PRODUCTION AND CHARACTERISATION OF A β-GLUCOSIDASE FROM A THERMOPHILIC BACTERIUM AND INVESTIGATION OF ITS POTENTIAL AS PART OF A CELLULASE COCKTAIL FOR CONVERSION OF LIGNOCELLULOSIC BIOMASS TO FERMENTABLE SUGARS

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A thesis submitted in fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

In the

FACULTY OF SCIENCE AND AGRICULTURE,

(School of Molecular and Life Sciences)

At the

UNIVERSITY OF LIMPOPO, SOUTH AFRICA.

SUPERVISOR: I. NCUBE

NOVEMBER, 2019

DECLARATIONS

I declare that this thesis hereby submitted to the University of Limpopo for the degree Doctor of Philosophy, is my own work and has not been previously submitted by me for the award of a degree at this or any other university. Neither does this thesis contain any material published or written previously, by any other person and that all material herein has been duly acknowledged.

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DEDICATIONS

This work is dedicated to two people without whom this achievement would not have been possible. You were both an indelible part of a journey that started at undergraduate. I wish you were both here to witness the culmination of all that hard work but your teachings and memories will live with me for as long as I am alive:

To my late mother Joyce Tsakan Masingi, a woman who believed in me, right from the start. Her love, support and the values she instilled in me have made me who I am today. She would be proud of this milestone in my life. Mommy I made it!

My late supervisor, Ignatious Ncube, who passed on a few months before this submission. He was a supervisor, mentor and a friend. He taught me everything I know about research, student supervision and academia in general. I will forever be grateful to have been supervised by him and all the opportunities he presented me with.

ACKNOWLEDGEMENTS

I would like to acknowledge the following:

Dr D LaGrange for assisting with finalizing the thesis and Prof KLM Moganedi for facilitating the submission process. I am truly grateful for the support and advice they willingly provided.

My husband and children; Creswell Phasha, Lehlogonolo Phasha and Lethabo Phasha, for their love, support, patience and all the sacrifices they had to make to allow me to concentrate on my research.

My siblings; Kulani, Musa and Angel for always believing in me. Kulani, you are my rock, the one constant in my life. I am grateful and blessed to have you in my life, your prayers keep me afloat.

My friend Gloria Ngoepe; where do I even begin? Thank you for the calm you bestowed on me each time I made a frantic call because something was not working in the lab. The sleepless nights we spent in the lab and sleeping in the office, just so we could meet a deadline. The many hours we spent on the phone strategising and problem solving.

The following interns, who were of much assistance to me during this study: Katlego Mashego, Lizzy Mohuba, Whitney, Ziwa and Nelisiwe Mthiyane. You were all a blessing to me.

Lesetja Lekgodi and Maphuti Matlala for technical assistance, as well as the whole Biotechnology team for a good working environment.

Prof Hlengani Siweya, the Executive Dean of the Faculty of Science and Agriculture; for granting me paid study leave that allowed me to concentrate on my work without worrying about finances.

University of Limpopo staff development programme for funding my studies.

VLIR for funding my research through funds for purchasing of laboratory reagents.

Most Importantly, I would like to thank the All Mighty God for his grace and mercy. He is the author and finisher of my faith. None of this would have been possible without Him.

ABSTRACT

The use of lignocellulosic biomass for bioethanol production is largely dependent on cost effective production of cellulase enzymes and most importantly, the availability of cellulases with sufficient β-glucosidase activity for complete hydrolysis of cellulose to glucose. Commercial cellulase preparations are often inefficient in the complete hydrolysis of cellulose to glucose. The addition of β-glucosidases to commercial cellulase preparations may enhance cellulolytic activity in the saccharification of cellulose to fermentable sugars.

A β-glucosidase producing thermophilic bacterium, *Anoxybacillus* sp. KTC2 was isolated from a hot geyser in the Zambezi Valley, Zimbabwe. The bacterium identified through biochemical tests and 16S rDNA sequencing, had an optimal growth temperature and pH of 60˚C and pH 8, respectively. The β-glucosidase enzyme had an optimal temperature of 60˚C and a broad pH range for activity, between 4.5 and 7.5 with an optimum at pH 7. The β-glucosidase enzyme retained almost 100% activity after 24 hours' incubation at 50˚C.

The *Anoxybacillus* sp. KTC2 β-glucosidase was partially purified and a partial amino acid sequence obtained through MALDI-TOF analysis. The whole genome of *Anoxybacillus sp* KTC2 β-glucosidase was sequenced and a β-glucosidase gene identified. The deduced amino acid sequence corresponded to the peptide sequences obtained through MALDI-TOF, confirming the presence of the a β-glucosidase on the genome of *Anoxybacillus sp* KTC2. Analysis of the deduced amino acid sequence revealed that the β-glucosidase enzyme belongs to the GH family 1. The β-glucosidase gene was isolated by PCR and successfully cloned into an *E. coli* expression system.

The saccharification efficiency of the β-glucosidase enzyme was evaluated through the creation of enzyme cocktails with the commercial cellulase preparation, Celluclast™. Celluclast™ with the *Anoxybacillus sp* KTC2 β-glucosidase were used to hydrolyse pure Avicel cellulose, at 50˚C over a 96 hour reaction time. The *Anoxybacillus sp* KTC2 β-glucosidase enabled a 25% decrease in the total cellulose loading without a decrease in the amount of glucose released.

The bacterial strain, *Anoxybacillus* sp. KTC2 is a good source of thermostable enzymes with potential industrial application. The β-glucosidase is a thermostable enzyme that has potential of reducing the cost of enzymes in bioethanol production.

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CHAPTER 1

1. INTRODUCTION

1.1 Background of the study

Lignocellulosic biomass is an abundant and renewable resource (Kalyani et al. 2013, Liu et al. 2014, Ndlovu and Wyk 2019, Wakai et al. 2019), and its hydrolysis and use in biofuel production has gained considerable attention in recent years due to the rapid rate of depletion of fossil fuel (Khare et al. 2015, Pancha et al. 2016), and the resultant hikes in fossil fuel prices (Van Dyk and Pletschke 2012, Banerjee et al. 2016). In addition to the increased fuel prices, the combustion of fossil fuels causes greenhouse gas emission which results in environmental pollution (Sukumaran et al. 2009, Cheng and Timilsina 2011, Nigam and Singh 2011, Menon and Rao 2012, Wakai et al. 2019).

Biofuels produced from lignocellulosic biomass include bio-ethanol, bio-butanol, and methane (Menon and Rao 2012). Bio-ethanol production is of particular importance in the transport industry (Lewis Liu et al. 2012, Banerjee et al. 2016), serving as an excellent liquid transportation fuel, which is currently used as a blend with petrol. The United States of America and Brazil are the world's leaders in biofuel production and they are currently using 10 and 22% ethanol-petrol blends, respectively (Banerjee et al. 2016). The USA and Brazil mainly use maize and sugarcane as feedstock, respectively (Senthilkumar and Gunasekaran 2005, Balat et al. 2008). However, the use of maize and sugarcane for biofuel production raises food safety concerns as it competes for land with food and feed production (Yeoman et al. 2010, Bhalla et al. 2013a). The ethical issues raised have led to a need to find alternative feedstocks for bioethanol production. Lignocellulosic biomass is currently being considered as a suitable alternative as it is renewable, does not compete directly with food production and every country can use what is readily available in their region, which would in turn keep production costs low (Menon and Rao 2012).

Sources of lignocellulosic biomass include agricultural waste such as municipal wastes, wood, rice straw, wheat, corn stover, corn cobs and bagasses (Harnpicharnchai et al. 2009, Khalil and Sivakumar 2015), molasses (Banerjee et al. 2016) and waste paper (Yanase et al. 2005). Bioethanol production from

lignocellulose requires three steps: pre-treatment, saccharification of the pre-treated biomass and fermentation of the resultant sugars to bioethanol (Khare et al. 2015). Pre-treatment of lignocellulose is required to break the lignin component between the plant material fibres and release hemicellulose and cellulose. Depending on the pre-treatment method used, hemicelluloses are either hydrolysed during pre-treatment or have to be hydrolysed enzymatically after pre-treatment. Cellulose has to be hydrolysed enzymatically to produce glucose. Pre-treatment of lignocellulosic biomass can be achieved through various methods such as; acid or alkaline treatment, steam explosion or ammonia fiber/freeze explosion (Berlin et al. 2005, Balat et al. 2008, Zhuang et al. 2016).

Utilisation of lignocellulosic biomass for bioethanol production requires cellulases; a group of enzymes that work together synergistically to hydrolyse cellulose, found in lignocellulose. These enzymes include endoglucanases which cleave internal β-1,4-glycosidic bonds, cellobiohydrolases, which are exo-acting enzymes that release cellobiose from the reducing or non-reducing ends of cellulose, and β-glucosidases, that hydrolyses cellobiose to glucose. In addition to hydrolysing cellobiose to glucose, β-glucosidase reduces cellobiose inhibition on endoglucanases and cellobiohydrolases (Florindo et al. 2018). β-glucosidase is the rate-limiting enzyme in the cellulose hydrolysis system, making it a key enzyme in biofuel production from lignocellulosic biomass (Bai et al. 2013, Diogo et al. 2015, Florindo et al. 2018).

