Characterisation of recombinant beta-glucosidases from *Thermomyces lanuginosus* SSBP and investigation of their synergistic potential in cellulose hydrolysis

Submitted in complete fulfilment for the Degree of Master of Applied Science in Biotechnology in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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REFERENCE DECLARATION

I, Ms C.N Langa- 21202140, Prof. K. Permaul and Dr. N.P. Mchunu do hereby declare that in respect of the following thesis:

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This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this thesis was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of Prof. Kugen Permaul and Dr. Nokuthula Mchunu.

Student's signature

DEDICATION

I would like to dedicate this work to my late father, Mlomo Langa who passed on the 1st of September 2019.

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ABSTRACT

Beta-glucosidases are present in all domains of living organisms and catalyse various biological functions. They hydrolyse β-1,4 glycosidic linkages and synthesise glucosides through transglycosylation or reverse hydrolysis. β-glucosidases are an important class of enzymes having significant prospects in industrial biotechnology. However, cellulolytic microorganisms produces this enzyme in insufficient amounts. This presents a great obstacle in large-scale biotechnology applications. Therefore, the search for novel β-glucosidases is ongoing to counteract this issue. Genome sequencing of *Thermomyces lanuginosus* SSBP revealed multiple β-glucosidase genes. This study was aimed at characterising five *T. lanuginosus* SSBP β-glucosidases (Bgls) expressed in *Pichia pastoris*. Minimal methanol medium (MM) is commonly used for induction of expression in *P. pastoris*. In this medium, only Bgl2 was expressed after 144 hours. An activity of 71.9 U/ml was obtained whereas Bgl1, Bgl3, Bgl4 and Bgl5 were not detectable. Yeast extract, peptone and methanol (YPM) was then used as an alternative medium. In YPM, all the enzymes were produced after 168 hours of induction of expression. Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 activities were 1.5 U/ml, 310.8 U/ml, 0.9 U/ml, 1.8 U/ml and 0.9 U/ml, respectively. The sizes of Bgls were determined using nucleotide sequences. Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 sizes were 99.9 kDa, 46.5 kDa, 46.8 kDa, 68.9 kDa and 54.3 kDa, respectively. After partial purification, the specific activities of 50.4 U/mg for Bgl1, 553.7 U/mg for Bgl2, 72.0 U/mg for Bgl3, 111.6 U/mg for Bgl4 and 44.0 U/mg for Bgl5 were obtained. The Bgls performed optimally at pH 6.0 and temperature of 50-60°C. Bgl2 and Bgl4 hydrolysed all the tested natural substrates of different linkages, indicating broad substrate specificity. Bgl1, Bgl3 and Bgl5 selectively hydrolysed β-1,3/6-linked substrates (gentiobiose and laminarin). Bgl2 was the dominating recombinant enzyme and it showed the ability to work in synergy with a commercial

cellulase to hydrolyse microcrystalline (MCC) and carboxymethyl cellulose (CMC). Upon supplementation of Bgl2, a 58% and 91% increase in glucose production was achieved from MCC and CMC, respectively. Therefore, this enzyme has potential to be used in cellulose degradation for valorisation of waste lignocellulosic biomass.

1. INTRODUCTION

Beta-glucosidases (E.C. 3.2.1.21) catalyse hydrolysis of β-1,4-glycosidic bonds to release non-reducing terminal glucosyl residues from glycosides and short-chain oligosaccharides. They are found in all domains of living organisms (Archaea, Bacteria and Eukaroytes) in which they play a variety of roles (Bhatia *et al*., 2002; Cairns *et al*., 2015; Singhania *et al*., 2013). β-glucosidases are an important class of enzymes having significant prospects in industrial applications. They are used in the food industry to release aroma from wine grapes during winemaking, hydrolysis of isoflavone glycosides in soy foods, pre-digestion of cellulose-based feeds for monogastric animals and in synthesis of oligosaccharides for pharmaceuticals (Krisch *et al*., 2010). Most importantly, β-glucosidase is a component of the multi-enzyme complex involved in cellulose hydrolysis, the most-abundant organic homo-polymer on Earth (Cheng and Timilsina, 2011; Tiwari *et al*., 2013).

The global energy demand and predicted depletion of fossil fuels has resulted in the search for alternative sources of energy. Wind, solar, geothermal energy as well as biomass-derived biofuels are renewable and promising replacements of fossil fuels (Owusu and Asumadu-Sarkodie, 2016). However, biofuels are considered the most sustainable (Abas *et al*., 2015; Sarkar *et al*., 2012; Zabed *et al*., 2017). Bioethanol is the most widely-employed liquid biofuel. It is produced through fermentation of sugars to ethanol (Amigun *et al*., 2006). Due to food security concerns raised against the use of food-based materials such as corn and sugarcane, plant biomass presents an alternative feedstock for bioethanol production (Aditiya *et al*., 2016; Gallezot, 2008; Mohapatra *et al*., 2017; Renzaho *et al*., 2017; Sims *et al*., 2010). These materials

consist of 40-50% cellulose, a polysaccharide of repeating glucose molecules (Gupta and Verma, 2015). The key to commercialisation of cellulosic bioethanol lies on the improvement of cellulose-degrading enzymes (Chandel *et al*., 2018; Ho *et al*., 2014).

The complete depolymerisation of cellulose is achieved by synergistic action of endoglucanases (E.C 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91) and β-glucosidase (E.C. 3.2.1.21). The latter plays a major role during this process as the rate-limiting enzyme. However, β-glucosidases are mostly produced in low amounts. *Trichoderma reesei*, a commercial cellulase source, produces 18% of endoglucanase, 72% of cellobiohydrolase and < 1% of β-glucosidase. This presents a great stumbling block in large-scale hydrolysis. Addition of exogenous β-glucosidase has been practiced to circumvent this challenge (Ahmed *et al*., 2017; Cairns *et al*., 2015; Tiwari *et al*., 2013).

Beta-glucosidases from different organisms have been heterologously expressed to enhance production (Singh *et al*., 2016). Genetic engineering approaches have also been used to improve properties of some available β-glucosidases (Fang *et al*., 2016; Guo *et al*., 2016; Lebbink *et al*., 2000; Lee *et al*., 2012; Singhania *et al*., 2017). Furthermore, screening for new sources of β-glucosidase is still ongoing for better extrapolation on improvement of cellulase efficiency at industrial level (Li *et al*., 2017).

2. LITERATURE REVIEW

2.1. Beta-glucosidases and their functions in biological systems

Under physiological conditions, β-glucosidases hydrolyse β-glycosidic linkages present between carbohydrate residues in aryl-amino-, aryl-β-D-glucosides, cyanogenic glucosides, disaccharides and short-chain oligosaccharides (Singh *et al*., 2016). The cleavage of the bonds between these molecules occurs via three steps. Firstly, the anomeric (C1) center of the substrate glycoside is attacked by an enzymic nucleophile. This results in formation of covalently linked α-glycosyl enzyme intermediate through an oxocarbenium ion-like transition state. Secondly, another active residue of the enzyme serves as the acid-base catalyst and donates a hydrogen ion to the glycosidic oxygen. This assists in the release of the aglycone group. Finally, the glycosyl-enzyme intermediate is hydrolysed via general base-catalysed attack by water at the anomeric center to release β-glucose as the product (Bhatia *et al*., 2002).

Under defined conditions, β-glucosidases also catalyse synthesis of glycosyl bonds between different molecules. This reaction can happen in two different modes, reverse hydrolysis and transglycosylation (Bhatia *et al*., 2002; Cairns and Esen, 2010; Cairns *et al*., 2015; Singhania *et al*., 2013). In the first approach, reverse hydrolysis, modification of reaction conditions such as lowering of water activity, trapping of product or high substrate concentration results to a shift in the equilibrium of reaction towards synthesis (Wang *et al*., 2018). This reaction is under thermodynamic control. In transglycosylation mode, a preformed donor glycoside is first hydrolysed by β-glucosidase forming an enzyme-glycosyl intermediate. This is then trapped by a nucleophile other than water (such as monosaccharide, disaccharide, aryl- amino- or alkyl-

alcohol or monoterpene alcohol) to yield a new elongated product. This reaction is under kinetic control (Bhatia *et al*., 2002).

2.2. Beta-glucosidase functions in mammals

Lactase phloridzin hydrolase (LPH), glucocerebrosidases (human and bile acid β-glucosidases, GBA1 and GBA2), broad substrate specific cytosolic β-glucosidase and pyridoxine glucoside hydrolase are β-glucosidases found in mammals. These enzymes play a pivotal role in metabolism of glycolipids and dietary glucosides. Lactase phloridzin hydrolase is a β-glucosidase localised in the brush borders of small intestines. It hydrolyses glycosides before adsorption. Lactase phloridzin hydrolase also possess a β-galactosidase activity, catalysing hydrolysis of lactose from milk and its deficiency leads to lactose intolerance (Berrin *et al*., 2002; Cairns and Esen, 2010; Day *et al*., 2000).

A well-studied mammalian β-glucosidase is human acid β-glucosidase (GBA1). This is a membrane-associated lysosomal enzyme which catalyses hydrolysis of glucosylceramides to glucose and ceramide. Inactivity of this enzyme causes accumulation of glucosyceramides in lysosomes resulting in chronic multisystem disorder, Gaucher's disease (Brumshtein *et al*., 2009; Grace and Grabowski, 1990; Harzer *et al*., 2012; Jung et al., 2019; Xu *et al*., 2003). Bile acid β-glucosidase (GBA2) is a second glucocerebrosidase. This is a non-lysosomal β-glucosidase associated with liver microsomes that hydrolyses both glucosylceramides and bile acid-3-O-glucoside. In a study by Yildiz *et al*. (2006), mutating GBA2 caused accumulation of glucosylceramides in the endoplasmic reticulum, thus a storage disorder in this tissue and infertility in mice (Yildiz *et al*., 2006).

Human cytosolic β-glucosidase is present in the liver, kidney, intestine and spleen. It catalyses hydrolysis of plant flavonoid glucosides (Tribolo *et al*., 2007). Pyridoxine glucoside hydrolase is another human β-glucosidase that plays a role in vitamin B6 bioavailability by hydrolysing pyridoxine-5-β-D-glucopyranoside (Berrin *et al*., 2002; McMahon *et al*., 1997).

2.3. Beta-glucosidase functions in insects

Insects feed on plants; their major dietary components are β-glycans. These are then hydrolysed by β-glucosidase found in their guts. Uchima *et al*. (2012) characterised this enzyme from a cellulose-decomposing higher termite, *Nasutitermes nakasagoensis*. The presence of β-glucosidases in insects also aid in their ability to metabolise compounds that are released by plants for defence against them to non-toxic constituents (Marana *et al*., 2001; Pentzold *et al*., 2015; Zagrobelny *et al*., 2008). Furthermore, Zhang *et al*. (2017) identified and characterised a β-glucosidase from pinewood nematode, *Bursaphencus xylophilus* using metagenomic analysis. Feeding on the pine tree of this parasite results in lethal wilt which suggested the presence of cell wall-degrading enzymes (Zhang *et al*., 2017).

2.4. Beta-glucosidase functions in plants

Multiple β-glucosidases catalysing diverse biological functions are present in plants. They are involved in defence, symbiosis, signalling, plant secondary metabolism and cell wall synthesis as well as lignification. Plant β-glucosidase functions are determined by substrate-specificities, tissue and subcellular localisation (Cairns and Esen, 2010; Cueto *et al*., 2018; Tian *et al*., 2013). Cyanogenic glucosides are present in various plant species, mainly for defence against pathogens and herbivores (Cairns *et al*., 2015; Pentzold *et al*., 2015; Zagrobelny *et al*., 2008). Upon cell

disruption, these are hydrolysed by β-glucosidases to toxic compounds such as cyanide and hydroxamic acids (4-hydroxy-1,4-benzaxazin-3-ones). Morant *et al*. (2008) referred to β-glucosidases as "detonators" of these chemical "bombs". The defence cyanogenic glucosides are generally stored in the vacuole and the corresponding β-glucosidases in the apoplasts or plastids to avoid their premature hydrolysis (Morant *et al*., 2008).

The β-glucosidase-mediated defence system is also exploited during the development of symbiotic relationships between endophytic fungi and plants. This was demonstrated by Sherameti *et al*. (2008) where the enzyme prevented the endophytic fungus, *Piriformospora indica* from colonising the roots of *Arabidopsis thaliana.* Since the major component of plant cell wall is β-linked glucosides, β-glucosidases also play a pivotal role in cell wall development and remodelling (Cairns *et al*., 2015). They hydrolyse oligosaccharides generated during cell wall turnover and remove β-glucosyl residues from monolignols glycosides for secondary cell wall lignification and stabilisation. Furthermore, β-glucosidases are also involved in activation of phytohormone glucosides and secondary metabolism (Cairns and Esen, 2010).

2.5. Beta-glucosidase functions in microorganisms

Although β-glucosidases are present in all forms of microorganisms, research has focused more on their application instead of their functions in these systems. Archaea are known for possessing functionally-stable β-glucosidases. This is due to the ability of these enzymes to withstand harsh conditions, e.g. high temperatures and pH levels (Li *et al*., 2013). Microbial β-glucosidases play fundamental roles in various biological processes. These include, degradation of cellulose and other carbohydrates for nutrient in-take, regulation of cell wall synthesis, host-pathogen

interactions and symbiotic associations (Singh *et al*., 2016). β-glucosidases enable phytopathogenic fungi to colonise their host plant tissues through hydrolysis of toxic glucosides to harmless intermediates (Collins *et al*., 2007). In bacteria, β-glucosidases are components of large complexes called cellulosomes. Furthermore, as a part of the cellulase multi-enzyme complex in cellulolytic microorganisms, these play the most important role during cellulose hydrolysis. The presence of β-glucosidases in soil microbes also contributes in maintenance of soil quality. This is because they aid in cycling of soil organic matter (Singh *et al*., 2016).

2.6. Classification of β-glucosidases

Beta-glucosidases are a heterogeneous group of well-characterised biologically important enzymes. They have been classified using various criteria and characteristics, namely, substrate specificity, amino acid sequence and structural identity. There are three groups based on substrate specificity; namely (a) aryl-β-glucosidases, which acts on aryl glucosides, (b) cellobiases, which hydrolyse cellobiose to release glucose and (c) broad substrate specific β-glucosidases, which hydrolyse a wide range of substrates (Henrissat and Bairoch, 1996). The second classification system is based on amino acid sequence and structural similarities. It has been used for all glycoside hydrolases (GH). It is the most suitable because it also reflects their evolutionary relationships and catalytic mechanisms. The related families are then grouped into clans (Cantarel *et al*., 2008; Singhania *et al*., 2017).

Of 156 glycoside hydrolase (GH) families, β-glucosidases are found in families GH1, GH3, GH5, GH9, GH30 and GH116 (Lombard *et al*., 2014). The largest clan is GH-A, it is comprised of GH1, GH5 and GH30 β-glucosidases. These families including GH116 that is grouped in GH-

O clan are characterised by a retaining catalytic mechanism. GH1, GH5 and GH30 have $(\beta/\alpha)_8$ barrel structures. In contrast, the active site of GH3 enzymes comprises two domains, while GH9 enzymes and GH116 have $(\alpha/\alpha)_6$ barrel structures. Numerous β-glucosidases from GH1 have been characterised and GH3 is comprised of fungal β-glucosidases making these two families the most studied (Singhania *et al*., 2017; Singhania *et al*., 2013). Although the mechanism by which GH1 enzymes recognise and hydrolyse substrates with different specificities is still an area of intense study, GH1 β-glucosidases have shown to have a great potential for application in biofuel production (Singhania *et al*., 2017).

2.7. Beta-glucosidase applications based on hydrolytic activity: Food and feed industry

Flavonoid and isoflavonoid glycosides are phenolic and phytoestrogen secondary metabolites that naturally occur in fruits, vegetables, tea, red wine, soybeans and leguminous plants. However, the glycosides are stored in the plant in their inactive forms like daidzin, genistin and glycitin. They are then hydrolysed by β-glucosidases to biologically effective aglycones such as daidzein, genistein and glycitein (Bhatia *et al*., 2002; Pei *et al*., 2016). These have health-enhancing functions such as prevention of sex hormone-dependent cancers, lowering risk of cardiovascular diseases and reduction of the bone mineral loss (Bhatia *et al*., 2002; Krisch *et al*., 2010; Pei *et al*., 2016; Yang *et al*., 2009).

Beta-glucosidases are also associated with removal of bitterness from citrus fruit juices by hydrolysing naringenin (4,5,7-trihydroxyflavanone-7-rhamoglucoside) to prunin (Gous, 2012;

Patil and Dhake, 2014). Apples, grapes, fruit juices and wines contains a free fraction of volatile terpenols as well as natural non-odourous and non-volatile glycoside precursors. These are important sources of fragrant compounds (Singh *et al*., 2016). The aglycone moiety is normally a volatile phenol such as vanillin, aliphatic or cyclic alcohol such as hexanol, 2-phenylethanol, benzylalcohol or terpenols such as nerol, linalool, geraniol and citronellol. These are linked to the disaccharides such as 6-O-α-rhamnopyranosyl β-glucoside and 6-O-α-L-arabinopyranosyl β-D-glucoside (Bhatia *et al*., 2002; Perera and Owen, 2008). The hydrolysis of glucosidic linkages release the aglycone moiety, resulting in aroma enhancement, thus benefiting winemaking process (Ovalle *et al*., 2018; Swangkeaw *et al*., 2011). In addition, nutritional value can be improved by the release of vitamins and antioxidants from glycosides. For instance, pyridoxine glucoside present in rice in its predominant form is hydrolysed by β-glucosidase to pyridoxine (vitamin B6) (Gregory III, 1998; McMahon *et al*., 1997).

Cassava is the fourth largest source of calories in the tropics, β-glucosidases are also used in its detoxification. These catalyse the hydrolysis of cyanogenic glucosides such as linamarin, cyanohydrin and lotaustralin to release the toxic -CN moiety. Thus making cassava fit for human consumption (Birk, 1996; Burns *et al*., 2010). Furthermore, the application of gellan, an exopolysaccharide produced by *Sphingomonas paucimobilis* is limited by its high viscosity and low solubility. Hydrolytic activity of β-glucosidases is used in production of low-viscosity gellan foods (Singh *et al*., 2016). Generally, there are native β-glucosidases present in source-plant tissues that can hydrolyse these precursors (Bi *et al*., 2019). However, the natural process by endogenous β-glucosidases is time-consuming. Therefore, exogenous enzymes can be

supplemented before or during processing of these plants to enhance the release of beneficial compounds (Gueguen *et al*., 1998; Wang *et al*., 2013).

In the feed industry, treatment of cellulose-based feeds with crude β-glucosidases enhanced digestibility of cellulose-based feeds for monogastric animals such as chickens and pigs (Krisch *et al*., 2010).

2.8. Beta-glucosidase applications based on synthetic activity

Beta-glucosidase transferase activity is used in the biosynthesis of oligosaccharides and alkyl-glucosides. Oligosaccharides can be used as therapeutic agents, diagnostic tools and promoting agents for probiotic bacteria (Bankova *et al*., 2006; Singh *et al*., 2016). They have important biological functions such as fertilisation, embryogenesis and cell proliferation. Alkyl-glucosides are highly biodegradable non-ionic surfactants. They have good antimicrobial properties and have potential application in pharmaceutical, chemical, cosmetic, food and detergent industries (Bhatia *et al*., 2002). The use of chemical methods to synthesise these compounds is slow and non-specific, therefore the enzymatic synthesis using the transglycosylation activity of β-glucosidases provides an alternative method (Mangas-Sánchez and Adlercreutz, 2015). This method involves a single step instead of several protection-deprotection steps required in chemical synthesis. Enzymes are also highly selective and reactions occur under mild conditions, thus can be applied at a large-scale (Bankova *et al*., 2006).

2.9. The significance of β-glucosidase in biofuel production

As a component of cellulase complex, β-glucosidase is largely exploited for the conversion of plant biomass to simple sugars for biofuel production. It catalyses the rate-limiting step of hydrolysis of cellobiose to glucose. Therefore, β-glucosidase is considered the key in determining efficiency of cellulase in biomass saccharification (Rani *et al*., 2014; Singhania *et al*., 2013).

2.9.1. The use of plant biomass for biofuel production

Bioenergy accounts for the majority of the global renewable energy supply. It is the energy content in solid, liquid and gaseous products from biological materials. These includes biofuels produced from plant biomass, heat and electricity produced from wood chips and pellets as well as biogas (biomethane and biohydrogen) (Ho *et al*., 2014). Biofuels are the major bioenergy fraction in the world and bioethanol is the most employed, either as a fuel or as gasoline enhancer (Amigun *et al*., 2006; Gupta and Verma, 2015; Sa´nchez and Cardona, 2007). Although the production cost is high, many countries have implemented programs for improvement of cost-effective technologies (Guo *et al*., 2015). Brazil and United States of America are the leading producers of bioethanol using sugarcane and corn grain. However, the use of food sources will limit the supply in the near future (Gray *et al*., 2006). More interest has been put on second generation biofuels. These are produced from the abundant plant biomass such as agricultural residues, wood, municipal solid wastes and dedicated energy crops. Therefore presenting sustainable feedstocks for efficient biofuel production (Geddes *et al*., 2011; Zabed *et al*., 2017). As the carbon dioxide emissions from fossil fuels are predicted to increase to 37

gigatonnes (Gt) in 2035, the use of plant biomass will result in a carbon neutral era (Ho *et al*., 2014). In addition, it will also allow for the application of a bio-refinery concept whereby biomass conversion will produce other value-added chemicals and regenerate energy for the process (Chen and Zhang, 2015; Sarkar *et al*., 2012). Plant biomass is rich in lignocellulose (Cragg *et al*., 2015; Sajith *et al*., 2016).

2.9.2. Lignocellulose structure

Lignocellulose is a composite of polymers such as cellulose, hemicellulose and lignin. In secondary plant cell walls, these are organised in non-uniform three dimensional structures to different degrees and varying composition. Lignocellulosic materials contain 40-50% cellulose, 25-35% hemicellulose and 15-20% lignin (Figure 2.1) (Cheng and Timilsina, 2011; Haghighi *et al*., 2013). Lignocellulose is resistant to degradation due to the crystallinity of its major component, cellulose. The rigidity of its structure is also due to the hydrophobicity of lignin and encapsulation of cellulose by the lignin-hemicellulose matrix (Isikgor and Becer, 2015).

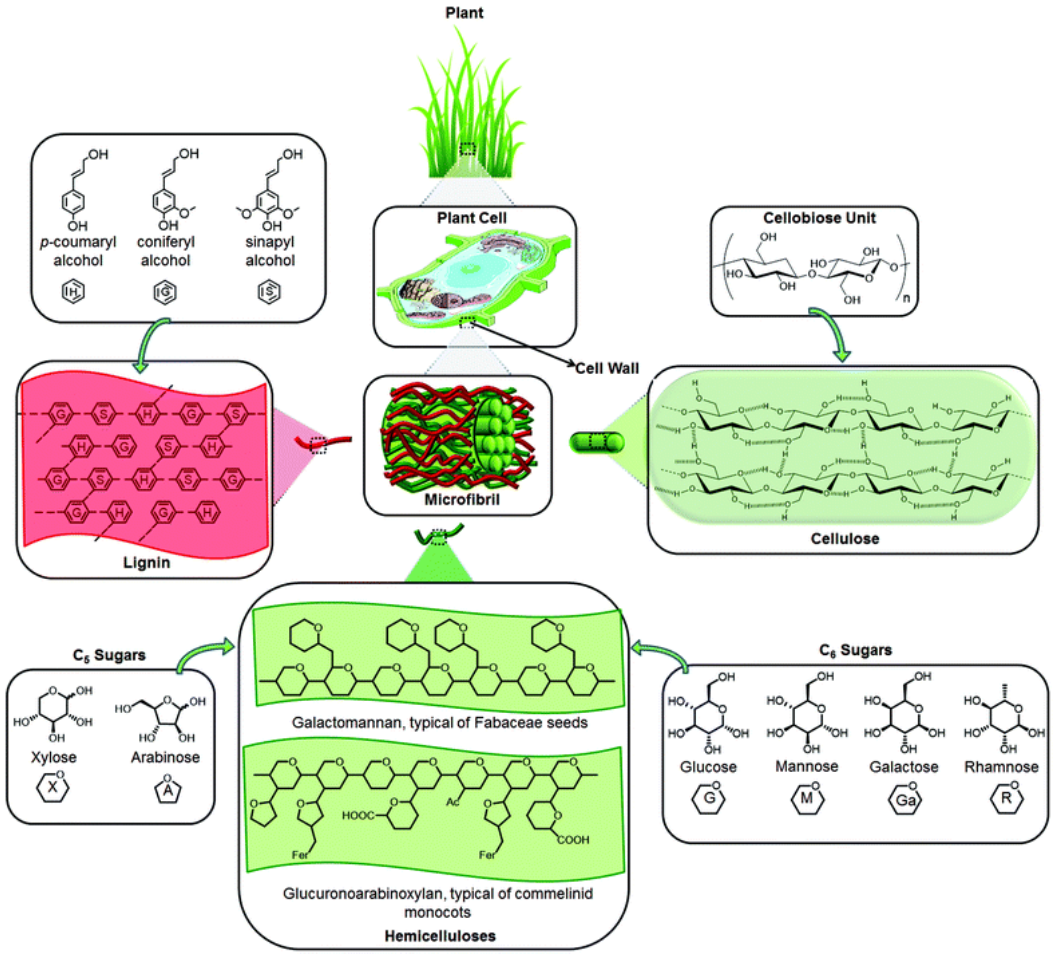

Figure 2.1: Plant cell wall components and their structures: cellulose, hemicellulose and lignin (Isikgor and Becer, 2015).

