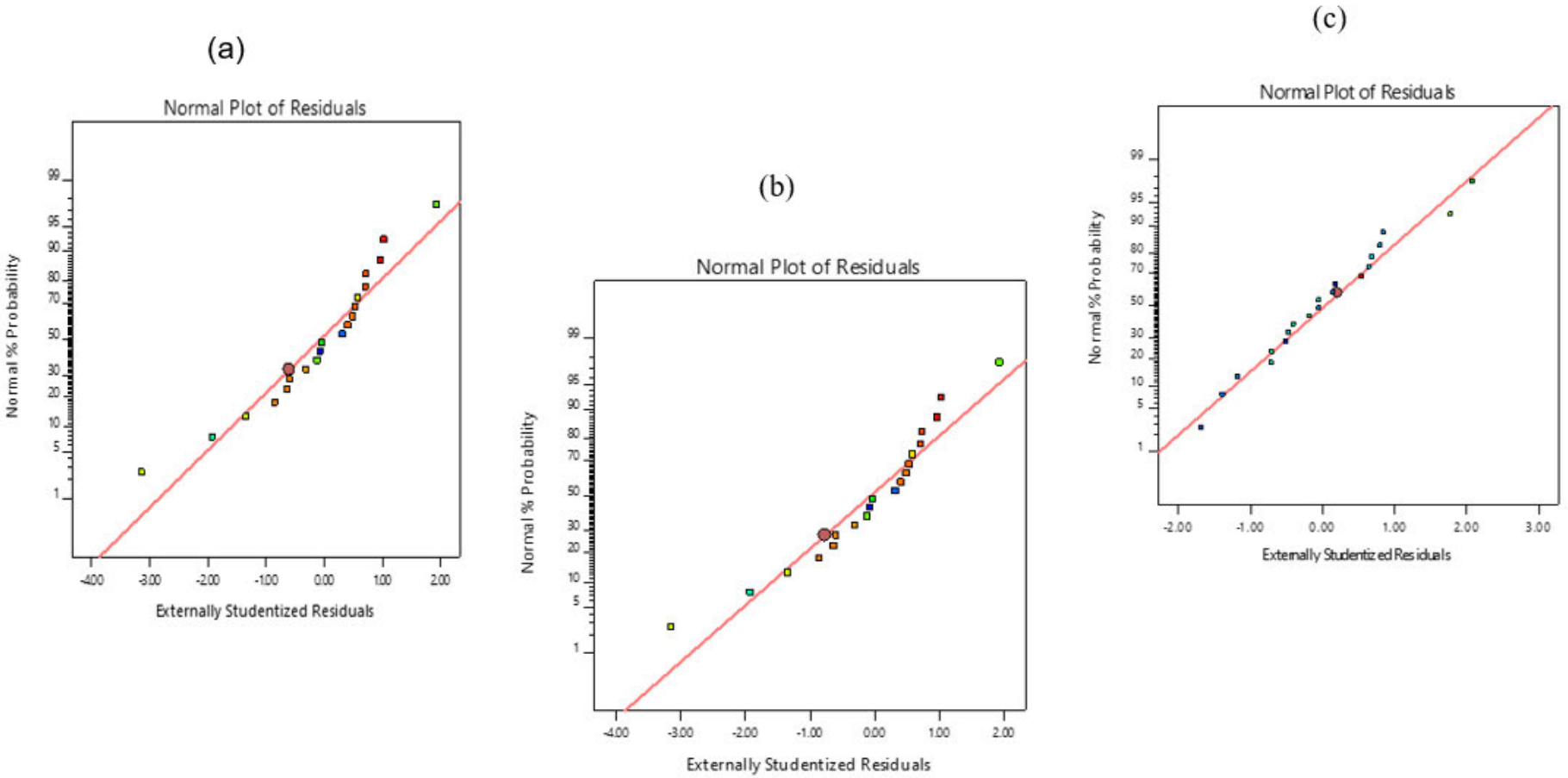

Figure 3.2: Normal probability plot of studentized residuals for (a) xylose yield (b) glucose yield and (c) total inhibitor concentration

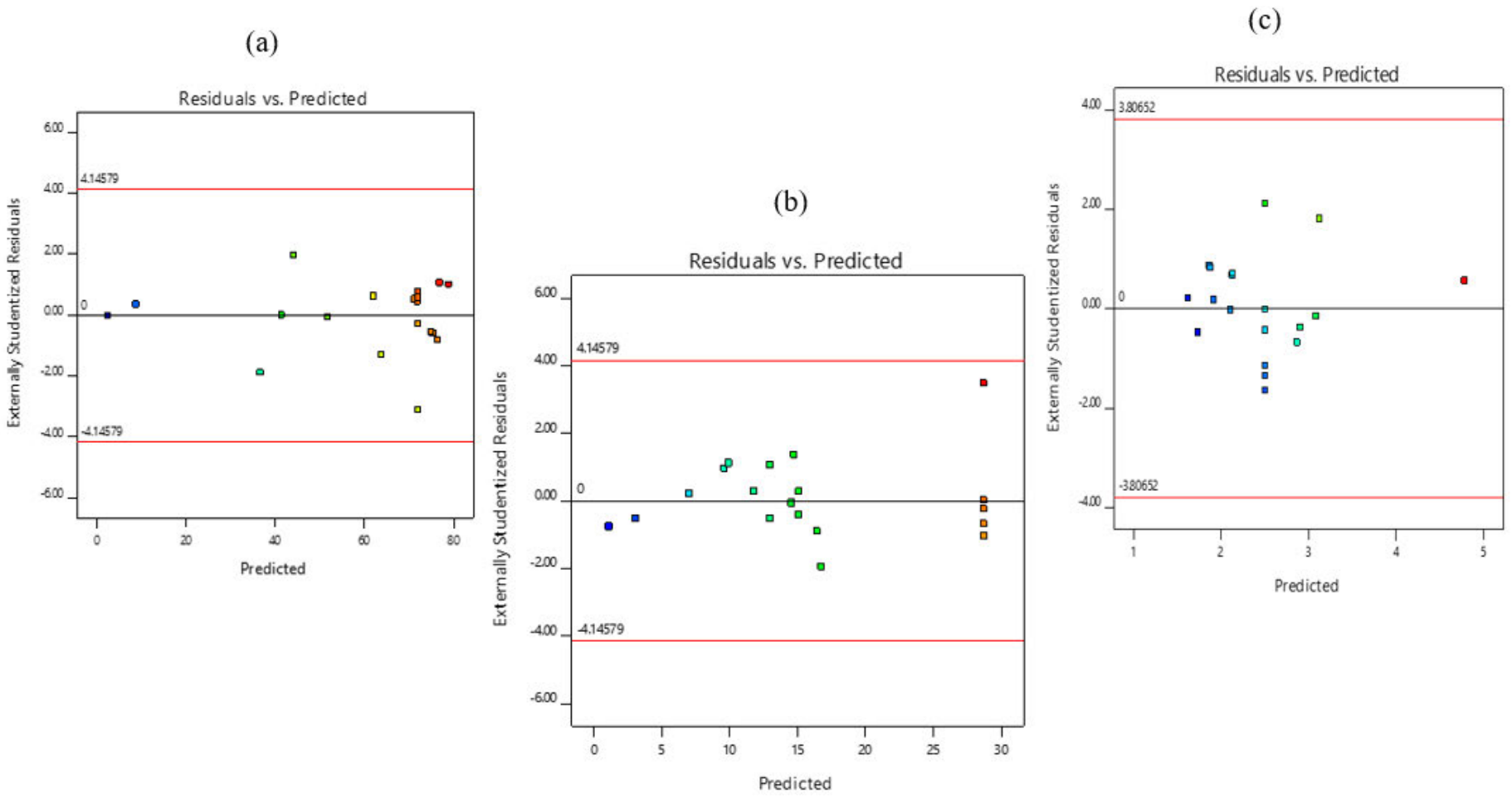

Figure 3.3: Plot of externally studentized residuals vs predicted response for (a) xylose yield (b) glucose yield and (c) total inhibitor concentration

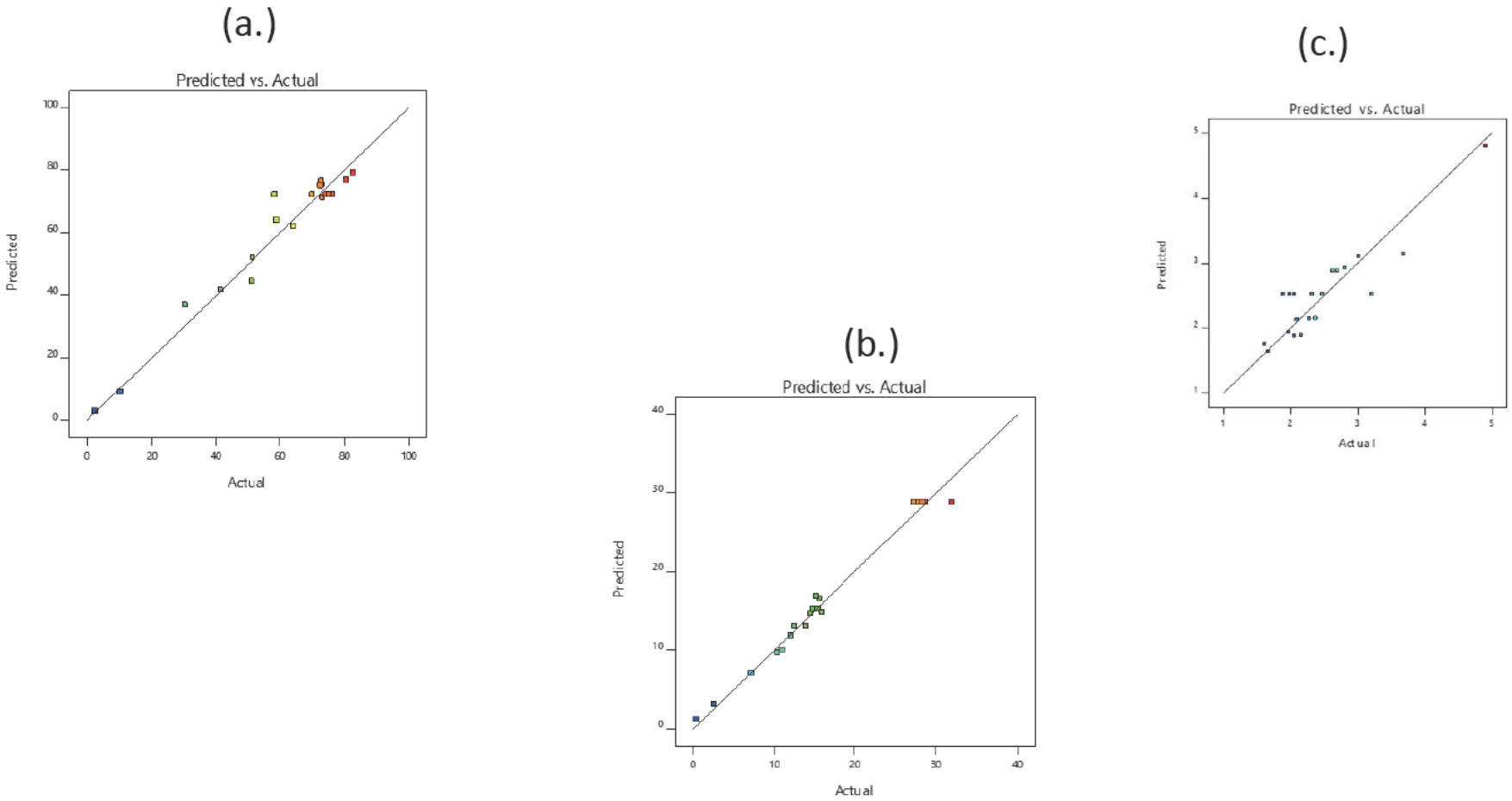

Figure 3.4: Plot of predicted vs actual values for (a) xylose yield (b) glucose yield and (c) total inhibitor concentration

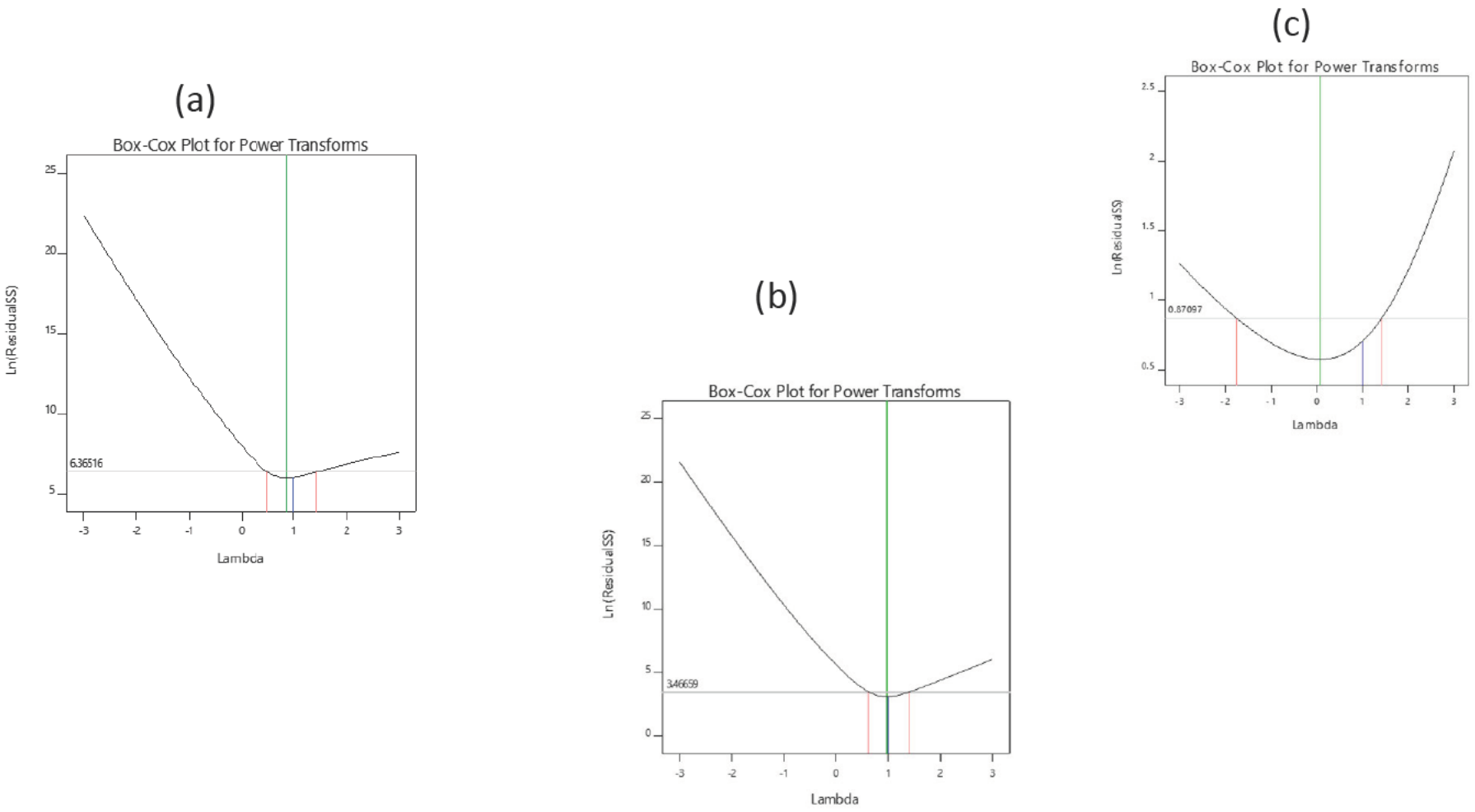

Figure 3.5: Box-Cox plot for (a) xylose yield (b) glucose yield and (c) total inhibitor concentration

3.3.4 Validation of model

The quadratic model predicted that the optimum conditions for maximum xylose (76.5%) and glucose yield (28.6%) at minimum inhibitor concentration (2.34 g/L) were temperature (110°C), acid concentration (3.1%) and time of reaction (55 min). Nguyen *et al.* (2018) had reported that optimal conditions for thermal acid pretreatment of soybean residue were 270 mM/L H_2SO_4 at 121°C for 60 min. To validate the predicted conditions and check the adequacy of the model equation, further pretreatment experiments were conducted in triplicates under these conditions and the mean values were presented. The experimental values of 79%, 27.2%, 2.53 g/L for xylose yield, glucose yield and minimum inhibitor concentration matched well with the predicted values demonstrating the accuracy of the model. In a previous study, Botella *et al.* (2019) also reported xylose and glucose yield in the range of 35 – 79% and 21-24% respectively from α-hydroxyethane sulfonic acid (HESA) pretreatment of ensiled sweet sorghum.