A significant proportion of commercial preparations of cellulases possess insufficient levels of β-glucosidase. These commercial cellulase preparations are inefficient for the complete hydrolysis of cellulose to glucose (Wen et al. 2005, Ahamed and Vermette 2008, Harnpicharnchai et al. 2009, Xia et al. 2018a). Therefore, the addition of β-glucosidases to commercial cellulase preparations should enhance cellulolytic activity in the saccharification of cellulose to fermentable sugars (Sánchez and Cardona 2008, Harnpicharnchai et al. 2009). In addition to low levels of β-glucosidase in commercial cellulase preparations, these commercial enzymes generally require long hydrolysis times. This leads to saccharification reactions being prone to microbial contamination, along with the enzymes generally being unstable and tend to lose activity during incubation (Haki and Rakshit 2003). The use of thermostable enzymes in cellulosic biomass conversion would be advantageous

since thermostable enzymes are active at higher temperatures, providing improved hydrolysis of the cellulosic substrates with lower risk of contamination (Rastogi et al. 2010).

In addition, the use of thermostable enzymes in conversion of cellulosic biomass to fermentable sugars offers several advantages such as improved hydrolysis, higher mass-transfer rates leading to better substrate solubility, as well as increased flexibility on process design which in turn lowers production costs (Rastogi et al. 2010, Bai et al. 2013). While the higher operational temperatures increase energy costs, the advantages compensate for these increased costs.

The increased need for thermostable enzymes has stimulated the search for thermophilic microorganisms from the environment for the isolation of thermostable enzymes (Patel et al. 2019). High temperature habitats such as hot geysers are a potential source of thermostable enzymes from thermophilic bacteria found in these habitats. Thermophilic organisms have been isolated from compost heaps, soil and wastewater (Rastogi et al. 2010), as well as hot springs (Wang et al. 2010).

Depending on the substrate and technology used, cellulase production is often the most expensive step in bioethanol production from lignocellulosic material (Haki and Rakshit 2003, Zheng et al. 2017). The employment of techniques to improve enzyme production in order to lower production costs would improve the prospects of bioethanol production from lignocellulosic biomass. The isolation of organisms capable of producing higher levels of extracellular β-glucosidases, with higher end product tolerance and the development of expression systems capable of high β-glucosidase expression, through recombinant DNA technologies may help lower the enzyme costs.

The present study focused on screening, isolation and characterisation of a thermophilic β-glucosidase producing bacterium; isolation and characterisation of the thermostable β-glucosidase enzyme; evaluation of the β-glucosidase enzyme's saccharification potential and ultimately, identification of the gene coding for the β-glucosidase.

1.2. Aims and Objectives

1.2.1. Aim

The study aimed to isolate and characterise a novel β-glucosidase from a thermophilic bacterium purified from a hot geyser in the Zambezi Valley of Zimbabwe and investigate its potential as part of a cellulase cocktail for conversion of lignocellulosic biomass to glucose.

1.2.2. Objectives of the study:

i. To identify the β-glucosidase-producing bacterial strain through biochemical and molecular biology techniques.
ii. To optimize production of the β-glucosidase enzyme from the bacterial strain.
iii. To partially purify the β-glucosidase enzyme through a combination of chromatography and electrophoretic techniques.
iv. To develop an enzyme cocktail for the saccharification of cellulose.
v. To determine the GH family of the β-glucosidase enzyme.

1.3. Hypothesis

Combining a thermostable β-glucosidase with commercial cellulase preparations that have low β-glucosidase activity will reduce the enzyme load needed for efficient hydrolysis of lignocellulosic biomass.

1.4. Significance of the study

The use of lignocellulosic biomass for bioethanol production is largely dependent on the availability of cellulases with sufficient β-glucosidase activity for complete hydrolysis of cellulose to glucose. Production of a thermostable β-glucosidase and combining it with a commercial cellulase preparation to form an enzyme cocktail should result in more efficient use of lignocellulosic biomass for bioethanol production.

CHAPTER 2

2. LITERATURE REVIEW

Successful and cost-effective use of lignocellulosic biomass as feedstock for bioethanol production is highly dependent on the structural composition of the substrate, a good pre-treatment method, the availability of hydrolytic enzymes for complete hydrolysis and fermentative microorganisms (Sukumaran et al. 2005, Khare et al. 2015, Zhuang et al. 2016, Artola et al. 2019, Wakai et al. 2019). The hydrolytic enzymes should include cellulases as well as accessory enzymes.

2.1. Lignocellulose

Lignocellulose accounts for more than 60% of plant biomass on earth (Tengerdy and Szakacs 2003). Lignocellulose is made up of lignin, cellulose and hemicellulose (Sánchez 2009, Zhuang et al. 2016, Lu et al. 2019) as well as pectin, proteins, ash salts and minerals in small qualities (Van Dyk and Pletschke 2012). The exact structure of each component varies among different lignocellulosic feedstock's (Narra et al. 2015). Lignocellulose is highly resistant to microbial breakdown due to the presence of lignin, the high degree of crystallinity of cellulose and polymerisation of the polysaccharides (Meehnian et al. 2016).

2.1.1. Lignin

Lignin is the most abundant heterogeneous cross-liked aromatic polymer in nature, with a three dimensional structure that covers cellulose in lignocellulosic biomass. The rigid structure is resistant to biodegradation and accounts for 10-25% of the mass of lignocellulosic material (Senthilkumar and Gunasekaran 2005, Travaini et al. 2016, Zhuang et al. 2016, Alves et al. 2018). The composition varies between hard and soft woods. Woodier plants as well as older plants contain higher levels of lignin, which makes the cell walls waterproof and provides a barrier against pathogens (Van Dyk and Pletschke 2012). Lignin plays a big role in the ease of hydrolysis of lignocellulose. Studies have shown a correlation between the percentage of lignin in a substrate and the amount of glucose that is subsequently released. Lignin forms a physical barrier that limits the accessibility of hydrolytic enzymes to cellulose and hemicellulose and may also inhibit these enzymes directly.

2.1.2. Cellulose

Cellulose is a water-insoluble glucose polymer and is the most abundant polysaccharide in nature. It consists of alternating crystalline and amorphous regions with a diameter of 20 nm and several micrometres in length. Cellulose accounts for about 40 - 50% of the total dry plant cell mass (Senthilkumar and Gunasekaran 2005, Yeoman et al. 2010, Menon and Rao 2012, Travaini et al. 2016, Ndlovu and Wyk 2019, Saha et al. 2019, Yassin et al. 2019). The glucose residues are linked together by β-1,4-glycosidic linkages with a degree of polymerization that ranges from a few hundred D-glucose units to more than a thousand (Chen et al. 2013, Alves et al. 2018). Glucose is the preferred substrate for fermentation to produce ethanol (Banerjee et al. 2016).

Cellulose has a compact crystalline structure resulting from the hydroxyl groups of D-glucose units joined by hydrogen bonds, making it hard to degrade. In addition to the compact crystalline structure, cellulose is surrounded by hemicellulose and rigid lignin structures. This limits enzyme and sometimes water accessibility to cellulose (Berlin et al. 2005, Sukumaran et al. 2005, Yeoman et al. 2010, Alves et al. 2018).

Cellulose hydrolysis can be achieved by either enzymatic or acid hydrolysis (Senthilkumar and Gunasekaran 2005, Artola et al. 2019). When partially hydrated, the cellulose fibres can swell enough to allow penetration by enzymes, resulting in cellulose hydrolysis (Sukumaran et al. 2005). Enzymatic hydrolysis of cellulose is achieved through the action of cellulases; a group of enzymes that work together synergistically to hydrolyse cellulose (Bernardes et al. 2019, Ndlovu and Wyk 2019, Saha et al. 2019). Acid hydrolysis of cellulose involves the application of sulphuric acid to cleave the glycosidic bonds within the cellulose chains. There is however a downside to using acid hydrolysis; the corrosive nature of acid modifies cellulose fibre and produces chemical waste. Enzymatic hydrolysis of cellulose provides an eco-friendly alternative to acid hydrolysis (Yassin et al. 2019).

2.1.3. Hemicellulose

Hemicelluloses vary in structure and composition (Barr et al. 2012). Hemicellulose is composed of xylan, mannan, galactan and arabinan containing polymers (Cheng and Timilsina 2011, Khare et al. 2015). Xylan is the most abundant hemicellulose

and consists of β-D-xylopyranosyl residues that are linked together by β-1,4 glycosidic bonds. Xylan accounts for 30 - 35% of a plant cell wall's total dry weight, making it an important part of the cell wall structure. The exact xylan percentage and composition of xylan present differ from one plant to another plant. Hardwoods typically have higher xylan content, while in softwoods hemicelluloses have a higher mannan composition (Van Dyk and Pletschke 2012).

Mannan is a major component of hemicellulose and it contains mannose, galactose and glucose residues. Mannan include glucomannas in hardwood and galactoglucomannans for softwood (Van Dyk and Pletschke 2012).