Cellulose is a water-insoluble homogeneous molecule that consists of D-glucose subunits interlinked by β-1,4-glucosidic bonds. Each glucan chain length can contain up to 25 000 glucose molecules. Chains of glucan (14-15) form a microfibril in a regular crystalline arrangement. A group of these microfibrils then forms a macrofibril or cellulose structure. Intra- and inter-molecular hydrogen bonds stabilises cellulose microfibrils (Nagarajan *et al*., 2017; Sajith *et al*., 2016). In nature, cellulose exists as a paracrystalline form with alternative crystalline and amorphous regions. Cellulose fibres also exist in other forms such as irregular twists or voids,

increasing their total surface area. Cellulose is the most prevalent component of lignocellulose (Juturu and Wu, 2014; Zabed *et al*., 2017).

In contrast to cellulose, hemicellulose is a heterogeneous, highly branched molecule with a random amorphous structure. It is composed of several heteropolymers such as xylan, galactomannan, glucuroarabinoxylan, glucomannan and xyloglucan. It also differs in composition. Hardwood hemicellulose contains mostly xylans whereas softwood hemicelluloses contains mostly glucomannans (Agbor *et al*., 2011; Mattéotti *et al*., 2011). The heteropolymers of hemicellulose are composed of mixed sugars and their derivatives (Figure 2.1). It contains hexose (glucose, galactose, mannose and rhamnose) and pentose (xylose and arabinose) and acetylated sugars. Hemicelluloses are embedded in the plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibres into microfibrils and cross-linking with lignin (Anwar *et al*., 2014).

Lastly, lignin is the most complex hydrophobic polymer that is made up of phenylpropane units such as coumaryl alcohol, coniferyl alcohol and sinapyl alcohol linked in a three-dimensional structure. It is tightly bound to cellulose and hemicellulose, providing compressive strength to the plant cell wall (Figure 2.1) (Sajith *et al*., 2016).

2.9.3. Lignocellulosic biomass feedstocks

The global production of plant biomass in the form of non-food agricultural and forest residues, municipal and industrial solid wastes as well as dedicated energy crops is at least 200 billion tonnes per year (Zabed *et al*., 2017). Corn, wheat, rice and sugarcane are major crops and

therefore a great portion of agricultural residues are obtained during their harvest and processing (Kim and Dale, 2004). The most promising agro-residue is corn stover. It is made up of stalks, leaves, cobs and husks that results from harvesting corn grains. Rice straw is also the most abundant lignocellulosic waste material in the world (Gupta and Verma, 2015). It is generated during production of rice worldwide. Rice straw comprises of remains of stems, leaf blades, leaf sheaths and panicle after threshing. It is estimated that 5.1 billion dry tons of agro-residues are produced globally (Ho *et al*., 2014).

Forest residues are also a tremendous source of readily available biomass. The global annual production of forest residues is estimated to be 501 million dry tons. They are made up of hardwood (angiosperms) and softwood (gymnosperms) as well as forest wastes (sawdust, pruning, bark thinning residues, wood chips and branches from dead trees) (Singh *et al*., 2010). Softwood comprises of evergreen species such as pine, cedar, spruce, cypress, fir, hemlock and redwood. They are associated with low density and rapid growth. Poplar, willow, oak, cottonwood and aspen are types of hardwood which has a low amount of lignin compared to softwood. Furthermore, its hemicellulose mostly consists of hexose sugars in contrast to softwood which contains more pentose sugars. Thus its use is advantageous because pentoses present a challenge in bioethanol production using current fermentation technologies. Fermenting microbes at commercial scale lack pentose sugar metabolism (Cheng and Timilsina, 2011).

Municipal solid waste is obtained from residential and non-residential sources. Although municipal solid wastes composition is highly variable, the organic fraction can also be used as a

feedstock for biofuel production. It includes putrescibles, papers, cardboards and plastics. Furthermore, the food and paper industries produce a huge amount of residues that can be used as biomass for biofuel production (Taherzadeh and Karimi, 2008). Industrial waste materials consist of peelings and scraps from fruits and vegetables, meat and poultry waste, pulp and fibre from sugar and starch extraction as well as coffee grounds. Waste management practices have moved away from direct disposal towards recovery, reuse, recycling and reduction (Ho *et al*., 2014). In addition to the application in bioenergy production, it also contributes to minimising the use of landfills and reduce emission of greenhouse gases (GHGs). Occasionally, due to contamination and diverse components these are not considered ideal. Compositional analysis must be carried out before utilisation and complex pre-treatment is required (Yang *et al*., 2015).

Waste materials are obtained at low cost and could be a solution for economical bioethanol production to meet the energy demand. However, these will not provide a continuous and reliable source of biomass. For instance, agro-residues are generated from seasonal crops (rice, wheat, corn and sugarcane) (Zabed *et al*., 2017). On the other hand, energy crops are the most feasible supply of lignocellulosic materials (Sims *et al*., 2010). These are mainly dedicated for biofuel production including perennial grasses (miscanthus, switchgrass and reed canary) and short-rotation wood crops (willows, poplar and eucalyptus) (Boateng *et al*., 2007; Heaton *et al*., 2008). Energy crops are being developed to grow in a short period of time and to be water as well as fertiliser efficient. Perennial grasses such as switchgrass and miscanthus are the best choices in terms of low input bioenergy production (Allwright and Taylor, 2016). These can tolerate cool temperatures, minimal water and can grow on a broad range of land types without the agronomic input of fertilisers (Haghighi *et al*., 2013; Koçar and Civaş, 2013; Yang *et al*.,

2015). Short rotation forest trees have also demonstrated their potential in the production of biofuels owing to their geographic distribution, low cost and less labour compared to annual crops (Mohapatra *et al*., 2017).

2.9.4. Pre-treatment of lignocellulosic biomass

Pre-treatment is firstly applied on lignocellulosic materials in order to separate lignin from cellulose and hemicellulose (Sarkar *et al*., 2012). It reduces the crystallinity of cellulose and increases the material porosity. Physical, biological and chemical processes are the most commonly used pre-treatment methods. However, they are associated with high energy- and time consumption as well as production of inhibitory compounds, respectively (Cheng and Timilsina, 2011). Enzymatic hydrolysis thus presents an alternative, environmentally friendly method of lignocellulose pre-treatment. Although the other pre-treatment stages are relevant for the process, extensive research is currently being conducted on the improvement of enzyme-based methods (Alvira *et al*., 2010; Eugenio *et al*., 2017; Guo *et al*., 2015).

Enzymatic hydrolysis involves degradation of cellulose to glucose and hemicellulose to hexose and pentose sugars. Hemicellulose can also be hydrolysed by chemical (acid hydrolysis) pre-treatment. If alkaline pre-treatment is applied, hemicellulases, for example, xylanases and xylosidases are used for hydrolysing xylan to xylose, a pentose sugar. Furthermore, it increases porosity of the lignocellulosic material, thus easing enzyme penetration and access to cellulose (Alvira *et al*., 2010). Cellulose degradation to single glucose units by synergistic action of endoglucanase (Figure 2.2: orange enzyme shape), exoglucanase (cellobiohydrolase) (Figure 2.2: red enzyme shape) and β-glucosidase (Figure 2.2: grey enzyme shape) is diagrammatically

presented in Figure 2.2. Firstly, endoglucanases randomly attack cellulose at amorphous regions to release cellooligosaccharides of different chain lengths. These are then cleaved at reducing and non-reducing ends by cellobiohydrolases (exoglucanase) to release cellobiose. β-glucosidase hydrolyses cellobiose to single glucose molecules that can then be used by fermenting microbes. β-glucosidase plays an important role during this process as it prevents feed-back inhibition of other cellulases (Singh *et al*., 2016).

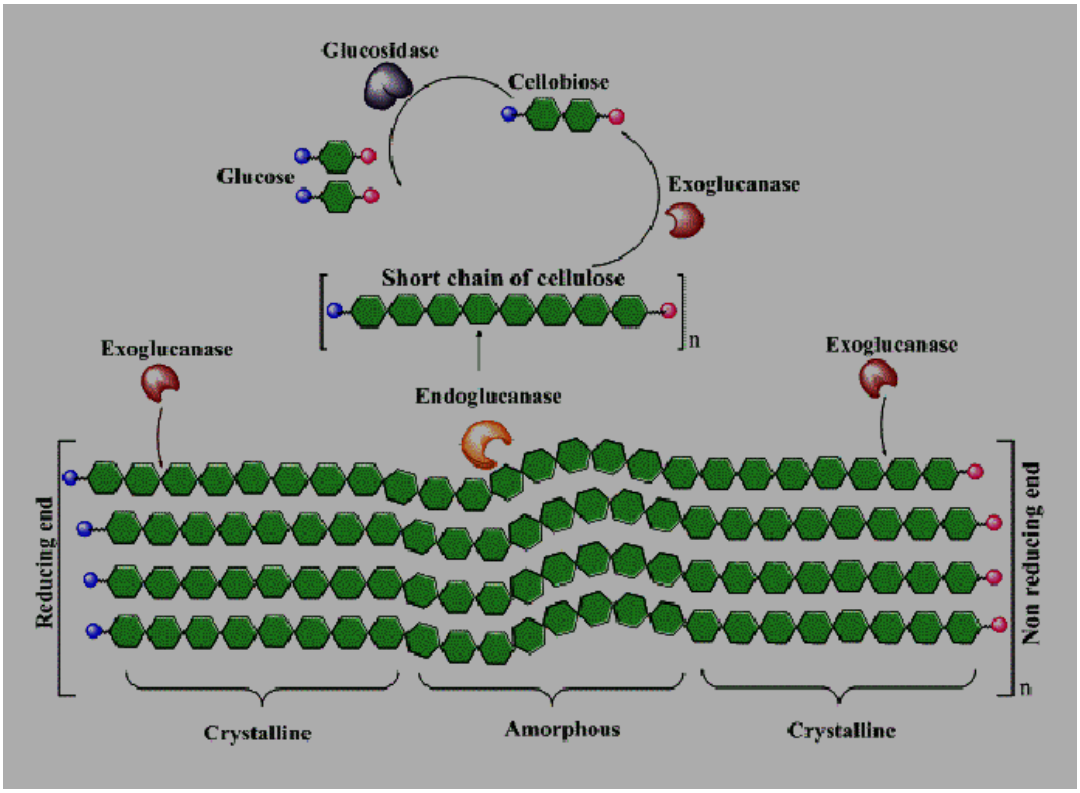

Figure 2.2: Diagrammatic presentation of cellulose depolymerisation by synergistic action of endoglucanase, exoglucanase (cellobiohydrolase) and β-glucosidase (Sajith *et al*., 2016).

2.10. Microbial production of β-glucosidases

Although β-glucosidases are present in all living organisms, microbes are considered the best choice for large-scale production. This is because of their rapid growth that accelerates enzyme production. Microorganisms are also easier to handle than higher Eukaryotes, therefore their use results in cost-effective processes. Genetic engineering methods such as mutagenesis and directed evolution can be performed easily to improve enzyme productivity (Gonza´ lez-Blasco *et al*., 1999; Lebbink *et al*., 2000; Singhania *et al*., 2017; Sørensen *et al*., 2013). Bacteria and fungi have been immensely exploited for β-glucosidase production. However, fungi are a preferred source of cellulase enzymes. This is because of the ability of these microorganisms to produce high titres of cellulolytic enzymes under both solid state and submerged fermentations (Amore *et al*., 2013; Nigam, 2013; Singhania *et al*., 2013).

2.11. Fungal production of β-glucosidase

Many extracellular GH3 fungal β-glucosidases have been produced, purified and characterised. Microscopic fungi are the most important source of β-glucosidase. However, filamentous fungi are preferred for the production of β-glucosidases for biomass hydrolysis purposes. These have an added advantage of growing on solid substrates and produce high titres of enzymes. Filamentous fungal sources that have been exploited for β-glucosidase production includes, *Aspergillus oryzae* (Riou *et al*., 1998), *Thermomyces lanuginosus* SSBP (Lin *et al*., 1999), *Aspergillus niger* A20 (Abdel-Naby *et al*., 1999), *Thermoascus aurantiacus* (Parry *et al*., 2001), *Chaetomium thermophilum* var. *coprophilum* (Venturi *et al*., 2002), *Penicillium purpurogenum* (Dhake and Patil, 2005), *Daldinia eschscholzii* (Karnchanatat *et al*., 2007), *Melanocarpus* spp.

MTCC 3922 (Kaur *et al*., 2007), *Monascus purpureus* (Daroit *et al*., 2008), *Phoma* spp. KCTC11825BP (Choi *et al*., 2011), *Neocallimastix patriciarum* W5 (Chen *et al*., 2012), *Aspergillus fumigatus* Z5 (Liu *et al*., 2012), *Penicillium italicum* (Park *et al*., 2012), *Fusarium proliferatum* NBRC109045 (Gao *et al*., 2012), *Penicillium purpurogenum* KJS506 (Bai *et al*., 2013), *Fusarium solani* (Bhatti *et al*., 2013), *Aspergillus saccharolyticus* (Sørensen *et al*., 2014), *Flammulina velutipes* (Mallerman *et al*., 2015), *Monascus sanguineus* (Dikshit and Tallapragada, 2015), *Sporothrix schenckii* (Hernández-Guzmán *et al*., 2016), *Gongronella butleri* (Santos *et al*., 2016) and *Fusarium oxysporum* (Olajuyigbe *et al*., 2016). Yeast strains have been also reported for β-glucosidase production; mostly *Candida* species (Singhania *et al*., 2017).

The inability of *T. reesei* to produce β-glucosidase optimally necessitated for the use of other sources. *Aspergillus* species are known to produce high titres of β-glucosidase (Singhania *et al*., 2011). A major commercial β-glucosidase, Novozyme 188 is from *A. niger* (Ahmed *et al*., 2017). Under both submerged and solid substrate fermentation, substrates such as cellulose, crude and pre-treated biomass have been used as carbon sources for the production of β-glucosidases. Submerged fermentation is used for many commercial enzymes due to its inherent advantages of better sterility, heat and mass transfer, ease of process monitoring and automation. Solid substrate fermentation is used for fungal fermentations for low-value enzymes (Singhania *et al*., 2010).

2.11.1. Multiplicity of β-glucosidases in fungal species

Many reports have revealed the differential expression of β-glucosidase genes such as multiplicity in genomes of filamentous fungi (Eugenio *et al*., 2017). *A. niger* NII-08121

produced four isoforms of β-glucosidases, Bgl1 being the major protein. Different carbon sources appeared to be playing a role in this phenomenon. For instance, the presence of lactose induced expression of glucose-tolerant isoforms (Singhania *et al*., 2011). Furthermore, genome sequencing of a cellulolytic fungus *Talaromyces amestolkiae* revealed a higher number of genes encoding for glycoside hydrolases, particularly β-glucosidases. BLASTP analysis against characterised GHs showed that this fungus possess twenty four β-glucosidases belonging to GH1, GH3 and GH5. However, in this case β-glucosidase activity was detected in the culture supernatant regardless of the carbon source. This suggested a central role of these enzymes in the metabolic strategy adopted by *T. amestolkiae* along evolution (Eugenio *et al*., 2017).

The possible difference in biological functions of multiple β-glucosidase in one organism was observed in a study by Xia *et al*. (2016). Three β-glucosidases from a cellulolytic thermophile, *Humicola insolens* Y1 were characterised. The enzymes showed different substrate specificities, *Hi*Bgl3A and *Hi*Bgl3C were active towards both cellobiose and aryl β-glucosides while *Hi*Bgl3B was a typical aryl β-glucosidase. Site directed mutagenesis further proved the vital role of three residues in recognising subsites of different substrates (Xia *et al*., 2016).

2.12. Genetic modification

Genetic modification is a widely used approach to combine multiple desirable traits into a single organism (Singhania *et al*., 2017). Microbes can be engineered to hyper-produce enzymes. However, there are drawbacks that are caused by possible complexities of organisms or proteins to be expressed. Prokaryotic genes can be cloned and expressed easily in bacterial systems whereas eukaryotic genome sequences are more complex. Nevertheless, there is a requirement

for enhancement of β-glucosidase production. This could be achieved by heterologous expression and genetically engineering of existing β-glucosidases to improve their properties (Rani *et al*., 2014; Sørensen *et al*., 2013). Genetic engineering approaches include, classical methods and specifically targeted improvements. Classical mutagenesis improves the enzyme activity at its regulatory level that leads to increased production of the gene of interest or decreases the expression of other genes. This applies on the production strains improvement not changes to enzyme itself for activity. The directed mutagenesis targets the amino acid sequence and changes are observed on the mutants (Sørensen *et al*., 2013). Several reports exist on this method for non-fungal β-glucosidases. For example, error prone PCR used for amino acid substitutions that were further recombined by gene shuffling was found to improve thermostability of *Paenibacillus polymyxa* β-glucosidase (Gonza´ lez-Blasco *et al*., 1999). Furthermore, another method was successfully performed on *Polycoccus furiosus* β-glucosidase that resulted in improvement of cellobiose hydrolysis at low temperature (Lebbink *et al*., 2000).

To perform advanced mutagenesis such as rational design, genetic information provided by genome sequencing is crucial. There is only few filamentous fungal β-glucosidases that have been genetically engineered. Lee *et al*. (2012) conducted an advanced mutagenesis study on *T. reesei* β-glucosidase where specific amino acids were mutated in the outer channel of an enzyme active site to improve its thermal stability (Lee *et al*., 2012). Furthermore, mutagenesis of only two amino acid residues (167/172) in CellA, another *T. reesei* β-glucosidase was carried out by Guo *et al*. (2016). This resulted in improved glucose tolerance and stimulation as well as pH and thermal stabilities. There was also a significant increase in the enzyme specific activity. Thus enhancing cellulose saccharification efficiency (Guo *et al*., 2016).

2.13. Cloning and heterologous expression of β-glucosidase genes

Metagenomic, bacterial and fungal sources are being extensively searched for β-glucosidases with desirable properties. For hyper-production, these have been cloned and expressed in *Escherichia coli* and eukaryotic systems such as filamentous fungi (*A. niger* and *T. reesei*) and yeasts (*Saccharomyces cerevisiae* and *P. pastoris*) (Ahmed *et al*., 2017; Singhania *et al*., 2017).

2.13.1. Metagenomic screening

Microorganisms are the most diverse living organisms on Earth. Soil and marine environments contains huge amounts of microbial cells. However, only 1% of these microorganisms are culturable and 99% are unculturable by available laboratory technologies. This leaves these microbes unexplored to investigation for their benefits to mankind (Singh, 2010). Metagenomics is the culture-independent technique utilised to analyse the genetic material present in an environmental sample (Thomas *et al*., 2012). In this approach, the following steps are carried out, namely, environmental DNA extraction and digestion, metagenomic DNA library construction and screening of libraries for gene and sequences of interest. Function-based screening, gene specific screening and direct sequencing are techniques used for metagenomic library screening. The gene of interest is then cloned and expressed. Metagenomic strategies have been used to find novel genes encoding for enzymes with superior characteristics (Ahmed *et al*., 2017).

Numerous β-glucosidases have been investigated from different metagenomes including wetland soil (Soo-Jin *et al*., 2007), buffalo rumen (Guo *et al*., 2008), rabbit cecum (Feng *et al*., 2009),

alkaline polluted soil and termite gut (Jiang *et al*., 2011), cow rumen (Del Pozo *et al*., 2012), agricultural soil (Biver *et al*., 2014; Li *et al*., 2012), *Globitermes brachycerastes* gut (Liu *et al*., 2018; Wang *et al*., 2012), Baltic seawater (Wierzbicka-Woś *et al*., 2013), compost microbial community (Uchiyama *et al*., 2013), Amazon soil (Bergmann *et al*., 2014), hydrothermal spring (Schröder *et al*., 2014), rumen of cattle feeding with *Miscanthus sinensis* (Li *et al*., 2014), Kusaya gravy (Uchiyama *et al*., 2015), mangrove soil metagenome (Mai *et al*., 2016) and Lake Poraquê in the Amazon (Toyama *et al*., 2018),

2.13.2. Bacterial recombinant β-glucosidases

Many bacterial enzymes exhibit desirable properties such as thermostability, glucose tolerance and alkaline adaptability. However, they are produced intracellularly. To ease purification and enhance production, numerous β-glucosidases have been investigated from bacteria. These have been cloned, over-expressed and characterised (Crespim *et al*., 2016; Florindo *et al*., 2018; Fusco *et al*., 2018; Liew *et al*., 2018; Long *et al*., 2016; Mattéotti *et al*., 2011; Sathe *et al*., 2017; Yan *et al*., 2018). For instance, the gut of the termite *Reticulitermes santonensis* contains an interesting number of prokaryotic microorganisms not found elsewhere. With the aim to discover new bacterial enzymes involved in cellulose digestion in the gut of *R. santonensis*, Mattéotti *et al*. (2011) studied a novel β-glucosidase. This enzyme was then cloned and expressed in *Escherichia coli*. The recombinant β-glucosidase was purified and performed optimally at pH 6.0 and 40°C. This enzyme also showed β-xylosidase activity, thus demonstrating applicability in both cellulose and hemicellulose degradation (Mattéotti *et al*., 2011).

Enzyme thermostability is one of the most prominent properties for industrial applications. A β-glucosidase from a thermophilic bacteria, *Thermotoga thermarum* was cloned and expressed in *E. coli*. In comparison to other β-glucosidases from thermophiles, the enzyme showed high thermostability. Recombinant *Tth*Bgl performed optimally at the temperature of 85℃ and retained 80% of its original activity at 80℃ for 120 minutes. It was also able to hydrolyse cellobiose at 75℃ for 9 hours (Long *et al*., 2016). On the other hand, the concept of simultaneous saccharification and fermentation (SSF) is one of the solutions in reducing the cost of bioethanol production. However, the major bottleneck is the diversity of optimum conditions for organisms involved in the overall process. Normally, cooling down stage is required because the fermentation step is carried out at 30℃. Therefore, the availability of psychrophilic enzymes is also advantageous. *Exoguobacterium antarcticum* B7 is a bacterium isolated from Antarctica soil samples. Crespim *et al*. (2016), recombinantly over-expressed its β-glucosidase in *E. coli* cells and it was characterised. Recombinant *Ea*Bgl1A was stable at the temperature range of 15-35℃ and was also optimal at pH 7.0. It demonstrated adaptation to both cold and alkaline conditions compared to other β-glucosidases. More importantly, *Ea*Bgl1A showed high tolerance to glucose than commercial enzymes indicating its potential in application in the SSF process (Crespim *et al*., 2016).

More reports on recombinant β-glucosidases from bacterial sources exhibiting the above-mentioned traits and expressed in *E. coli* are available. These include glucose-tolerant and thermostable β-glucosidases from *Thermoanaerobacterium thermosaccharolyticum* DSM 571 (Pei *et al*., 2012), *Pyrococcus furiosus* (Cota *et al*., 2015), *Thermotoga* spp. (Cota *et al*., 2015; Yang *et al*., 2018), *Methylococcus capsulatus* (Sathe *et al*., 2017), *Dictyoglomus turgidum*

(Fusco *et al*., 2018), *Jeotgalibacillus malaysiensis* (Liew *et al*., 2018) and *Bifidobacterium* spp. (Florindo *et al*., 2018; Yan *et al*., 2018). Furthermore, a recent study by Ichikawa *et al*. (2019) demonstrated the applicability of biological simultaneous enzyme production and saccharification (BSES). *Clostridium thermocellum* is the best known member of the anaerobic, thermophilic, cellulolytic and ethanol-producing bacterium. It also have great potential in SSF for bioethanol production. Its cellulase system is exploited for saccharification of both crystalline and amorphous cellulose (Khan and Akhtar, 2010). A β-glucosidase from *Thermoanaerobacter brockii* was cloned and expressed in *E. coli*, *Bacillus subtillis* and *Brevibacillus choshinensis*. These bacterial cells were then supplemented into the *Clostridium thermocellum* cellulose hydrolysis preparation. After three days of incubation, 37.3 g/l of glucose was released from cellulose and Avicel. The same amount of glucose was produced when the purified β-glucosidase was used. This indicated that purification costs can be reduced by direct supplementation of β-glucosidase-producing bacterial cells. Thus demonstrating the potential of BSES approach in improving cellulose degradation (Ichikawa *et al*., 2019).