3.3.5 Interactive effect of process variables on responses

The response models are mapped against two experimental factors while the other factor is maintained constant at its central value. In view of the parameters investigated in this study, the maximum xylose and glucose yield in the prehydrolysate was attained.

The maximum xylose yield (76%) could be obtained when acid concentration ranged from 2.67 – 3.9% and pretreatment time ranged from 63 – 110 min. On the other hand, the maximum glucose yield (28.6%) could be obtained when acid concentration ranged from 1.8 – 3.9% and pretreatment time ranged from 40 – 80 min. It was observed that higher xylose and glucose yield could be obtained at either short pretreatment time and high acid concentration or long pretreatment time and low acid concentration (Figure 3.6 and 3.7 a, b). Too low or too high values of both factors resulted in low sugar yield. The positive effect of acid concentration and time was also reported during dilute acid pretreatment of dried distillers grains with soluble DDGS (Cekmecelioglu and Demirci 2019).

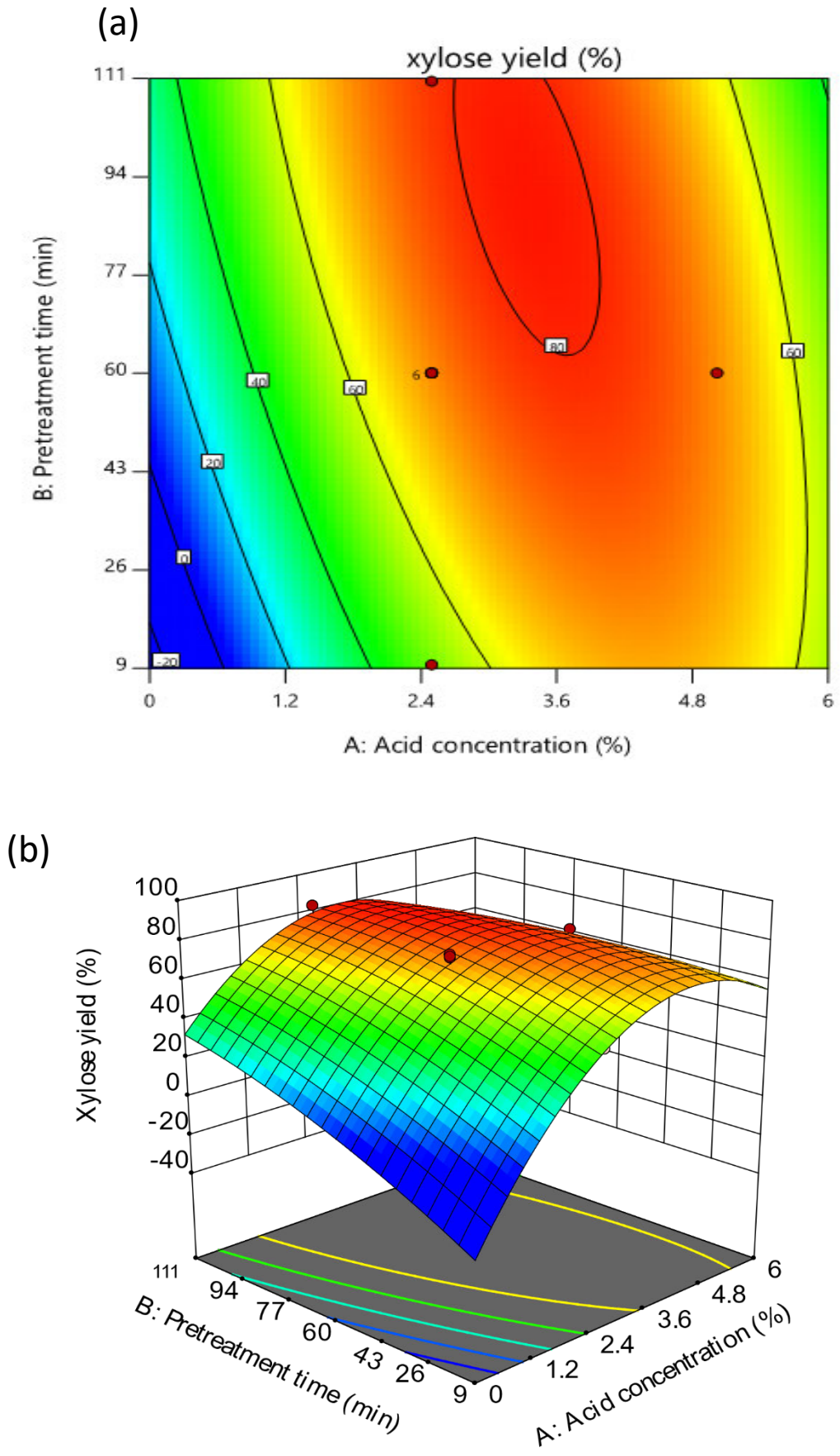

Figure 3.6: Response surface graph showing the interactive effect of acid concentration and time on xylose yield; (a) contour plot (b) 3D graphs

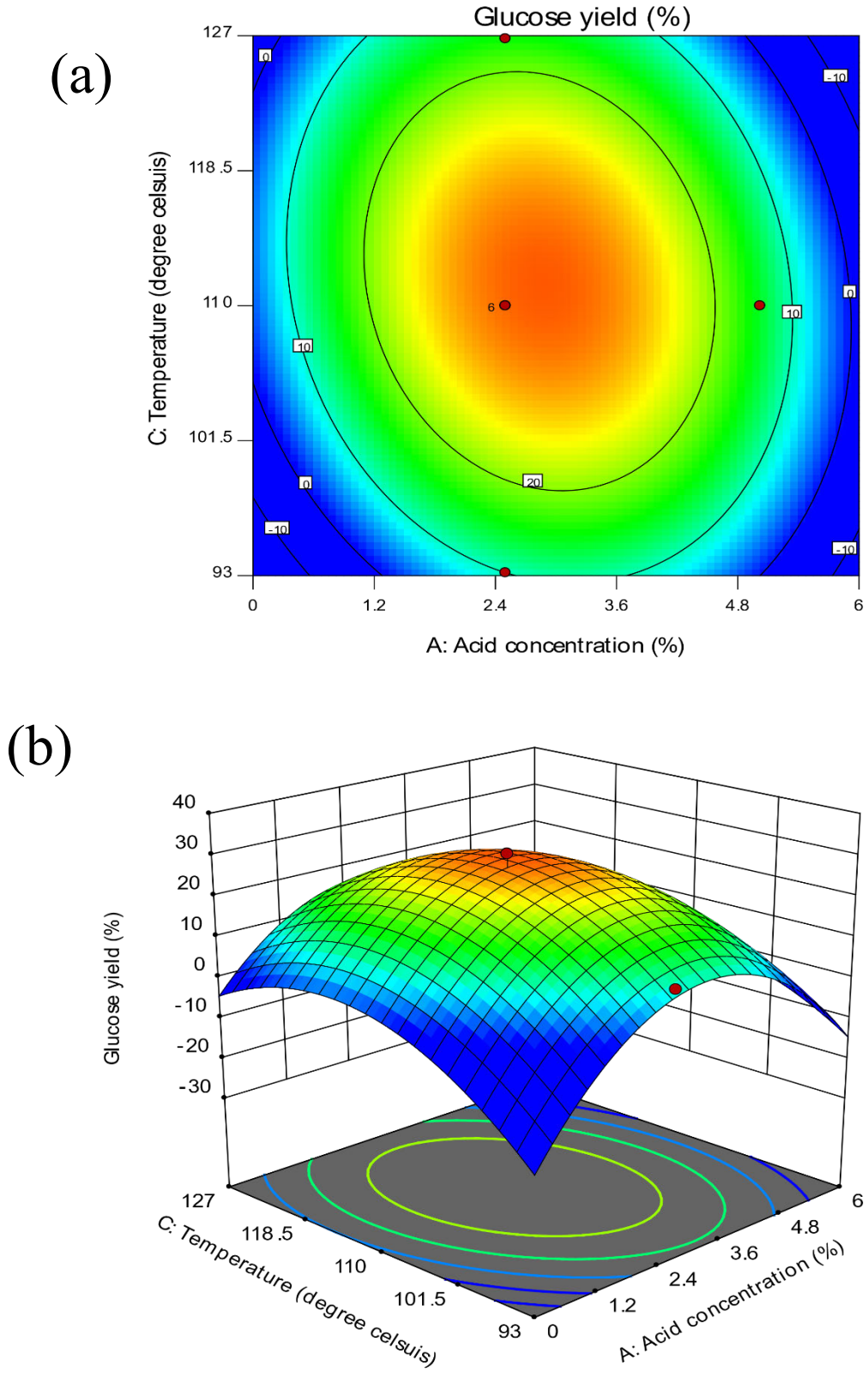

Figure 3.7: Response surface graph showing the interactive effect of acid concentration and time on glucose yield; (a) contour plot (b) 3D graphs

The observation is attributed to the fact that in an aqueous environment, dilute acids dissociate into protons (H^+) which diffuse through the lignocellulosic matrix, cleaving the ether bonds between hemicellulose and lignin and the glycosidic linkage between sugar monomers. This results in the solubilization of hemicelluloses, amorphous cellulose into glucose and acid-soluble lignin (Loow *et al.* 2016). Under low acid conditions, fewer protons (H^+) are available to catalyse the reaction. Hence, longer contact time with the lignocellulosic material is required to instigate bond cleavage and improve sugar yield. At a higher acid concentration, more protons (H^+) are available to cleave the ether and glycosidic bonds, reducing the required contact time. Therefore, long pretreatment time with high acid concentration enables further catalytic reaction of the solubilized sugars by the free protons (H^+) to by-products, leading to lower xylose and glucose yield (Aguilar *et al.* 2002).

Also, for this study, a similar trend was observed in the interaction between acid concentration and temperature. Under low-temperature conditions, the increase in acid concentration enhanced xylose and glucose yield, while at high temperatures, high sugar yield was obtained at low acid concentration. (Figure 3.8 and 3.9 a, b). Contour lines indicate that the maximum xylose yield was obtained when acid concentration and the temperature were in the range of 2.9 – 3.99% and 110 – 122°C respectively. At less than 2.9% acid concentration, there was a steady increase in xylose yield with temperature up to 122°C. A similar trend was observed for glucose yield. The observed trend could be because temperature influences the rate of reaction during DAP. Hence, increasing the temperature facilitates faster diffusion of protons and hydrolysis of bonds leading to an increase in sugar yield even at low acid concentration. However, as the acid concentration increases with temperature, the solubilised sugars are speedily converted to inhibitors leading to a decrease in the sugar yield (Loow *et al.* 2016). This trend was also observed by Cao *et al.* (2018), who reported enhanced xylose yield when phosphoric acid concentration was high and the temperature was low or phosphoric acid concentration was low and the temperature was high.