2.2. Enzymes for cellulose degradation

2.2.1. Cellulases

Cellulases are a group of enzymes that work together synergistically to hydrolyse cellulose. Endoglucanases (1,4- β-D-glucan 4-glucanohydrolase; EC 3.2.1.4), cleave internal β-1,4-glucosidic bonds, cellobiohydrolases (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) are exo-acting enzymes that release cellobiose from the reducing and non-reducing ends of cellulose (Figure 2.1) and β-glucosidases (EC 3.2.1.21), which hydrolyse cellobiose to glucose (Bai et al. 2013, Kalyani et al., 2013, Santa-Rosa et al. 2017, liew. et al. 2018, Ndlovu and Wyk 2019). The action of β-glucosidases helps reduce cellobiose-inhibition on endoglucanases and cellobiohydrolases (Xue et al. 2012, Diogo et al. 2015, Florindo et al. 2018).

Cellulases are inducible enzymes; their production and secretion is only achieved in the presence of an inducing substrate. Lignocellulosic biomass is used as an inducer for cellulase enzyme production and the β-glucosidase enzyme is also directly involved in cellulase induction through the trans-glycosylation activity of this enzyme. Trans-glycosylation activity of the β-glucosidase enzyme on cellulose degradation products results in the formation of sophorose; a potent cellulase inducer. Lignocellulosic substrates such as corn stover, rice straw, bran and bagasse have been well studied and documented as cellulose inducers (Sukumaran et al. 2005, Zhou et al. 2012, Lu et al. 2019).

Cellulases are highly specific biocatalysts that are classified as glycoside hydrolases by the International Union of Biochemistry and Molecular Biology Committee (NC-IUBMB) (Santa-Rosa et al., 2017). Cellulolytic enzymes have a saccharification efficiency of between 90% and 98% and produce environmentally friendly by-products (Ahamed and Vermette 2008). Cellulases have found use in industrial sectors such as the textile, food, detergent, paper, packaging, agricultural, and biofuel industries (da Costa et al. 2015, Artola et al. 2019, Yassin et al. 2019).

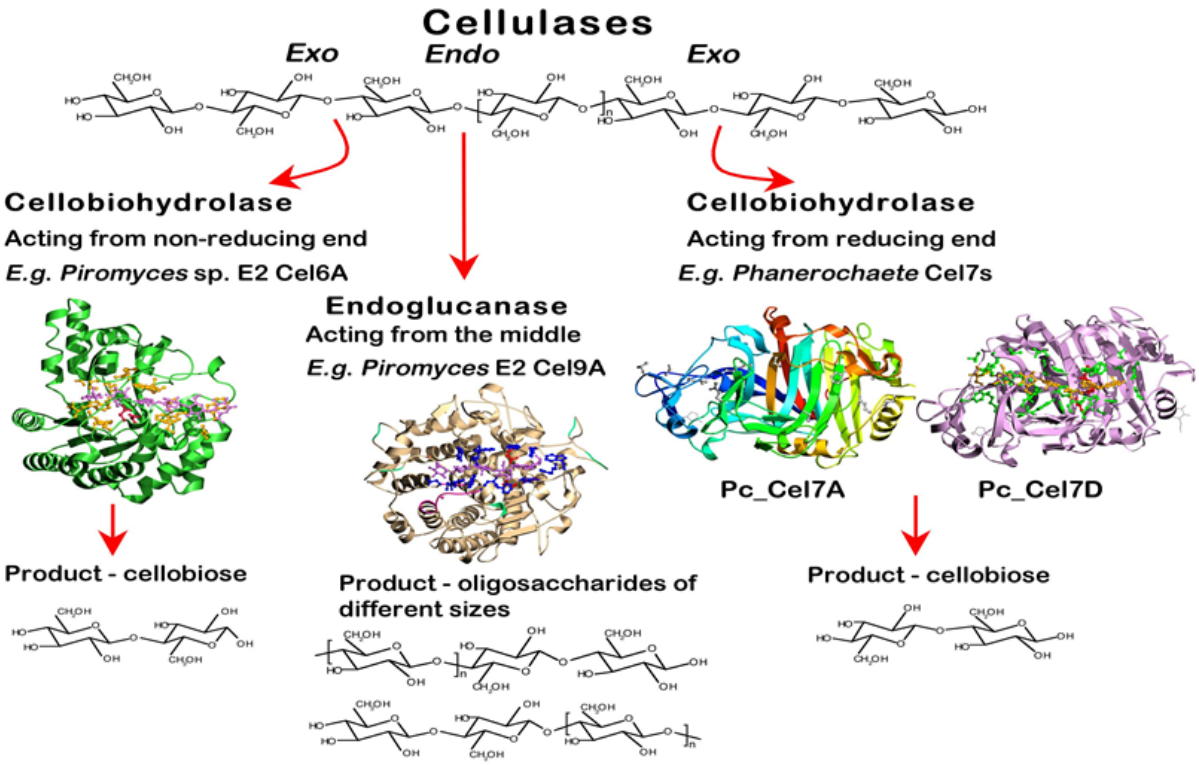


Figure 2.1. Schematic representation of the action of cellobiohydrolase and endoglucanase enzymes on cellulose. The action of each cellulase enzyme and hydrolysis products are illustrated. Cellobiohydrolases act from reducing and non-reducing ends to produce cellobiose while endoglucanases act from the middle of the cellulose polymer to produce oligosaccharides (Ubhayasekera 2005).

Sections 2.2.2 to 2.2.4 will address the sources, substrate specificity and the classification of β-glucosidase to provide a deeper understanding of this enzyme family. The discussion will be limited to β-glucosidases as the focus of the current study is on the production of this specific cellulase enzyme.

2.2.2. Sources of β-glucosidases

β-glucosidases are found in a broad range of organisms, which include plants, animals and microorganisms (Pal et al. 2010, Ndlovu and van Wyk 2019). The activity of β-glucosidases is largely dependent on their location, physiological function and the biological system where they are found (Roy et al. 2005, Daroit et al. 2007).

2.2.2.1. Plant β-glucosidases

In plants, β-glucosidases play a variety of roles from lignification, synthesis of β-glucan during cell wall development, cell wall degradation in the endosperm during germination, and phytohormone activation for growth and development. Furthermore, these enzymes are involved in pigment metabolism, fruit ripening as well as providing protection against pathogens (Roy et al. 2005).

2.2.2.2. Mammalian β-glucosidases

Mammalian β-glucosidases, commonly known as glucocerebrosidases, are responsible for catalysing the hydrolysis of glucosylceramides in the lysosome (Brumshtein et al. 2009, Luan et al. 2010, Alfonso et al. 2013). A glucocerebrosidase deficiency leads to Gaucher's disease, which is hereditary. The glucocerebrosidase enzyme acts in lysosomes on the sphingolipid glucocerebroside, catalysing its conversion to glucose and ceramide. Glucocerebrosidase play an important role in the metabolism of glycolipids and its absence leads to accumulation of glucosylceramide in macrophages, resulting in "foam cells" in the spleen, liver and bone. Type 1 Gaucher disease is the most common form of this disease. The tissue damage is mostly limited to the liver, spleen and bone, but may sometimes involves the lungs and kidneys. In type 2 and 3 Gaucher disease, there is also neurologic accumulation of foam cells and damage, leading to severe neurologic outcomes in infancy or childhood. The main treatment for Gaucher disease is enzyme

replacement therapy, in which the defective GlcCerase is supplemented with an active enzyme, administered to patients intravenously usually every two weeks. Infusions of glucocerebrosidase have been shown to increase the activity of this enzyme intracellularly, to decrease foam cells and to ameliorate the signs of symptoms of type 1 Gaucher disease (Brumshtein et al. 2009, Luan et al. 2010, Alfonso et al. 2013).

2.2.2.3. Microbial β-glucosidases

Microbial β-glucosidases have been isolated from both bacterial and fungal species. Microbial β-glucosidases are localized as intracellular, extracellular and cell wall-associated enzymes. These enzymes are responsible for the hydrolysis of cellulose and carbohydrates for nutrient up-take and carbon recycling. In addition to these functions, β-glucosidases are responsible for cellulase gene induction, cell wall metabolism, host–pathogen interactions, and symbiotic association (Gutierrez-Correa et al. 1999, Roy et al. 2005, Chen et al. 2013, Ahmed et al. 2017). β-Glucosidases have been used in biotechnological applications such as biofuel production, the beverage industry, food industry, cassava detoxification and oligosaccharides synthesis. These β-glucosidases are preferred for industrial uses due to their robust activity, ease of production and the novel properties they exhibit (Ahmed et al. 2017).