However, in *E. coli*, recombinant enzymes are still expressed intracellularly. This system also has a drawback in that the formation of disulphide bonds is hampered by a highly reducing environment in the cytoplasm (Çelik and Çalık, 2012; Yang *et al*., 2013). In contrast, some eukaryotic systems secrete recombinant proteins into the medium. This also allows for post-translational modifications thereby increasing the possibility of retaining protein native structures (Liu *et al*., 2013; Singhania *et al*., 2017).

2.13.3. Eukaryotic recombinant β-glucosidases: Filamentous fungi

Filamentous fungi are known to be good producers of β-glucosidase from GH3. Their ability to utilise inexpensive substrates and extracellular production of proteins have made them attractive for industrial applications. Most of the filamentous fungi provides stable transformants as they integrate the transformed plasmid into their genomes. Therefore, they can be used as hosts for recombinant DNA. *A. oryzae* and *T. reesei* have been employed for expressing both fungal and non-fungal β-glucosidase genes (Singhania *et al*., 2017).

Uchima *et al*. (2011) expressed a β-glucosidase (G1NkBG) from *Neotermes koshunensis* in *A. oryzae*. This lower termite secretes endogenous β-glucosidase in the salivary glands (Uchima *et al*., 2011). In addition to hydrolysing cellobiose efficiently, G1NkBG was stimulated by glucose demonstrating its applicability in improving cellulose degradation for bioethanol production (Uchima *et al*., 2011). Furthermore, twenty three genes encoding putative β-glucosidases were found in the genome database of *A. oryzae*. Ten of these belonging to GH3 were over-expressed in this fungus using the improved glucoamylase (*glaA*) gene promoter. Only three of these β-glucosidases had not been characterised such as BglA, BglF and BglJ. Although their properties were not exceptional, this study demonstrated the possibility of mining of novel β-glucosidases from microbial genome sequences and the use of *A. oryzae* as an expression system (Kudo *et al*., 2015).

Engineering of *T. reesei* to improve β-glucosidase production would be of high value. Ma *et al*. (2011) expressed a β-glucosidase from *Penicillium decumbens* in *T. reesei* Rut-C30 under a

strong inducible cellobiohydrolase 1 promoter (*cbh1*). The enzyme was glycosylated properly and its activity increased 6-8 fold compared to the non-recombinant strain. The pBGL1 also improved saccharification efficiency of cellulase preparation from *T. reesei* Rut-C30 due to the increase in its β-glucosidase activity (Ma *et al*., 2011). In another study, a thermostable β-glucosidase gene from *Periconia* spp. was successfully engineered into the genome of *T. reesei* QM9414 strain. The β-glucosidase activity increased 10.5 fold, from 2.2 U/mg to 23.9 U/mg and total cellulase activities increased after 24 hours of incubation. Compared to the parent strain, the transformed *T. reesei* also released more reducing sugars from untreated and pre-treated barley as well as microcrystalline cellulose. These features suggest that the transformants can be used for β-glucosidase production and improving biomass conversion (Dashtban and Qin, 2012).

2.13.4. Recombinant β-glucosidases from yeasts

Yeasts are popular industrial hosts for expression of recombinant enzymes. This is due to advantages associated with these systems such as ease of genetic manipulation, rapid growth and ability to perform eukaryotic post-translational modifications (e.g. glycosylation). The commonly used yeast expression systems are *P. pastoris, S. cerevisiae, Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe, Yarrowia lipolytica*, *Arxula adeninivorans*, and *Candida boidinni* (Chen *et al*., 2018; Palabiyik *et al*., 2006). These utilise different carbon sources, rapidly reach high densities and are thermo- and halo-tolerant. Some yeast systems have been engineered for added advantages. For example, lack of proteases, optimised transformation and expression efficiency (Liu *et al*., 2013). In addition, yeast strains have also been exploited as sources of β-glucosidases (Hernández *et al*., 2003; Mai and Thanh, 2010). Dan *et al*. (2000) expressed a β-glucosidase gene (Bgl1) from *A. niger* in both *S. cerevisiae* and *P. pastoris*. Low

level of expression was obtained in *S. cerevisiae*, 1.9 U/mg. In another study, *Thermoascus aurantiacus* (*Ta*BGL) and *Phanerochaete chrysporium* (*Pcc*Bgl1) β-glucosidases were expressed in *S. cerevisiae* Y294. The *Pcc*Bgl1 demonstrated a high activity of 3.3 U/ml. Since the host organism was cultivated on cellobiose that was hydrolysed to glucose, 4.8 g/l of ethanol was produced (Njokweni *et al*., 2012). Several more studies have been conducted on *S. cerevisiae* strains over-expressing β-glucosidase for improved cellulosic bioethanol production (Larue *et al*., 2016; Wilde *et al*., 2012; Yamada *et al*., 2013).

In addition to engineering *S. cerevisiae* for consolidated bioprocessing, it is also a generally regarded as safe (GRAS) strain. Therefore, it can be used as a host for expressing enzymes that are used in the food and pharmaceutical industries (Liu *et al*., 2013). In this context, a β-glucosidase with the ability of glycosylating piceid, a stilbenoid glucoside and a major resveratrol derivative in grape juices was characterised from *Dekkera bruxellensis*. This enzyme was then expressed in *S. cerevisiae exg1D* mutants. This β-glucosidase may be relevant for future application in the production of bioactive compounds and aroma enhancement during winemaking (Kuo *et al*., 2018). However, expression of heterologous proteins in *S. cerevisiae* is not always optimal for large-scale production due to loss of the plasmid during scale-up, hyper-glycosylation and low protein yield (Ahmed *et al*., 2017). A methylotrophic yeast, *P. pastoris* has been developed for expression as an alternative to *S. cerevisiae*.

2.13.5. Expression in *Pichia pastoris*

Pichia pastoris possess highly inducible promoters and its expression methods, host strains and vectors are commercially available. The commonly used expression strains such as *P. pastoris* GS115, X-33, KM71H and protease-deficient ones (*P. pastoris* SMD 1163 and SMD 1168H) were derived from the wild-type *P. pastoris* NRRL-Y 11430 (Liu *et al*., 2013). The genome of *P. pastoris* contains two copies of the alcohol oxidase gene such as *AOX1* and *AOX2*. These allow *P. pastoris* to utilise methanol as the sole carbon. The *AOX1* promoter regulates 85% of the alcohol oxidase activity in the cell that drives heterologous protein expression in *Pichia*. Transformation in *P. pastoris* may result in three phenotypes related to methanol utilisation, namely, methanol utilisation plus (Mut^+), methanol utilisation slow (Mut^S) and methanol utilisation minus (Mut^-) (Liu *et al*., 2013). The Mut^+ strains grow on methanol at the wild-type rate whereas the Mut^S have a disruption in the *AOX1* gene. The Mut^S strains then rely on the weaker *AOX2* gene for methanol metabolism. These grow slower and utilise methanol at a slow rate. The Mut^- phenotype strains are unable to grow on methanol. They are defective in both the *AOX1* and *AOX2* genes (Daly and Hearn, 2005; Zhang *et al*., 2000). The use of Mut^- strains can be advantageous in the expression of recombinant proteins where low growth rates are desirable for production. Methanol utilisation plus phenotypes are the most exploited strains. Glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter is also used for constitutive production of heterologous proteins in *P. pastoris*. This approach does not require methanol induction (Daly and Hearn, 2005; Macauley-Patrick *et al*., 2005; Várnai *et al*., 2014).

Using *P. pastoris* system is advantageous due to its ability to grow to very high cell densities in minimal medium and ease of genetic manipulation such as gene targeting, high-frequency DNA

transformation as well as cloning by functional complementation (Macauley-Patrick *et al*., 2005). Additionally, this system has a perfect processing mechanism including signal peptide cleavage, protein folding and post-translational modifications inside the cell. *P. pastoris* also secretes its products into the medium with normal function (Liu *et al*., 2013). Simple purification of secreted recombinant proteins is possible due to the relatively low levels of extracellular native proteins (Singhania *et al*., 2013).

The first fermentation protocol for Mut^{+} and Mut^{S} strains was developed by Brierley *et al*. (1990). In this study, bovine lysozyme c2 was expressed as a model system up to 600 mg/l. *P. pastoris* fermentation includes two separate phases. The cells are initially grown on glycerol to the exponential phase of growth. This is followed by a fed-batch phase, in which methanol is fed at a limited rate or maintained at some level to induce the *AOX* promoter for protein expression (Zhang *et al*., 2000).

Pichia pastoris expression system for research purposes is supplied by Invitrogen along with the product manual. Many heterologous proteins including β-glucosidases have been expressed. *P. pastoris* is normally cultivated on buffered minimal medium at 28-30℃ with shaking at 200-250 rpm. The expression is usually carried out between 3 to 10 days with methanol added (0.5% v/v) every 24 hours to maintain induction (Chen *et al*., 2011; Harhangi *et al*., 2002; Karnaouri *et al*., 2013; Li *et al*., 2013; Mai and Thanh, 2010; Meko'o *et al*., 2010; Uchima *et al*., 2012; Yan *et al*., 2012). Different levels of β-glucosidase expression have been achieved. Properties of some fungal β-glucosidases expressed in *P. pastoris* are summarised in Table 2.1.

Table 2.1: Fungal β-glucosidases expressed in *Pichia pastoris*

Source organism	Properties of the recombinant β-glucosidase	Reference
Periconia spp.	672.0 U/mg activity, thermostable and broad substrate specific activity	Harnpicharnchai *et al*., 2009
Saccharomycopsis fibuligera BMQ 908	60.0 U/ml cellobiase activity	Mai and Thanh, 2010
Aspergillus niger	51.0 U/mg cellobiase activity	Meko'o *et al*., 2010
Trichoderma reesei	60.0 activity and intermediate temperature stability	Chen *et al*., 2011
Nasutitermes takasagoensis	5.8 U/mg activity with broad substrate specificity	Uchima *et al*., 2012
Paecilomyces thermophila	9.6 U/ml activity and broad substrate specificity	Yan *et al*., 2012
Myceliphthora thermophila	41.0 U/ml activity and broad substrate specificity	Karnaouri *et al*., 2013
Phialophora spp.	1.2 U/ml activity and aryl β-glucosidase	Li *et al*., 2013
Aspergillus nidulans AN2227	5.50 U/mg activity with good pH and temperature	Auta *et al*., 2016
Trichoderma viride	143.0 U/ml hydrolytic activity and gentiooligosaccharide synthesis	Wang *et al*., 2018

2.14. Biochemical characterisation of β-glucosidases

Biochemical characterisation involves simple kinetic measurements that are conducted to provide details on the enzyme's mode of action. These include determination of enzymes substrate specificity, pH and temperature optima as well as stability.

2.14.1. Beta-glucosidase purification

Protein purification is an essential first step in understanding enzymes biochemical functions. Purification separates the protein of interest from a mixture of various molecules, yielding a single molecule sample. The enzyme assay is then performed to confirm the presence of the protein in the preparation. It is based on the reaction that the enzyme catalyses. The first step in purifying intracellular proteins involves cell disruption to form a homogenate. It is followed by fractionation using centrifugation, which applies as a first step in extracellular protein purification. Usually the pellet consists of unwanted cell components leaving the enzyme on the supernatant, which then serves as a source of protein for subsequent purification steps (Berg *et al*., 2002).

Proteins are mostly purified according to their solubility, size, charge and binding affinity. A series of separations are conducted to yield a pure protein from the supernatant. On the basis of solubility, it is known that proteins are less soluble at high salt concentrations. This phenomenon is referred to as salting out. Proteins precipitates at different salt concentrations. Upon addition of salt to the mixture, the surface tension of water increases. This gives rise to hydrophobic interaction between the protein and water. In response to this situation, the protein decreases its surface area to minimise contact with the solvent by folding and self-association which leads to precipitation (Berg *et al*., 2002). Ammonium sulphate [$(NH_4)_2SO_4$] has a much higher solubility than any of the phosphate salts and is mostly used for protein precipitation (Wingfield, 2001).

The precipitated protein is re-suspended in the buffer and dialysed to remove the residual salt. The mixture is subjected to a semipermeable membrane which allows transversion of small molecules and ions through the pores. The proteins are retained inside the bag and then resuspended in the buffer for subsequent steps (Berg *et al*., 2002). Gel filtration chromatography which is based on size exclusion, ion exchange chromatography utilising net charge of proteins and affinity chromatography based on binding of specific DNA sequences are the more discriminating purification techniques that have been used for many enzymes including β-glucosidases (Harnpicharnchai *et al*., 2009; Long *et al*., 2016; Uchima *et al*., 2012; Yan *et al*., 2012). The enzyme activity and protein concentration are determined at each step of purification. This also aids in the calculation of the overall yield of the process (Berg *et al*., 2002).

The commonly used accurate and easy to perform methods are Lowry method utilising Folin's phenol reagent (Lowry *et al*., 1951); Bradford assay, based on the principle of protein-binding dye (Coomassie Brilliant Blue G-250) (Bradford, 1976); BCA protein assay using bicinchoninic acid (Smith *et al*., 1985) and ultraviolet (UV) absorbance by spectrophotometry at 280 nm. The latter is based on the presence of tyrosine and tryptophan in proteins (Scopes, 1974). The specific activity is then determined as the ratio of enzyme activity to the protein amount in the assay. As the purification progresses, the specific activity increases. The degree of purification attained is mostly determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This technique is also used in molecular weight determination (Eyzaguirre *et al*., 2005; Purwanto, 2016).

2.14.2. Beta-glucosidase assay

Sensitive and simple methods have been developed for determination of β-glucosidase activity. Alkyl- or aryl-glucosides are commonly used as substrates. Upon enzyme hydrolysis, either a coloured or fluorescent product is released. The most utilised chromogenic substrate is 4-nitrophenyl β-D-glucopyranoside (pNPG), which releases 4-nitrophenol. The activity is then measured spectrophotometrically. The fluorogenic substrate 4-methylumbelliferyl β-D-glucopyranoside (MUG) is used for β-glucosidase qualitative assays. The enzyme activity is indicated by fluorescence under UV light. Other β-glucosidase substrates used are natural glycosides such as salicin, esculin, amygdalin, gentiobiose, laminaribiose and the disaccharide cellobiose (Eyzaguirre *et al*., 2005).

If the criterion for assay is activity in cellulose degradation, cellobiose should be the substrate of choice. Interaction of β-glucosidase with its substrates is not fully understood yet. However, it has been stated that enzyme activity is low on cellobiose compared to artificial substrates. It was reported that enzyme kinetics depends on the configuration of the substrate. In this case, cellobiose requires conformational change for hydrolysis (Singhania *et al*., 2013). In addition, β-glucosidase has a very rigid structure in substrate binding site that accommodate one glucose of cellobiose but the second one changes the conformation using rotation of the alpha bond of the glycoside to fit in the substrate binding site. Since artificial substrates do not undergo this process, it is the main reason for low enzyme activity towards cellobiose (Sørensen *et al*., 2013). β-glucosidase activity towards cellobiose is measured by determining free glucose concentration using glucose oxidase-peroxidase assay (Elshafei *et al*., 2011). Furthermore, coupled hexokinase-glucose-6-phosphate dehydrogenase assay and high-performance liquid

chromatography (HPLC) are also used to detect released glucose (Chan *et al*., 2016; Mukherjee *et al*., 2016). Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) are used for routine assays (Baba *et al*., 2017; Gao *et al*., 2014; Meleiro *et al*., 2017). These methods also allow for the identification of β-glucosidase synthetic activity (Eyzaguirre *et al*., 2005).

Fungal β-glucosidases are often expressed in multiple forms that differ in some physiochemical properties. Native PAGE analysis are based on gel electrophoresis followed by visualisation of the activities *in situ* in the gel. The most commonly used substrates are 6-bromo-2-naphtyl-β-D-glucoside and 4-methylumbelliferyl β-glucopyranoside (MUG) (Kim *et al*., 2007; Rajoka *et al*., 2015).

2.14.3. Effect of pH and temperature on β-glucosidase activity

In all biological processes the enzyme performance is mostly influenced by pH and temperature. Variations in pH and temperature may lead to inactivation of the enzyme, depending on its tolerance and time of exposure. Ionic groups that are involved in enzyme catalysis such as acid-base catalyst in β-glucosidase active site, protonation state of carboxylic acid residue and carboxylate nucleophile are essential for the enzymatic reaction i.e. changes in pH can damage the catalytic mechanism (Sørensen *et al*., 2013).

The majority of β-glucosidases display optimum pH over the range of 4.0 to 6.0 and stable a high pH levels. This allows a choice of enzymes for a variety of applications (Eyzaguirre *et al*., 2005; Singh *et al*., 2016). The optimum temperature of β-glucosidases from all domains of living organisms ranges from 30-90℃ (Crespim *et al*., 2016; Ma *et al*., 2015; Schröder *et al*., 2014; Singh *et al*., 2016). Evidently, β-glucosidases from thermophilic fungi have higher temperature optima and stability compared to those from mesophiles (Mehta *et al*., 2016). The maximal stability is also influenced by the medium pH in which the enzyme is kept. It was initially suggested that the carbohydrate moiety also affects enzyme thermostability. The enzymes with higher carbohydrate content were stable. However, this was further declared to be inconclusive since low-carbohydrate-content β-glucosidase also shown to be thermostable (Eyzaguirre *et al*., 2005). Furthermore, protein surface compactness and hydrophilic/hydrophobic balance was suggested to play a vital role in thermostability, pH stability and substrate specificity (Kumwenda *et al*., 2013; Wijma *et al*., 2013).

2.14.4. Beta-glucosidase substrate specificity

Besides hydrolysing short-chain cellooligosaccharides, β-glucosidases show activity toward both natural and synthetic aryl-β-glucosides with a variety of aglycones. The relative activities towards these glucosides vary among enzymes. Based on substrate specificity, β-glucosidases have been classified as cellobiases (Harhangi *et al*., 2002; Liew *et al*., 2018; Mai and Thanh, 2010; Meko'o *et al*., 2012; Pei *et al*., 2012), aryl-β-glucosidases (Crespim *et al*., 2016; Florindo *et al*., 2018; Li *et al*., 2013; Long *et al*., 2016) and broad substrate specific β-glucosidases (Gao *et al*., 2014; Harnpicharnchai *et al*., 2009; Karnaouri *et al*., 2013; Kudo *et al*., 2015; Uchima *et al*., 2012; Yan *et al*., 2012). Many β-glucosidases belong to the last group. Broad substrate

specific β-glucosidases are able to hydrolyse β-1,4; β-1,2; β-1,3 and β-1,6 glycosidic bonds (Krisch *et al*., 2010). For instance, Dhr1 and Glu1 are *Soghurm bicolor* β-glucosidase isoenzymes which shares 72% similarity. Dhr1 is strictly specific for its natural substrate, dhurrin (p-hydroxy-(S)-mandelonitrile-β-D-glucoside). On the other hand, Glu1 hydrolyse broad spectrum of substrates in addition to its natural substrate, 2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxaxin-3-one. Based on structural data from enzyme-substrate complexes, the substrate specificity is defined by different binding modes of glycone and aglycone moieties (Verdoucq *et al*., 2004).

2.15. *Thermomyces lanuginosus* and multiple β-glucosidase genes

Thermomyces lanuginosus is a non-cellulolytic thermophilic fungus that is widely distributed in self-heating masses of organic debris. Tsiklinskaya first isolated it in 1899 from a potato that had been inoculated with soil in the garden (Singh *et al*., 2003). Thermophilic fungi are characterised by their ability to grow at temperatures up to 60℃ and are unable to grow below 20℃. Compared to mesophiles, they are also known for the production of thermostable enzymes with great potential for industrial application (Maheshwari *et al*., 2000; Mehta *et al*., 2016; Yeoman *et al*., 2010).

Numerous *T. lanuginosus* strains have been reported for hyper-production of xylanases and other hemicellulases (Berg *et al*., 1998; Gomes *et al*., 1993; Puchart *et al*., 1999; Purkarthofer *et al*., 1993). They are used to degrade xylan, a major hemicellulose, with high specificity. Xylanases from *T. lanuginosus* are characterised by high thermostability and stability over a broad pH range. They are commonly used in pulp and paper industry to substitute chemicals used in pulp

bleaching (Kantelinen *et al*., 1993). The levels of hemicellulase expression vary among *T. lanuginosus* strains. Other glycosyl hydrolases are expressed in very low amounts. Carbon and nitrogen sources used for growth have been reported to have great effect on enzyme production by this fungus (Singh *et al*., 2003). In addition to cellulase-free thermostable xylanase, *T. lanuginosus* also secretes other hydrolytic enzymes such as α-amylases, glucoamylases, pectinases, proteases and lipase that are used in different industrial processes for example starch saccharification, depectinisation of fruit based products, in detergents and transesterification (Maheshwari *et al*., 2000; Singh *et al*., 2003).

Although *T. lanuginosus* is non-cellulolytic, cellulose-degrading enzymes were still identified in its genome. These includes β-1,4-endoglucanases that shared similarity with *T. reesei* and *A. kawachii* cellulases (Mchunu *et al*., 2013). This fungus is suggested to rely on commensalism with cellulolytic fungi in composts. In retrospect, Lin *et al*. (1999) produced a β-glucosidase with high specificity from *T. lanuginosus* SSBP. The purified enzyme exhibited similar specific activities against both cellobiose and aryl-glucosides. It was stable at 50℃ and at pH levels above 5.0 (Lin *et al*., 1999). Furthermore, genome sequencing revealed the presence of six β-glucosidases in this fungus (Mchunu *et al*., 2013).

The predicted gene sizes, number of deduced amino acids, signal peptide sequences and molecular masses of the enzymes are shown in Table 2.2. The *bgl1* (2568 bp) was the largest, followed by *bgl4*, *bgl5* and *bgl3* while *bgl2* (1260 bp) was the smallest. Signal peptides were only detected on *bgl2* and *bgl3* not in other three β-glucosidases. According to Singh *et al*.

(2013), signal peptides can modulate protein stability and aggregation in a sequence dependent manner.

Table 2.2: Summary of the sequence analysis of β-glucosidase genes from *T. lanuginosus* SSBP

	bgl1	***bgl2***	***bgl3***	***bgl4***	***bgl5***
Size (bp)	2586	1260	1344	1938	1410
Amino acids	861	419	447	645	469
Signal peptide (aa)	ND	1-23	1-17	ND	ND
Molecular mass (kDa)	99.9	46.5	46.8	68.9	54.3

ND: not detected

Following molecular characterisation, cloning of five selected β-glucosidase genes (designated *bgl1* to *bgl5* in this report) was conducted at the Enzyme Technology Laboratory, Durban University of Technology (N.P Mchunu 2018 - personal communication). The genes were isolated, amplified and ligated between the EcoR I/Not I (*bgl1*, *bgl3* and *bgl5*) or SnaB I/Avr II (*bgl2* and *bgl6*) sites of the pPIC9K vector (Figure 2.3) which was then cloned into *E. coli* JM 109. The recombinant plasmids selected from positive transformants were then transformed into *P. pastoris* GS115 for expression (Mchunu, 2014).