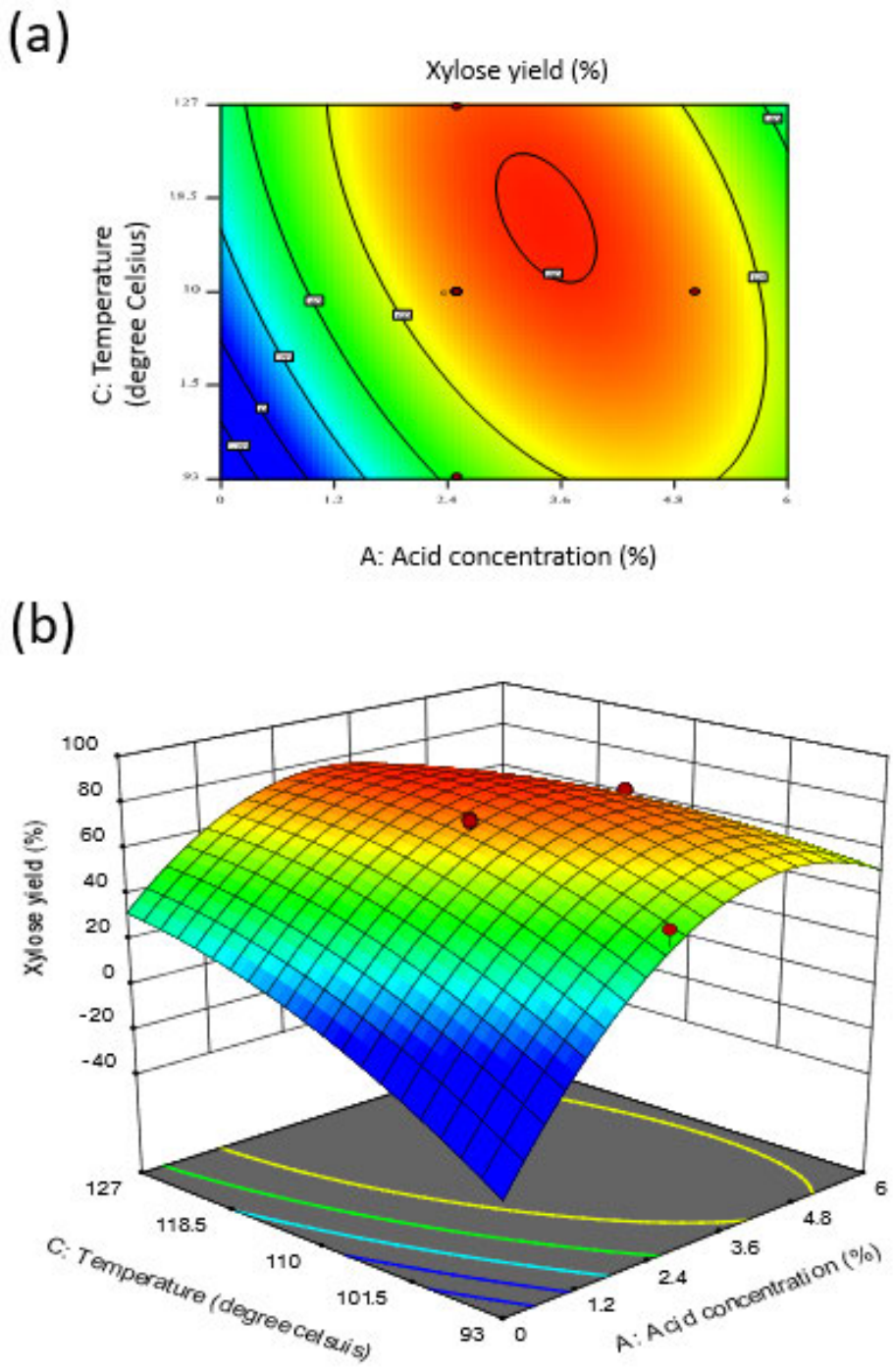

Figure 3.8: Response surface graph showing the interactive effect of acid concentration and temperature on xylose yield; (a) contour plot (b) 3D graph

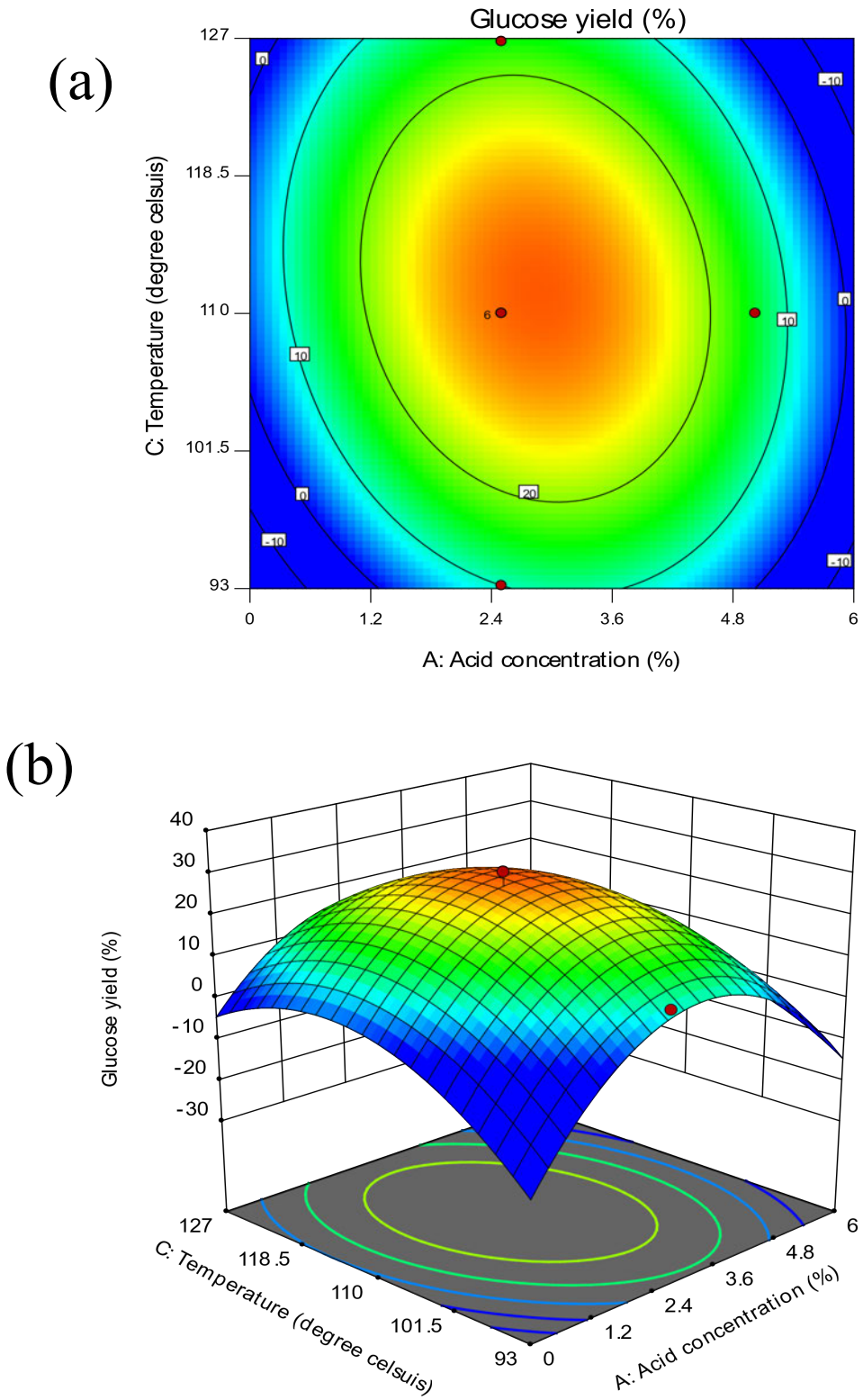

Figure 3.9: Response surface graph showing the interactive effect of acid concentration and temperature on glucose yield; (a) contour plot (b) 3D graphs

The interaction between temperature and time in this study was insignificant for sugar yield. The contour plot (Figure 3.10 and 3.11 a,b) indicates that under similar temperatures, increasing the time above 1 h did not lead to any significant increase in sugar yield. This may be attributed to the fact that the temperature range within the scope of this study was not sufficiently high to instigate hemicellulose solubilization without the effect of the acid catalyst (≥2.9%). Hemicellulose and lignin decomposition had been shown to occur above 120°C (Yang *et al.* 2007). Hence, several dilute acid pretreatment studies were carried out at a temperature greater than 120°C (Solarte-Toro *et al.* 2019). Therefore, heating alone irrespective of the duration of pretreatment did not lead to significant sugar yield.

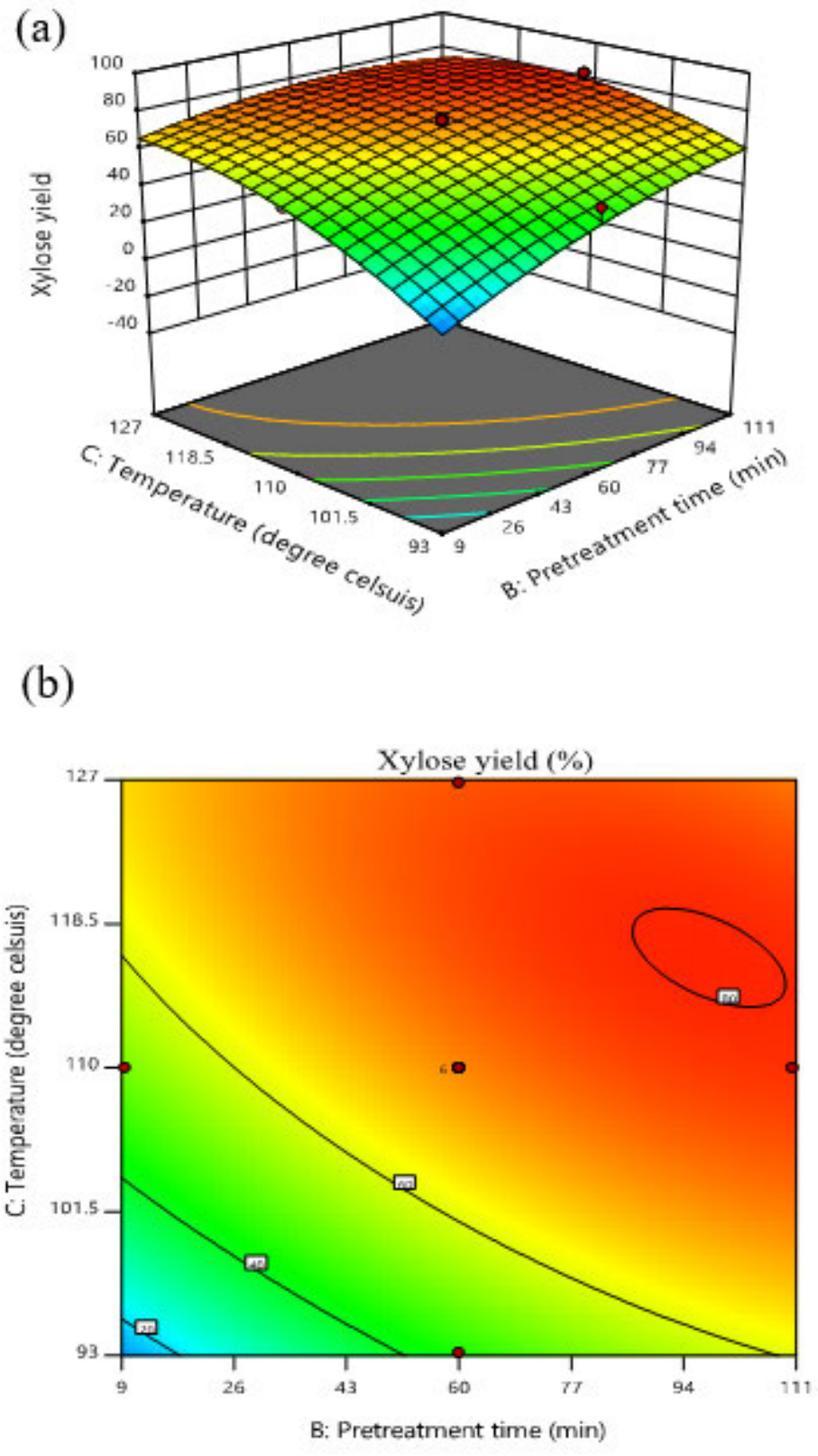

Figure 3.10: Response surface graph showing the interactive effect of acid concentration and temperature on xylose yield; (a) contour plot (b) 3D graph

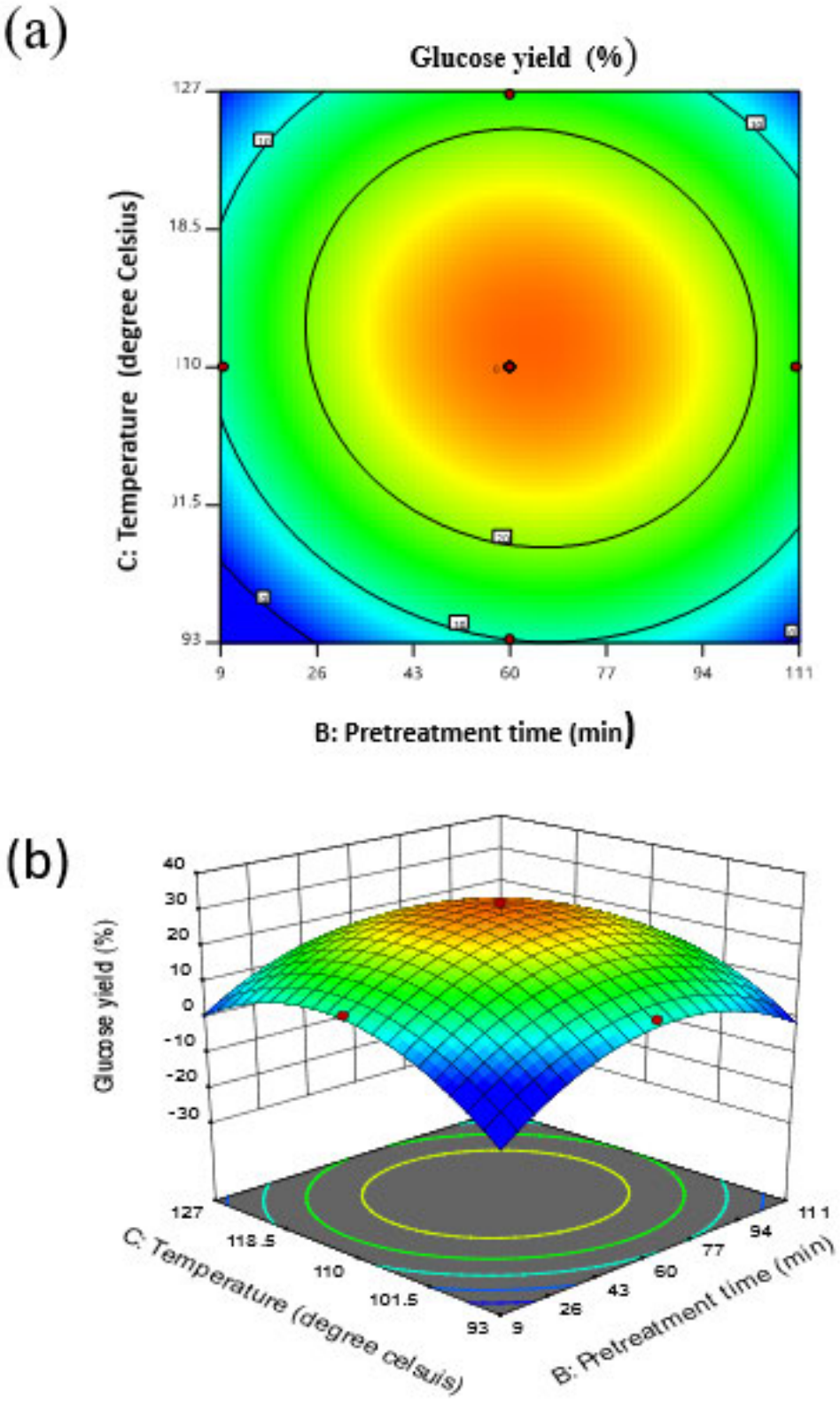

Figure 3.11: Response surface graph showing the interactive effect of pretreatment time and temperature on glucose yield; (a) contour plot (b) 3D graphs

The concentration of inhibitors increased steadily when any two interacting factors increased from the lowest to the highest values. Maximum inhibitors were formed when any two factors were at the maximum (Figure 3.12 – 3.14). This trend is related to the severity of the pretreatment which influenced by acid concentration, temperature and time (Solarte-Toro *et al.* 2019). When any two factors were at maximum, the severity of the pretreatment was high and under severe conditions, the conversion of solubilised sugars into inhibitors (formic acid, acetic acid, furans) accelerated (Jönsson and Martín 2016).