2.2.3. β-glucosidase substrate specificity

The hydrolysis of natural polysaccharides or glycoconjugates by enzymes cocktails or purified enzymes is a complex task. Substrate specificity is dependent on the recognition of more than one glycosidic moiety, sometimes an aglycone moiety, and the linkages between them. Both polymers and commercial substrates are used to study substrate specificity of the hydrolases (Mazzaferro et al. 2012). β-glucosidases catalyse the hydrolysis of cellobiose and other glycosides (Gonzilez-candelas et al. 1990). Experimentally, the activity of β-glucosidase enzyme is measured using *p*-nitrophenol β-D-gucoparanoside (pNPG) or 4-methyl umbelliferyl β-D-glucoside (MUG) and in some cases cellobiose has been used. The artificial substrates pNPG and MUG are preferred over the natural substrate cellobiose, because several β-

glucosidases have shown a higher catalytic activity (K_{cat}) and Michaelis constant (K_m) on the artificial substrates than that of the natural substrate (Rani et al. 2012).

The kinetics of β-glucosidases are dependent on the configuration of the substrate and a conformational change is essential for cellobiose hydrolysis. The rigid structure of the substrate binding site of β-glucosidase accommodates the first glucose unit of cellobiose but not the second unit. The second glucose unit has to change conformation by rotation of the δ-bond of the glucoside in order to fit into the substrate binding site, while this conformation change is not necessary for pNPG. The conformational change needed for cellobiose hydrolysis is the reason for β-glucosidase's lower K_{cat} and K_m towards cellobiose (Rani et al. 2012).

2.2.4. β-glucosidase classification

β-glucosidase classification can either be based on substrate specificity or the nucleotide sequence identity scheme. Classification by substrate specificity can be (i) aryl β-glucosidase, which acts on the aryl-glucosides, (ii) true cellobiases, which hydrolyse cellobiose to glucose and (iii) broad substrate specificity β-glucosidases, which act on a wide substrate spectrum (Liew et al., 2018). Most β-glucosidases are classified as having broad spectrum specificity (Hong et al. 2009, Singhania et al. 2013, Yang et al. 2013, Asha et al. 2016, Florindo et al. 2018, Liew et al., 2018).

The classification based on nucleotide sequence identity is considered the best method. This method is based on the sequence and folding similarities (hydrophobic cluster analysis (HCA) of the enzymes. The sequence based classification is used to characterize the enzyme from a structural point of view while the substrate specificity method is useful for isolating and characterizing new or structurally undefined glucosidases (Singhania et al. 2013).

β-glucosidases have been classified into glucoside hydrolase (GH) families GH1, GH2, GH3, GH5, GH9, GH30 and GH116 based on their amino acid sequences. However, GH3 and GH5 are the most relevant β-glucosidases for biotechnological applications. This classification is based on the grouping criteria specified in the CaZy database (Harnpicharnchai et al. 2009, Fan et al. 2011, Diogo et al. 2015, Kar et al. 2017, Florindo et al. 2018). GH family enzymes, are enzymes that hydrolyse glycosidic bonds between carbohydrates or between carbohydrates and non-

carbohydrate moieties. Although GH enzymes have similar catalytic functions, the different families have different 3D folds (Pozzo et al. 2010, Garvey et al. 2013, Florindo et al. 2018). GH1 β-glucosidases are mainly found in archaebacteria, plants (Kar et al. 2017) and mammals. These β-glucosidases also possess galactosidase activity (Harnpicharnchai et al. 2009) while GH3 β-glucosidases are mostly found in bacteria, fungi and yeast (Harnpicharnchai et al. 2009, Yang et al. 2013). The conserved catalytic centre of GH1 enzymes typically has an amino acid sequence NEP or TENG with E being the catalytic amino acid (Figure 2.2) (Fan et al. 2011, Jun et al. 2018).

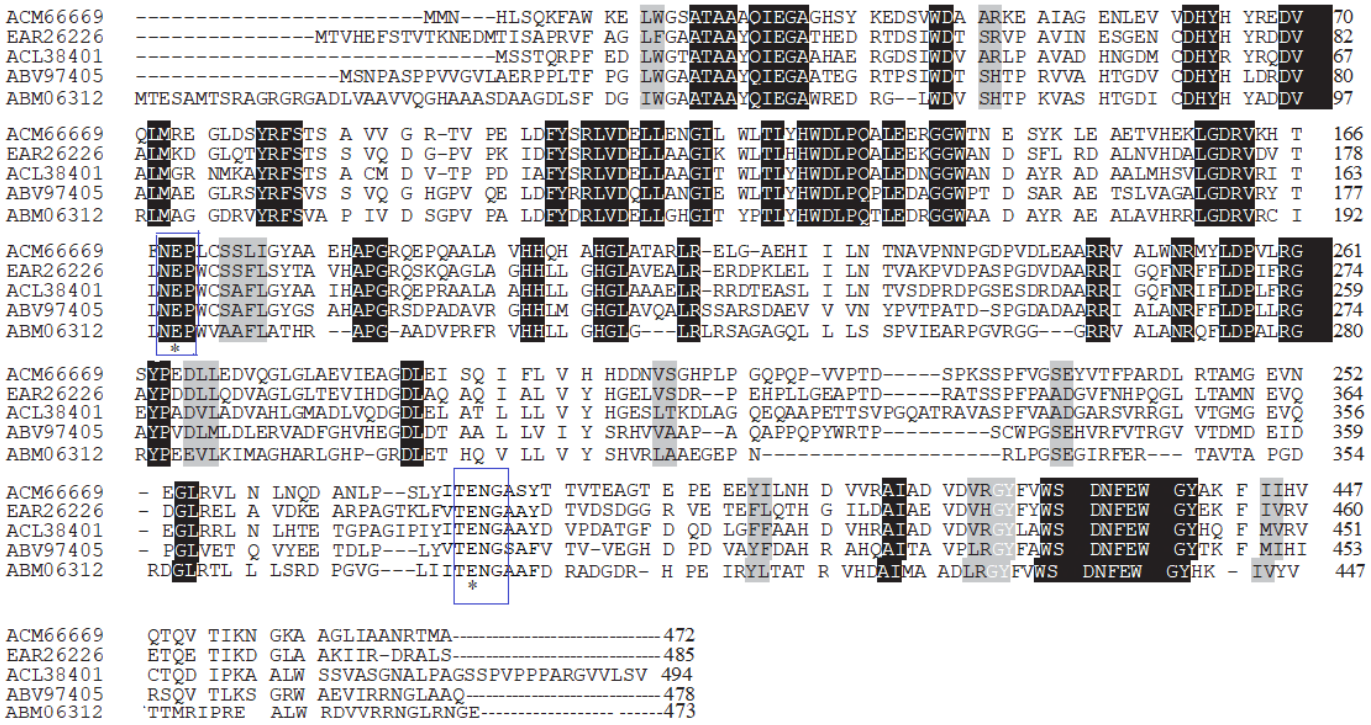


Figure 2.2. Sequence alignment of GH1 enzymes. *Micrococcus antarcticus* ACM66669), *Arthrobacter aurescens* TC1 (EAR26226), a marine actinobacterium (ACL38401), *Arthrobacter chlorophenolicus* (ABV97405) and *Salinispora arenicola* (ABM06312). The conserved regions for the catalytic central in GH1 are boxed in blue, and the catalytic amino acid is marked with an asterisk. Identical residues are shown on a black background and similar residues are shown in grey. The sequences were aligned using CLUSTAL W (Fan et al. 2011).

2.3. Thermophilic microorganisms as a source of thermostable enzymes

Thermophilic organisms are organisms found in extreme habitats such as geothermal geysers, hot springs and deep sea vents (Belkova et al., 2007). Thermophilic and extremophilic organisms are a good source of thermostable enzymes as their metabolic processes occur under extreme temperatures. The thermophilic organisms are adapted to tolerate extreme environments better than mesophilic organisms (Khare et al. 2015). Enzymes can be isolated from a variety of sources, however, microbial enzyme sources are preferred as they can easily be cultured in large quantities and they are capable of producing large quantities of enzyme in a short period of time (Acharya and Chaudhary 2012). Thermophilic bacteria have been found to be good sources of thermostable enzymes, including β-glucosidases. At 60°C β-glucosidases from bacteria have been found to exhibit higher enzyme activity compared to β-glucosidases of archaebacterial origin. This is true for β-glucosidases isolated from *Clostridium thermocellum*, *Microbispora bispora*, *Thermoanaerobicbacter brockii* and *Thermobifida fusca*. However, β-glucosidases from these bacterial species were unstable at 60°C (Hong et al., 2009)

2.4. Bioethanol production

Section 2.4 briefly outlines the sequential steps followed in bioethanol production from lignocellulosic biomass, namely; pre-treatment, saccharification and ultimately, fermentation or ethanol production.

2.4.1. Pre-treatment

Pre-treatment of lignocellulosic biomass is essential for effective enzymatic hydrolysis of plant material (Bhalla et al. 2013, Zhuang et al. 2016, Artola et al. 2019, Wakai et al. 2019). Untreated biomass contains indigestible polymers that yield less than 20% glucose upon hydrolysis. Pre-treatment improves the enzymatic hydrolysis process (Cheng and Timilsina 2011, Khare et al. 2015, Wakai et al. 2019), by disrupting the lignin structure and its linkages on plant material, removing hemicellulose, which limits access to cellulose by cellulases, reducing cellulose crystallinity, and reducing the degree of polymerisation of cellulose (Senthilkumar and Gunasekaran 2005, Balat et al. 2008). Enhanced lignocellulose hydrolysis makes pre-treatment one of the key steps for efficient saccharification, in biofuel

production (Khare et al. 2015, Zhuang et al. 2016). Lignocellulosic biomass pre-treatment increases the accessibility of the cellulose polymer to enzymatic hydrolysis. This step may lower the amount of enzyme required for saccharification. Several pre-treatment methods have been developed. These are grouped as follows; biological pre-treatment, physical pre-treatment and chemical treatment using acid, alkali, ozone or organic solvents (Liu et al. 2014, Zhuang et al. 2016, Wakai et al. 2019).