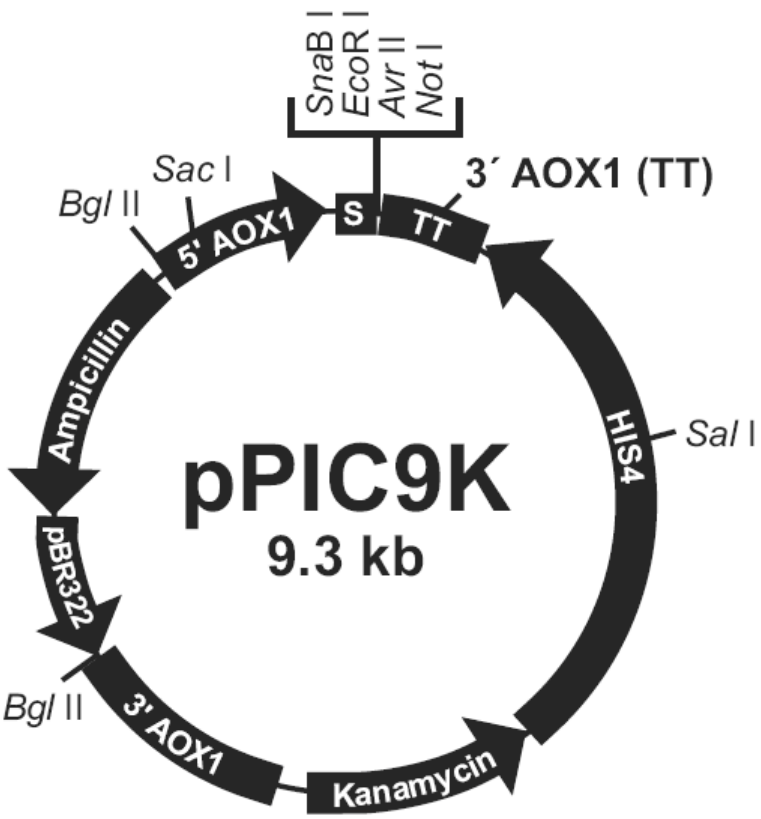

Figure 2.3: Vector map of the pPIC9K plasmid used for cloning of *bgl* genes into *P. pastoris* expression system (Invitrogen, 2010).

2.16. Scope of the study

The important role played by β-glucosidase in cellulose degradation along with the addressed issue of its production in low amounts necessitate for the search of different sources of this enzyme to improve its productivity. It is evident that thermophilic fungi are good sources of industrial enzymes. The presence of multiple β-glucosidase genes in *T. lanuginosus* SSBP required their further characterisation to assess their potential in cellulose degradation.

2.16.1. Aim

To conduct biochemical characterisation of five recombinant β-glucosidases from *T. lanuginosus* SSBP expressed in *P. pastoris* and to assess their potential in cellulose hydrolysis.

2.16.2. Objectives

- To conduct production of five β-glucosidases from *T. lanuginosus* SSBP expressed in *P. pastoris* using shake flask fermentations.
- To partially purify produced β-glucosidases using ammonium sulphate precipitation.
- To conduct biochemical characterisation of the recombinant β-glucosidases such as pH and temperature optima as well as stability.
- To determine substrate specificity of recombinant β-glucosidases using various β-linked aryl glucosides and disaccharides.
- To investigate the synergistic potential of the highly expressed enzyme in improving cellulose hydrolysis.

3. MATERIALS AND METHODS

3.1. Maintenance of recombinant strains

Five *P. pastoris* GS115 recombinant strains containing pPIC9K plasmids with β-glucosidase genes (Bgl1-Bgl5) were obtained from the Enzyme Technology Research Group, Durban University of Technology. The organisms were sub-cultured on yeast extract (1% w/v), peptone (2% w/v), glucose (2% w/v) and technical agar (1.5% w/v) [YPG agar] containing 2 mg/ml Geneticin (Sigma Aldrich) and incubated at 30℃ for 48 hours. For short-term storage, the plate cultures were kept at 4℃. For long-term storage, 15% glycerol stocks of the recombinant strains were prepared and stored at -80℃ (N.P Mchunu, 2018 - personal communication).

3.2. Growth medium and enzyme substrates

Geneticin-containing YPG agar plates were used to resuscitate recombinant strains from the stock cultures. Minimal medium (MGY and MM) was used for biphasic expression of β-glucosidases. Minimal glycerol medium (1% v/v) (MGY) containing yeast nitrogen base (YNB) without amino acids (1.34% v/v) [Sigma Aldrich], drop-out mix minus histidine (7.5% of YNB) [US Biological] and biotin (0.0004% v/v) was used for biomass accumulation and minimal methanol medium (MM) [prepared the same way as MGY except that glycerol was substituted with methanol (0.5% v/v)] was then used for induction of expression. Yeast extract, peptone and glucose/methanol medium (YPG/YPM) was alternatively used for enzyme production. The media components were weighed and dissolved in distilled water and sterilised at 121℃ for 30

minutes. Geneticin, methanol, biotin, YNB and drop-out mix were sterilised separately using 0.22 μm sterile filters and added to flasks prior to inoculation and for induction of expression.

The substrate 4-methylumbelliferyl β-D-glucopyranoside (MUG) [Sigma Aldrich] was used for qualitative enzyme and in-gel protein assays and 4-nitrophenyl β-D-glucopyranoside (pNPG) [Sigma Aldrich] was used for quantitative enzyme assays. All substrates (10 mM) for the standard enzyme assays were dissolved in sodium citrate buffer, pH 5.0. Disaccharides such as cellobiose, gentiobiose and salicin as well as a polysaccharide, laminarin (Sigma Aldrich) were used to determine the enzymes specific activities. Microcrystalline cellulose (Sigma Aldrich) and carboxymethyl-cellulose (CMC) [Sigma Aldrich] were used to determine the effect of β-glucosidases on cellulose degradation.

3.3. Qualitative screening for β-glucosidase expression

For qualitative screening of enzyme expression, a single colony of each *P. pastoris* recombinant strain was spotted on minimal methanol agar plates and incubated at 30℃ for 48 hours. The non-recombinant strain was grown as a negative control. The cultures were overlaid with 4 ml of 1% agarose containing 10 mM MUG substrate and incubated at 50℃ for 30 minutes. These were then viewed under UV light and digitally photographed in a ChemiDoc™ XRS+ imager (Bio-Rad).

3.4. Methanol-induced expression of β-glucosidases

The five *P. pastoris* strains containing *bgl* genes and non-recombinant *P. pastoris* GS115 (negative control) were sub-cultured onto YPG agar plates and incubated at 30℃ for 48 hours for

resuscitation. *P. pastoris* uses methanol as a carbon source. It was essential to determine the rate at which the strain utilises methanol as this information is needed to select the method to be used for heterologous protein expression. The methanol-utilisation phenotype of the strains was determined by streak plating on both minimal glucose and minimal methanol agar plates which were incubated at 30℃ for 48 hours and rate or extent of growth examined. Minimal medium is universally used for expression in *P. pastoris*. Induction of expression was therefore performed according to the phenotype of the recombinant strains as described in the *Pichia* expression kit (Invitrogen, 2014). To grow the cells to the exponential phase of growth, single colonies of each strain from 48-hour cultures were aseptically transferred into sterile 100 ml minimal glycerol medium minus histidine (MGY) in 500 ml Erlenmeyer flasks and incubated at 30℃ in a shaking incubator (250 rpm) until the culture reached an OD_{600}= 2-6 (18 hours).

To initiate methanol induction, the cells were harvested by centrifugation at 3000 x *g* for 5 minutes. The supernatants were discarded and the pellets were washed twice with sterile ice-cold minimal methanol medium minus histidine (MM) and then re-suspended in 20 ml of the same medium in 100 ml Erlenmeyer flasks and incubated at 30℃ for 168 hours with shaking at 250 rpm. Induction of expression was maintained by addition of 100% sterile methanol to a final concentration of 0.5% (v/v) every 24 hours.

Alternatively, yeast extract, peptone and glucose medium (YPG) was used for inoculum development as mentioned above, except that single colonies were aseptically inoculated into 10 ml of sterile YPG, in 100 ml Erlenmeyer flasks. The flasks were also incubated at 30℃ in a shaking incubator (250 rpm) until the culture reached an OD_{600} = 2-6. To initiate methanol-

induction, the cells were also harvested by centrifugation at 3000 x *g* for 5 minutes. The supernatants were discarded and the pellets were washed twice with sterile distilled water and then re-suspended in 100 ml of sterile yeast extract, peptone and methanol (YPM) in 1 litre Erlenmeyer flasks and incubated at 30℃ for 168 hours shaking at 250 rpm with addition of 100% methanol to a final concentration of 0.5% (v/v) every 24 hours to maintain induction. In both growth media, 2 ml samples were removed from fermentation flasks at 24-hour intervals for measuring enzyme production. The enzyme expression levels (activities) were compared in a graph against induction time. The growth medium that promoted higher enzyme expression was then used for scaling-up in 1 litre Erlenmeyer flasks for further characterisation.

3.5. Beta-glucosidase quantitative enzyme assay

The enzyme activity was measured by hydrolysis of 4-nitrophenyl β-D-glucopyranoside (pNPG) whereby 4-nitrophenol was released. This resulted in formation of yellow colour that can be quantified spectrophotometrically. The reaction mixture contained 100 µl of the enzyme and 250 µl of 10 mM pNPG dissolved in 0.1 M citrate-phosphate buffer (pH 5.0). It was then incubated for 30 minutes in a water bath (Julabo TW2, Sigma Aldrich) set at 50℃. Thereafter, 1 ml of 1 M sodium carbonate was added to stop the reaction (Karnchanatat *et al*., 2007; Uchima *et al*., 2011). The mixture was then transferred into 1.5 ml cuvettes and 4-nitrophenol concentration was measured at 405 nm using a spectrophotometer (Biochrom Libra S21). One unit of activity was defined as the amount of enzyme which released 1 µmol of 4-nitrophenol per minute under assay conditions (Kim *et al*., 2007). The mean absorbance derived from three independent repeats was used in the equation below to calculate enzyme activity (Meko'o *et al*., 2010; Uchiyama *et al*., 2013).

$$\text{β-glucosidase activity (U/ml)} = \frac{\Delta OD(OD_{test}\text{-}OD_{blank}) \times V_t \times df}{\varepsilon \times l \times V_s \times t}$$

V_t: Total volume (1.35 ml)

V_s: Sample volume (0.1 ml)

ε: Extinction coefficient of 4-nitrophenol under the assay condition (18.5 mM^{-1} cm^{-1}).

ℓ: Light path length (1 cm)

t: Reaction time (30 minutes)

df: Dilution factor

3.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The sizes of recombinant β-glucosidases predicted from gene sequences were confirmed using SDS-PAGE (Eyzaguirre *et al*., 2005). Samples were prepared by adding 5 µl of Laemmli buffer (Laemmli, 1970) to 20 µl enzyme solutions obtained during β-glucosidase expression and after ammonium sulphate precipitation. Thereafter, samples were immersed in a boiling water bath for 5 minutes at 100℃ and separated in 12% acrylamide gel resolving gels. Proteins were separated according to their molecular weights at a constant current of 20 mA, using a running buffer prepared by dissolving 30 g of Tris base, 144 g of glycine and 10 g of SDS in 1 litre of distilled water. The gels were then stained for 15 minutes using Coomassie stain (prepared by dissolving 1 g of Coomassie Brilliant Blue G-250 dye in 40 ml of acetic acid, 180 ml ethanol, and 180 ml distilled water) and de-stained overnight using a mixture of 100 ml acetic acid, 300 ml ethanol and 600 ml distilled water. The gels were then viewed under epi-white illumination and digitally

photographed in the ChemiDoc™ XRS+ imager (Bio-Rad). Protein molecular weight markers (ThermoFisher Scientific) were used as size references for separated proteins.

3.7. Partial purification of β-glucosidases

A range from zero to ninety percent (0-90%) ammonium sulphate saturation was used to precipitate the extracellular proteins at 4℃. Ammonium sulphate precipitation calculator was used to determine the amount of the salt to be added at different fractions of 0-25%, 25-50%, 50-75% and 75-90% $(NH_4)_2SO_4$ saturation (EnCor Biotechnology Inc, 2016). The initial volume of each enzyme's crude extract was 1 litre. Ammonium sulphate was weighed and ground using a pestle and mortar to very fine particles to increase its surface area and it was then gradually added to the enzyme extract and left stirring at 4℃ overnight. The mixtures were then centrifuged at 12 000 x *g* for 15 minutes and the pellets re-suspended in 100 ml of 0.1 M sodium citrate buffer, pH 5.0 and 1 ml of the supernatant was removed to measure enzyme activity. This was repeated incrementaly until 90% $(NH_4)_2SO_4$ saturation was reached. The enzyme activities were then measured on both the supernatants and pellets of all fractions after dialysis. Dialysis membrane tubes were saturated in 300 ml of boiling distilled water, sodium bicarbonate (0.05% w/v) and ethylene-di-amine-tetra-acetic acid (EDTA) disodium (0.03% w/v) solution. Precipitated enzymes (100 ml) were each transferred into saturated dialysis tubes which were then immersed in 20 mM sodium citrate buffer (pH 5.0) in a 5 litre beaker that was kept at 4℃ for 12 hours with continuous stirring. The dialysed enzymes were then further concentrated using Amicon Ultra-15 Centrifugal Filter Units with a 30 kDa membrane cut-off (Sigma Aldrich). Protein concentrations were determined using the Bradford assay (section 3.8). Total activities,

specific activities, purification folds and yields were determined using the following equations (Berg *et al*., 2002).

Total protein (mg) = protein concentration (mg/ml) x total volume (ml)

Total activity (U) = enzyme activity (U/ml) x total volume (ml)

$$\text{Specific activity (U/mg)} = \frac{\text{total activity (U)}}{\text{total protein (mg)}}$$

$$\text{Purification (fold)} = \left(\frac{\text{final specific activity}}{\text{initial specific activity}}\right)$$

$$\text{Yield (\%)} = \left(\frac{\text{total units}}{\text{initial total units (first purification step)}}\right) \text{x } 100$$

3.8. Protein concentration determination

The protein concentration of the dialysed enzyme fractions was determined using the Bradford assay described by Bradford (1976), with slight modifications. The enzyme solutions (20 µl) were mixed with 1 ml of Bradford reagent (Bio-Rad) and the mixture was left to stand for 5 minutes at room temperature. Thereafter, the absorbance was measured at 595 nm using a spectrophotometer (Biochrom Libra S21) and the protein concentration was estimated using a bovine serum albumin (0.1-1 mg/ml) [Bio-Rad] standard curve prepared under the same conditions (Bradford, 1976).

3.9. Native PAGE analysis

3.9.1. Zymography

Native PAGE was also used to analyse activities of the partially-purified enzymes. Samples were prepared by adding 5 µl of native gel loading dye (Laemli, 1970) to 20 µl of the enzyme

solution. Proteins were separated using 12% native acrylamide gel (prepared the same manner as SDS-PAGE described in section 3.6, except that SDS was excluded in all gel preparations) and electrophoresis at 20 mA. The gel was then soaked in 10 mM MUG solution in 0.1 M sodium citrate buffer, pH 5.0 and incubated at 50℃ for 30 minutes and then viewed under UV illumination and digitally photographed in the ChemiDocTM XRS+ imager (Bio-Rad). The gel was then observed for fluorescing protein bands, indicating enzyme activity.

3.9.2. Coomassie staining

Samples were prepared in the same manner as described in section 3.9.1 and proteins were separated using 12% native acrylamide gel and electrophoresis at 20 mA. Thereafter, the gel was stained, de-stained and digitally photographed the same way as described in section 3.6. Native protein molecular weight markers (ThermoFisher Scientific) were used as size references for separated proteins.

3.10. Characterisation of β-glucosidases

3.10.1. Beta-glucosidase pH optimum and stability

The pH optima of the partially-purified enzymes were determined by measuring activities as described in section 3.5 at 50℃ over the pH range of 3.0-9.0 for 30 minutes. The substrate, pNPG was dissolved in 0.1 M sodium citrate buffer for testing the pH range 3.0-6.0 and 0.1 M Tris-HCl for testing the pH range 7.0-9.0 (Karnaouri *et al*., 2013). The stability at different pH levels was determined after incubating the enzymes in the above buffer solutions at room temperature for

240 minutes with sampling at 30 minute-intervals and then measuring the remaining activities using pNPG at optimum pH and temperature (Meko'o *et al*., 2010).

3.10.2. Beta-glucosidase temperature optimum and stability

Temperature optima were determined by measuring the activities of the partially-purified enzymes as described in section 3.5 at pH 5.0 in the temperature range of 30-90℃ for 30 minutes. For thermostability, the enzymes were pre-incubated at temperature range (30-90℃) for 240 minutes, with sampling at 30-minute intervals. Thereafter, the residual activities were measured using pNPG at the optimum pH and temperature (Li *et al*., 2013).

3.10.3. Beta-glucosidase substrate specificity

Beta-glucosidase substrate specificities were determined by measuring the amount of released glucose by enzyme hydrolysis of cellobiose (β-1,4), gentiobiose (β-1,6), salicin (β-1,2) and laminarin (β-1,3/6). An aliquot of 0.25 ml of each substrate (10 mM) solution in 0.1 M sodium citrate (pH 6.0) buffer was mixed with 0.25 ml of the enzyme solution. In negative control tubes (reaction blanks), the enzymes were omitted. The reaction mixtures were then incubated at 50℃ for 30 minutes and the reactions stopped by immersing test tubes in a water bath (Julabo TW2, Sigma Aldrich) set at 100℃ for 5 minutes. The glucose oxidase-peroxidase assay kit (Sigma Aldrich) was then used to measure the liberated glucose. Glucose oxidase-peroxidase reagent (glucose oxidase-peroxidase and o-dianisidine) [0.5 ml] was added to 0.25 ml of the above-mentioned reaction mixture. It was incubated at 37℃ for 30 minutes, followed by addition of 0.5 ml of 12 N sulphuric acid to stop the reaction. The mixture was then transferred to 1.5 ml cuvettes and read at 540 nm against the reagent blank (distilled water) using a spectrophotometer

(Biochrom Libra S21). The glucose concentration was determined using a standard curve (0.1-1 mg/ml of glucose standard).

3.10.4. Effect of addition of Bgl on hydrolysis of artificial cellulose

Artificial cellulose (carboxymethyl cellulose and microcrystalline cellulose) was hydrolysed using commercial cellulase from *A. niger* (C1184) [Sigma Aldrich]. The partially purified Bgl2 was supplemented to the preparation to determine its effect on the hydrolysis rate. The commercial β-glucosidase, Novozyme 188 (C6105) [Sigma Aldrich] was used as a positive control for β-glucosidase activity during hydrolysis. In test tubes, 5 ml of 1% (w/v) of each substrate dissolved in 0.1 M citrate buffer (pH 5.0) was mixed with 5 ml of cellulase (0.3 U/mg) supplemented with β-glucosidase [Bgl2 (553.7 U/mg) or Novozyme 188 (0.25 U/mg)]. The effect of increment of Bgl2 to a fixed concentration of cellulase [1:2, 1:3 and 1:4 (v/v)] was also investigated. The reaction mixtures were incubated at 50℃ and 100 rpm in a shaking water bath for 7 hours and samples analysed at hourly intervals. The reaction was stopped by immersing the sample tubes in boiling water (100℃) for 5 minutes. The amount of liberated glucose was determined using the GOD-POD assay as described in section 3.10.3.

3.11. Statistical analyses

For enzyme expression experiments, each recombinant strain was cultivated in three different flasks. Three enzyme reaction and three enzyme blank tubes were prepared from each of these flasks for enzyme assays and activities were calculated using average absorbance values. The hydrolysis reactions and assays were also performed in triplicates. The data was derived from averages of three independent repeats.

4. RESULTS

4.1. Qualitative screening for β-glucosidase expression

Beta-glucosidase activity on MUG, a fluorogenic substrate is indicated by fluorescence. The non-recombinant *P. pastoris* strain had no fluorescence whereas all five strains harbouring Bgl genes on MUG-containing MM agar plate had fluorescent halos (Figure 4.1). It was clearly evident that Bgl2 had the largest and brightest fluorescent zone compared to the other Bgls. This was an initial indication of the higher activity of Bgl2 (Figure 4.1C). The fluorescence of Bgl2 was followed by Bgl4, Bgl5 and Bgl1 (Figure 4.1 E, F and A, respectively). Bgl3 had the lowest intensity of fluorescence compared to the other enzymes (Figure 4.1D).

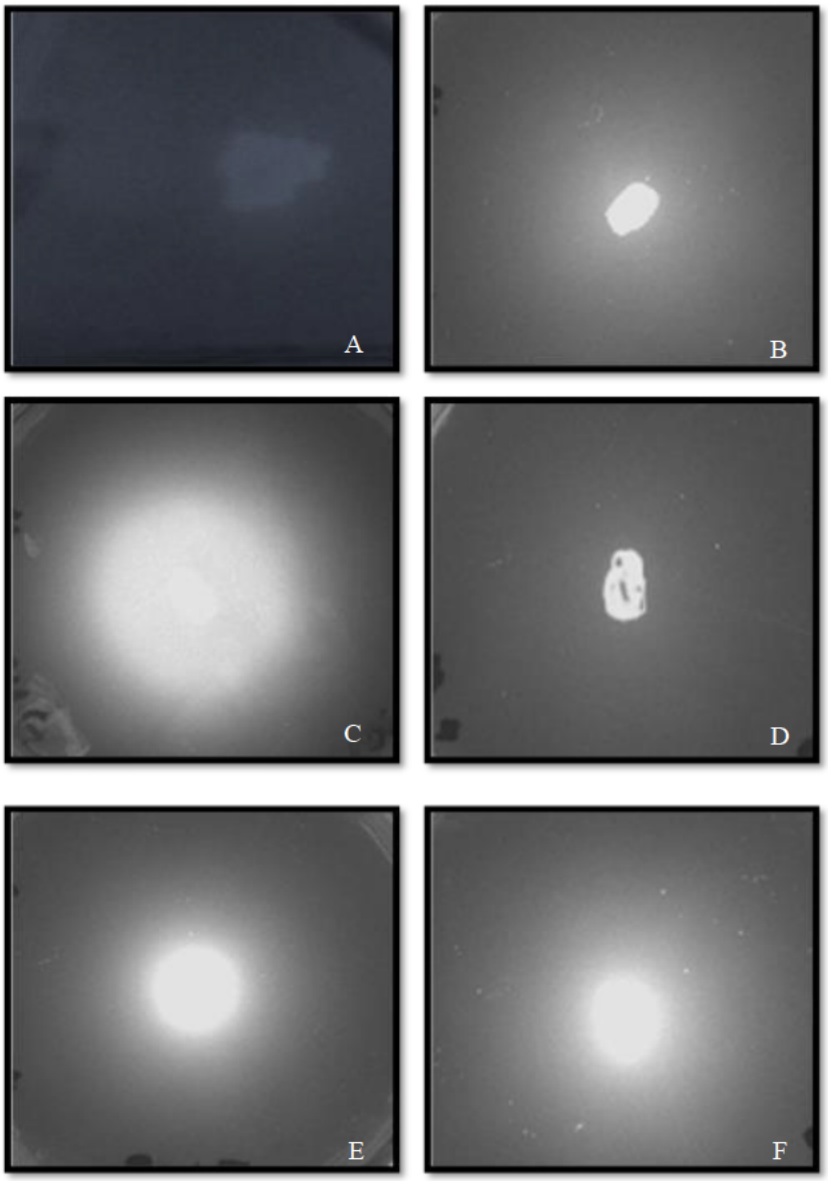

Figure 4.1: Analysis of five *P. pastoris* recombinant strains (Bgl 1-5) grown on MUG-containing MM agar plates at 30°C for 48 hours and viewed under UV light for qualitative screening of enzyme expression. A: non-recombinant *P. pastoris* GS115 strain; B-F: recombinant strains, Bgl1-5.

4.2. Methanol-induced expression of β-glucosidases

4.2.1. Minimal medium

The poor growth on minimal methanol (Figure 4.2 right hand side) compared to minimal dextrose agar plates indicated that the strains utilises methanol at a slower rate (Figure 4.2 left hand side) thus their phenotype was methanol utilisation slow (Mut^S) (Figure 4.2). Enzyme production was initially conducted using minimal methanol medium for 168 hours and very low activity was detected. Only Bgl2 had high activity (Figure 4.3). This was observed using a qualitative assay where the supernatants were loaded in wells of a MUG-containing plate. Bgl2 was the only one that had a clear visible fluorescent halo. With a quantitative assay using pNPG, there was no expression in all five Bgls, after 0 hour to 48 hours of induction. Activity of 3.4 U/ml was detected after 72 hours with Bgl2 only. There was a sharp increase to 31.5 U/ml after 120 hours followed by a further increase to 71.9 U/ml after 144 hours of induction of expression (Figure 4.4). The enzyme activity slightly decreased (14 units) by the 168^{th} hour. Bgl1, Bgl3, Bgl4 and Bgl5 had no detectable activity for the entire induction period (168 hours), and showed similar profiles to the negative control (Figure 4.4).