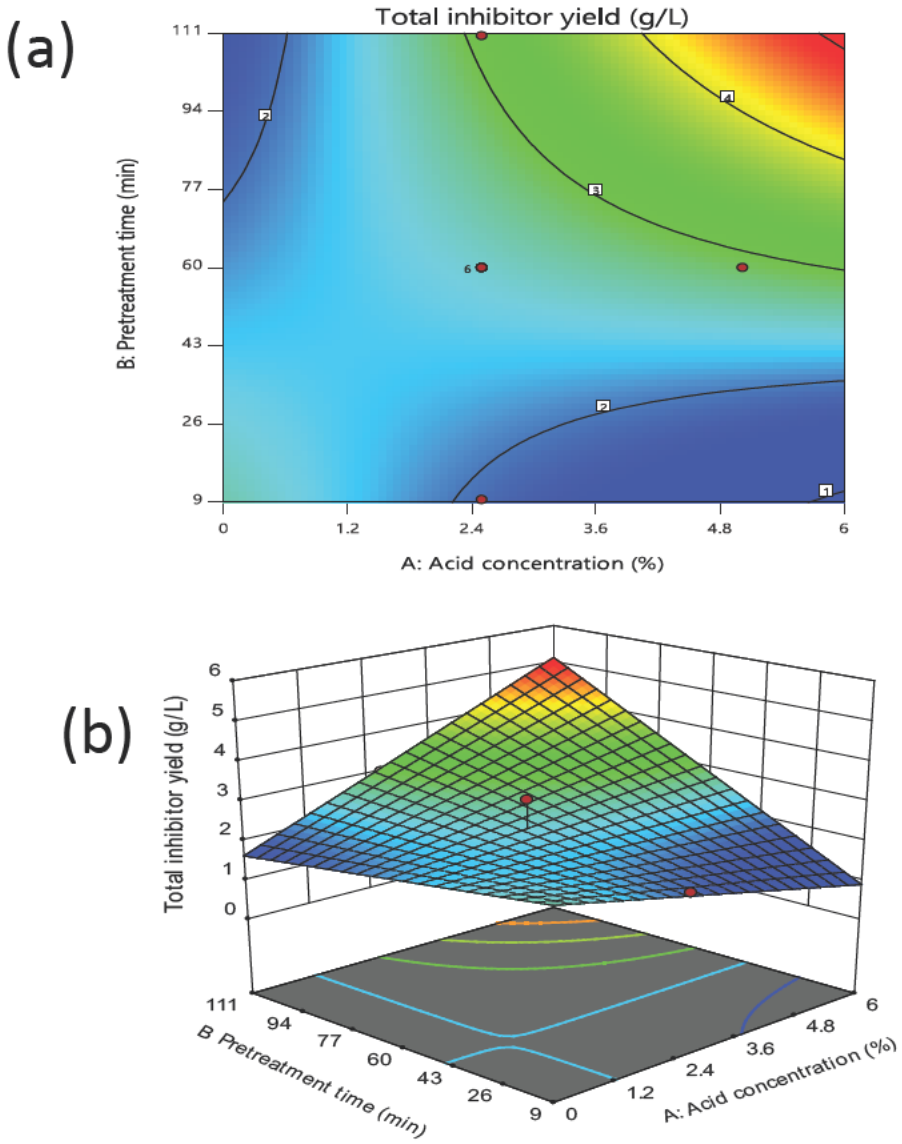

Figure 3.12: Response surface graph showing the interactive effect of acid concentration and time on inhibitor concentration (a) contour plot (b) 3D graph

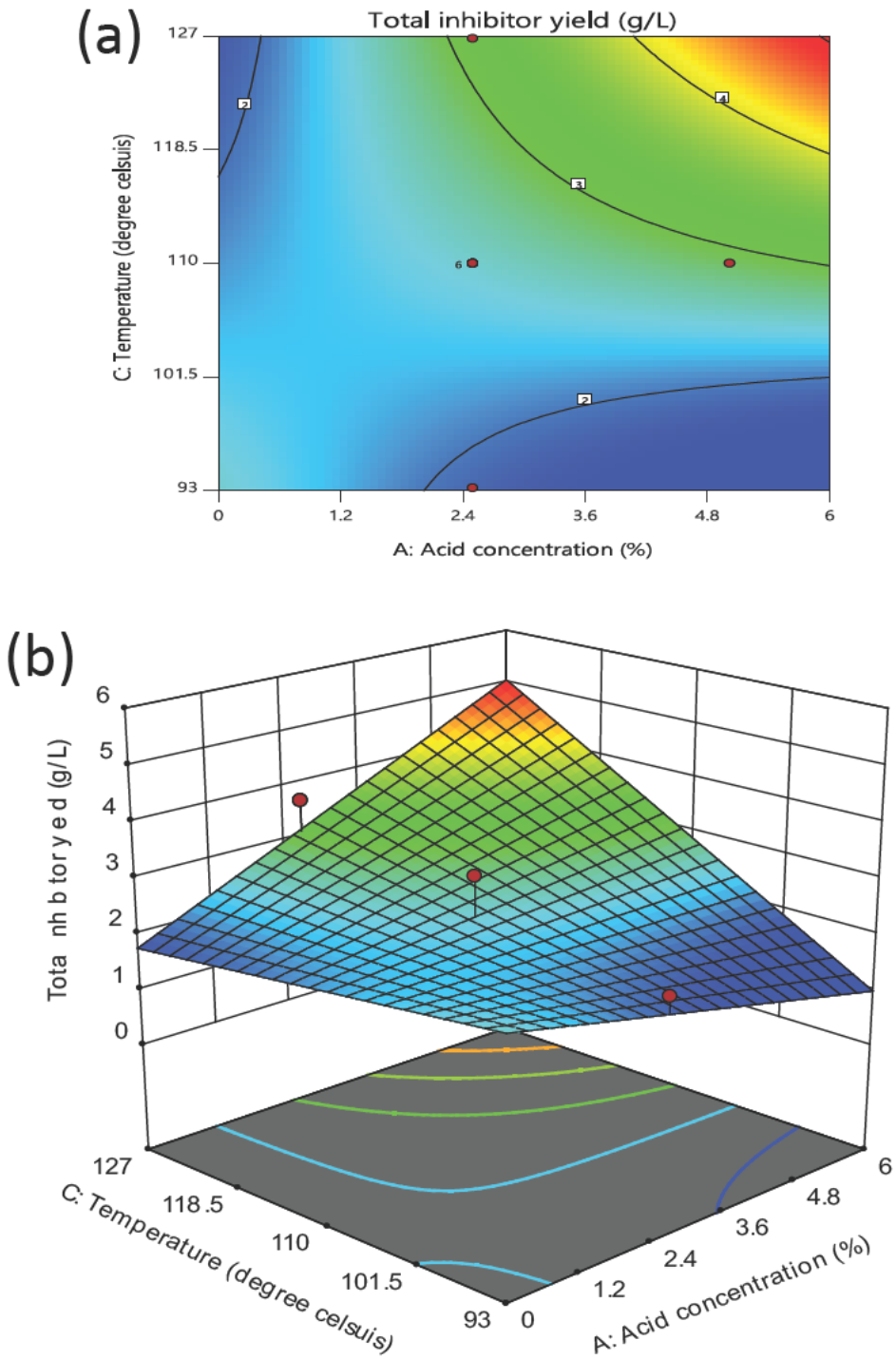

Figure 3.13: Response surface graph showing the interactive effect of acid concentration and temperature on inhibitor concentration (a) contour plot (b) 3D graph

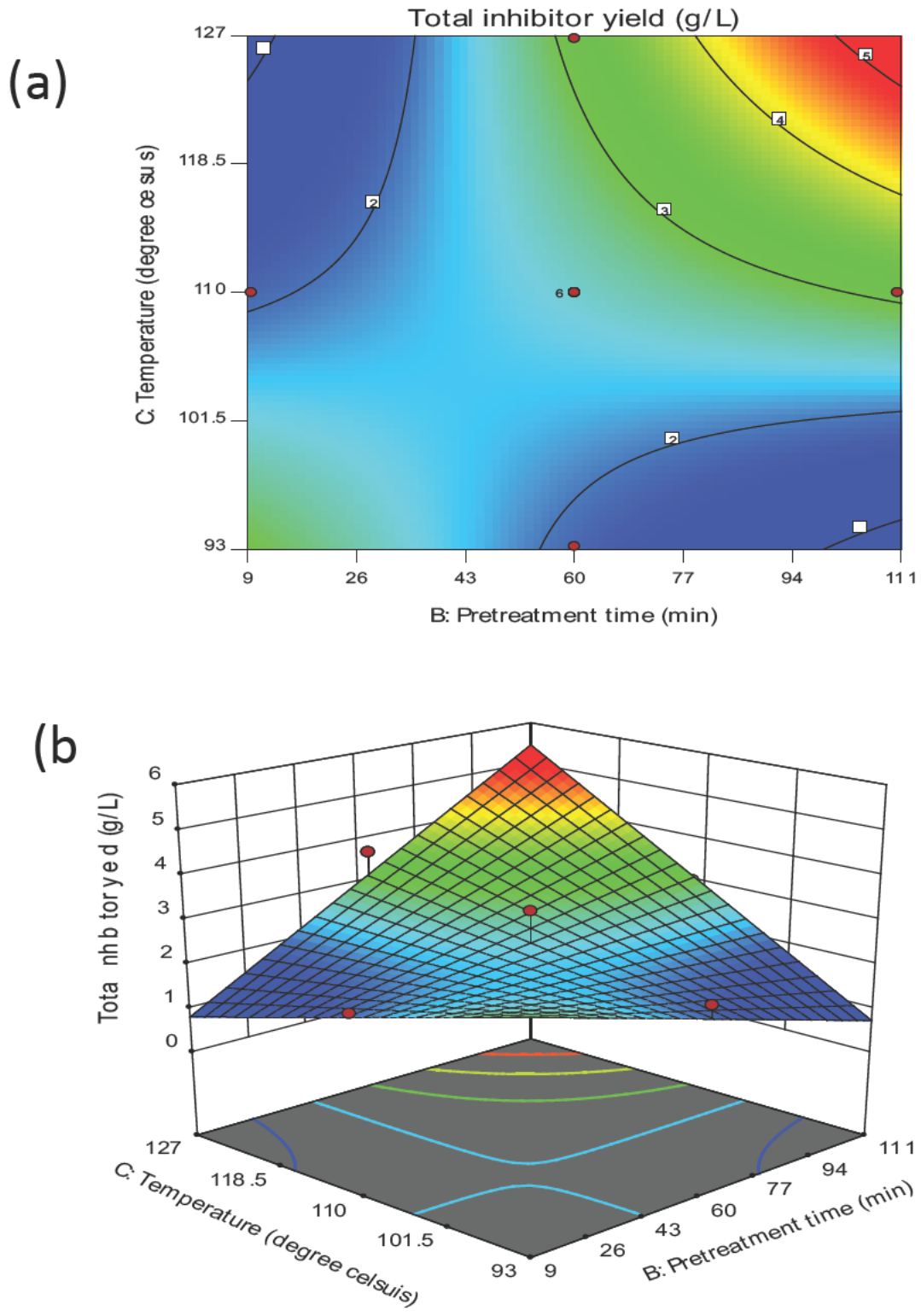

Figure 3.14: Response surface graph showing the interactive effect of temperature and time on inhibitor concentration (a) contour plot (b) 3D graph

3.3.6 Characteristics of solid residue pretreated at optimal conditions

The solids recovered after dilute acid pretreatment of CH at optimal conditions contained 31% cellulose, 2.5% hemicellulose and 4.1% lignin. This is equivalent to about 72% of the initial cellulose remaining in the pretreated solids which could be saccharified enzymatically and fermented to bioethanol.

3.3.7 X-ray diffraction

X-ray diffraction was conducted to determine crystallinity differences in untreated and dilute acid pretreated CH samples. The crystallinity of lignocellulosic biomass is determined by the composition of cellulose, hemicellulose, and lignin (Wiselogel, Tyson and Johnson 2018). Cellulose is the source of crystalline regions (well, ordered, recalcitrant regions); however, it also contains amorphous domains (less ordered and reactive regions). On the other hand, hemicellulose and lignin are wholly amorphous. Some studies indicate that crystallinity imparts recalcitrance to biomass hindering digestibility (Bichot *et al.* 2018). Compared to the untreated CH, diffractogram results from this study show that the crystalline peak present at an angle $2\Theta = 22.5°$ (crystalline cellulose) becomes extended and sharper, in addition to multiple new crystalline peaks in the pretreated sample (PCH) (Figure 3.15). The crystallinity index increased from 55% to 65% in untreated and pretreated CH respectively. This increasing trend in the crystallinity index is consistent with previous studies and has been attributed to the removal of amorphous portions in the biomass leading to an improvement in digestibility. The crystallinity index of dilute phosphoric acid pretreated (4% v/v) cauliflower wastes were slightly increased from 41.31% to 45.28% and 30.95% to 32.57% for cauliflower stalk and leaf respectively (Majumdar *et al.* 2019). Also, the crystallinity index and crystallite size of dilute acid pretreated rice straw increased from 40.84 to 51.49% and 2.60 to 3.08 nm compared to native samples (Kshirsagar *et al.* 2015). A similar trend was observed for cotton stalk (Gaur *et al.* 2016).