Each pre-treatment method has both advantages and disadvantages, however, the choice of pre-treatment method, is dependent on the biomass feedstock. The best pre-treatment method should provide high sugar yields, low inhibitor and by-product concentrations while being simple to scale up. Acid, alkali. liquid explosion and ammonia fibre expansion (AFEX) are considered good pre-treatment methods, based on the above criteria. Pre-treatment methods such as acid and liquid hot water have drawbacks. Steam explosion produces high amounts of inhibitors, while, alkaline pre-treatment typically produces less inhibitors but takes longer, with high salt formation (Khare et al. 2015).

2.4.1.1. Physical Pre-treatment

Physical Pre-treatment of lignocellulosic material can be achieved by either mechanical comminution, steam explosion or AFEX. Mechanical comminution employs a combination of chipping, grinding and milling of lignocellulosic material to particle size of 0.2-2 mm. This reduces the material's crystallinity while increasing the surface area for enzyme hydrolysis (Cheng and Timilsina 2011, Zhuang et al. 2016). Pre-treatment reduces the degree of polymerization of cellulose resulting in the formation of more cellulose chain ends, available to exoglucanases (Karimi and Taherzadeh 2016).

Steam explosion is one of the simplest and most environmentally friendly forms of pre-treatment methods. The method has a high efficiency and involves subjecting the lignocellulosic material to high temperature (160-260˚C) and pressure (2000-5000 kPa). The pressure is rapidly dropped to atmospheric pressure which causes an explosion of the lignocellulosic material (Mood et al. 2013). The explosion breaks down lignin causing the release of cellulose and hemicellulose. Hemicellulose is hydrolysed by acetic acid and other acids released during the steam explosion (Balat

et al. 2008). The advantage of using steam explosion are; the low energy requirement compared to mechanical comminution (mechanical comminution requires 70% more energy) and does not involve any recycling or environmental cost. Steam explosion is the most cost-effective methods for hardwood but less effective on softwood. The only disadvantage of using thermal pre-treatment, is the formation of compounds that may be inhibitory to the subsequent ethanol production process (Luiza et al. 2011).

AFEX is similar to steam explosion, the lignocellulosic material is soaked in liquid ammonia at high temperature (100˚C) for a period of time. The pressure is quickly dropped, breaking lignin bonds and increasing the material's permeability (Balat et al. 2008). This is a short and simple method, effective for the treatment of corn stover and sugarcane, but less effective on materials with high lignin content (Mood et al. 2013). This method does not hydrolyse hemicellulose but only serves to release hemicellulose and cellulose for enzymatic hydrolysis. AFEX yields high hydrolysis rates for pre-treated lignocellulose but requires efficient recovery of ammonia to make the process economical (Balat et al. 2008).

2.4.1.2. Chemical pre-treatment

Acid pre-treatment can be achieved through the use of sulphuric acid, hydrochloric acid, peracetic acid, nitric acid and phosphoric acid. Acid treatment can be done using either concentrated or dilute acids. Dilute acid methods are the most studied and widely used (Sánchez and Cardona 2008, Narra et al. 2015).This method hydrolyses hemicellulose and releases cellulose for enzymatic hydrolysis. Between 80 and 95% of sugars from hemicellulose can be recovered through the use of dilute acid.

Alkaline pre-treatment methods are generally effective for agricultural waste material such as rice straw and herbaceous crops. Alkaline pre-treatment method uses lower temperature and pressures, compared to most pre-treatment methods (Mood et al. 2013, Narra et al. 2015). Alkaline treatment swells the lignocelullosic material, increasing the internal surface area while decreasing crystallinity and disrupting lignin (Sánchez and Cardona 2008, Narra et al. 2015). The disruption and removal of lignin, and the altered state of cellulose amongst other things, results in cellulose that can be easily digested by enzymes (Bali et al. 2014). However, the drawback is that

some alkali is converted to irrecoverable salts or the salts are incorporated into the biomass. These salt formation effects do not occur with acid pre-treatment. The use of dilute NaOH is usually more economical than concentrated NaOH. Calcium hydroxide has also been used in alkaline pre-treatment of wheat straw, poplar wood and switch grass (Mood et al. 2013).

2.4.2. Enzymatic saccharification

Lignocellulosic biomass presents a sustainable feedstock for biofuel production. Lignocellulose saccharification is a critical step in bioethanol production, as fermentation product yields are largely dependent on the concentration of reducing sugars released. Enzymatic saccharification is an effective and environmentally friendly method for the production of reducing sugars from lignocellulosic biomass (Guo et al. 2018, Hyun et al. 2019). Cellulases and accessory enzymes hydrolyse cellulose to fermentable sugars. These sugars are then used as substrate by yeast for bioethanol production (Hyun et al. 2019).

An effective saccharification step requires a highly active enzyme cocktail, optimal hydrolysis temperature as well as optimal enzyme loading. An optimal enzyme cocktail for industrial saccharification should have sufficient activities of all three major cellulases for complete hydrolyses of lignocellulosic biomass to reducing sugars (Oleson and Schwartz 2019). Reaction temperature is crucial in enzymatic hydrolysis as high temperatures facilitate the breakdown of complex sugars to simple sugars and many cellulases are effective at higher temperatures (45 - 60˚C). The saccharification process is carried out in an open system with the saccharification temperature maintained mostly at 50˚C to prevent contamination by lactic acid bacteria. Depending on the optimal temperature of the cellulase enzyme cocktail used, the saccharification reactions are kept at temperatures below 60˚C to avoid enzyme denaturation (Oguro et al. 2019). Enzyme loading is yet another crucial parameter to be considered during saccharification. Very high enzyme loading leads to high enzyme costs as well as very high glucose titres which may in turn cause product inhibition of cellulases, resulting in low glucose yields. Optimization of enzyme loading, results in reduced enzyme costs, optimal glucose production and minimal hydrolysis time.

Pre-treatment of lignocellulosic biomass is an essential step in enzymatic hydrolysis. The pre-treatment step removes lignin and provides enzyme access to the cellulose component of the biomass (Xia et al. 2018a, Hyun et al. 2019). Increased glucose yields are observed following pre-treatment however, the pre-treatment step may introduce inhibitors that reduce the saccharification efficiency. Inhibitors can be removed or neutralized by adding extra enzymes, washing, a pressing step or the addition of neutralizing agents. The neutralizing step may lead to improved saccharification efficiency (Keshav et al. 2018, Oleson and Schwartz 2019). However, the choice of neutralization step used should be thoughtfully considered, as methods such as those requiring the addition of extra enzymes, lead to increased enzyme costs.

2.4.3. Fermentation

Conversion of reducing sugars produced during biomass hydrolysis to ethanol, is the last step in biofuel production process. Fermentation is carried out by industrial ethanol-producing yeast strains. The fermentation process can either be performed through separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) or semi simultaneous saccharification and fermentation process (SSSF) (Onay 2019).

The three methods each have their own advantages and drawbacks. SHF is mostly used during research and process optimisation, where parameters such as the effects of pH, temperature and time, are studied. SHF is difficult to operate, expensive and requires higher enzyme loading for tolerable ethanol yields. SSF is often more advantageous due to the low costs associated with the process, because reactions are generally easier and occur in a single bioreactor, making the process less expensive than the SHF. The drawbacks of SSF include difficulties associated with downstream processing, where yeast and enzyme recovery creates a challenge for scaling up. SSSF eliminates some of the disadvantages associated with SSF. The process is characterized by a short pre-saccharification stage followed by SSF. An optimised pre-saccharification step results in higher product yields during subsequent SSF (Onay 2019).

Effective fermentation requires the availability of ethanol tolerant microbial strains that can utilize both hexoses (glucose) and pentoses (xylose). Naturally occurring yeast species that can effectively ferment xylose for bioethanol production include *Scheffersomyces stipitis*, *Candida shetahae* and *Pachchysolen tannophilus*. Additionally, *S. stipitis* has shown potential for industrial applications due to its ability to ferment both hexoses and pentoses (Keshav et al. 2018).

Yeasts from the genus *Saccharomyces* are commonly used as ethanol producers from hexose sugars. However, other microorganisms such as *Kluyveromyces* marxianus and even the bacterial strain, *Zymomonas mobilis* have been used (Alfenore and Molina-Jouve 2016).

2.5. References

Acharya, S. and Chaudhary, A., 2012. Bioprospecting thermophiles for cellulase production: A review. *Brazilian Journal of Microbiology*. 43 (3), 844–856.

Ahamed, A. and Vermette, P., 2008. Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor. *Biochemical Engineering Journal*, 42 (1), 41–46.