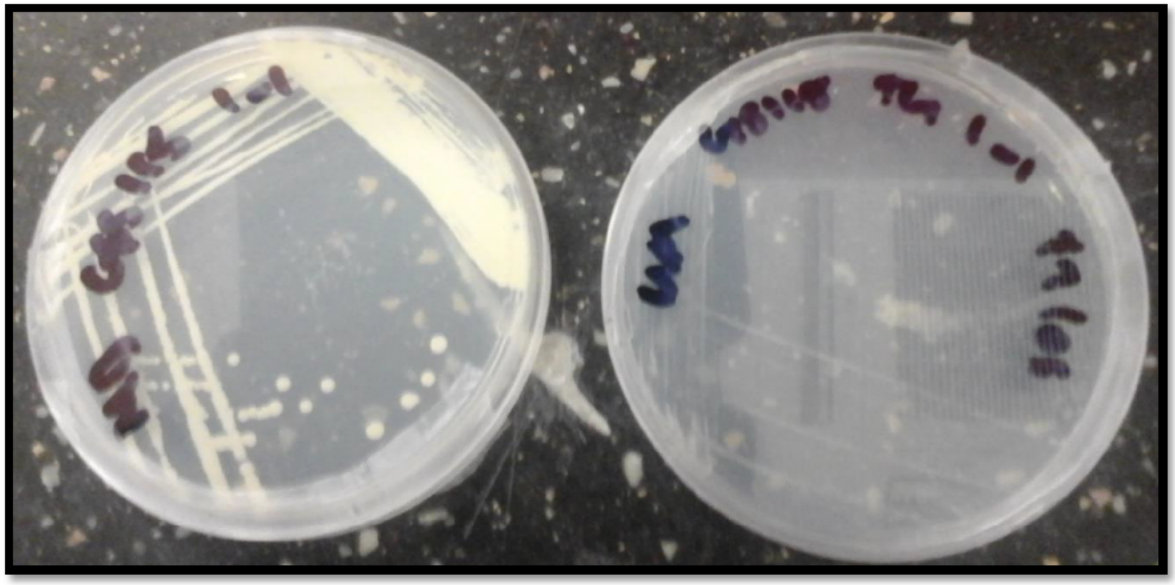

Figure 4.2: *P. pastoris* recombinant strain (Bgl1) grown on minimal dextrose and minimal methanol agar plates at 30°C for 48 hours. The growth was similar for all recombinant strains, therefore only the set of Bgl1 plates were presented.

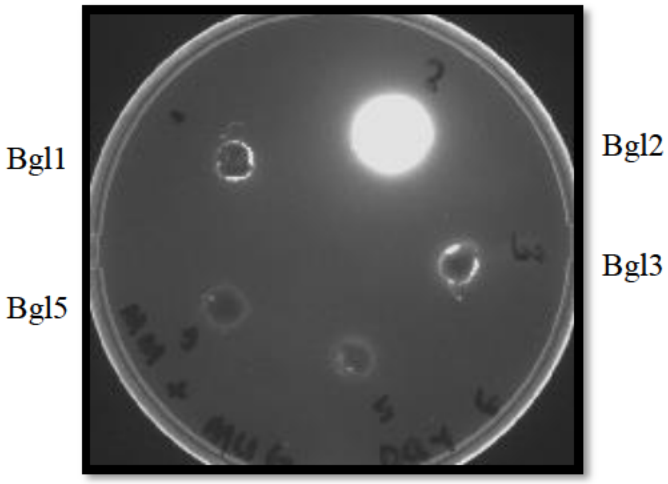

Figure 4.3: Fluorescence of Bgl 1-5 supernatants of *P. pastoris* clones after 168 hours of growth in MM medium on MUG-containing MM plates under UV light.

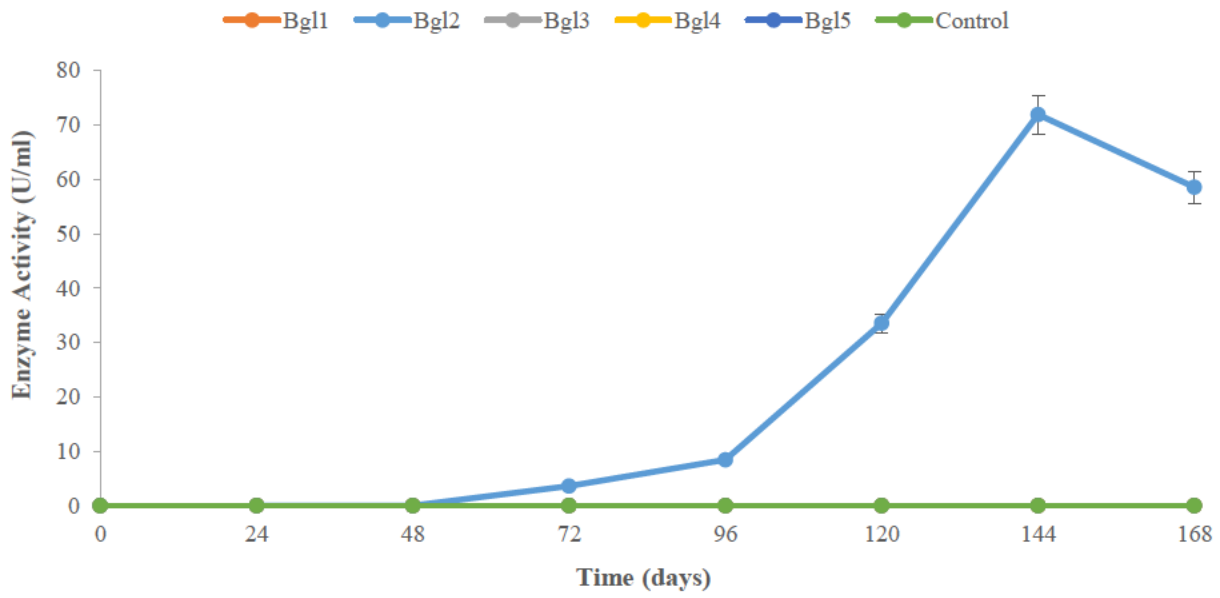

Figure 4.4: Induction of expression of five Bgls in minimal methanol medium (MM) without histidine at 30°C with shaking at 250 rpm for 168 hours. Enzyme expression was quantified using pNPG. Negative control: non-recombinant *P. pastoris* GS115.

4.2.2. Yeast extract, peptone and methanol (YPM) medium

Minimal methanol medium supported expression of Bgl2 only. Bgl1, Bgl3, Bgl4 and Bgl5 activities in this medium were undetectable in both qualitative and quantitative assays. Yeast extract, peptone and methanol (YPM) was then used for enzyme production. In the qualitative assays, faint fluorescent halos were visible around wells of Bgl1, Bgl3 and Bgl5, whereas the Bgl4 halo was as large and bright but smaller than that of Bgl2. This was a clear indication of an increase in enzyme activities in this medium (Figure 4.5).

In the quantitative assay, enzyme activities gradually and consistently increased until 168 hours of induction of expression. Bgl2 expression was the highest with an activity of 310.8 U/ml. Bgl1, Bgl3, Bgl4 and Bgl5 activities were considerably lower; 1.5 U/ml, 0.9 U/ml, 1.8 U/ml and 0.9

U/ml, respectively. The fermentation was continued to 192 hours. However, there was a decrease in all enzyme activities at this point (Figure 4.6). It was also observed that in YPM, enzyme expression started immediately after induction whereas in MM medium it only began after 72 hours (Figure 4.4). Bgl2 activity was 300 times than that of Bgl1, Bgl3, Bgl4 and Bgl5. The negative control confirmed that background proteins did not contribute to the observed activities.

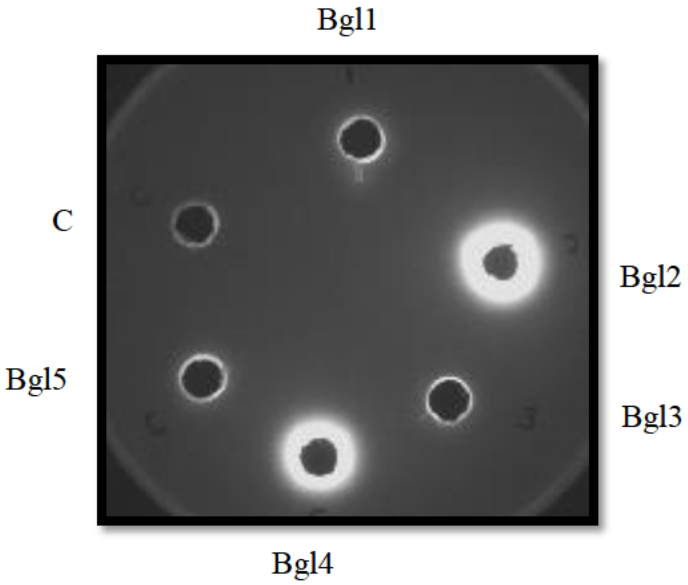

Figure 4.5: Fluorescence of Bgl 1-5 supernatants of *P. pastoris* clones after 168 hours of growth in YPM medium on MUG-containing plates under UV light. C: negative control, non-recombinant *P. pastoris* GS115 culture supernatant.

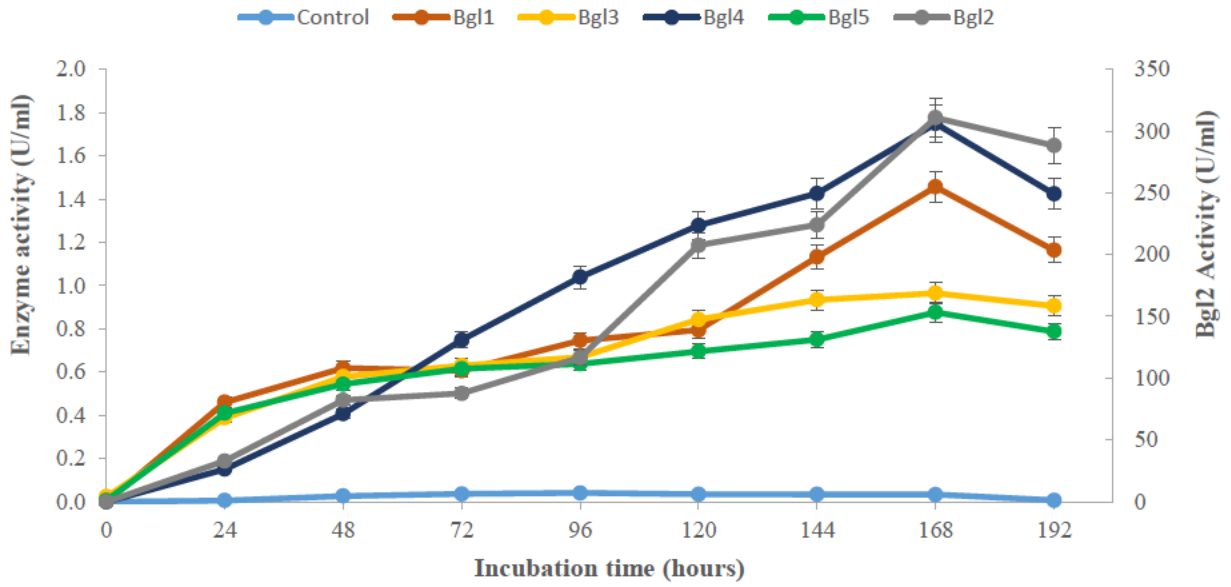

Figure 4.6: Induction of expression of Bgls in YPM medium at 30°C with shaking at 250 rpm for 192 hours. Enzyme expression was quantified using pNPG. Bgl2 is shown on a secondary axis on the right. Negative control: non-recombinant *P. pastoris* GS115.

4.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to confirm sizes of the five Bgls. According to their gene sequences, Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 sizes were calculated to be: 99.9 kDa, 46.5 kDa, 46.8 kDa, 68.9 kDa and 54.3 kDa, respectively. Protein bands corresponding to Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 were present close the predicted molecular weights. For Bgl2, Bgl3 and Bgl5, the enzymes appeared to be the dominant bands on the gel (Figure 4.7).

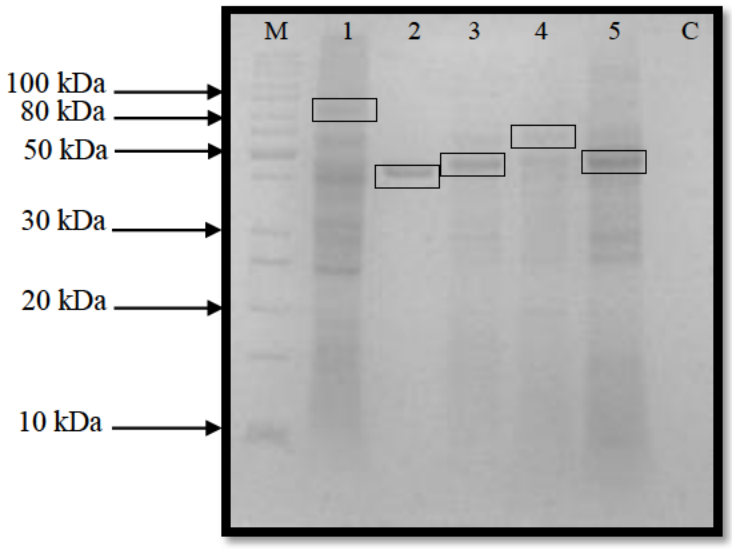

Figure 4.7: SDS-PAGE analysis of expression of Bgls in YPM medium. Proteins were separated using 12% acrylamide gel containing SDS and electrophoresis at 20 mA. The gel was stained with Coomassie stain and photographed under epi-white illumination. M: protein molecular weight markers (kDa); 1: Bgl1; 2: Bgl2; 3: Bgl3; 4: Bgl4 and 5: Bgl5. C: negative control.

4.4. Partial purification of β-glucosidases

Salting out using ammonium sulphate precipitation followed by ultrafiltration was used to partially-purify Bgls produced in YPM. The highest enzyme activities were obtained at the 25-50% fractions. Bgl2 had the highest total protein concentration (1 730.8 mg), followed by Bgl1 (531.0 mg), Bgl3 (367.5 mg) and Bgl4 (212.9 mg). Bgl5 had the lowest protein concentration of 124.4 mg (Tables 4.1-4.5). After ultrafiltration, the resulting protein concentrations for Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 were 1.4 mg, 27.7 mg, 1.4 mg, 1.6 mg, and 1.6 mg, respectively. Nevertheless, all enzyme specific activities increased. Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 specific activities after ultrafiltration were 50.4 U/mg, 553.7 U/mg, 72.0 U/mg, 111.6 U/mg, 44.0 U/mg, respectively (Tables 4.1-4.5). The purification-folds also increased for all the enzymes. Bgl2, Bgl3 and Bgl5 appeared as the major protein bands after SDS-PAGE analysis. However, Bgl1 and Bgl4 bands were still faint (Figure 4.8). Native PAGE analysis revealed two different forms

of Bgl2. Both these bands showed enzyme activity in zymography (Figure 4.9 A and B). Bgl1, Bgl3, Bgl4 and Bgl5 were not detected after native PAGE analysis.

Table 4.1: Summary of partial purification steps for Bgl1

Purification step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	1000	531.0	1106.1	2.1	-	100
Ammonium sulphate	100	39.9	417.1	10.4	5.0	37.7
Ultrafiltration	10	1.5	75.2	**50.4**	4.7	6.8

Table 4.2: Summary of partial purification steps for Bgl2

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	1000	1 730.8	136 917.2	79.1	-	100
Ammonium sulphate	100	151.3	21 752.6	143.8	1.8	15.9
Ultrafiltration	10	27.7	15 447.3	**553.7**	3.9	11.3

Table 4.3: Summary of partial purification steps for Bgl3

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	1000	367.5	1 312.8	3.6	-	100
Ammonium sulphate	100	25.1	345.1	13.7	3.8	26.3
Ultrafiltration	10	1.4	104.5	**72.0**	5.4	8.0

Table 4.4: Summary of partial purification steps for Bgl4

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	1000	212.9	2 635.9	12.4	-	100
Ammonium sulphate	100	35.2	1 064.6	30.2	2.4	40.4
Ultrafiltration	10	1.6	185.2	**111.6**	3.8	7.0

Table 4.5: Summary of partial purification steps for Bgl5

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	1000	124.4	1 363.1	11.0	-	100
Ammonium sulphate	100	14.8	348.4	23.5	2.1	25.6
Ultrafiltration	10	1.6	69.2	**44.0**	1.8	5.1

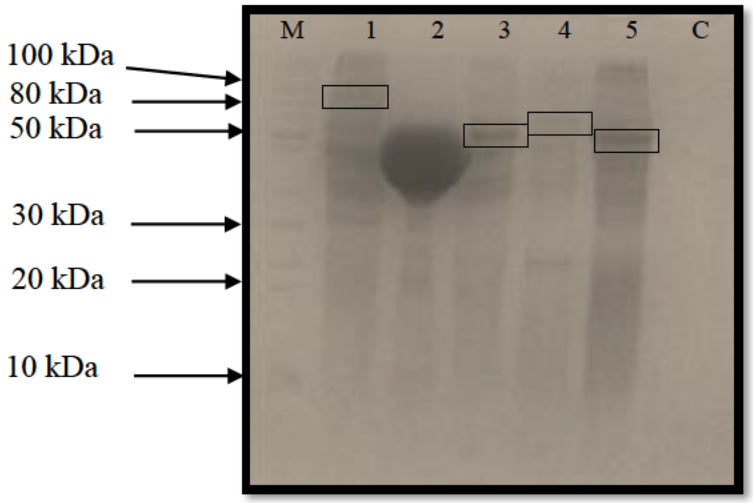

Figure 4.8: SDS-PAGE analysis of Bgls after partial purification. Proteins were separated using 12% acrylamide gel containing SDS and electrophoresis at 20 mA. The gel was stained with Coomassie stain and photographed under epi-white illumination. M: protein molecular weight markers (kDa); 1: Bgl1; 2: Bgl2; 3: Bgl3; 4: Bgl4 and 5: Bgl5. C: negative control.

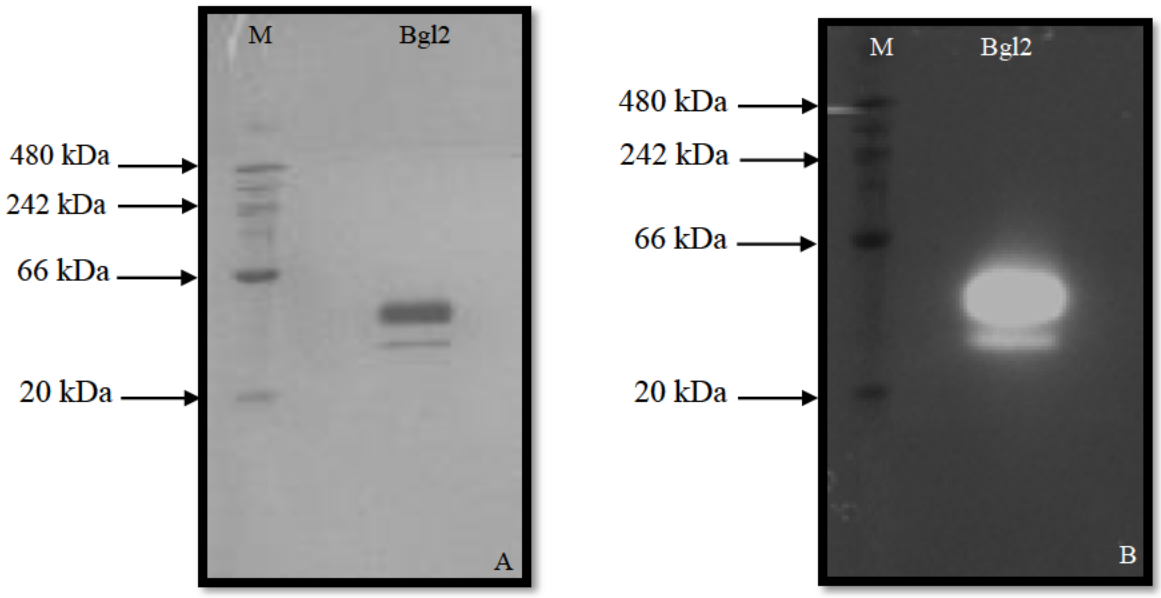

Figure 4.9: Native PAGE analyses of Bgl2 after partial purification. Proteins were separated using 12% native acrylamide gel and electrophoresis at 20 mA. The gel was stained with Coomassie stain (A) and photographed under epi-white illumination and in-gel MUG assay gel (B) under UV illumination. M: native molecular weight markers (kDa).

4.5. Characterisation of β-glucosidases

4.5.1. Beta-glucosidase pH optimum and stability

Both low and high pH levels had a negative effect on all enzyme activities. At pH 3.0 Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 activities were 0.3 U/ml, 81.2 U/ml, 0.7 U/ml, 0.5 U/ml and 0.1 U/ml, respectively. Activities were still relatively low at pH 4.0 and increased at pH 5.0 and 6.0. The highest activities were obtained at pH 6.0. At the optimum pH, Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 activities reached 23.8 U/ml, 429.9 U/ml, 13.3 U/ml, 33.1 U/ml and 18.1 U/ml, respectively. At pH 7.0-9.0 the enzyme activities drastically decreased to 1.3 U/ml, 52.4 U/ml, 0.7 U/ml, 0.9 U/ml and 0.5 U/ml for Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5, respectively (Figure 4.10).

Bgl1 and Bgl2 were more stable at neutral pH, since they retained 73% and 84% of their optimal activities at pH 7.0 after 150-240 minutes of incubation, respectively (Figures 4.11-4.12). These enzymes were less stable at acidic pH levels. After 150 minutes of incubation at pH 4.0, pH 5.0 and pH 6.0, Bgl1 retained 11%, 32% and 42% of its activity, respectively. This enzyme was moderately stable at basic pH levels, as it retained 63% and 49% of its activity after 150 minutes of incubation at pH 8.0 and pH 9.0, respectively (Figure 4.11).

After 240 minutes of incubation, only 9%, 40% and 45% of Bgl2 activity was retained at pH 4.0, pH 5.0 and pH 6.0, respectively (Figure 4.12). Bgl2 was also fairly stable at basic pH levels. The enzyme retained 77% and 65% of its activity as after 240 minutes of incubation at pH 8.0 and pH 9.0, respectively (Figure 4.12).

Bgl3 also followed a similar trend as Bgl1 and Bgl2. This enzyme was more stable at neutral pH as 68% of its activity was retained after 150 minutes of incubation (Figure 4.13). The enzyme was also fairly stable at pH 8.0 and pH 9.0. It retained 59% and 46% of its activities at these pH levels, respectively. Bgl3 was less stable at pH 6.0, pH 5.0 and pH 4.0 as only 46%, 32% and 13% of its activity was retained after 150 minutes of incubation (Figure 4.13).

Bgl4 demonstrated a broad pH stability as above 60% of its activity was retained after 60 minutes of incubation at pH 4.0 to pH 9.0. Bgl4 retained 65% of its activity after 150 minutes of incubation at pH 5.0 and 48% at pH 4.0. Furthermore, at pH 6.0, pH 7.0, pH 8.0 and pH 9.0, the enzyme retained 41%, 29%, 25% and 18% of its activity after 150 minutes of incubation, respectively (Figure 4.14).

Bgl5 followed the same trend as Bgl4. After 60 minutes of incubation, the enzyme retained 71% and 75% of its activity at pH 4.0 and pH 5.0, respectively (Figure 4.15). Bgl4 retained 37%, 43%, 36%, 32%, 20% and 13% of activity after 150 minutes of incubation at pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0 and pH 9.0, respectively.