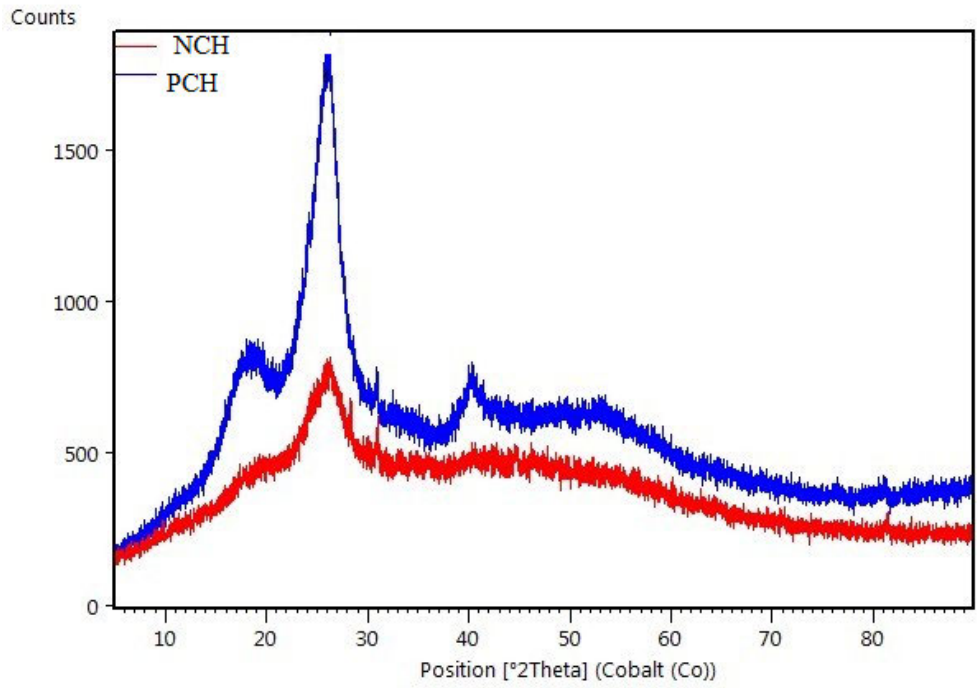

Figure 3.15: X-ray diffraction peaks for native CH (NCH) and pretreated CH (PCH)

3.3.8 Fourier transform infrared (FTIR) analysis

Chemical changes in the native and pretreated samples were observed using FTIR (Figure 3.16). The peak at 1507 cm^{-1}, attributed to the C – C widening in the aromatic ring of lignin, occurred only in the pretreated sample. Other characteristic lignin bands at 1600 cm^{-1} (C═C stretching vibration in lignin), 1372 cm^{-1} (phenolic hydroxyl group), 1320 cm^{-1} (C – O stretching of the syringyl ring) as well as ether (ar–C–O–C–al) and ester linkage between hemicellulose and lignin assigned to peaks 1234 cm^{-1} and 1727 cm^{-1} respectively exhibited weaker bonds in the pretreated sample compared to native samples, indicating a cleavage of the linkages between lignin and carbohydrates and a disruption in the lignin structure due to dilute acid treatment (Anita *et al.* 2019). Absorption peaks at 897 cm^{-1} (β-1,4-glycosidic linkages associated with crystalline cellulose) and 1153 cm^{-1} (asymmetric elongation of C–O–C within cellulose) occurring only in the pretreated sample indicate damage to β-1,4-glycosidic linkages within the cellulose structure (Kumar, Upadhyay and Mishra 2019). Other peaks associated with cellulose and hemicellulose observed around 1027 cm^{-1} (C–O, C═O stretching of hemicellulose or cellulose), 1364 cm^{-1} (C – H bending vibration in cellulose and hemicellulose), 1422 cm^{-1} (CH_2 scissoring motion in cellulose), 2900 cm^{-1} (C

– H and CH_2 stretching) and 3200 - 3300 cm^{-1} (OH stretching) also exhibited weaker bonds in the pretreated sample compared to the native sample.

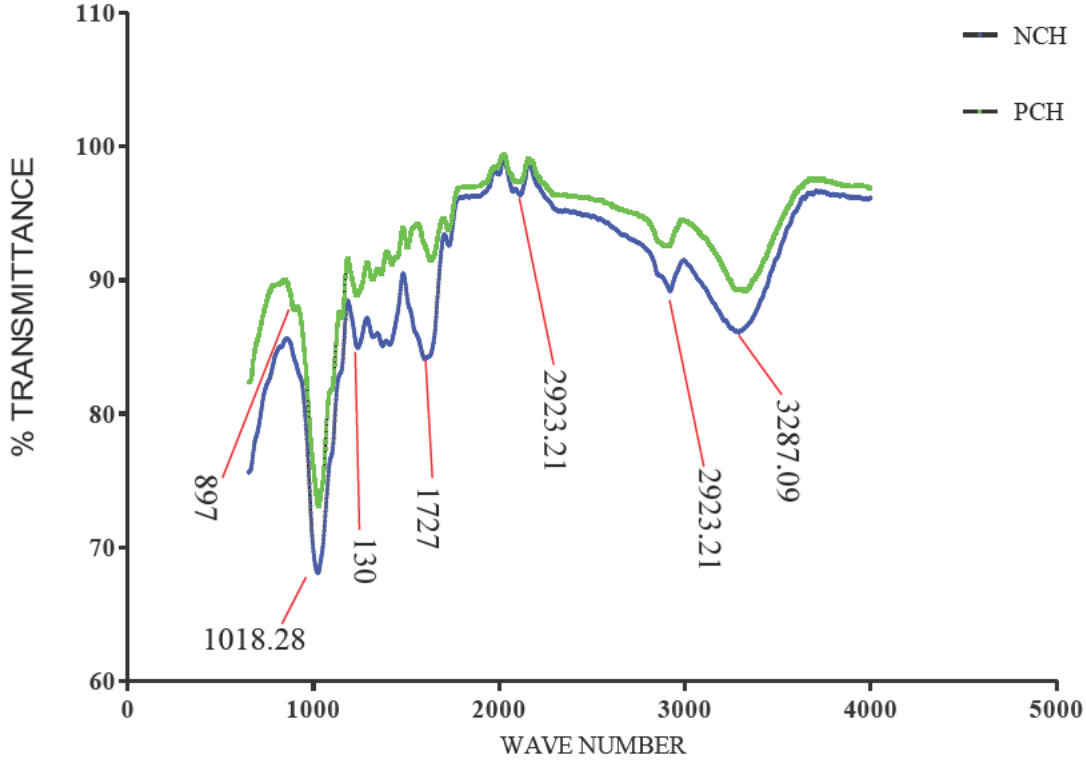

Figure 3.16: Fourier transform infrared spectroscopy of native and pretreated CH

3.3.9 Scanning electron microscopy

SEM images reveal a complete and compact lignocellulosic structure in the untreated cowpea haulm (Figure 3.17 a). Multiple cracks and fibre porosity of cowpea haulm was observed in the SEM image after DAP pretreatment under the optimized conditions (Figure 3.17 b). Peng *et al.* (2019) report similar fibre disruption in rice straw after acetic acid pretreatment. Similarly, dilute sulphuric acid pretreated cassava residues exhibited fragmented surfaces compared to the untreated residues which were compact and intact (Zhang *et al.* 2011). The abrasion and fibre disruption in dilute acid pretreated biomasses may be attributed to the solubilization of the amorphous portion correlated with the enhanced acid effects on the biomasses with the help of high temperature and pressure from the pretreatment (Elsayed *et al.* 2018).

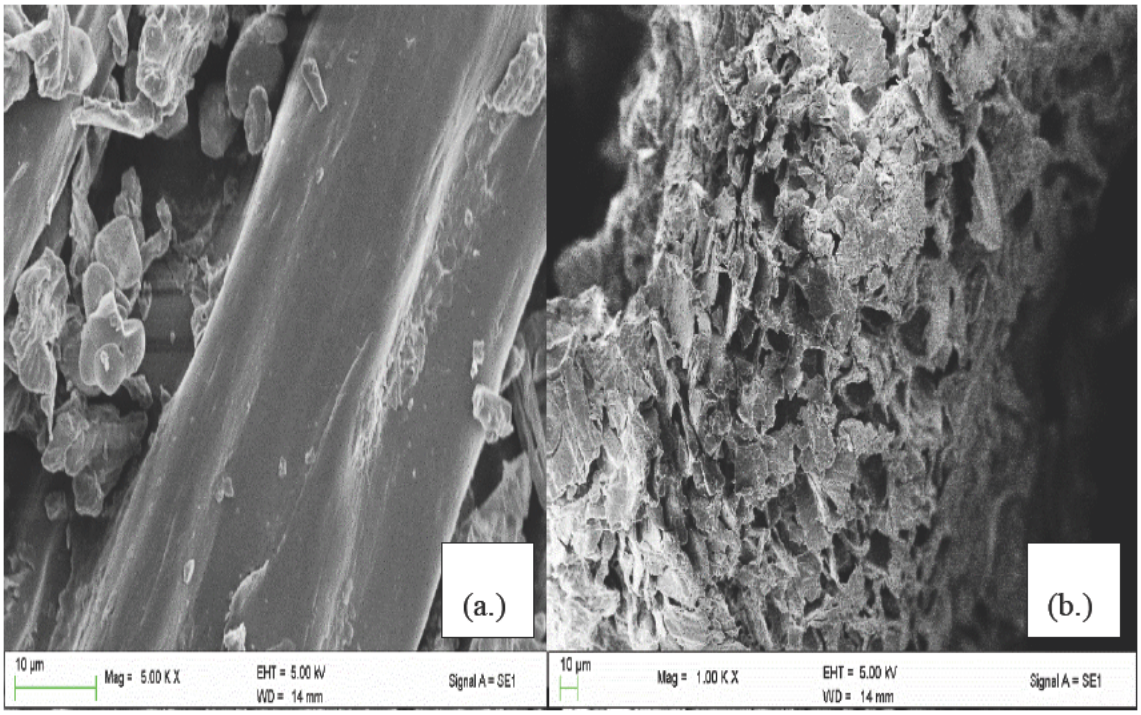

Figure 3.17: Scanning electron micrograph of (a.) native and (b.) pretreated cowpea haulm

3.3.10 Detoxification and fermentation of prehydrolysate

The prehydrolysate obtained under optimal conditions contained glucose 13 g/L, xylose 6.5 g/L and arabinose 2.03 g/L. The inhibitors furfural 0.8 g/L, 5-HMF 0.2 g/L, formic acid 1.44 g/ L and acetic acid 0.05 g/ L were also detected. The concentration of inhibitors obtained in this study was lower than in some other studies. Díaz-Blanco *et al.* (2018) reported concentration (g/L) of 0.22, 1.78 and 2.51, for furfural, HMF, and acetic acid respectively in dilute acid pretreated agave hydrolysate. The yields of formic acid, acetic acid, furfural, and HMF in dilute sulphuric acid prehydrolysate of cassava residue were 0.76, 0.44, 0.05, and 0.02 g/L respectively (Yu *et al.* 2018b). The amount of acetic acid and furans quantified in this study was lower than inhibitory concentrations for ethanologens. Acetic acid is inhibitory to *S. cerevisiae* when the concentration exceeds 1.5 g/L while concentrations of furfural and HMF > 1 g/L would inhibit the bioethanol production process (Wikandari *et al.* 2010). Weak acids such as acetic or formic acid disrupt cellular energy generation by collapsing pH gradients. This leads to an influx into the cytosol and improper ion transportation, thereby inhibiting cell growth and productivity (Kim 2018). Furfural and HMF inhibit glycolysis, especially interfering with the activity of dehydrogenases, causing a reduction in growth rates and cell yields (Pienkos and Zhang 2009). In addition, inhibitors exert varying synergistic effects on ethanologens depending on the inhibitor composition

(Greetham, Hart and Tucker 2016; Hu *et al.* 2019). Moreover, the highly varied composition of inhibitory compounds that could be produced during pretreatment hinders an accurate quantification of all inhibitors (Zhang *et al.* 2018). Hence, detoxification is an essential and commonly applied step after DAP to remove these inhibitory substances. Overliming is a common procedure to reduce the toxicity of prehydrolysate through the removal of inhibitory compounds by precipitation (Andary *et al.* 2012; Wikandari *et al.* 2019). It is usually performed by the addition of alkali ($Ca(OH)_2$ or NaOH) to increase the pH up to 12, followed by an adjustment to the cultivation pH (Ranatunga *et al.* 2000). The alkaline conditions facilitate aldol reactions between ketones and aldehydes and the oxidation of carbonyl compounds mitigate their toxicity (Zhang *et al.* 2018). The detoxified hydrolysate in this study contained glucose 10.8 g/L, xylose 4.6 g/L, arabinose 1.03 g/L, furfural 0.1 g/L and formic acid 0.29 g/ L. There was complete removal of acetic acid, 5-HMF, and 80% removal of furfural and formic acid after the detoxification step. Deshavath *et al.* (2017) had reported a 23.7%, 20%, 11.9% and 12.9% reduction in furfural, 5-HMF, formic acid, and acetic acid after overliming of dilute acid pretreated sorghum stalks. However, there was approximately 23% and 29% loss of glucose and xylose. In the studies of Zhang *et al.* (2018), 7.2% sugar loss was reported following overliming of poplar prehydrolysates. Mohagheghi, Ruth and Schell (2006) also observed that xylose loss in corn stover hydrolysate rose with increasing overliming pH from 7% at pH 9 to 34% at pH 11.

Fermentation of detoxified acid hydrolysate using coculture of *S. cerevisiae* BY4743 and *S. stipitis* wild type (PsY633) produced maximum ethanol titer of 6.22 ± 0.17 g/L (Figure 3.18), after 36 h incubation, which corresponds to an ethanol yield of 0.38 g/g sugar consumed, with 0.17 g/L/h productivity and 74% fermentation efficiency. About 88% of the initial glucose was consumed within 24 h and 3.7 ± 0.3 g/L ethanol was produced, which represents 59% of the total ethanol produced. Xylose utilization started after glucose was exhausted. The results obtained in this study are comparable with previous literature. In a study conducted on sugarcane straw, about 12.67 g ethanol/L was produced from 33.45 g/L glucose, corresponding to 62.74% fermentation efficiency after dilute acid pretreatment and enzymatic hydrolysis (Mesa *et al.* 2017). Keshav *et al.* (2016) reported maximum ethanol of 11.64 ± 0.48 g/L from the fermentation of detoxified acid pretreated cotton stalk hydrolysate using coculture of *S. cerevisiae* VS3 and *P. stipitis* NCIM3498. In his study, the maximum ethanol yield of 0.47 g/g with a productivity of 0.24 g/L/h was attained at 48 h incubation.

Similar studies by Láinez et al. (2019) reported maximum ethanol concentrations of 22.79 g/ L after 24 h fermentation by *S. cerevisiae* from 50.88 ± 0.54 g /L glucose, corresponding to 87.63% fermentation efficiency.