Ahmed, A., Aslam, M., Ashraf, M., Nasim, F., Batool, K., and Bibi, A., 2017. Microbial β -Glucosidases : Screening , Characterisation , Cloning and Applications. *Journal of Applied & Environmental Microbiology*, 5 (2), 57–73.

Alfenore, S. and Molina-Jouve, C., 2016. Current status and future prospects of conversion of lignocellulosic resources to biofuels using yeasts and bacteria. *Process Biochemistry*, 51 (11), 1747–1756. Available from: http://dx.doi.org/10.1016/j.procbio.2016.07.028. Online.

Alfonso, P., Andreu, V., Pino-angeles, A., Moya-, A. A., García-moreno, M. I., Rodríguez-rey, J. C., and Sµnchez-jimønez, F., 2013. Bicyclic Derivatives of l -Idonojirimycin as Pharmacological Chaperones for Neuronopathic Forms of Gaucher Disease, (Scheme 1), 1–7.

Almeida, M. N. De, Falkoski, D. L., Guimarães, V. M., and Rezende, S. T. De, 2019. Industrial Crops & Products Study of gamba grass as carbon source for cellulase production by Fusarium verticillioides and its application on sugarcane bagasse sacchari fi cation. *Industrial Crops & Products*, 133 (March), 33–43. Available from: https://doi.org/10.1016/j.indcrop.2019.03.008. Online

Alves, L. D. F., Meleiro, L. P., Silva, R. N., Westmann, C. A., Guazzaroni, M., and Hickey, W. J., 2018. Novel Ethanol- and 5-Hydroxymethyl Retrieved From a Brazilian Secondary Atlantic Forest Soil Metagenome, 9, 1–17.

Artola, A., Marín, M., and Antoni, S., 2019. Production and recovery of cellulases through solid-state fermentation of selected lignocellulosic wastes. Journal of Cleaner Production, 209, 937–946.

Asha, P., Jose, D., and Singh, I. S. B., 2016. Bioresource Technology Purification

and characterisation of processive-type endoglucanase and b -glucosidase from Aspergillus ochraceus MTCC 1810 through saccharification of delignified coir pith to glucose. *Bioresource Technology*, 213, 245–248. Available from: http://dx.doi.org/10.1016/j.biortech.2016.03.013. Online.

Ay, F., Karaoglu, H., Inan, K., Canakci, S., and Belduz, A. O., 2011. Cloning, purification and characterisation of a thermostable carboxylesterase from Anoxybacillus sp. PDF1. *Protein Expression and Purification*, 80 (1), 74–79. Available from: http://dx.doi.org/10.1016/j.pep.2011.06.019. Online.

Bai, A., Zhao, X., Jin, Y., Yang, G., and Feng, Y., 2013. A novel thermophilic β-glucosidase from Caldicellulosiruptor bescii: Characterisation and its synergistic catalysis with other cellulases. *Journal of Molecular Catalysis B: Enzymatic*, 85–86, 248–256.

Balat, M., Balat, H., and Öz, C., 2008. Progress in bioethanol processing. *Progress in Energy and Combustion Science*.

Bali, G., Meng, X., Deneff, J. I., Sun, Q., and Ragauskas, A. J., 2014. The Effect of Alkaline Pretreatment Methods on Cellulose Structure and Accessibility. *ChenSusChem Communications*, 2200, 1–6. DOI: 10.1002/cssc.201402752. Online

Banerjee, D., Mukherjee, S., Pal, S., and Khowala, S., 2016. Enhanced saccharification efficiency of lignocellulosic biomass of mustard stalk and straw by salt pretreatment. *Industrial Crops and Products*, 80, 42–49.

Barr, C. J., Mertens, J. A., and Schall, C. A., 2012. Critical cellulase and hemicellulase activities for hydrolysis of ionic liquid pretreated biomass. *Bioresource Technology*, 104, 480–485.

Bartholomew, J. W. and Mittwer, T. O. D., 1883. municipal hospital, 1–29.

Belkova, N, Tazaki, K, , Zakharona, R. J, Zakharona, V, P., 2007. activity of bacteria in water of hot springs from southern and central Kamchatskaya Geothermal Provinces, Kamchatka Peninsula, Russia. *Microbial Research*, 162, 99–107.

Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Markov, A., Skomarovsky, A., Okunev, O., Gusakov, A., Maximenko, V., Gregg, D., Sinitsyn, A., and Saddler, J., 2005.

Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates - Evidence for the role of accessory enzymes. *Enzyme and Microbial Technology*, 37 (2), 175–184.

Bernardes, A., Pellegrini, V. O. A., Curtolo, F., Camilo, C. M., Mello, B. L., and Johns, M. A., 2019. Carbohydrate binding modules enhance cellulose enzymatic hydrolysis by increasing access of cellulases to the substrate. *Carbohydrate Polymers*, 211, 57–68. Available from: https://doi.org/10.1016/j.carbpol.2019.01.108. Online.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., and Sani, R. K., 2013a. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, 128, 751–759. Available from: http://dx.doi.org/10.1016/j.biortech.2012.10.145. Online.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., and Sani, R. K., 2013b. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*.

Brijwani, K., Oberoi, H. S., and Vadlani, P. V., 2010. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochemistry*, 45 (1), 120–128.

Brumshtein, B., Aguilar-moncayo, M., García-moreno, M. I., Mellet, O., Fernµndez, J. M. G., Silman, I., Shaaltiel, Y., Aviezer, D., and Sussman, J. L., 2009. Synthesis, Biological Evaluation , and Crystal Structure in Complex with Acid β-Glucosidase, 1480–1485.

Chan, C. S., Sin, L. L., Chan, K. G., Shamsir, M. S., Manan, F. A., Sani, R. K., and Goh, K. M., 2016. Characterisation of a glucose-tolerant β-glucosidase from Anoxybacillus sp. DT3-1. *Biotechnology for Biofuels*, 9 (1), 1–11.

Chen, M., Qin, Y., Cao, Q., Liu, G., Li, J., Li, Z., Zhao, J., and Qu, Y., 2013. Promotion of extracellular lignocellulolytic enzymes production by restraining the intracellular β-glucosidase in Penicillium decumbens. *Bioresource Technology*, 137, 33–40.

Cheng, J. J. and Timilsina, G. R., 2011. Status and barriers of advanced biofuel

technologies: A review. *Renewable Energy*, 36 (12), 3541–3549.

da Costa, J. A., Marques, J. E., Gonçalves, L. R. B., and Rocha, M. V. P., 2015. Enhanced enzymatic hydrolysis and ethanol production from cashew apple bagasse pretreated with alkaline hydrogen peroxide. *Bioresource Technology*, 179, 249–259.

Daroit, D. J., Silveira, S. T., Hertz, P. F., and Brandelli, A., 2007. Production of extracellular β-glucosidase by *Monascus purpureus* on different growth substrates. *Process Biochemistry*, 42, 904–908.

Diogo, A., Cota, J., Corre, T. L. R., Hoffmam, Z. B., Garcia, W., Oliveira, L. C., Prade, R. A., and Squina, F. M., 2015. Comparative analysis of three hyperthermophilic GH1 and GH3 family members with industrial potential. *New Biotechnology*, 32, 13–20.

Fan, H. X., Miao, L. L., Liu, Y., Liu, H. C., and Liu, Z. P., 2011. Gene cloning and characterisation of a cold-adapted β-glucosidase belonging to glycosyl hydrolase family 1 from a psychrotolerant bacterium *Micrococcus antarcticus*. *Enzyme and Microbial Technology*, 49 (1), 94–99. Available from: http://dx.doi.org/10.1016/j.enzmictec.2011.03.001. Online.

Florindo, R. N., Souza V. P., Mutti, H. S., Camilo, C., Manzine L. R., and Marana, S. R., 2018. Structural insight into beta-glucosidase transglycosylation based on biochemical, structural and computational analysis of two GH1 enzymes from Tricoderma hararzium. *New Biotechnology*, 40, 218–227.

Garvey, M., Klose, H., Fischer, R., Lambertz, C., and Commandeur, U., 2013. Cellulases for biomass degradation: Comparing recombinant cellulase expression platforms. *Trends in Biotechnology*, 31, 581-593

Ghose, T. K., 1987. Measurement of cellular activities. *Pure & Applied. Chem*, 59, 257–268.

Goh, K. M. and Kahar, U. M., 2013. Recent discoveries and applications of Anoxybacillus, 97, 1475–1488.

Gonzilez-candelas, L., Ramn, D., Polaina, J., Agroquimica, I. De, and Alimenws, T. De, 1990. Sequences and homology analysis of two genes encoding p-

glneosidases from *Bacillus polymyxa*. Gene, 95, 31–38.

Guo, H., Chang, Y., and Lee, D., 2018. Enzymatic sacchari fi cation of lignocellulosic biorefinery : Research focuses. Bioresource Technology, 252, 198–215.

Gutierrez-Correa, M., Portal, L., Moreno, P., and Tengerdy, R. P., 1999. Mixed culture solid substrate fermentation of Trichoderma reesei with Aspergillus niger on sugar cane bagasse. *Bioresource Technology*, 68 (2), 173–178.