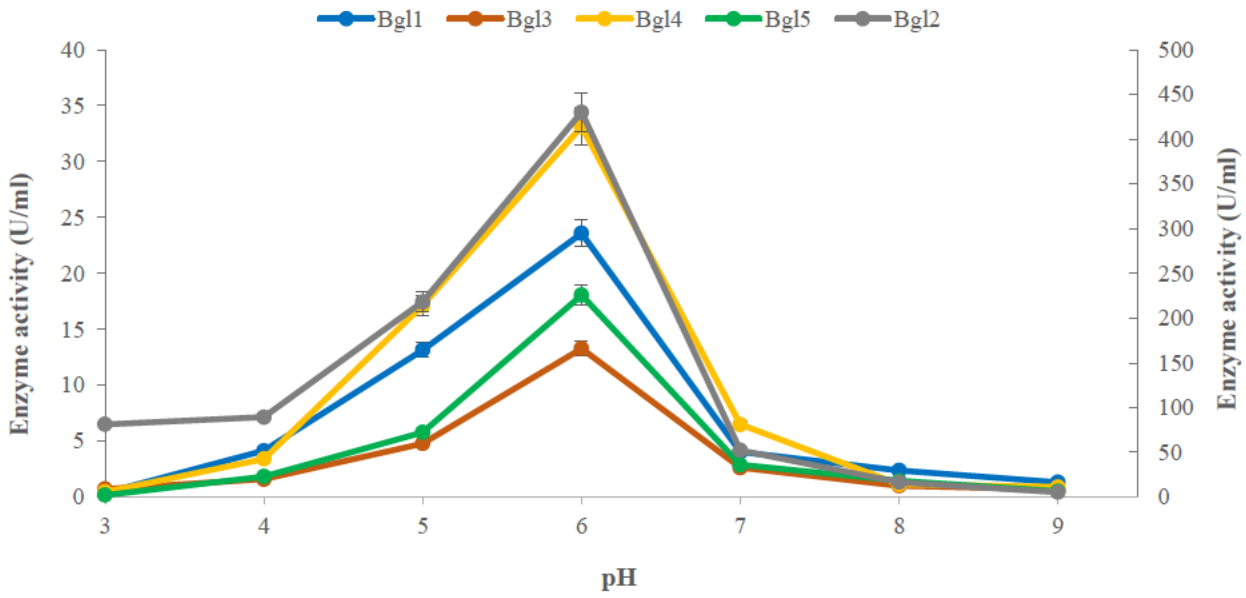

Figure 4.10: The effect of pH (3.0-9.0) on Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 activities under standard assay conditions (50℃). Bgl2 activity is presented on the secondary axis.

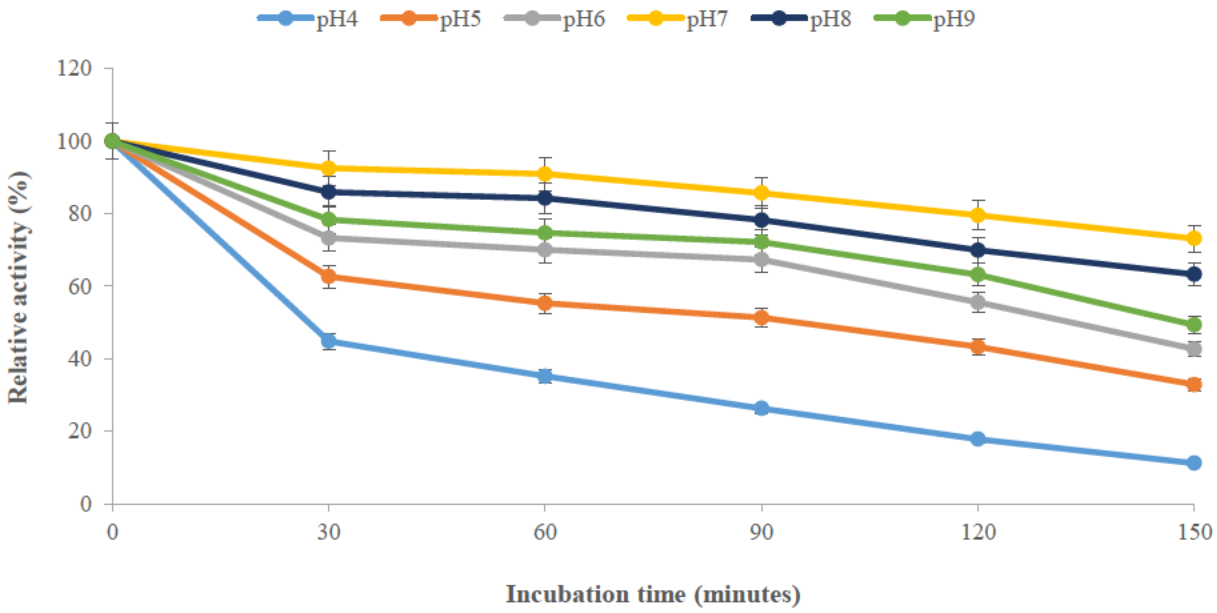

Figure 4.11: pH stability of Bgl1. Pre-incubation was conducted at pH 4.0-9.0 for 150 minutes, with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 60°C).

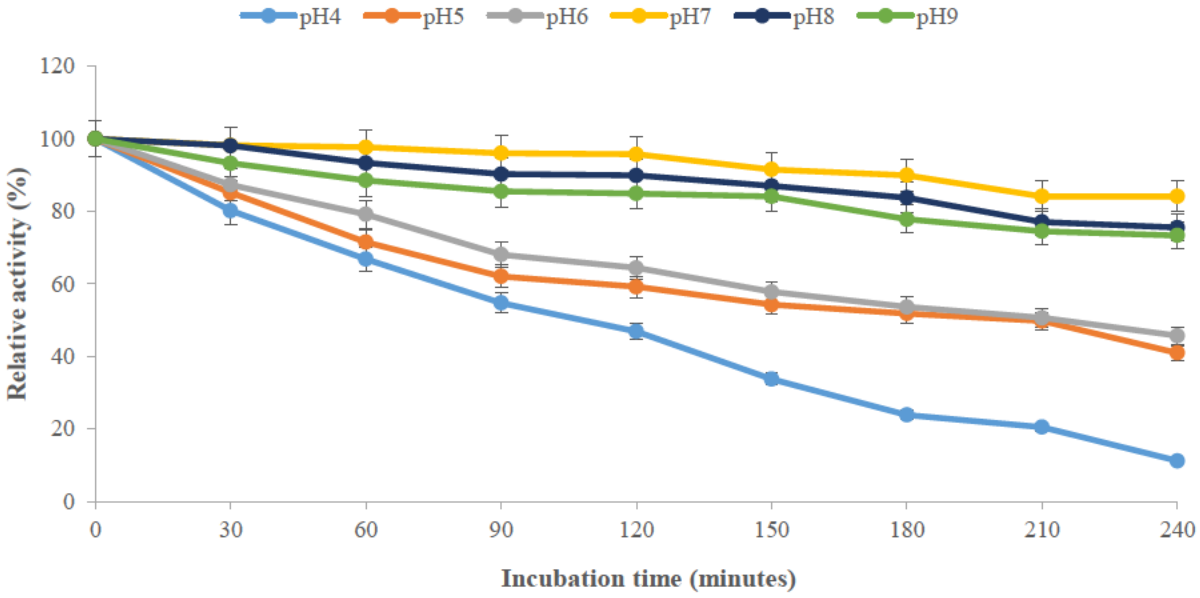

Figure 4.12: pH stability of Bgl2. Pre-incubation was conducted at pH 4.0-9.0 for 240 minutes, with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 60°C).

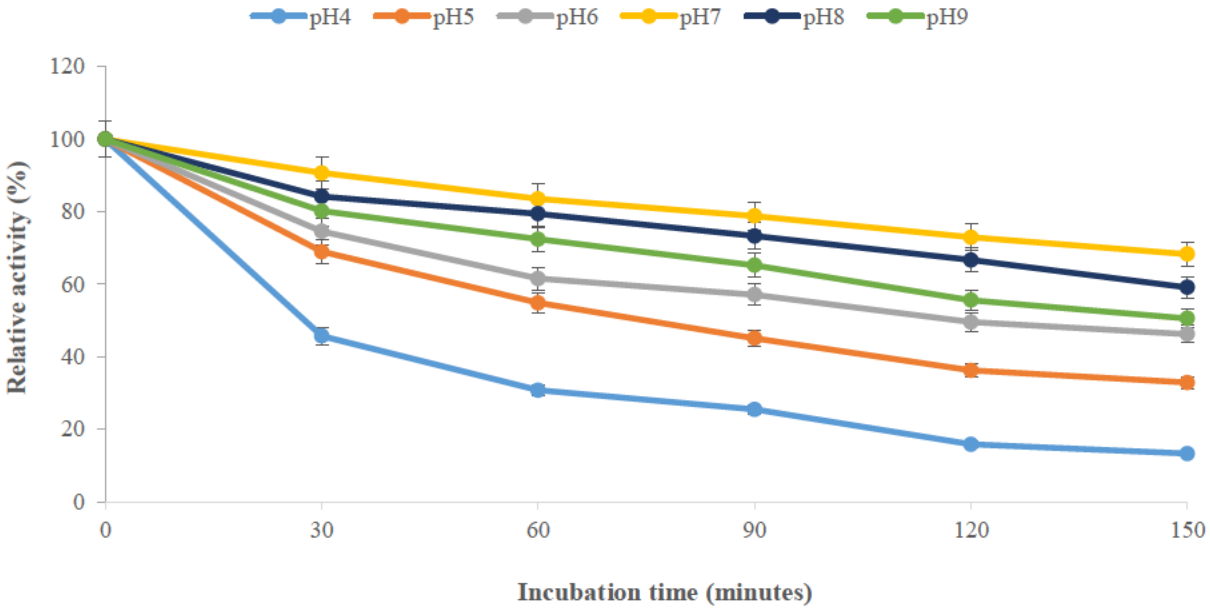

Figure 4.13: pH stability of Bgl3. Pre-incubation was conducted at pH 4.0-9.0 for 150 minutes, with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 60°C).

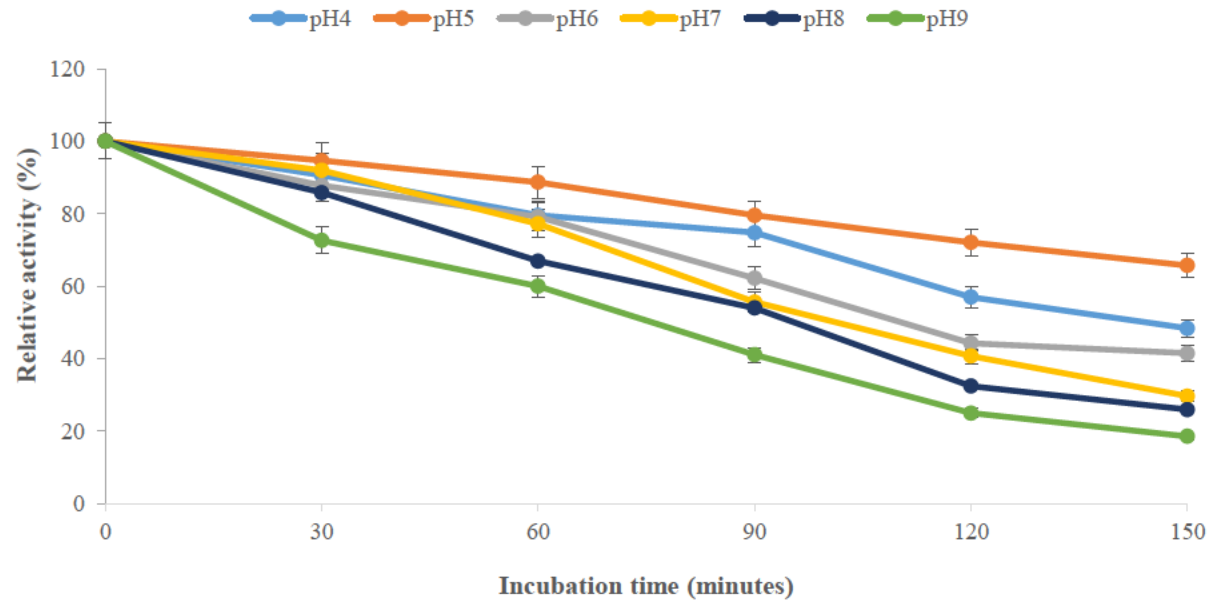

Figure 4.14: pH stability of Bgl4. Pre-incubation was conducted at pH 4.0-9.0 for 150 minutes, with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 60°C).

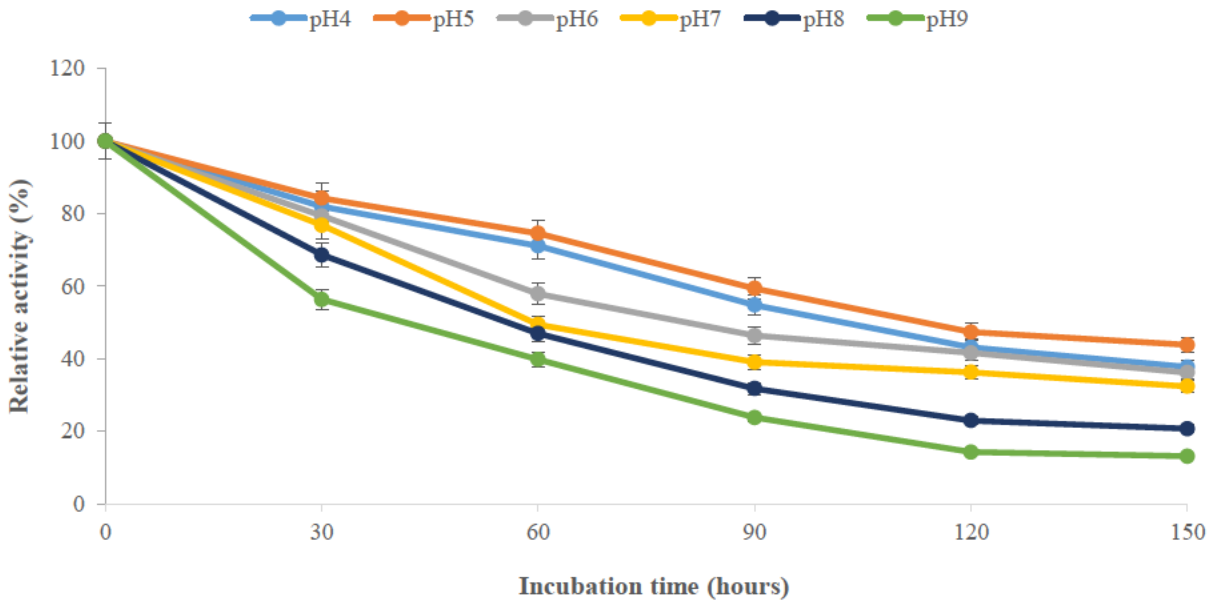

Figure 4.15: pH stability of Bgl5. Pre-incubation was conducted at pH 4.0-9.0 for 150 minutes, with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 60°C).

4.5.2. Beta-glucosidase temperature optimum and stability

The optimum temperature for Bgl1, Bgl3, Bgl4 and Bgl5 was 50°C. Their activities decreased as the temperature levels rose above 50°C. At optimum conditions, Bgl1, Bgl3, Bgl4 and Bgl5 activities increased to 22.9 U/ml, 14.7 U/ml, 35.0 U/ml, 17.4 U/ml, respectively (Figure 4.16). Low enzyme activity (66.4 U/ml) was obtained at 30°C for Bgl2. The activity gradually increased with increase in temperature. The optimum temperature of this enzyme was 60°C and the highest activity of 467.1 U/ml was obtained at this temperature. The enzyme activity also decreased as the temperature increased to 70°C (51.9 U/ml), 80°C (36.3 U/ml) and 90°C (33.2 U/ml) (Figure 4.16).

Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 thermostability trends were similar. The enzymes were more stable at 30-40°C. However, there were differences in their residual activities and they appeared

to be less stable at higher temperatures. Bgl1 retained 65% to 61% of its optimal activity at 30 and 40 ℃ after 150 minutes of incubation, respectively. Bgl1 also retained 27% of activity at 50℃ after 150 minutes of incubation. At 60℃ only 3% remained after 30 minutes (Figure 4.17). During the 240-minute incubation period for stability testing, there was a gradual loss of Bgl2 activity at 30℃, 40℃ and 50℃. At these temperature levels, this enzyme retained 80%, 60% and 50% of its activity, respectively. After 30 minutes of incubation, Bgl2 lost 31%, 41% and 69% of its activity and after 240 minutes only 10%, 3% and 1% activity was retained at 60℃, 70℃ and 80℃, respectively (Figure 4.18). Bgl3 retained 83% and 76% of its activity at 30℃ and 40℃, respectively (Figure 4.19). This was slightly higher than all other Bgls at these temperature levels. Bgl4 residual activity after 150 minutes of incubation at 30℃ and 40℃ was 73% and 62%, respectively. The enzyme retained up to 11% of its activity after 150 minutes at 50℃ (Figure 4.20). Bgl5 retained 75% and 65% of its activity after 150 minutes of incubation at 30℃ and 40℃, respectively. The residual activity of 14% was retained after 150 minutes of incubation at 50℃ (Figure 4.21). The dramatic decrease in residual activities was observed at 60℃ after 30 minutes of incubation (Figures 4.17, 4.19, 4.20 and 4.21).

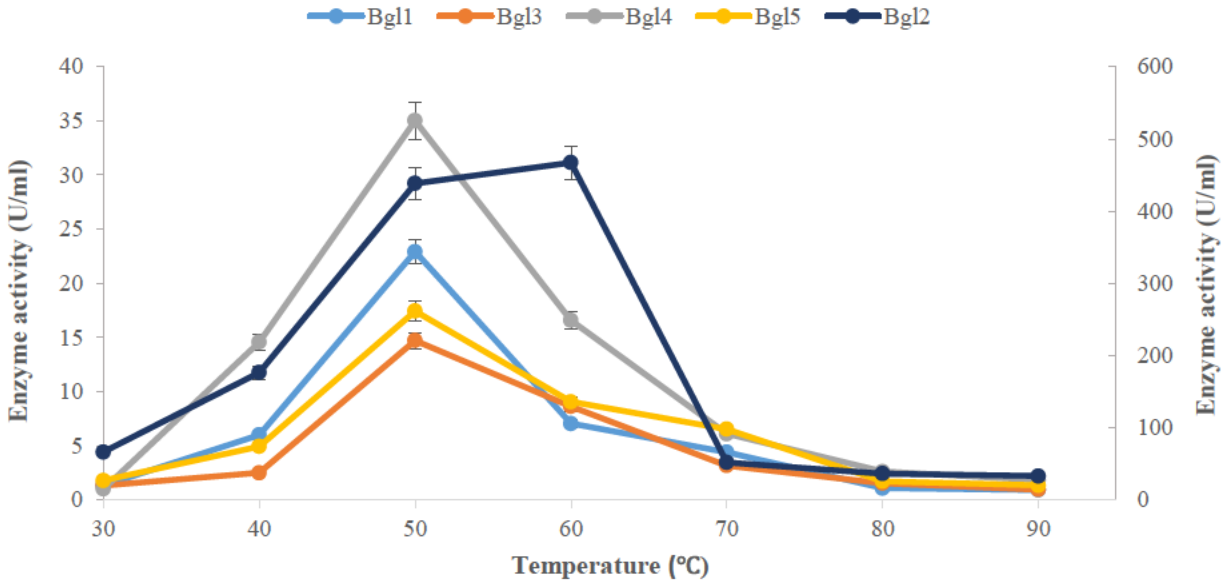

Figure 4.16: The effect of temperature (30-90°C) on Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 activities under optimal assay conditions (pH 6.0). Bgl2 activity is presented on the secondary axis.

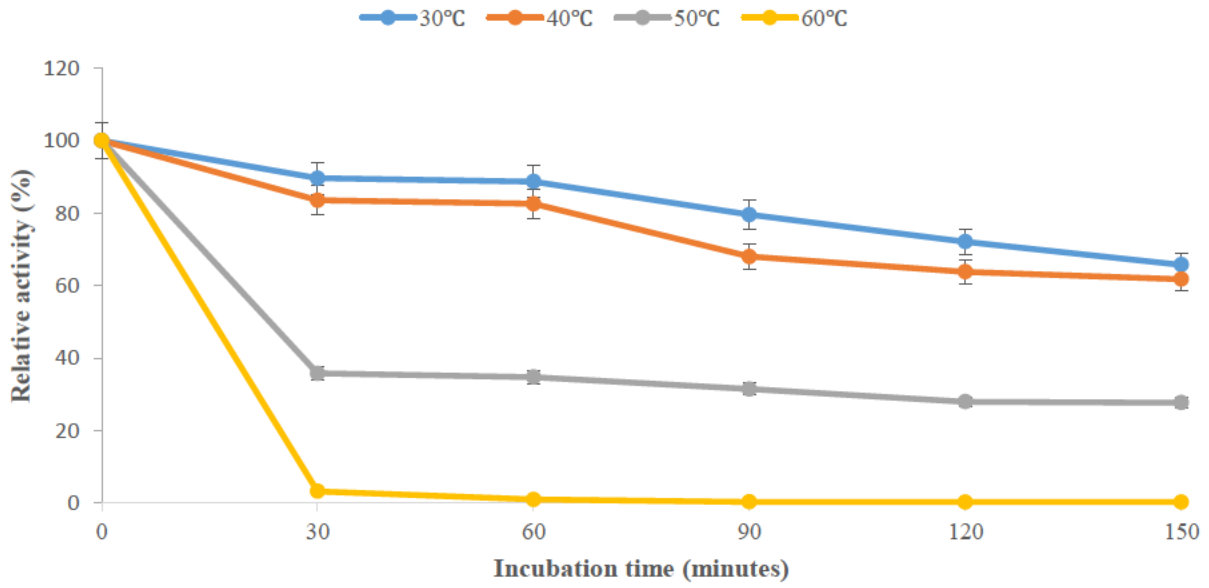

Figure 4.17: Bgl1 thermostability by pre-incubation at the temperature range of 30-60°C for 150 minutes with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 50°C).

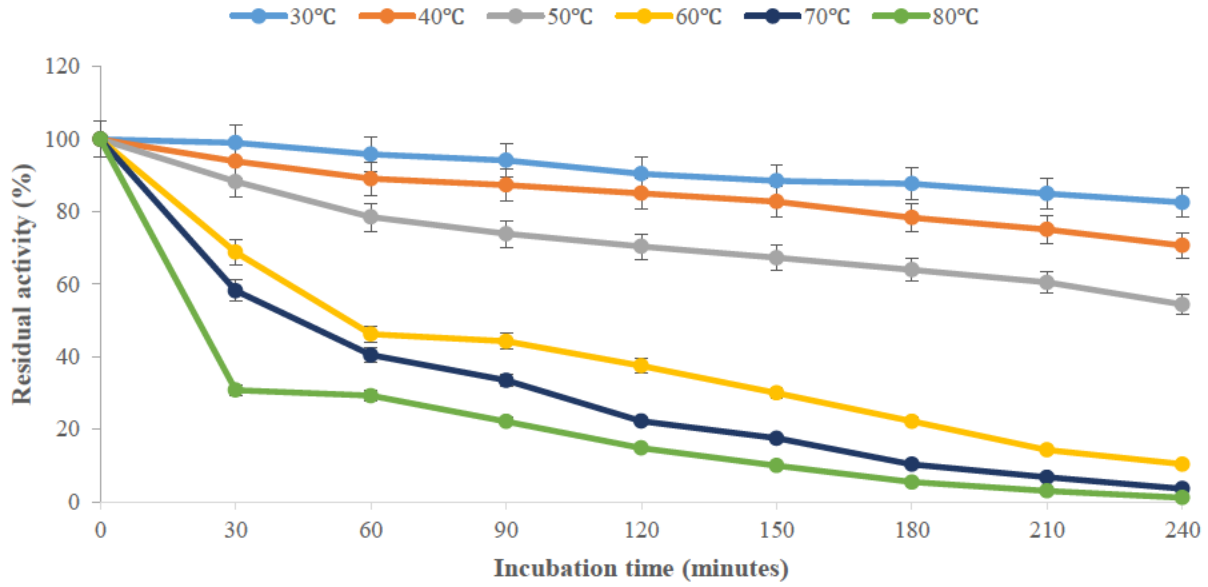

Figure 4.18: Bgl2 thermostability by pre-incubation at the temperature range of 30-80°C for 240 minutes with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 60°C).

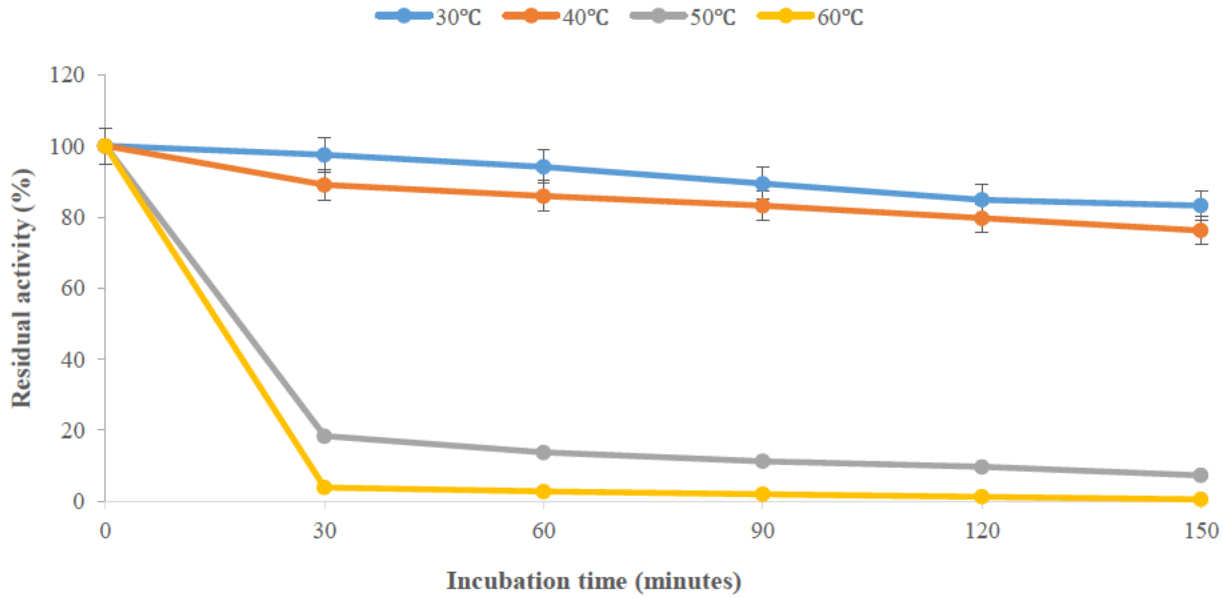

Figure 4.19: Bgl3 thermostability by pre-incubation at the temperature range of 30-60°C for 150 minutes with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 50°C).