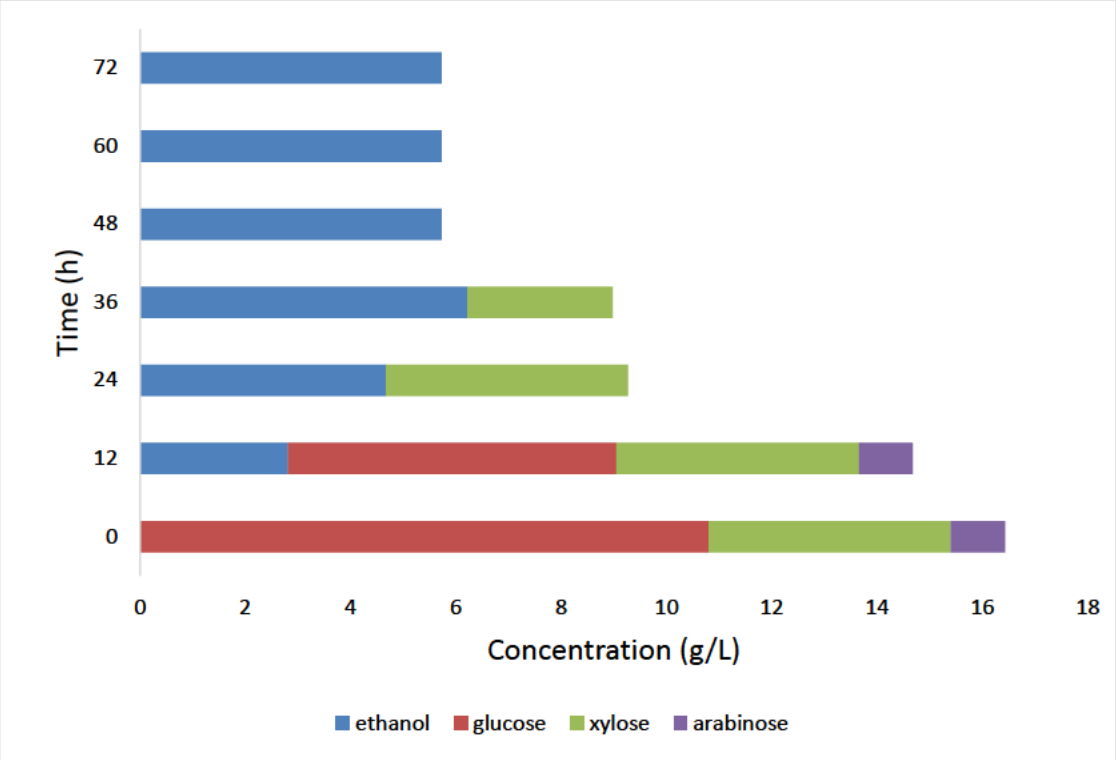

Figure 3.18: Time course for sugar consumption and ethanol production in the SHF process

3.3.11 Simultaneous saccharification and fermentation with prehydrolysis

S. cerevisiae BY4743 was introduced following a 24 h presaccharification that resulted in an initial sugar concentration of 14.8 g/L glucose (53% of the theoretical yield) and 2.01 g/L xylose (90% of the theoretical yield) accumulated in the fermentation broth. The maximum ethanol titer of 9.45 g/L obtained in this study within 18 h of fermentation corresponded to an ethanol yield of 0.42 g/g glucose and productivity of 0.53 g/L/h (Figure 3.19).

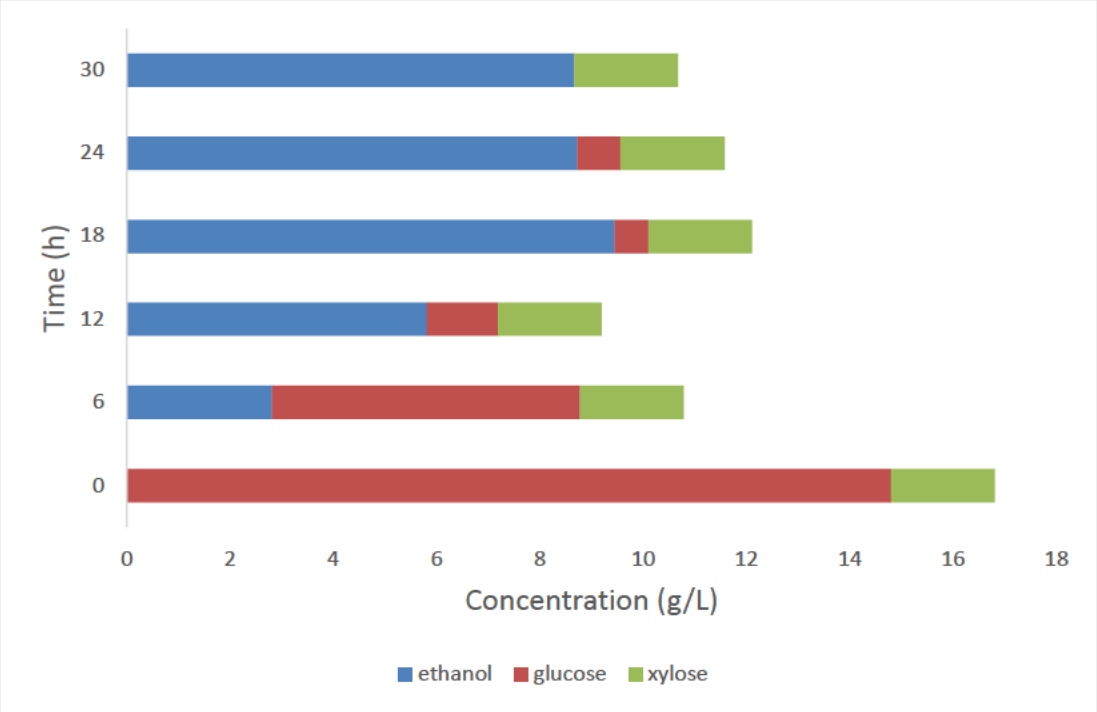

Figure 3.19: Time course for sugar consumption and ethanol production in the SSF process

The results obtained in this study agrees with earlier reports which have indicated that the application of a prehydrolysis step prior to SSF, facilitated an ethanol yield ≥ 50% of the theoretical yield (McIntosh *et al.* 2016; Fernandes *et al.* 2018). Following a 24 h prehydrolysis and SSF of sugar straw hydrolysate, Mesa *et al.* (2017) observed an ethanol concentration of 14.47 g/L corresponding to 72.37% of glucose utilization. Fernandes *et al.* (2016) observed an ethanol concentration of 5.7 g/L corresponding to 58% of ethanol yield from PSSF of dilute acid pretreated olive pomace. Bioethanol concentrations and bioethanol conversions for the PSSF of sequential alkalic salt and dilute acid pretreated corncob was 36.92 g/L and 62.36 % respectively (Sewsynker-Sukai and Gueguim Kana 2018). The variation in the ethanol concentration in various studies is due to the differences in the amount of fermentable sugar subjected to the fermentation process (Li *et al.* 2016a).

Considering the pretreatment and enzymatic hydrolysis step in this study, a total sugar concentration of 44.9 g/L corresponding to 93% of the theoretical sugar in CH was hydrolysed, indicating the effectiveness of DAP. However, only 84.5% of the hydrolysed sugar was recovered for fermentation, due to sugar loss in the pretreatment and detoxification stage. High sugar yields above 90% have been reported for DAP of biomasses

such as cotton, agave, etc. (Gaur *et al.* 2016; Díaz-Blanco *et al.* 2018). The fermentation of both the hydrolysates from DAP and the enzymatic stage in this study gave a total ethanol titre of 15.67 g/L corresponding to 75% conversion efficiency. This was similar to the ethanol yield of 73.3% observed from the fermentation of mixed sugars obtained from dilute acid hydrolysis and enzymatic hydrolysis of *Agave lechuguilla* using *Escherichia coli* MM160 (Díaz-Blanco *et al.* 2018). The conversion of both pentose and hexose sugars has been shown to improve ethanol titers (Li *et al.* 2018a; Wu *et al.* 2018b).

3.4 Conclusion

The physicochemical composition, pretreatment, and fermentation of CH was investigated in this study. Response surface methodology was employed to investigate optimal sugar recovery from dilute acid pretreatment of CH. Our findings suggested that CH was a promising feedstock for bioethanol due to its high polysaccharide and low lignin composition. In addition, 85% of the theoretical sugar yield in CH could be recovered with dilute acid hydrolysis indicating the effectiveness of this pretreatment strategy for biosugar production from CH. Furthermore, the fermentation of sugar hydrolysates obtained from both the pretreatment and enzymatic stage was useful to improve bioethanol yield from CH. However, further studies on optimisation of the enzymatic and fermentation conditions could improve the yield and should be explored.

4 CHAPTER FOUR
DEEP EUTECTIC SOLVENT PRETREATMENT OF BAMBARA HAULM FOR ENHANCED SACCHARIFICATION AND BIOETHANOL PRODUCTION

4.1 Introduction

Bambara haulm is the above-ground residual biomass remaining after the pod harvest. It is an underutilised yet promising lignocellulosic feedstock for valorisation to bioethanol. Typically, in the conversion of lignocellulosic biomass to bioethanol, a pretreatment step precedes saccharification, in order to overcome the recalcitrance of the lignocellulosic feedstock. To date, a series of chemicals have been applied to overcome biomass recalcitrance including acid, alkali, inorganic salt, ionic liquid, etc., each presenting inherent advantages and disadvantages (Behera *et al.* 2014). More recently, a nascent group of chemicals called deep eutectic solvents (DES) have shown high applicability in lignocellulosic biomass processing (Zdanowicz, Wilpiszewska and Spychaj 2018). DES is in the class of ionic liquids; however, they are preferred for their ease of synthesis, low toxicity, lower cost, recyclability, etc. (Liu *et al.* 2018b). DES is a eutectic mixture obtained from binary or ternary combination of hydrogen bonding donors (HBDs) and hydrogen bonding acceptors (HBAs) at a specific molar ratio. DES can be prepared by simultaneously heating and stirring the HBA-HBD mixture until a clear liquid is formed (Loow *et al.* 2017). The DES remains a liquid at ambient temperature due to the formation of intermolecular hydrogen bonds that leads to a depression in the melting point (Pätzold *et al.* 2019).

Choline chloride is the most commonly used HBA because it is inexpensive and easily accessible (Zdanowicz, Wilpiszewska and Spychaj 2018). Studies have shown that several DES have improved the digestibility of biomass (Xu *et al.* 2016; Zhang, Xia and Ma 2016; Procentese and Rehmann 2018). Notably, DES produced using choline chloride and lactic acid, formic acid, acetic acid, glycerol, urea, etc., have reportedly enhanced the digestibility of lignocellulosic biomass three to four times more than untreated biomass (Xu *et al.* 2016). However, like other pretreatment methods, DES pretreatment is also feedstock-dependent.

For example, Gunny *et al.* (2015) reported the effectiveness of ChCl:urea and ChCl:glycerol in the pretreatment of rice husk. However, ChCl:urea and ChCl:glycerol was ineffective in the pretreatment of corn cob (Xu *et al.* 2016). Such differences have been attributed to varying recalcitrance of lignocellulosic biomass. Moreover, DES pretreatment has been shown to be affected by factors such as pretreatment time, HBA to HBD ratio, temperature and solid loading (Procentese and Rehmann 2018; Liu *et al.* 2019c). Studies on the pretreatment of BGH have not been earlier reported. The application of DES could facilitate clean fractionation of BGH and enhance its digestibility for conversion to ethanol. Thus, it is worth investigating the effectiveness of several DES in the pretreatment of BGH for cellulosic ethanol production and optimising conditions for their maximum digestibility.

4.2 Materials and methods

4.2.1 Materials

BGH was obtained from the Agricultural Research Council (ARC), VOPI, Pretoria, South Africa, dried, milled and sieved to a particle size of ≤ 0.5 mm. The milled BGH was stored at room temperature in airtight plastic containers. Choline chloride, lactic acid, formic acid, acetic acid, glycerol, and urea were of the highest purity and purchased from Sigma Aldrich, South Africa.

4.2.2 DES synthesis

Choline chloride was dried at 80°C for 6 h and cooled to room temperature in a desiccator prior to use. Six DES were synthesized by combining choline chloride and the various hydrogen bond donors at specific molar ratios (Table 4.1). The mixture was heated at 60°C with continuous stirring (180 rpm), in an Erlenmeyer flask until a homogenous colourless liquid was formed. The synthesized DES was further heated at 80°C for 6 h to remove the unreacted free acid, cooled and kept for 24 h in a vacuum desiccator before use to ascertain that the solution remained clear.

Table 4.1: List of deep eutectic solvents used in the study.

HBA	HBD	Molar ratio	Acronym
Choline chloride	Acetic acid	1:2	ChCl:AA
Choline chloride	Formic acid	1:2	ChCl:FA
Choline chloride	Lactic acid	1:2	ChCl:LA
Choline chloride	Glycerol	1:2	ChCl:GLY
Choline chloride	Urea	1:2	ChCl:UREA
Choline chloride	Imidazole	3:7	ChCl:IM

4.2.3 Pretreatment of BGH with DES

Each DES was weighed into an Erlenmeyer flask, preheated to 100°C in a water bath and mixed with BGH (3 g) at a solid to liquid ratio of 1:10 (w/w). The pretreatment was conducted by heating the slurry at 100°C for 3 h with continuous stirring (180 rpm). After pretreatment, the flasks were taken out and cooled to 70°C by placing the flask in cold water. The slurry was then mixed with 200 ml of hot deionised water (antisolvent), stirred for 1 h and vacuum filtered to recover the pretreated solids. The washing step was repeated until the wash water pH was neutral, indicating complete removal of residual DES. The solid residue was dried at 30°C for further use. The filtrate was combined and centrifuged at 10,000 g for 5 min to precipitate lignin. The recovered lignin was further washed with 15 ml ethanol thrice, dried at 30°C to constant weight and stored in airtight vials in a desiccator. Lastly, the DES-water mixture was vacuum evaporated at 50°C to recover the pure DES.

4.2.4 Enzymatic hydrolysis

Untreated and DES pretreated BGH (1 g) was enzymatically hydrolysed at 10% solid loading in 2 ml Eppendorf tubes containing citrate buffer (50 mM, pH 4.8), 0.01 wt.% NaN_3, and the enzyme cocktails, Accellerase 1500 and Accellerase XC (Genencor, Netherland). The

enzymes were loaded at a ratio of 1:1 and an enzyme load of 30 mg protein/g biomass (Kothari *et al.* 2018). Enzymatic hydrolysis was conducted in a water bath at 50°C and 150 rpm for 48 h. The hydrolysate was boiled for 5 min to deactivate the enzymes and then centrifuged at 17,000 g for 2 min. The resulting supernatant was collected and analysed for total reducing sugar by the DNS method (Miller 1959). The enzymatic sugar yield (g sugars/ g pretreated biomass) was calculated as the ratio between the mass of sugars produced by the enzymatic hydrolysis and the theoretical potential mass of sugar obtainable from the pretreated biomass subjected to the enzymatic hydrolysis process (Xu *et al.* 2019).

4.2.5 Optimization of DES pretreatment conditions

Based on the results obtained, ChCl:LA pretreatment was further optimised due to its high efficiency in lignin removal and digestibility. To minimise cellulose removal, the impact of temperature (80, 100, and 120°C) and pretreatment time (1, 2, and 3 h) on the pretreated solid composition and enzymatic sugar yield was further studied (Table 20). The pretreatment method was the same as described previously except that the antisolvent was changed to ethanol. Enzymatic hydrolysis was also performed as stated earlier using 7 g of biomass, pretreated under optimal conditions (ChCl:LA, 100°C, 1 h).

4.2.6 Bioethanol production

4.2.6.1 Microorganism and inoculum preparation

S. cerevisiae BY4743 (Discipline of Microbiology, University of KwaZulu-Natal, South Africa) previously maintained on a yeast peptone dextrose (YPD) agar (10 g/L of yeast extract, 20 g/L of bacteriological peptone, 20 g/L of glucose and agar 15 g/L) was used as inoculum. *S. cerevisiae* BY4743 was grown on YPD broth at 30°C for 18 h in an orbital shaker (120 rpm) prior to use. Cell growth was measured using a spectrophotometer (Genesys 150 UV-Vis, Thermo Fisher Scientific, USA) at a wavelength of 600 nm.