Haki, G. D. and Rakshit, S. K., 2003. Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, 89, 17-34

Harnpicharnchai, P., Champreda, V., Sornlake, W., and Eurwilaichitr, L., 2009. A thermotolerant β-glucosidase isolated from an endophytic fungi, Periconia sp., with a possible use for biomass conversion to sugars. *Protein Expression and Purification*, 67 (2), 61–69.

Hong, M. R., Kim, Y. S., Park, C. S., Lee, J. K., Kim, Y. S., and Oh, D. K., 2009. Characterisation of a recombinant β-glucosidase from the thermophilic bacterium Caldicellulosiruptor saccharolyticus. *Journal of Bioscience and Bioengineering*, 108 (1), 36–40.

Hyun, D., Min, H., Hoon, Y., Sukyai, P., and Heon, K., 2019. Bioresource Technology Pretreatment and enzymatic saccharification of oak at high solids loadings to obtain high titers and high yields of sugars. *Bioresource Technology*, 284, 391–397. Available from: https://doi.org/10.1016/j.biortech.2019.03.134. Online.

Ichikawa, S., Ichihara, M., Ito, T., Isozaki, K., Kosugi, A., and Karita, S., 2019. Glucose production from cellulose through biological simultaneous enzyme production and sacchari fi cation using recombinant bacteria expressing the b - glucosidase gene. *Journal of Bioscience and Bioengineering*, 127 (3), 340–344. Available from: https://doi.org/10.1016/j.jbiosc.2018.08.008. Online.

Joo, A. R., Jeya, M., Lee, K. M., Lee, K. M., Moon, H. J., Kim, Y. S., and Lee, J. K., 2010. Production and characterisation of β-1,4-glucosidase from a strain of Penicillium pinophilum. *Process Biochemistry*, 45 (6), 851–858.

Jun, K., Lim, L., Ying, H., Chan, K., Shahir, M., and Mau, K., 2018. International

Journal of Biological Macromolecules Purification and characterisation of a novel GH1 beta-glucosidase from *Jeotgalibacillus malaysiensis*. *International Journal of Biological Macromolecules*, 115, 1094–1102. Available from: https://doi.org/10.1016/j.ijbiomac.2018.04.156. Online.

Kalyani, D., Lee, K. M., Kim, T. S., Li, J., Dhiman, S. S., Kang, Y. C., and Lee, J. K., 2013. Microbial consortia for saccharification of woody biomass and ethanol fermentation. *Fuel*, 107, 815–822.

Kar, B., Verma, P., Patel, G. K., and Sharma, A. K., 2017. Molecular cloning, characterisation and in silico analysis of a thermostable β-glucosidase enzyme from Putranjiva roxburghii with a significant activity for cellobiose. *Phytochemistry*, 140, 151–165. Available from: http://dx.doi.org/10.1016/j.phytochem.2017.04.021. Online.

Karimi, K. and Taherzadeh, M. J., 2016. Bioresource Technology A critical review on analysis in pretreatment of lignocelluloses : Degree of polymerization , adsorption / desorption , and accessibility. *Bioresource Technology*, 203, 348–356. Available from: http://dx.doi.org/10.1016/j.biortech.2015.12.035. Online.

Keshav, P. K., Banoth, C., and Anthappagudem, A., 2018. Industrial Crops & Products Sequential acid and enzymatic saccharification of steam exploded cotton stalk and subsequent ethanol production using Scheffersomyces stipitis NCIM 3498. *Industrial Crops & Products*, 125 (September), 462–467. Available from: https://doi.org/10.1016/j.indcrop.2018.08.060. Online.

Khalil, A. and Sivakumar, N., 2015. Genome Sequence of Anoxybacillus flavithermus Strain AK1 , a Thermophile Isolated from a Hot Spring in Saudi Arabia, 3, 207713-207713.

Khare, S. K., Pandey, A., and Larroche, C., 2015. Current perspectives in enzymatic saccharification of lignocellulosic biomass. *Biochemical Engineering Journal*, 102, 38–44.

Khattak, W. A., Khan, T., Ha, J. H., Ul-Islam, M., Kang, M. K., and Park, J. K., 2013. Enhanced production of bioethanol from waste of beer fermentation broth at high temperature through consecutive batch strategy by simultaneous saccharification and fermentation. *Enzyme and Microbial Technology*, 53 (5),

322–330.

Kikani, B. A. and Singh, S. P., 2012. The stability and thermodynamic parameters of a very thermostable and calcium-independent α-amylase from a newly isolated bacterium, Anoxybacillus beppuensis TSSC-1. *Process Biochemistry*, 47 (12), 1791–1798. Available from: http://dx.doi.org/10.1016/j.procbio.2012.06.005. Online.

Kim, Y., Mosier, N. S., Ladisch, M. R., Ramesh Pallapolu, V., Lee, Y. Y., Garlock, R., Balan, V., Dale, B. E., Donohoe, B. S., Vinzant, T. B., Elander, R. T., Falls, M., Sierra, R., Holtzapple, M. T., Shi, J., Ebrik, M. A., Redmond, T., Yang, B., Wyman, C. E., and Warner, R. E., 2011. Comparative study on enzymatic digestibility of switchgrass varieties and harvests processed by leading pretreatment technologies. *Bioresource Technology*, 102 (24), 11089–11096.

Kumar, S. and Nussinov, R., 2001. Cellular and Molecular Life Sciences How do thermophilic proteins deal with heat ? **, 58, 1216–1233.

Lewis Liu, Z., Weber, S. A., Cotta, M. A., and Li, S. Z., 2012. A new β-glucosidase producing yeast for lower-cost cellulosic ethanol production from xylose-extracted corncob residues by simultaneous saccharification and fermentation. *Bioresource Technology*, 104, 410–416.

Li, C., Li, D., Feng, J., Fan, X., Chen, S., Zhang, D., and He, R., 2019. Duckweed (Lemna minor) is a novel natural inducer of cellulase production in Trichoderma reesei. *Journal of Bioscience and Bioengineering*, 127 (4), 486–491. Available from: https://doi.org/10.1016/j.jbiosc.2018.09.017. Online

Liew, K. J., Lim, L., woo, H. Y., Chan, K., Shamsir, M., Mau, S. K.G., 2018. Purification and characterisation of a novel GH1 beta-glucosidase from Jeotgalibacillus malaysiensis. *International Journal of Biological Macromolecules*, 115 (https://doi.org/10.1016/j.ijbiomac.2018.04.156), 1094–1102.

Lim, Y. L., Chan, K. G., Ee, R., Belduz, A. O., Canakci, S., Kahar, U. M., Yaakop, A. S., and Goh, K. M., 2015. Complete genome of the potential thermozyme

producer Anoxybacillus gonensis G2Tisolated from the Gönen hot springs in Turkey. *Journal of Biotechnology*, 212, 65–66. Available from: http://dx.doi.org/10.1016/j.jbiotec.2015.08.007. Online.

Liu, J. F., Cao, Y., Yang, M. H., Wang, X. J., Li, H. Q., and Xing, J. M., 2014. Enhanced saccharification of lignocellulosic biomass with 1-allyl-3-methylimidazolium chloride (AmimCl) pretreatment. *Chinese Chemical Letters*, 25 (11), 1485–1488.

Lu, M., Li, J., Han, L., and Xiao, W., 2019. Bioresource Technology An aggregated understanding of cellulase adsorption and hydrolysis for ball- milled cellulose. *Bioresource Technology*, 273 (October 2018), 1–7. Available from: https://doi.org/10.1016/j.biortech.2018.10.037. Online.

Luan, Z., Higaki, K., Aguilar-moncayo, M., and Li, L., 2010. A Fluorescent sp 2 - Iminosugar With Pharmacological Chaperone Activity for Gaucher Disease : Synthesis and Intracellular Distribution Studies, 01605, 2453–2464.

Luiza, A., Santos, F., Yuri, K., Kawase, F., Luiz, G., and Coelho, V., 2011. The Journal of Supercritical Fluids Enzymatic saccharification of lignocellulosic materials after treatment with supercritical carbon dioxide. *The Journal of Supercritical Fluids*, 56 (3), 277–282. Available from: http://dx.doi.org/10.1016/j.supflu.2010.10.044. Online.

Manickam, N. K., Rajarathinam, R., and Muthuvelu, K. S., 2018. Bioresource Technology New insight into the e ff ect of fungal mycelia present in the bio-pretreated paddy straw on their enzymatic sacchari fi cation and optimization of process parameters, 267 (May), 291–302.

Mazzaferro, L. S., Piñuel, L., Erra-balsells, R., Giudicessi, S. L., and Breccia, J. D., 2012. and its application to the synthesis of the new fluorogenic substrate. *Carbohydrate Research*, 347 (1), 69–75. Available from: http://dx.doi.org/10.1016/j.carres.2011.11.008. Online.

Meehnian, H., Jana, A. K., and Maiti, M., 2016. Effect of particle size , moisture content , and supplements on selective pretreatment of cotton stalks by Daedalea flavida and enzymatic saccharification. *Biotech*, 6 (2), 1–13.