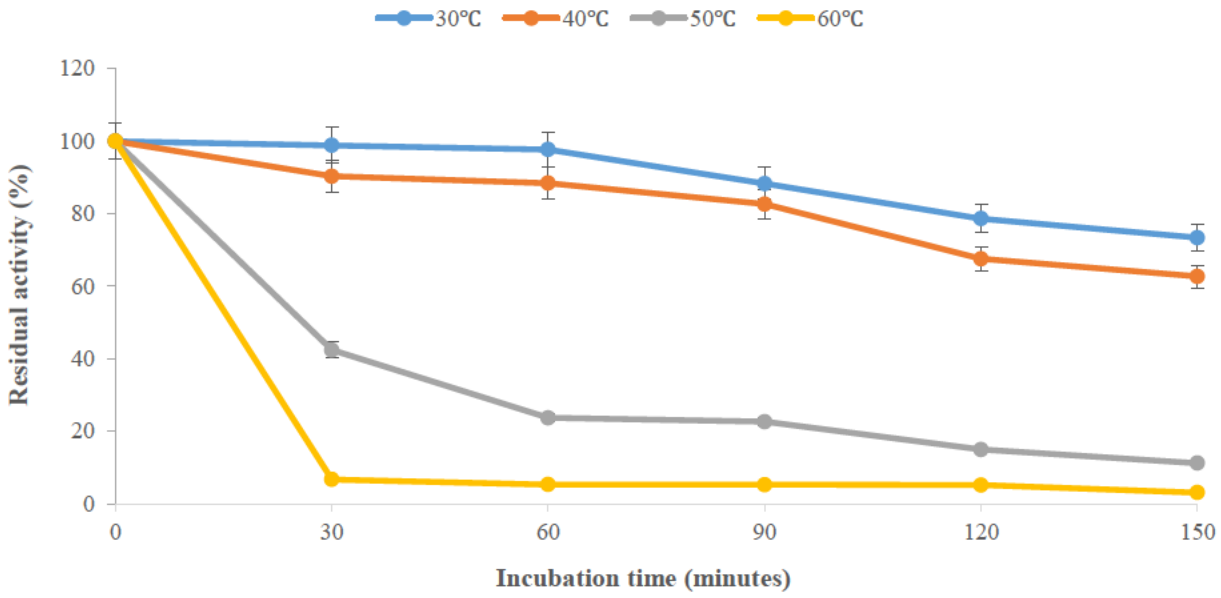

Figure 4.20: Bgl4 thermostability by pre-incubation at the temperature range of 30-60°C for 150 minutes with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 50°C).

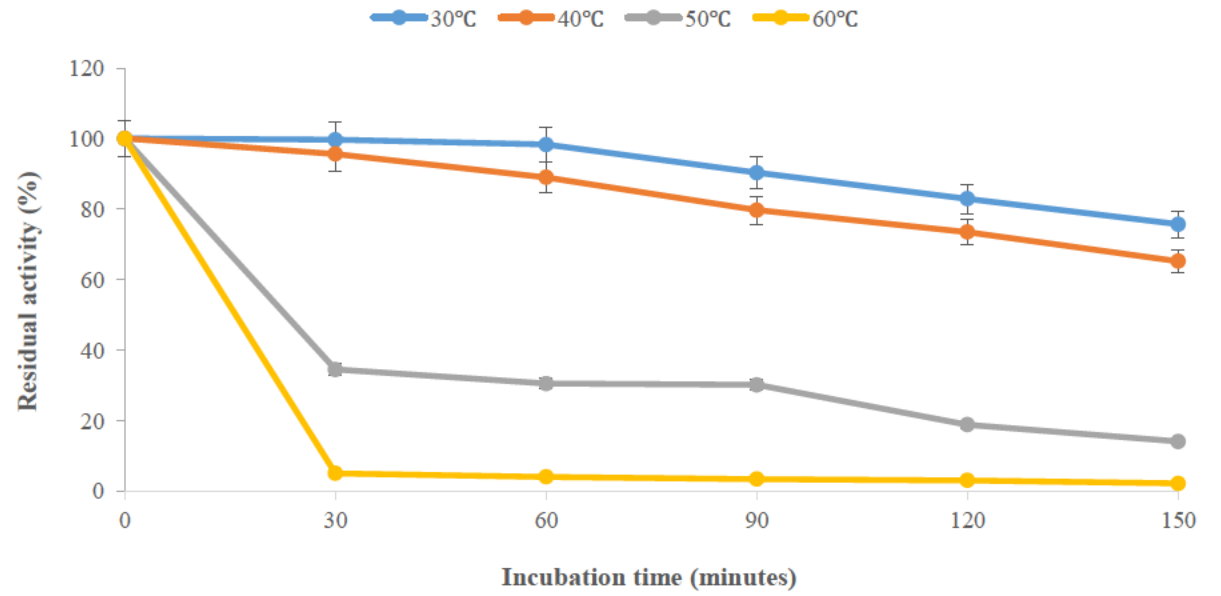

Figure 4.21: Bgl5 thermostability by pre-incubation at the temperature range of 30-60°C for 150 minutes with the residual activity measured at 30-minute intervals under optimal assay conditions (pH 6.0 and 50°C).

4.5.3. Beta-glucosidase substrate specificity

Using the glucose oxidase-peroxidase (GOD-POD) assay, glucose was detected after hydrolysis of all four tested substrates (cellobiose, gentiobiose, salicin and laminarin) for Bgl2 and Bgl4. These were the only enzymes that were active on cellobiose. The highest concentration of glucose produced from this substrate was 19.9 mM and 17.9 mM for Bgl2 and Bgl4, respectively (Figure 4.18). Gentiobiose was hydrolysed by all Bgls. Highest glucose concentration from this substrate was obtained for Bgl5 (18.5 mM), followed by Bgl2 (17.2 mM), Bgl1 (16.8 mM), Bgl4 (11.8 mM) and Bgl3 (11.5 mM). Salicin was the least hydrolysed disaccharide. Bgl2 released 11.6 mM of glucose followed by Bgl4 (4.0 mM), Bgl3 (1.1 mM) and Bgl5 (0.2 mM). There was no detectable activity of Bgl1 on salicin. The amount of glucose released was 19.3 mM for Bgl2, 18.8 mM for Bgl3, 18.7 mM for Bgl1, 18.3 mM for Bgl5 and 16.1 mM for Bgl4. Bgl2 and Bgl4 demonstrated a broad substrate specificity as they were active on substrates having different glycosidic linkages (β-1,2, β-1,3, β-1,4 and β-1,6). Bgl1, Bgl3 and Bgl5 were more active on β-1,3- and β-1,6-linked substrates (gentiobiose and laminarin) (Figure 4.22).

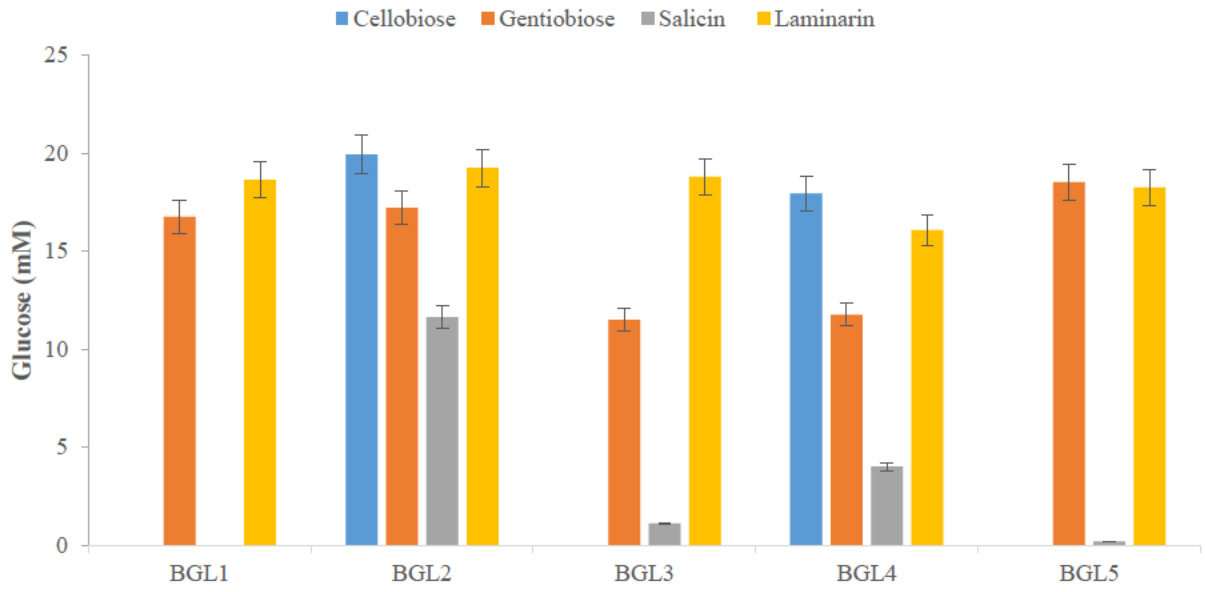

Figure 4.22: Glucose liberated by action of Bgls on natural substrates (cellobiose, gentiobiose, salicin and laminarin), measured using the glucose oxidase-peroxidase (GOD-POD) assay.

4.5.4. Hydrolysis of artificial cellulose by Bgl2

After 1 hour of incubation of microcrystalline cellulose with cellulase only, 5.2 mM of glucose was produced. It gradually increased to 8.9 mM after 7 hours of incubation (Figure 4.23). Supplementation of Bgl2 greatly improved the hydrolysis rate as 12.8 mM of glucose was released after 1 hour of incubation with the highest concentration of 14.1 mM obtained after 7 hours. The positive control, cellobiase also showed a synergistic effect as 15.0 mM of glucose was liberated after 1 hour of incubation with the highest concentration being 16.3 mM. The sharp increase in glucose concentration was observed in the first hour which was followed by a minor increase during the 7 hours of incubation (Figure 4.23).

On carboxymethyl cellulose, the cellulase produced 5.6 mM and 10.6 mM of glucose after 1 and 7 hours of incubation, respectively (Figure 4.24). When Bgl2 was added, saccharification

substantially increased as 16.2 mM of glucose was released after 1 hour. The activity then gradually increased and remained constant after 3 hours of incubation and the highest obtained glucose was 20.3 mM after 7 hours. The *A. niger* cellobiase had an effect slightly higher than that of Bgl2, the highest glucose released was 21.4 mM (Figure 4.24).

The use of different ratios of enzyme constituents is commonly found in enzyme cocktails. The 1:2, 1:3 and 1:4 ratios of cellulase to Bgl2 were used for further hydrolysis of CMC. As expected, this greatly increased the hydrolysis rate. After 1 hour of incubation, 27.0 mM, 36.9 mM and 52.3 mM of glucose was released at enzyme cocktail ratios of 1:2, 1:3 and 1:4, respectively. Glucose concentration remained constant after 3 hours of incubation for all the preparations. At this time point, the highest glucose concentrations obtained were 32.5 mM, 42.6 mM and 59.6 mM for the 1:2, 1:3 and 1:4 enzyme cocktails, respectively (Figure 4.25).

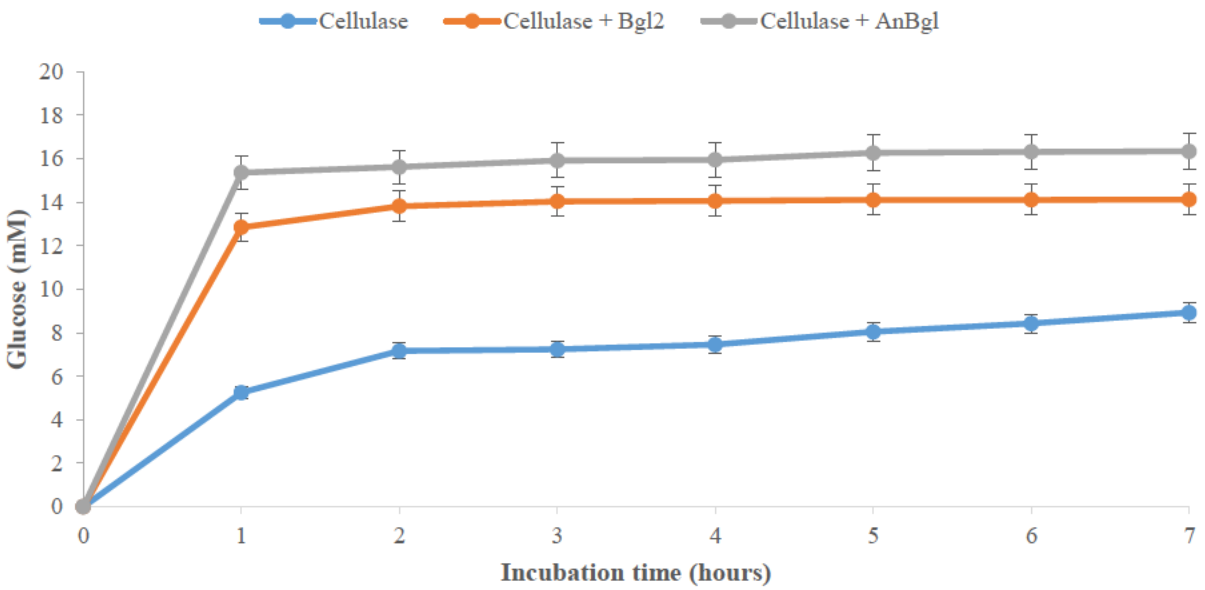

Figure 4.23: The effect of addition of Bgl2 on the hydrolysis of 1% microcrystalline cellulose at 50°C and pH 5.0 for seven hours.

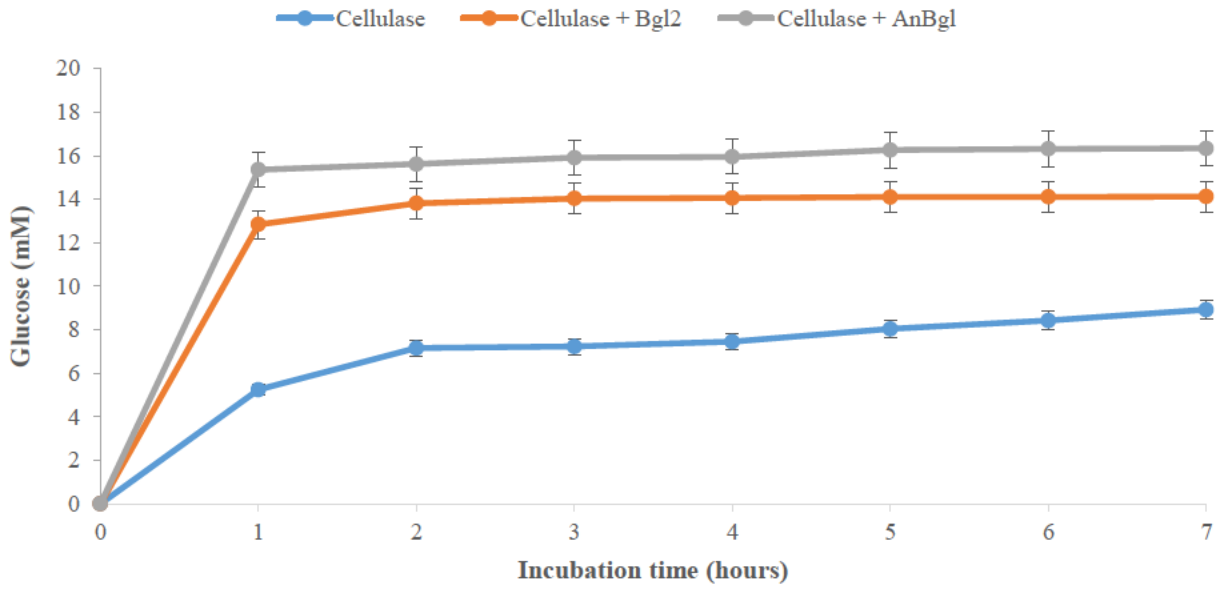

Figure 4.24: The effect of addition of Bgl2 on the hydrolysis of 1% carboxymethyl cellulose (CMC) at 50°C and pH 5.0 for seven hours.

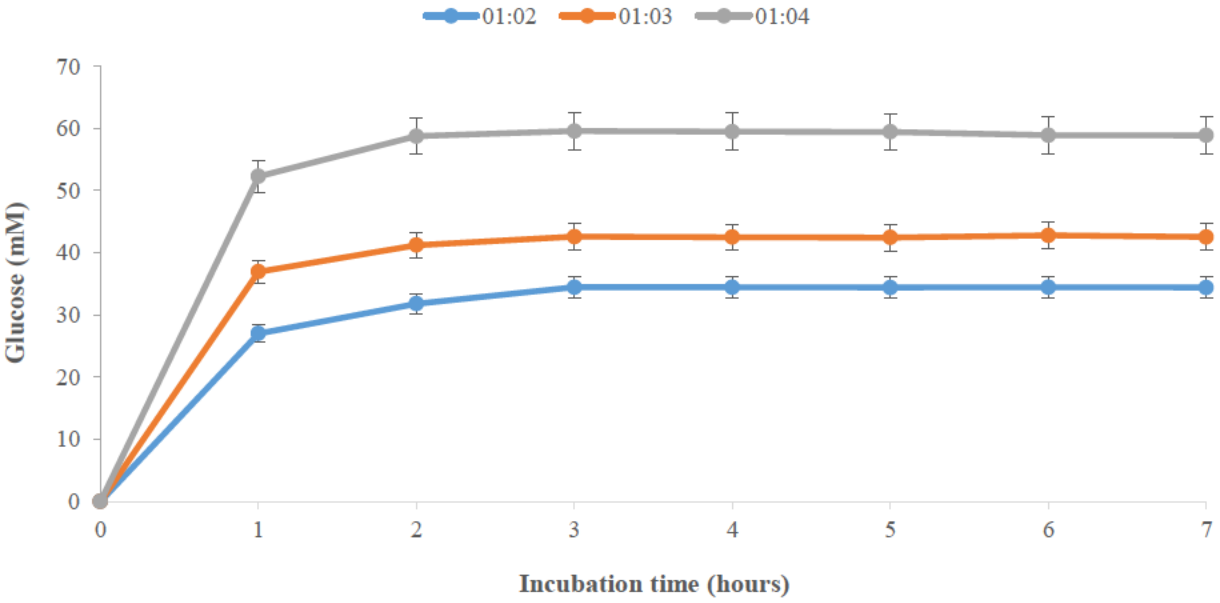

Figure 4.25: The effect of increased Bgl2 activity on the hydrolysis of 1% carboxymethyl cellulose at 50°C and pH 5.0 for seven hours.

5. DISCUSSION

The lack of optimal β-glucosidase production for efficient cellulose degradation is a major bottleneck for the commercialisation of cellulosic biofuels. Different sources of β-glucosidase are being explored for supplementation of cellulase preparations. Microorganisms present a feasible source for industrial enzymes owing to their ease of cultivation and downstream processing. Filamentous fungi are mostly considered a good source of cellulolytic enzymes. This is due to their ability to grow on lignocellulosic substrates (Passos *et al*., 2018). β-glucosidases from filamentous fungi have been produced and their industrial applicability demonstrated (Gao *et al*., 2014; Karnchanatat *et al*., 2007; Oh *et al*., 2018; Singhania *et al*., 2011; Yang *et al*., 2009). However, β-glucosidase yields differ among organisms. For instance, *Aspergillus* spp. and *Penicillium* spp. produce sufficient amounts of β-glucosidases compared to *T. reesei* (Singhania *et al*., 2011; Vaishnav *et al*., 2018). Furthermore, thermophilic fungi produce appreciably higher β-glucosidase titres in comparison to thermo-tolerant fungi (Sonia *et al*., 2008).

Sugarcane bagasse, microcrystalline cellulose, rice straw, whey permeate, wheat bran, carboxymethyl cellulose, quercetin, and corn cobs have been used as carbon sources for β-glucosidase production from fungal strains (Gao *et al*., 2014; Jeya *et al*., 2010; Sonia *et al*., 2008). These have supported different levels of β-glucosidase production. *T. lanuginosus* is an industrially-important thermophilic fungal strain (Singh *et al*., 2003). Although it is considered non-cellulolytic, β-glucosidases from this fungus were reported (Lin *et al*., 1999; Sonia *et al*., 2008). To date, these are the only studies available on β-1,4-glucosidase from *T. lanuginosus* and the enzyme activities were significantly low. Consistent enzyme hyper-production can be

achieved through heterologous expression. It has been considered a standard approach to enhance β-glucosidase production for industrial application (Singhania *et al*., 2013). For instance, *P. pastoris* expresses heterologous proteins through a highly inducible alcohol oxidase promoter. Furthermore, the α-signal in this system facilitates protein secretion into the medium, easing the purification process. The ability of *P. pastoris* to over-produce five novel β-glucosidases from *T. lanuginosus* SSBP for potential application in cellulose degradation was investigated.

The qualitative β-glucosidase MUG assay was based on fluorescent 4-methylumbelliferone that was released when the substrate was hydrolysed. The presence of fluorescent halos around all five colonies of Bgl-habouring *P. pastoris* strains in MUG-containing MM agar plates indicated that the enzymes were expressed. Although the inoculation was not uniform, the difference in the intensity of the zones also showed that the activities were not equal (Figure 4.1). This was further confirmed by quantitative assay using pNPG. It was also observed that for Bgl1 and Bgl3, only the colonies appeared to be fluorescing. This could mean that these enzymes were poorly transported out of the cells.

Pichia pastoris GS115 strains that were used in this study were His^- (auxotrophic for histidine). Consequently, the plasmid habours the *HIS4* (histidinol dehydrogenase) gene enabling only recombinant strains to grow on histidine-deficient media (Figure 2.6). The methanol utilisation phenotype is used for selection of the expression/induction method. It is determined by the manner or rate at which the strains grow on minimal dextrose compared to minimal methanol medium. All recombinant strains were found to have the slow methanol utilisation phenotype

(Mut^S) as they grew faster and to a greater extent on minimal dextrose compared to minimal methanol medium (Figure 4.3). The growth was similar for all five strains and only the set of Bgl1 plates were shown. This is also in line with the site at which the genes were inserted, *Bgl* II. According to the pPIC9K manual, gene insertion at this restriction site generates HIS^+ Mut^S phenotypes (Invitrogen, 2010). Furthermore, prior to determination of the phenotype, the use of Mut^+ protocol had been attempted and there was no detectable expression in all recombinant strains. Mut^+ strains are firstly grown to the exponential phase in small volumes and diluted to a specific concentration when initiating induction. Mut^S strains requires biomass accumulation before induction of expression (Invitrogen, 2014). Although Mut^+ strains are the most employed for expression in *Pichia,* it has been reported that Mut^S strains results in the highest production compared to other phenotypes (Dashtban and Qin, 2012). Charoenrat *et al*. (2015) reported that in Mut^S strains, *AOX1* is transcribed three times lower than in Mut^+. It was stated that recombinant protein production depends on methanol consumption rate, its availability and oxygen. The effect of different start-up cell concentrations was investigated and higher cell concentrations resulted in increased expression (Charoenrat *et al*., 2015).

When minimal medium was used for expression of Bgls, the induction was conducted according to the phenotype of the strains (Mut^S) for 168 hours. It was clear in the whole cell qualitative assay that Bgl2 had the highest activity. It was surprising that Bgl1, Bgl3, Bgl4 and Bgl5 were not detectable in MM medium for the entire 168 hours of induction of expression, as the quantitative assay used is more sensitive than the plate assay.