4.2.6.2 Bioethanol fermentation

The biomass hydrolysate (30 ml) was supplemented with fermentation medium (composed of yeast extract 5 g/L, peptone 5 g/L, $Mg_2SO_4 \cdot 7H_2O$ 1 g/L, $(NH_4)_2SO_4$ 1 g/L and KH_2PO_4 2 g/L, pH 6) and autoclaved at 121°C for 15 min and cooled (Sanusi, Faloye and Gueguim

Kana 2019). A 10% inoculum was added to the sterile broth and the fermentation was carried out at 30°C, 150 rpm, pH 5.5, with a total working volume of 50 ml. Samples were routinely taken for the analysis of sugars and ethanol.

4.2.7 Analytical methods and formulas

The solid yield (%) was defined as the ratio of pretreated BGH (g) to the raw material (g). Glucan, xylan and lignin content of all the samples were determined by the standard NREL protocols (Sluiter *et al.* 2008c). Sugar yield for enzymatic hydrolysis was calculated as the percentage of theoretical sugar yield of pretreated biomass. The solid recovery was calculated as the percentage of solid recovered after pretreatment.

The composition of untreated and DES pretreated BGH was determined using the NREL technical reports (Sluiter and Sluiter 2010). Monomeric sugars and ethanol were quantified as described in section 3.2.7. The glucose utilisation (%), ethanol yield, productivity and fermentation efficiency were calculated according to the Equations (9 – 12) respectively.

Fourier transform infrared (FTIR) spectrophotometry was performed using Perkin Elmer Spectrum 100 (Waltham, MA, USA) with attenuated total reflectance (ATR) sampling accessory. The spectrum was recorded between 380 and 4000 cm^{-1} (Kumar, Parikh and Pravakar 2016).

4.3 Results and discussion

4.3.1 Screening of DES for BGH pretreatment

Six deep eutectic solvents (DESs) were synthesized by mixing choline chloride and hydrogen donors, including acetic acid, formic acid, lactic acid, imidazole, glycerol and urea to form, ChCl:AA, ChCl:FA, ChCl:LA, ChCl:IM, ChCl:GLY and ChCl:UREA. Although a homogeneous and clear liquid was formed by ChCl and each of the six different HBDs at 60°C, a waxy solid precipitated upon cooling ChCl-IM (3:7). A stable eutectic point had been earlier reported at a molar ratio of ChCl-IM (3:7) by Ren *et al.* (2016), however, this was not achieved in this study. The proton affinities of the HBA and the HBDs at specific molar ratios determine their phase behaviours (Smith, Abbott and Ryder 2014). Hence it may be inferred that the eutectic point was not reached for ChCl:IM at molar ratio 3:7. Therefore, only five DESs (ChCl-AA, ChCl-FA, ChCl-GLY, ChCl-LA, and ChCl-UREA)

were examined for their capability to improve the digestibility of BGH. BGH was pretreated with the five DES and was treated under similar conditions (100°C, 3 h). The pretreatment resulted in the fractionation of the biomass into the following components: (1) a cellulose-rich substrate (pulp), (2) a regenerated lignin and (3) a recovered DES solution containing a range of solubilized sugars. The cellulose rich pulp recovered from the pretreatment is the desired intermediate which was subjected to enzymatic saccharification to release glucose for bioethanol fermentation.

4.3.2 Mass recovery and chemical composition of DES pretreated BGH solids

The solid yield decreased after the pretreatment and was in the range of 34.33% to 45.42% (Table 4.2). The low solid yield was attributed to the removal of cellulosic components mainly lignin and hemicellulose into the DES solution. DES pretreatment resulted in the solubilisation of cellulose (28.25 – 44.15%) and hemicellulose (> 50%). In addition, all five DES exhibited varying delignification ability (Table 4.2). ChCl:LA (67.3%) and ChCl:Urea (39.23%) exhibited the highest and lowest delignification respectively. Lignin serves as a protective covering to cellulose and hemicelluloses, conferring recalcitrance on the biomass (Bichot *et al.* 2018). Therefore, the partial removal of lignin could facilitate the enhanced digestibility of BGH (Zhang, Xia and Ma 2016). The delignification ability of the five DES investigated in this study has been previously reported (Zdanowicz, Wilpiszewska and Spychaj 2018). However, comparative evaluation of previous studies and the current study indicate that the delignification potential of each DES is feedstock dependent (Table 4.3). This could be attributed to the varying levels of recalcitrance among the feedstocks tested. The acidic DES showed higher capacity (>50%) for delignification compared to the neutral ChCl:GLY and the mildly alkaline ChCl:Urea (Table 4.2). The three acidic DES investigated in this study (ChCl:AA, ChCl:FA, ChCl:LA) has previously shown high selectivity to extract poplar wood lignin and enhance cellulose reactivity (Tian *et al.* 2019). Although the mechanism of DES action on lignin solubilisation is not fully understood, the high lignin selectivity of acidic DES has been attributed to the acidity and strong ionic properties of this class of DES. Esterification between the acids and the hydroxyl group in lignin as well as strong ionic interactions between the DES and lignin moieties have been proposed as a possible mechanism for the highly selective lignin solubilisation (Smink *et al.* 2019; Tian *et al.* 2019).

The changes in the chemical structure of untreated and DES-pretreated BGH was confirmed by FTIR analysis (Figure 4.1). Compared to the native sample, characteristic lignin bands at 1600 cm^{-1} (C═C stretching vibration in lignin), 1372 cm^{-1} (phenolic hydroxyl group), 1320 cm^{-1} (C – O stretching of the syringyl ring) exhibited weaker bonds indicating partial removal of lignin. Also, ether (ar–C–O–C–al) and ester linkage between hemicellulose and lignin assigned to peaks 1234 cm^{-1} and 1727 cm^{-1} respectively, exhibited weaker bonds suggesting cleavage and partial removal of hemicellulose and lignin (Chen and Wan 2018). The peak at 1507 cm^{-1} attributed to C – C widening in the aromatic ring of lignin, occurred only in the pretreated sample. This indicates a disruption of the lignin moieties. In addition, multiple new peaks around 1377 cm^{-1} (C – H bending vibration in cellulose) and 1153 cm^{-1} (asymmetric elongation of C–O–C within cellulose) were observed only in the pretreated sample which suggests that cellulose was exposed and deformed. Other peaks associated with cellulose and hemicellulose observed around 1027 cm^{-1} (C—O, C═O stretching of hemicellulose or cellulose), 1364 cm^{-1}, 1422 cm^{-1} (CH_2 scissoring motion in cellulose), 2900 cm^{-1} (C – H and CH_2 stretching) and 3200 - 3300 cm^{-1} (OH stretching) also exhibited weaker bonds in the pretreated sample compared to the native sample (Chen, Reznicek and Wan 2018).

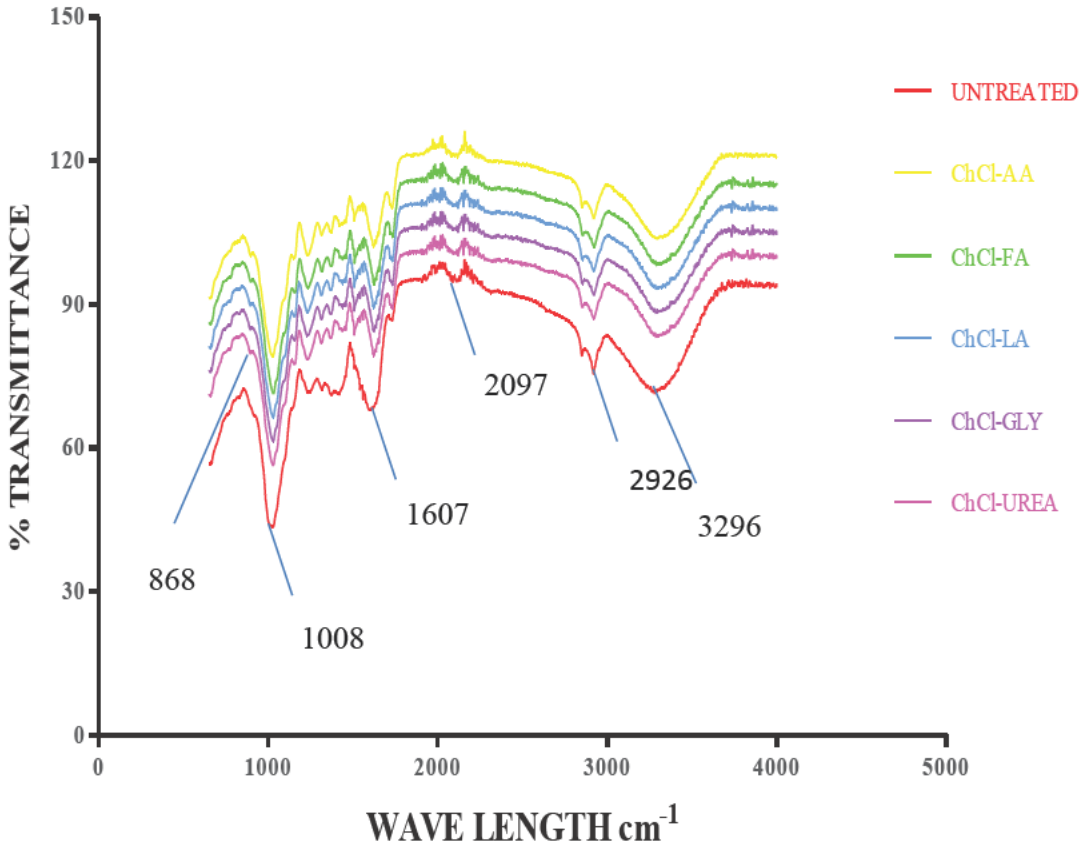

Figure 4.1: FTIR of untreated and DES pretreated BGH

Our initial characterisation studies had shown a high amount of amorphous region in BGH (78%) (Figure 2.2). The high polysaccharide solubilisation could be attributed to the dissolution of this amorphous region, in particular, cellulose and hemicelluloses, which are easily hydrolysed. High recovery of glucan and hemicellulosic sugars is necessary to improve the techno-economics of a bioethanol process (Lynd *et al.* 2017), hence, the sugar loss during DES pretreatment is not desirable and requires a more in-depth study. Although the solubility of pure cellulose and xylan in DES was reportedly low (<1%) (Lynam, Kumar and Wong 2017), studies utilising lignocellulosic biomass report hemicellulose removal above ≥ 50% and higher cellulose loss (Table 4.3). Chen, Reznicek and Wan (2018) observed hemicellulose and glucan removal of 90.06% and 3.65 – 5.62% respectively when switchgrass was pretreated with acidified or aqueous mixtures of ChCl:GLY. ChCl:FA pretreatment of corn stover led to 2.2% and 66.2% glucan and hemicellulose removal respectively (Xu *et al.* 2016). The recovery of the hemicelluloses and amorphous celluloses prior to DES pretreatment could improve sugar yield for bioconversion to ethanol. (Ong *et al.* 2019) achieved a 58% improvement in xylose recovery by ultrasonication of oil palm fronds in water medium prior to DES pretreatment. Steam pretreatment of poplar (190°C, 10 min) resulted in a 75% hemicellulose recovery and enhanced the subsequent DES pretreatment (Tian *et al.* 2017).

Table 4.2: Yield and composition of untreated and DES pretreated solid residue

Pretreatment	**Solid recovery (%)**	**Composition (%)**			**Removal (%)**			**Enzymatic TRS yield (%)***
		Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin	
Untreated		36.49	14.91	13.10				28.54 ± 0.5
ChCl-AA	38.20	61.17 ± 2.01	16.04 ± 0.2	14.53 ± 0.1	35.96 ± 1.1	58.89 ± 0.9	57.63 ± 0.7	79.44 ± 4.8
ChCl-FA	36.91	56.38 ± 1.3	13.84 ± 0.02	16.63 ± 0.2	42.97 ± 1.5	65.73 ± 0.7	53.1 ± 1.3	86.2 ± 1.1
ChCl-LA	34.33	59.38 ± 0.01	14.45 ± 0.2	12.48 ± 0.01	44.15 ± 1.6	65.67 ± 1.3	67.3 ± 1.0	98 ± 0.9
ChCl-GLY	45.42	57.65 ± 0.9	15.44 ± 0.6	15.12 ± 0.01	28.25 ± 1.2	52.98 ± 1.0	47.56 ± 1.3	71.84 ± 2.3
ChCl-UREA	39.08	59.75 ± 0.2	15.26 ± 0.2	20.36 ± 0.2	40.25± 0.5	60.94 ± 0.5	39.23 ± 0.5	84.65 ± 1.5

* TRS – Total reducing sugar

4.3.3 Effect of DES pretreatment on enzymatic sugar yield

The performance of DES pretreatment is best described by the fermentable sugars yield from enzymatic hydrolysis. Higher sugars yield suggests better cellulose digestibility by the enzyme. High enzymatic yields (> 70% of the theoretical yield) were obtained from all the pretreatment and the results were comparable to previous studies. ChCl:Imidazole pretreated corn cob gave a sugar yield of 76% (Procentese *et al.* 2015a). Ethylammonium chloride: ethylene glycol (EAC:EG) pretreated oil palm trunk had a glucose yield of 74%. The high sugar yield observed in this study is attributed to the solubilisation of lignin and hemicellulose from DES pretreatment enhancing the enzyme access to cellulose. Hou *et al.* (2017) observed that component loss in rice straw achieved by the synergistic action of choline chloride – malic acid and choline chloride – urea resulted in high enzymatic sugar yield. The pretreatment of waste lettuce leaves with ChCl:glycerol gave enzymatic glucose and xylose yields of 94.9% and 75.0% respectively (Procentese *et al.* 2017). ChCl-LA pretreatment gave the highest sugar yield (98%) in this study, although it also gave the highest cellulose, hemicellulose and lignin removal. ChCl-LA has previously exhibited high performance at improving the digestibility of corn cobs (Zhang, Xia and Ma 2016), corn straws (Liu, Wei and Wu 2019) and oil palm empty fruit bunch (Tan, Ngoh and Chua 2018; Thi and Lee 2019). Sugar yield above 80% was obtained from all pretreatments with hemicellulose removal ≥ 60% (Table 4.2). For instance, ChCl-Urea had the lowest lignin removal and 60% hemicellulose removal and achieved an 85% enzymatic sugar yield. This suggests that hemicellulose may be a major contributor to the recalcitrance of BGH than lignin. In a previous study by DeMartini *et al.* (2013), it was shown than xylan removal improved the digestibility of switchgrass compared to lignin removal. It was observed that hemicellulose branches were a major source of recalcitrance in rice straw which strengthens the primary cell wall by strongly coordinating the hydrogen bond donor sites on cellulose surface (Silveira *et al.* 2013; Li *et al.* 2015). Moreover, the high enzymatic sugar yield obtained in this study, was achieved under milder conditions compared to other studies (Table 4.3) which were conducted above 100°C, for 6 – 48 h (Tan, Ngoh and Chua 2018). However, even under the comparatively mild conditions employed in this study, cellulose loss was still evident. Therefore, further studies to examine the effect of pretreatment temperature and time on the digestibility and cellulose removal in BGH was conducted.

Table 4.3: Comparison of DES pretreatment of different agricultural residues.

DES	Molar ratio	Feedstock	Pretreatment conditions	Digestibility /sugar yield (%)	Lignin removal (%)	Hemicellulose removal (%)	Cellulose removal /loss (%)	Reference
ChCL-AA	1:6	Akebia herbal residue	100°C, 8 h, 10% solid load		33.8	55.4	28.9	(Yu *et al.* 2018a)
ChCL-AA	1:2	BGH	3 g biomass, 100°C, 3 h, 10% solid load	79.44	57.63	58.89	35.96	This study
ChCL-FA	1:5	Xylose residue	1 g, 80 – 100°C, 2 h	86 - 89	54 – 63.5		17.5 – 20	(Guo *et al.* 2018)
ChCL-FA	1:2	BGH	3 g biomass, 100°C, 3 h, 10% solid load	86.2	53.1	65.73	42.97	This study
ChCL-FA	1:2	Corn stover	5 g biomass, 130°C, 2 h, 20% solid load	91.5	23.8	66.2	NR	(Xu *et al.* 2016)
ChCL-FA	1:2	Akebia herbal residue	120°C, 8 h, 10% solid load	40.7	87	2.2	NR	(Yu *et al.* 2018a)
ChCl-LA	1:2	Oil palm empty fruit bunch	0.1 g biomass, 120°C, 3 h, 10% solid load	20.7	NR	NR	NR	(Thi and Lee 2019)

ChCl-LA	1:2	Corn cob	0.3 g biomass, 90°C, 24 h, 5% solid load	81.6	64.7	NR	NR	(Zhang, Xia and Ma 2016)
ChCl-LA	1:2	BGH	3 g biomass, 100°C, 3 h, 10% solid load	98	67.3	65.67	44.15	This study
ChCl-LA	1:2	Corn stover	2.5 g biomass, ramped up to 152°C, 45 sec, 10% solid load	78.5	79.60	90.06	24.89	(Chen and Wan 2018)
ChCl-GLY	1:2	Oil palm empty fruit bunch	0.1 g biomass, 120°C, 3 h, 10% solid load	20%	NR	NR	NR	(Thi and Lee 2019)
ChCl-GLY	1:2	Corn cob	0.3 g biomass, 90°C, 24 h, 5% solid load	96.4	71.3	NR	NR	(Zhang, Xia and Ma 2016)
ChCl-GLY	1:2	BGH	3 g biomass, 10°C, 3 h, 10% solid load	71.84	47.56	52.98	28.25	This study
ChCl-UREA	1:2	Oil palm empty fruit bunch	0.1 g biomass, 120°C, 3 h, 10% solid load	16.9%	NR	NR	NR	(Thi and Lee 2019)
ChCl-UREA	1:2	BGH	3 g biomass, 100°C, 3 h, 10% solid load	84.65	39.23	60.94	40.25	This study

NR – Not reported

4.3.4 Effect of temperature and time on ChCl:LA pretreatment of BGH

The effects of pretreatment temperature and time on the BGH pretreated by ChCl: lactic acid (1:2) were shown in Table 4.4. With the increase in pretreatment temperature and time, the solid recovery decreased correspondingly (35 – 47%) due to the removal of lignin, hemicellulose and amorphous cellulose. Lignin removal ranged from 51 – 67%, such that even under the mildest conditions (60°C, 1 h), ChCl: lactic could still remove half of the lignin present in the untreated biomass. This is indicative of the high lignin selectivity of ChCl: lactic (1:2) even at low temperatures and shorter time periods. The result obtained in this study is consistent with that of (Zhang, Xia and Ma 2016) in which 65% lignin removal from ChCl: lactic (1:2) pretreated corn cob resulted in 82% glucose yield. They also observed that lignin removal above 70% did not improve sugar yield.

Cellulose and hemicellulose removal ranged from 23 – 44% and 51 – 67% respectively. Even under the mildest condition (60°C, 1 h), 23.8% cellulose loss was observed. At 60°C, extending the pretreatment time form 2 h to 3 h did not increase cellulose loss beyond 30%, whereas, at a higher temperature, cellulose loss increased rapidly with longer pretreatment time (Table 4.4). This reinforces the need to remove the amorphous portion from BGH prior to DES pretreatment for maximum sugar recovery. The enzymatic sugar yield increased at a specific temperature when time progressed from 1 h to 3 h. ChCl:LA pretreatment at 100°C for 3 h gave the highest sugar yield of 96%. However, ChCl:LA pretreatment at 100°C for 1 h was chosen as the best option when lignin removal, sugar yield, cellulose loss and the energy cost of longer pretreatment time was considered.

Table 4.4: Yield and composition of ChCl-LA pretreated BGH at different temperature and time

Pretreatment temperature (°C)	Pretreatment time (h)	Solid recovery (%)	Composition (%)			Removal (%)			Enzymatic TRS yield (%)
			Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin	
Native			36.49	14.91	13.10				28.54
60	1	47	27.92	6.88	6.8	23.48	52.72	51.91	76.8
	2	43.5	25.50	7.05	6.25	30.12	53.86	52.29	79.4
	3	42	25.31	6.75	5.9	30.64	54.73	55.00	84.3
80	1	40	26.58	8.76	6.19	27.16	51.25	52.75	76.5
	2	39	25.46	6.10	5.78	30.22	53.31	55.88	82.65
	3	37	23.10	7.26	5.13	36.69	59.09	60.84	89.1
100	1	37	25.48	6.78	5.14	30.17	54.53	60.70	94.8
	2	35	21.91	6.43	4.83	39.96	56.8	63.10	95.7
	3	35	20.38	4.96	4.25	44.15	66.73	67.56	96.3

4.3.5 Bioethanol fermentation of ChCl:LA pretreated BGH

The fermentation of BGH enzymatic hydrolysate pretreated with ChCl-LA was conducted for 72 h with *S. cerevisiae* (Figure 4.2). The initial sugar concentration of the hydrolysate was 30.43 g/L glucose, 6.7 g/l xylose, and 2.18 g/L arabinose. Ethanol production started approximately after 12 h and the fermentation was completed within 60 h. The glucose concentration depleted within 60 h and arabinose and xylose began to deplete slowly after glucose was exhausted. About 75% of the initial pentose was not utilised at the end of the 72 h. A maximum ethanol concentration of 11.57 g/L was produced, corresponding to an ethanol yield of 0.38 g/g sugar and productivity of 0.19 g/L/h. DES is relatively new in the biomass pretreatment sphere, hence studies of DES pretreatment for subsequent bioethanol fermentation are limited. Nonetheless, Xu *et al.* (2018) reported a 77.5% conversion yield in a ChCl based one pot cellulosic ethanol production process. ChCl-formic acid pretreatments of Akebia' herbal residues led to 98.0% enzymatic saccharification and a 100% ethanol yield (Yu *et al.* 2018a).

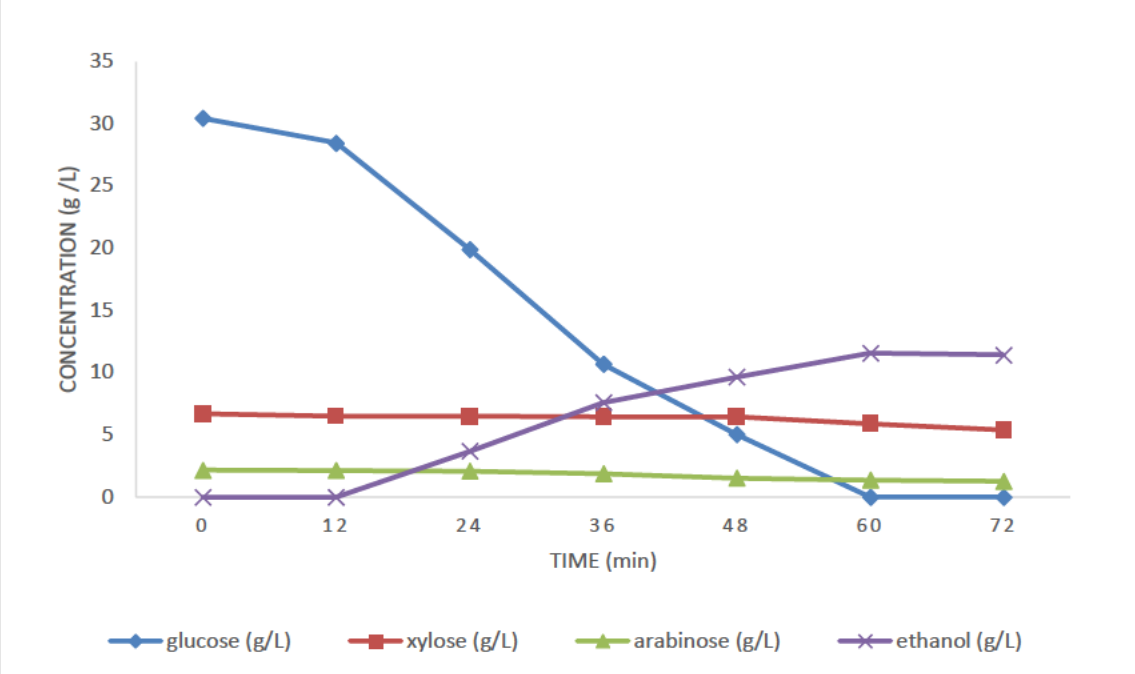

Figure 4.2: Time course for bioethanol fermentation of ChCl-LA pretreated BGH

4.4 Conclusions

The results from this study suggest that DES are excellent media for BGH pretreatment with high selectivity for lignin removal and digestibility of pretreated solid. However, due to the high percentage of amorphous regions in BGH, it is necessary to recover the amorphous cellulose and hemicellulose fraction first in a water or ultra-low acid medium, prior to the application of DES to prevent sugar loss.

5 CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

Efforts at promoting the utilisation of bambara groundnut and cowpea especially in water-stressed regions have stimulated research into the value addition of the seeds, whereas, the haulms are discarded off as waste. However, this study, for the first time, demonstrates that BGH and CH have characteristics that compare well with other commonly used bioethanol feedstocks. Compositional and structural characterisation of BGH and CH show that both biomasses have similar characteristics that could enable them to be used as single and or co-substrates in a bioethanol production process. Specifically, BGH and CH contain a large portion of polymeric sugar (≥50% of their dry weight) predominated by amorphous regions that can be converted to bioethanol. Moreover, their high extractive content offers a potential source for multiproduct valorisation in a biorefinery approach. It is also evident that a pretreatment step is necessary to facilitate the deconstruction of the lignocellulose architecture because pretreatment enhanced sugar recovery by three or four times on average. However, the presence of easily hydrolysable, amorphous regions indicate that only very mild pretreatment regimes may be required to facilitate biosugar production, which is advantageous in terms of the process economics.

So far, there have been no studies on the pretreatment of CH, hence an established pretreatment scheme such as dilute acid pretreatment was employed to improve the digestibility of CH and enhance biosugar production for bioethanol fermentation. Operational factors including temperature, time, and acid concentration on sugar yield and inhibitor formation was investigated and optimised using response surface methodology (RSM). The solid recovered after DAP was subjected to prehydrolysis with simultaneous saccharification and fermentation (PSSF). In addition, the pretreatment hydrolysate was detoxified and fermented to ethanol using cocultures of *S. cerevisiae* BY4743 and *S. stipitis* wild type (PsY633). The use of dilute acid in the pretreatment of CH was effective and facilitated 95% hydrolysis of the inherent sugar in CH, of which 85% was recoverable indicating the effectiveness of this pretreatment strategy for biosugar production from CH. The fermentation of sugar hydrolysates obtained from both the pretreatment and enzymatic stage was useful to improve bioethanol yield from CH. A total ethanol titre of 15.67 g/L was obtained corresponding to 75% conversion efficiency. However, further studies on

optimisation of the enzymatic and fermentation conditions could improve the yield and should be explored.

On the other hand, deep eutectic solvents are a nascent group of solvents showing high applicability in the fractionation of lignocellulosic biomass and were chosen for BGH pretreatment due to their reported high lignin selectivity leading to high sugar yield. Five deep eutectic solvents were prepared between choline chloride and acetic acid, formic acid, lactic acid, glycerol and urea at a molar ratio of 1:2. The synthesized DES were screened for their effectiveness in improving enzymatic sugar yield by pretreating BGH with each DES under the same conditions (100°C, 3 h), followed by a 48 h enzymatic saccharification. DES pretreatment showed high lignin selectivity in BGH, however, a significant amount of cellulose and hemicellulose was also removed in the process which could impact process economics. Evidently, irrespective of the pretreatment conditions employed, it was necessary to remove the easily hydrolysable portions in BGH prior to DES pretreatment. This is because BGH is a low recalcitrant feedstock compared to major biomasses such as rice straw, sugar cane bagasse or corn stover. Choline chloride – lactic acid (ChCl-LA) treatment provided the most promising result and was further optimised by investigating the effect of three temperatures and time on cellulose loss and enzymatic sugar yield. ChCl-LA pretreatment at 100°C for 1 h was observed to be the best condition for maximum sugar recovery. A maximum ethanol yield of 11.57 g/L was produced from enzymatic hydrolysate following a 72 h with *S. cerevisiae* BY4743. As DES pretreatment is relatively new in biomass pretreatment, there are currently limited reports on the fermentation of recovered DES hydrolysate and the techniques for recovery of non-lignin solutes. Overall, the ethanol production in this study is comparable with reports from other crops indicating the viability of BGH and CH as feedstock for bioethanol. However, the outcome of the yield is dependent on the recovery and use of both pentose and hexose sugars.

RECOMMENDATIONS

Based on the findings from this study, the following recommendations are made for further study: A detailed elucidation of the cell wall structure of bambara and cowpea haulm using high precision visual and molecular techniques are required to further understand the spatial links between cell wall components and aid pretreatment design. There is a need to study the correlation between lignin content in haulm and planting data. In addition, more studies

comparing the efficiency of the various gravimetric methods for determining the cell wall composition methods is required. Also, the impact of similar pretreatment conditions on both BGH and CH should be compared to justify their use as co-substrates in a pretreatment and fermentation scheme. In addition, the development of new DES and DES pretreatment methods (single or combined pretreatments) should be further explored to improve sugar recovery. Moreover, more studies fermenting DES hydrolysates to bioethanol must be undertaken to facilitate maximum recovery and conversion of sugars solubilised in the DES. Finally, other methods for pretreatment of BGH and CH should be explored. Meanwhile, valorisation studies must be targeted at the recovery of other products along with ethanol from the various components.

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