Expression of Bgl2 commenced after 72 hours of induction (Figure 4.4). Li *et al*. (2017) reported enzyme expression that started after 48 hours. In some studies, *P. pastoris* has expressed β-glucosidases after 24 hours (Chen *et al*., 2011; Karnaouri *et al*., 2013; Yan *et al*., 2012). It can be noted that the strains that were used in above-mentioned reports were Mut^+ phenotypes. However, in a study by Ramani *et al*. (2015) whereby a Mut^S phenotype strain (*P. pastoris* KM71H) was used, expression was detected after 24 hours. Nevertheless, crude Bgl2 activity obtained in this study (71.9 U/ml) was higher than some β-glucosidases expressed in *P. pastoris* after purification. For example, in a study by Ramani *et al*. (2015) the highest activity obtained was 52.0 U/ml and there was no further increase in expression after 96 hours of induction. Charoenrat *et al*. (2015) also achieved expression of 33.7 U/ml in shake flasks. The Mut^S phenotype wild type strain, *P. pastoris* KM71H was used in both these studies. On the other hand, Mut^+ strains have been used for expressing many β-glucosidases. Expression higher than Bgl2 in this study was obtained by Wang *et al*. (2018), 143.0 U/ml of a recombinant β-glucosidase from *Trichoderma viride*. Furthermore, (Chen *et al*., 2011) reported 60 U/ml of Bgl1 from *T. reesei*. This result is directly comparable because it was at similar small scale using the same vector, pPIC9. Using the same conditions, Pei *et al*. (2016) obtained only 20.0 U/ml of *N. crassa* β-glucosidase. The medium used in these studies was optimised whereas Bgl2 activity was higher using standard minimal medium. Even lower expression of 1.2 U/ml has also been reported using Mut^+ strains (Li *et al*., 2013).

The slight fluorescence in the qualitative assays using the culture supernatants indicated that the other Bgls (Bgl1, Bgl3, Bgl4 and Bgl5) were also active. Attempts were then made to investigate an alternative medium for their expression. Yeast extract, peptone and glucose/methanol

(YPG/YPM) medium had been successfully used in the Enzyme Technology Laboratory for expression in *P. pastoris*. Yeast extract, peptone and glucose (YPG) was used for biomass accumulation and glucose was then substituted with methanol for induction. In this medium, all Bgls were expressed. This was initially observed in the qualitative assay, as the fluorescent halos increased in size and intensity compared to that produced in MM (Figure 4.5). Interestingly, the Bgl4 fluorescence intensity was as bright as that for Bgl2.

The enzyme activities were then quantified and, as opposed to MM medium, expression was detected after 24 hours of induction. This could be due to the complexity of the medium which included yeast extract and peptone. However, Bgl1, Bgl3, Bgl4 and Bgl5 activities were all below 2 U/ml. Yeast extract, peptone and methanol (YPM) showed to be superior than MM medium as Bgl2 activity increased to 310.8 U/ml. This was four times higher than the 71.9 U/ml obtained in MM. The highest enzyme production was achieved after 168 hours of induction for all the strains, followed by a decrease by the 192nd hour. The superiority of a complex medium was confirmed by (Ma *et al*., 2015). In their study, buffered complex medium containing yeast extract, peptone, YNB and biotin (BMMY) as well as buffered (BMMH) and non-buffered (MMH) minimal media were investigated. The BMMY supported rapid growth of strains and highest enzyme expression compared to minimal media (Ma *et al*., 2015).

The successful use of YPM in this study greatly contributes to reducing the cost of enzyme production. According to recent Sigma price list (19/03/2020), 1 kg of YNB used as a nitrogen source in MM medium is R10 187.00 whereas the same quantity of yeast extract used in YPM is R3 807.00. The total cost for implementing production of these enzymes in MM would be

R18 358.00 whereas R7 292.00 would be required for YPM media components. This demonstrate the feasibility of industrial application of these enzymes since the production cost is one of the major bottlenecks in this arena (Ferreira *et al*., 2018).

The five recombinant β-glucosidases in this study were of different sizes: Bgl1: 99.9 kDa, Bgl2: 46.5 kDa, Bgl3: 46.8 kDa, Bgl4: 68.9 kDa and Bgl5: 54.3 kDa. These were calculated from their nucleotide sequences (Table 2.1). The protein bands close to the determined sizes were present in all enzyme lanes. For instance, on Bgl1 lane, a protein band between 100 kDa and 80 kDa markers appeared. Bgl2 and Bgl3 bands were below the 50 kDa molecular weight marker as well as Bgl4 and Bgl5 above. The β-glucosidase from *T. lanuginosus* SSBP reported by Lin *et al*. (1999) was a dimer, it had 200 kDa and 105 kDa subunits that were estimated by gel filtration and calculated using SDS-PAGE, respectively (Lin *et al*., 1999). This was slightly confusing since all the molecular weights of the recombinant enzymes were below 100 kDa, only Bgl1 size was close, 99.9 kDa. It is improbable to assume that the β-glucosidase reported by Lin *et al*. (1999) was among these enzymes. Calculations of exact molecular weights of recombinant Bgls and analysis of the enzyme from the parent strain could be conducted for conclusive comparisons. Nevertheless, molecular masses of β-glucosidases from other microorganisms have been characterised at the range of 39-480 kDa (Sonia *et al*., 2008).

Ammonium sulphate precipitation and ultrafiltration were used to concentrate the enzymes. In comparison to the crude extracts, Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 specific activities increased 24, 7, 20, 9 and 4 times respectively (Tables 4.1-4.5). The observed dramatic decrease in protein concentrations during ammonium sulphate precipitation could be due to the solubility of the

enzymes in various ammonium sulphate fractions. This was evident in SDS-PAGE analysis of Bgl2, as the enzyme precipitated in all ammonium sulphate fractions (Appendix 1). Dako *et al*. (2012) reported that the protein can be found in more than one fraction depending on its solubility. Nevertheless, Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 purification-folds also increased. The SDS-PAGE analysis of the partially-purified enzymes (Figure 4.8) appeared to be similar to that of the crude extracts (Figure 4.7). The notable difference was the increase in concentration of Bgl2, Bgl3 and Bgl5 except for Bgl1 and Bgl4. The problem with detecting active heterologous proteins using SDS-PAGE Coomassie staining was reported by Dan *et al*. (2000). The β-glucosidase from *A. niger* expressed in *S. cerevisiae* only appeared on Western Blotting.

There were two possible isoforms of Bgl2 in the native PAGE analysis (Figure 4.9). This was only observed with the use of complex YPM because when MM medium was used, there was only one active band. This could be due to post-translational modifications during growth in this medium. A similar phenomenon was reported by Yoneda *et al*. (2014). The presence and glycosylation of two isoenzymes of β-glucosidase (nD2S and nD2L) from *Chaetomella raphigera* was influenced by different media components. When the fungus was grown on glucose-rich agar medium, nD2S dominated whereas addition of cellobiose into the medium resulted in elevated nD2L levels. To further investigate this, the enzymes were treated with endoglycosidase H to remove N-glycans. It was reported that one variant had additional modifications that altered its molecular weight (Yoneda *et al*., 2014). In relation to Bgl2, the larger band is probably glycosylated whereas the second one is non-glycosylated. This could be further confirmed by its structural analysis or treatment with deglycosylation enzymes. Although the protein concentrations were above the minimum detection limit, Bgl1, Bgl3, Bgl4 and Bgl5

were not detected on the native PAGE analysis. Attempts to concentrate the enzymes using acetone were not successful.

More discriminating chromatographic methods for β-glucosidase purification have been used in many studies (Auta *et al*., 2016; Ma *et al*., 2015; Ramani *et al*., 2015; Xia *et al*., 2016). These have resulted in high enzyme specific activities and yields. However, in addition to increased yields, ease of purification is also the most important advantage in using *P. pastoris* as a heterologous expression system (Macauley-Patrick *et al*., 2005). In this study, high specific activities were obtained after partial purification. Lin *et al*. (1999) purified a β-glucosidase from *T. lanuginosus* SSBP to apparent homogeneity using ammonium sulphate, affinity and ion exchange chromatography. The resulting specific activity was only 9.7 U/mg. The principal aim of heterologous expression of β-glucosidases from *T. lanuginosus* SSBP was to enhance production. According to Karnchanatat *et al*. (2007), stated that β-glucosidase activities range from 5.0 to 979.0 U/mg. The highest activity of 672.0 U/mg was reported by Harnpicharnchai *et al*. (2009) for a β-glucosidase from *Periconia* spp. expressed in *P. pastoris*. Additionally, high expression of Bgl1-Bgl5 was also obtained at the collaborating laboratory in China (N.P Mchunu, 2018 - personal communication). Furthermore, another collaborator, Dong *et al*. (2018) achieved 2834.0 U/mg of the oligo-1,6-glucosidase that was identified in the genome of *T. lanuginosus* SSBP and expressed in *P. pastoris*.

Moreover, the positive impact for fewer purification steps in large-scale production was demonstrated by Pei *et al*. (2016). In their study, a β-glucosidase from *N. crassa* was expressed

in *P. pastoris* and it was the dominant protein in SDS-PAGE analysis. Surprisingly, the crude extract and purified enzyme specific activities were similar. The raw enzyme was used for further characterisation. It was suggested that this could reduce the cost at industrial level since purification is not required (Pei *et al*., 2016).

The characterisation of β-glucosidases from *T. lanuginosus* SSBP was conducted to assess their applicability. Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 performed optimally at pH 6.0. The enzymes were more active below this pH level as the activities sharply decreased when pH increased (Figures 4.10). The β-glucosidase from the parent strain was also optimal at pH 6.0 (Lin *et al*., 1999). In this study, all the enzyme assays were conducted at pH 5.0 (except for characterisation experiments). For Bgl2, the activity was 200 units lower than that at the optimum pH. Many β-glucosidases are optimal at the pH range of 4.0-5.5, however, pH 5.0 is used as the standard. This allowed for direct comparison with other enzyme activities reported in literature.

Bgl1, Bgl2 and Bgl3 were more stable under basic conditions for the entire incubation period. Alkaline pre-treatment is mostly used to render lignocellulose less recalcitrant to enzyme hydrolysis. Therefore, this is an advantage because these enzymes could strive better at those high pH levels. However, changes in pH during storage may affect the enzyme activities since they had been initially kept at pH 5.0. The enzymes lost 90% and 60-70% of their activities after 150-240 minutes of incubation at pH 4.0 as well as pH 5.0 and pH 6.0, respectively (Figures 4.11-4.13). Bgl1, Bgl2 and Bgl3 pH stability is comparable with β-glucosidase from the parent strain that was stable at levels above pH 5.0; it retained 90% of its activity (Lin *et al.*, 1999).

Furthermore, β-glucosidases from many microorganisms have also been reported to be stable at high pH levels (Sonia *et al*., 2008). Contrastingly, Bgl4 and Bgl5 demonstrated broad pH stability (Figures 4.14-4.15). These enzymes retained 40-70% and 60-80% of their activities at all tested pH levels after 60 minutes of incubation. This was however followed by gradual decrease in residual activity at basic pH levels. This suggests that *T. lanuginosus* SSBP has a sufficient arsenal of β-glucosidases that are stable over wide range of pH. β-glucosidases with similar properties have been reported from many microorganisms (Eyzaguirre *et al*., 2005; Li *et al*., 2018; Olajuyigbe *et al*., 2016; Zhang *et al*., 2017).

The optimum temperature of Bgl2 was 60℃ and when optimal conditions were used, an activity of 467.1 U/ml was achieved (Figure 4.16). Lin *et al*. (1999) reported that optimal conditions of β-glucosidases from thermophilic microorganisms are pH 6.0 and temperature of 45-65℃. Other β-glucosidases with high optimal activity at 70℃ have been reported, e.g. *Bg*1I from *T. reesei*, *PtBglu3* from *P. thermophila* and *Mt*Bgl3 from *M. thermophila* (Chen *et al*., 2011; Karnaouri *et al*., 2013; Yan *et al*., 2012). The optimum temperature for Bgl1, Bgl3, Bgl4 and Bgl5 was 50℃ (Figure 4.16). Similar findings were reported by Florindo *et al*. (2018) whereby two β-glucosidases (*Th*Bgl1 and *Th*Bgl2) from *Trichoderma harzianum* had different optimum temperatures at 35℃ and 40℃, respectively. In the same context, *H. insolens* Y1 β-glucosidases had different pH and temperature optima. *Hi*Bgl3A and *Hi*Bgl3C were optimal at 60℃ whereas the optimum temperature for *Hi*Bgl3B was 50℃ (Xia *et al*., 2016).

Bgl1, Bgl2, Bgl3, Bgl4 and Bgl5 were most stable at 30℃ and 40℃ as they retained more than 80-60% of their activities after 2-4 hours of incubation at these temperatures. Many enzymes from thermophiles are more stable at mesophilic temperatures (Eyzaguirre *et al*., 2005). Bgl2 stability decreased as the temperature increased and it retained 50% of its activity at 50℃ for 240 minutes compared to 60℃. After 60 minutes of incubation at this temperature, a dramatic decline in residual activity was observed (Figure 4.18). The loss of almost 70% of Bgl2 activity at 80℃ after 30 minutes of incubation indicates its poor stability at high temperatures. After 30 minutes of incubation at 50℃ and 60℃, Bgl1, Bgl3, Bgl4 and Bgl5 lost 60% and 80% of their activities (Figures 4.17, 4.19, 4.20 and 4.21). The enzymes demonstrated a lack of stability at their respective optimum temperature levels. This could be due to the long incubation period for the enzyme assay (30 minutes) and thermostability testing sampling intervals (30 minutes). Similar findings have been reported by Pei *et al*. (2016) and Xia *et al*. (2016). In the first study, the enzyme performed optimally at 60℃ and also lost its stability after 10 minutes of incubation at this temperature. Comparable to Bgls, it was stable at mesophilic temperatures (Pei *et al*., 2016). Furthermore, one β-glucosidase from a thermophilic fungus, *H. insolens* Y1 had thermostability slightly higher than the other two isoenzymes at 50-60℃, however, it lost 80% of its activity after 10 minutes of incubation at 70℃ (Xia *et al*., 2016). The failures associated with applications of enzymes are greatly influenced by variations in conditions at laboratory and industrial scales, e.g. pH and thermal stability tests conducted for shorter periods. According to Sørensen *et al*. (2013), this is one of the accelerators of challenges experienced in the use of β-glucosidases during cellulose degradation. In this study, this was taken into consideration as the stability tests were conducted for 2-4 hours compared to many reports where they were performed for only 60

minutes. These results can therefore be better extrapolated for potential successful industrial applications.

Bgl2 and Bgl4 hydrolysed all the tested natural substrates whereas Bgl1, Bgl3 and Bgl5 were active only on gentiobiose and laminarin (Figure 4.22). As per classification of β-glucosidases, Bgl2 and Bgl4 showed broad specificity as they hydrolysed different β-glycosidic linkages of cellobiose (β-1,4), gentiobiose (β-1,6), salicin (β-1,2) and laminarin (β-1,3/6). Bgl3 was also slightly active on salicin, a β-1,2 linked glucoside. It mostly hydrolysed gentiobiose and laminarin. Like Bgl1, Bgl3 and Bgl5, many β-glucosidases with higher activity on β-1,3/6-linked substrates than β-1,2 and β-1,4 disaccharides have been reported (Chen *et al*., 2010; Gao and Wakarchukb, 2014; Karnchanatat *et al*., 2007; Ramani *et al*., 2015; Yang *et al*., 2014). It is safe to assume that the Bgls are involved in catalysing different biological functions in the parent strain.

Functionally diverse and distantly related β-glucosidases from the same organism have been reported in some studies (Nazir *et al*., 2008; Oh *et al*., 2018). In particular, *H. insolens* Y1 β-glucosidases were expressed in *P. pastoris* at different levels. Two enzymes, *Hi*Bgl3A and *Hi*BglC had broad substrate specificity whereas *Hi*Bgl3B was an aryl β-glucosidase. Moreover, like Bgls, these isoenzymes belonged to different evolutionarily related clades. Functional diversity was further confirmed by unique amino acid residues in their sequences (Xia *et al*., 2016). The broad substrate specific β-glucosidases are the most advantageous for industrial use since their activity is equal on both natural and artificial substrates (Ramani *et al*., 2015). Sørensen *et al*. (2013) also suggested that using cellobiose for investigation of β-glucosidases

eliminates challenges that can be encountered during cellulose degradation for biofuel production.

Since Bgl2 was the dominant and most active recombinant enzyme (553.7 U/mg), its effect on hydrolysis of artificial forms of cellulose at pH 5.0 and 50℃ in combination with cellulase from *A. niger* was investigated. The amount of glucose released in the presence and absence of Bgl2 was measured by the GOD-POD assay. A substantial increase in hydrolysis rate was observed when Bgl2 was supplemented. After 1 hour of incubation, there was a 2.4 and 2.8 times increase in glucose production from microcrystalline (MCC) and carboxymethyl cellulose (CMC), respectively (Figures 4.23 and 4.24). Compared to MCC, CMC was hydrolysed quickly and highest glucose was obtained from this substrate. This may have been due to the insoluble nature of MCC as it was not completely dissolved in the solution. It was only aided by continuous shaking. Although the units were not equal, the concentration of glucose produced in the presence of the commercial *A. niger* cellobiase was only 1-2 mM higher than that of Bgl2 on CMC and MCC, respectively.

Since Bgl2 showed the ability to improve cellulose hydrolysis and using different enzyme ratios is common practice (Chen *et al*., 2010; Zhang *et al*., 2017), Bgl2 doses were also varied. As expected, the higher glucose concentrations were obtained within shorter periods of time. The development of economically feasible cellulose degradation process requires efficient enzymes able to increase reaction rates. At 1:1 cellulase to Bgl2 ratio, 16.2 mM of glucose was produced. With high Bgl2 activity, glucose yield increased to as high as 52.3 mM after 1 hour of incubation (Figure 4.25). However, the released glucose remained constant after 3 hours of incubation. This

could be due to the inhibition of the enzyme by high glucose concentrations and low stability at 50℃. Bgl2 lost 20% of its activity at this temperature after 1 hour and its glucose tolerance was not determined. Furthermore, according to the manufacturer, the optimum conditions for the commercial cellulase used are temperature of 37℃ and pH 5.0. This presented a challenge because Bgl2 performed poorly at 30-40℃ and this was the only cellulase enzyme available in the laboratory. The conditions were chosen because many reports on hydrolysis using commercial cellulases have been conducted at 50-55℃ (Saha and Bothast, 1996; Wang and Xia, 2011; Dotsenko *et al*., 2012). However, this could be solved by the use of a cellulase that complements the optimal characteristics of Bgl2 or optimisation of process conditions.

Nevertheless, Bgl2 performed better than *Hi*Bgl3C reported by Xia *et al*. (2016). Supplementation of *Hi*Bgl3C during hydrolysis of Avicel yielded only 4.85 mM of glucose after 96 hours of incubation. Furthermore, Saha and Bothast (1996) hydrolysed 2% microcrystalline cellulose using the commercial cellulase, Cytolase 123. Addition of 0.4 U/ml Bgl increased glucose production by 8.3% after 24 hours of incubation. Although the exact concentration of glucose was not reported, Bgl2 activity was higher compared to this enzyme (Saha and Bothast 1996). Recently, Yin *et al*. (2018) supplemented two recombinant β-glucosidases from *Actinomadura amylolytica* YIM 77502T in the hydrolysis of lignocellulosic substrates. On CMC, Bgl2 performed better than these enzymes as the released glucose was only 1.5 μmol/ml after 24 hours. However, *Aa*Bgl1 and *Aa*Bgl2 had high synergistic effect with cellulase on corncobs, birch sawdust and Avicel compared to CMC (Yin *et al*., 2018). In another study by Zhang *et al*. (2017), supplementation of a recombinant β-glucosidase from *Thermotoga naphthophila* RKU-

10 resulted in high conversion rate of corn straw compared to microcrystalline cellulose. This demonstrated the potential of Bgl2 on cellulose degradation.

6. CONCLUSION

The aim of this study was to characterise β-glucosidases that were identified in the genome of *T. lanuginosus* SSBP and heterologously expressed in *P. pastoris*. Due to its non-cellulolytic nature, this fungus has not been considered as a source of enzymes involved in cellulose degradation. It is mostly known for hyper-production of hemicellulases. The identification of multiple putative β-glucosidase genes in the genome of *T. lanuginosus* SSBP was intriguing and required investigation of their potential for industrial application. Furthermore, the use of *P. pastoris* expression system has been proven to enhance production of many enzymes. Recombinant β-glucosidases from *T. lanuginosus* SSBP were successfully expressed in *P. pastoris*.

Yeast extract, peptone and methanol medium supported hyper-production of Bgl2 and improved expression of Bgl1, Bgl3, Bgl4 and Bgl5 compared to the commonly used minimal methanol medium. This was also an added advantage as it demonstrated the potential in reducing the total production cost. The recombinant enzymes were partially-purified and Bgl2 had the highest specific activity of 553.7 U/mg without specialised purification. The enzymes were optimal at 50-60℃ and demonstrated stability at a broad pH range as well as adequate thermostability. As it was initially hypothesised that the multiple β-glucosidases play different roles in *T. lanuginosus* SSBP, the enzymes showed functional diversity in terms of substrate specificity. Two Bgls (Bgl2 and Bgl4) had broad substrate specificity whereas Bgl1, Bgl3 and Bgl5 were more active on β-1,3/6-linked glucosides. However, their functional diversity could be further confirmed by structural analysis.

The most efficient recombinant enzyme (Bgl2) demonstrated synergistic effect with cellulase on hydrolysis of microcrystalline and carboxymethyl cellulose as high glucose concentrations were released at the shortest period of time when it was added. Upon saccharification of a lignocellulosic substrate and optimisation of the process conditions, this enzyme has potential to be used in cellulose degradation for valorisation of waste lignocellulosic biomass.

7. RECOMMENDATIONS

Future work following on from this study will include optimisation of production of the recombinant enzymes in bioreactors. This will determine if the enzymes can be produced at a large scale for industrial applications. Although high activities were obtained after partial purification, chromatography-based methods will be used to conduct further characterisation and application of all the enzymes. Structural analysis or crystallography should be performed to gain insights of the enzymes biological functions. Another possible strategy would be to improve the enzymes stability characteristics through site directed mutagenesis. Furthermore, synergistic effect of Bgl2 was demonstrated on artificial cellulose. The use of a lignocellulosic substrate will further reveal its true potential in improving cellulose degradation for industrial purposes. *T. lanuginosus* has shown to be a source of enzymes with desirable properties. This fungus could also be considered for β-glucosidases that can be supplemented in cellulose degradation processes.

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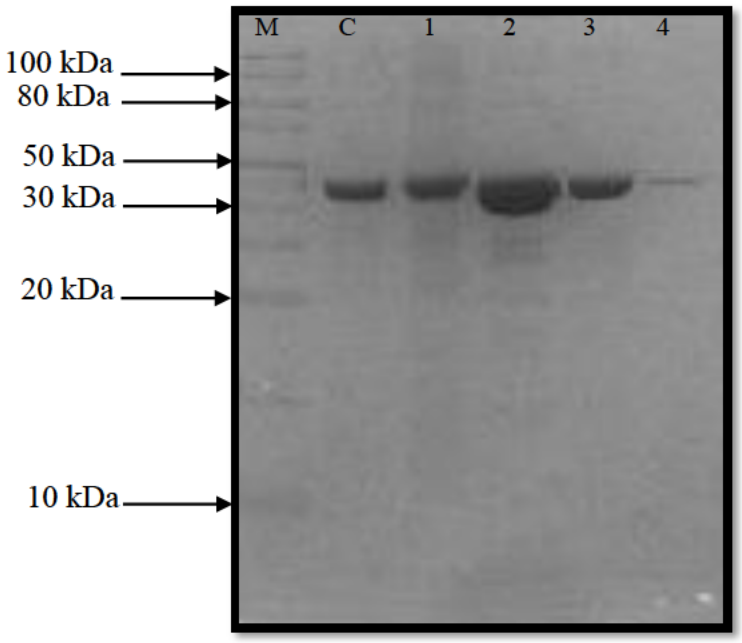
APPENDIX



Appendix 1: SDS-PAGE analysis of BGL2 after partial purification. Proteins were separated using 12% acrylamide gel containing SDS and electrophoresis at 20 mA. The gel was stained with Coomassie stain and photographed under epi-white illumination. M: protein molecular weight markers (kDa); C: crude enzyme extract; $(NH_4)_2SO_4$ fractions: 1: 0-25%; 2: 25-50%; 50-75%; 75-90%.