Mei, H., Xia, D., Zhao, Q., Zhang, G., and Qiu, Z., 2016. Molecular cloning , expression , puri fi cation and characterisation of a novel cellulase gene (Bh-EGase I) in the beetle Batocera hors fi eldi. *Gene*, 576 (1), 45–51. Available from: http://dx.doi.org/10.1016/j.gene.2015.09.057. Online.

Menon, V. and Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*.

Mood, S. H., Golfeshan, A. h., Tabatabaei, M., Jouzani, G. S., Najafi, G. H., Gholami, M., and Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renewable and Sustainable Energy Reviews*, 27, 77-93.

Narra, M., James, J. P., and Balasubramanian, V., 2015. Simultaneous saccharification and fermentation of delignified lignocellulosic biomass at high solid loadings by a newly isolated thermotolerant Kluyveromyces sp. for ethanol production. *Bioresource Technology*, 179, 331–338.

Ndlovu, T. M. and Wyk, J. P. H. Van, 2019. Relative saccharification of waste paper during successive treatment with garden snail (Cornu aspersum) cellulase. *Sustainable Chemistry and Pharmacy*, 11 (December 2018), 54–60. Available from: https://doi.org/10.1016/j.scp.2018.12.003. Online.

Nigam, P. S. and Singh, A., 2011. Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*.

Oguro, Y., Nakamura, A., and Kurahashi, A., 2019. Effect of temperature on sacchari fi cation and oligosaccharide production efficiency in koji amazake, 127 (5), 570–574.

Oleson, K. R. and Schwartz, D. T., 2019. Bioresource Technology Reports Douglas-fir extractives inhibit saccharification. *Bioresource Technology Reports*, 5 (October 2018), 335–338. Available from: https://doi.org/10.1016/j.biteb.2018.10.001. Online

Onay, M., 2019. Bioethanol production via different saccharification strategies from *H. tetrachotoma* ME03 grown at various concentrations of municipal wastewater

in a flat-photobioreactor. *Fuel*, 239, 1315–1323. Available from: https://doi.org/10.1016/j.fuel.2018.11.126. Online.

Pal, S., Banik, S. P., Ghorai, S., Chowdhury, S., and Khowala, S., 2010. Purification and characterisation of a thermostable intra-cellular β-glucosidase with transglycosylation properties from filamentous fungus Termitomyces clypeatus. *Bioresource Technology*, 101 (7), 2412–2420.

Pamella Santa-Rosa., Anita Souza, Rosemary Roque, Andrade Filho, Spartaco Edmar, Adolfo Mota, C. N.-S., 2017. Production of β-glucosidase and CMCase thermostables by Penicillium Sp. LMI01 isolated in the Amazon region. Electronic Journal of Biotechnology. *Electronic journal of biotechnology*, 31 (31. 10.1016/j.ejbt.2017.11.005.).

Pancha, I., Chokshi, K., Maurya, R., Bhattacharya, S., Bachani, P., and Mishra, S., 2016. Comparative evaluation of chemical and enzymatic saccharification of mixotrophically grown de-oiled microalgal biomass for reducing sugar production. *Bioresource Technology*, 204, 9–16.

Patel, A. K., Singhania, R. R., Sim, S. J., and Pandey, A., 2019. Thermostable cellulases : Current status and perspectives. Bioresource Technology 279, 385–392.

Poli, A., Esposito, E., Lama, L., Orlando, P., Nicolaus, G., de Appolonia, F., Gambacorta, A., and Nicolaus, B., 2006. Anoxybacillus amylolyticus sp. nov., a thermophilic amylase producing bacterium isolated from Mount Rittmann (Antarctica). *Systematic and Applied Microbiology*, 29 (4), 300–307.

Pozzo, T., Pasten, J. L., Karlsson, E. N., and Logan, D. T., 2010. Structural and Functional Analyses of β-Glucosidase 3B from Thermotoga neapolitana: A Thermostable Three-Domain Representative of Glycoside Hydrolase 3. *Journal of Molecular Biology*, 397 (3), 724–739.

Rani, R., Kumar, A., Sukumaran, R. K., Larroche, C., and Pandey, A., 2012. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource Technology*, 1–8.

Rastogi, G., Bhalla, A., Adhikari, A., Bischoff, K. M., Hughes, S. R., Christopher, L.

P., and Sani, R. K., 2010. Characterisation of thermostable cellulases produced by Bacillus and *Geobacillus strains*. *Bioresource Technology*, 101 (22), 8798–8806.

Roy, A., Kucukural, A., and Zhang, Y., 2010. I-TASSER : a unified platform for automated protein structure and function prediction, 5 (4), 725–738.

Roy, P., Mishra, S., and Chaudhuri, T. K., 2005. Cloning, sequence analysis, and characterisation of a novel β-glucosidase-like activity from *Pichia etchellsii*. *Biochemical and Biophysical Research Communications*, 336 (1), 299–308.

Saha, K., Verma, P., Sikder, J., Chakraborty, S., and Curcio, S., 2019. Synthesis of chitosan-cellulase nanohybrid and immobilization on alginate beads for hydrolysis of ionic liquid pretreated sugarcane bagasse. *Renewable Energy*, 133, 66–76. Available from: https://doi.org/10.1016/j.renene.2018.10.014. Online.

Sánchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, 27 (2), 185-194.

Sánchez, Ó. J. and Cardona, C. A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*, 99 (13), 5270-5295.

Senthilkumar, V. and Gunasekaran, P., 2005. Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. *Journal of Scientific and Industrial Research*, 64 (11), 845-853.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., and Pandey, A., 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource Technology*, 127, 500-507.

Srivastava, N., Srivastava, M., Mishra, P. K., Gupta, V. K., and Molina, G., 2018. Applications of fungal cellulases in biofuel production : Advances and limitations. *Renewable and Sustainable Energy Reviews*, 82 (August 2017), 2379–2386. Available from: https://doi.org/10.1016/j.rser.2017.08.074. Online.

Sukumaran, R. K., Singhania, R. R., Mathew, G. M., and Pandey, A., 2009. Cellulase production using biomass feed stock and its application in

lignocellulose saccharification for bio-ethanol production. *Renewable Energy*, 34 (2), 421–424.

Sukumaran, R. K., Singhania, R. R., and Pandey, A., 2005. Microbial cellulases Production , applications and challenges. *Bioresource Technology*, 64 , 832–844.

Sumiyo, K. S., Wakayama, J., Tsukamoto, K., Hayashi, N., Miyazaki, Y., Ohmori, H., Tajima, K., and Yokoyama, H., 2013. Degradation of microcrystalline cellulose and non-pretreated plant biomass by a cell-free extracellular cellulase/hemicellulase system from the extreme thermophilic bacterium Caldicellulosiruptor bescii. *Seibutsu-kogaku Kaishi*, 115 (1), 64–70.

Tengerdy, R. P. and Szakacs, G., 2003. Bioconversion of lignocellulose in solid substrate fermentation. *Biochemical Engineering Journal*, 13, 169-179.

Terme, A., 2009. Anoxybacillus thermarum sp . nov ., a novel thermophilic bacterium isolated from thermal mud in Euganean hot springs , 867–874.

Travaini, R., Martín-Juárez, J., Lorenzo-Hernando, A., and Bolado-Rodríguez, S., 2016. Ozonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. *Bioresource Technology*, 199, 2-12.

Ubhayasekera, W., 2005. *Structural Studies of Cellulose and Chitin Active Enzymes*. Advances in Applied Microbiology, 70, 1-55

Van Dyk, J. S., and Pletschke, B. I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy, Biotechnology Advances, 30 (6), 1458–1480,.

Veysi Okumus, S. O., 2015. Isolation of a Novel Thermophilic Anoxybacillus flavithermus SO-13, Production, Characterisation and Industrial Applications of its Thermostable alpha-Amylase. *Journal of Bioprocessing & Biotechniques*, 05 (07). Available from: https://www.omicsonline.org/open-access/isolation-of-a-novel-thermophilic-anoxybacillus-flavithermus-so13-production-characterisation-and-industrial-applications-of-itsthermostable-amylase-2155-9821-1000237.php?aid=56297. Online.

Wakai, S., Nakashima, N., Ogino, C., Tsutsumi, H., and Hata, Y., 2019. Bioresource Technology Modified expression of multi-cellulases in a filamentous fungus Aspergillus oryzae. *Bioresource Technology*, 276, 146–153. Available from: https://doi.org/10.1016/j.biortech.2018.12.117. Online.

Wang, Y., Wang, X., Tang, R., Yu, S., Zheng, B., and Feng, Y., 2010. A novel thermostable cellulase from Fervidobacterium nodosum. *Journal of Molecular Catalysis B: Enzymatic*, 66 (3–4), 294–301.

Wei, Y., Acquah, C., Obeng, E. M., Dullah, E. C., Jeevanandam, J., and Ongkudon, C. M., 2019. Biochimie Parametric study of immobilized cellulase-polymethacrylate particle for the hydrolysis of carboxymethyl cellulose. *Biochimie*, 157, 204–212. Available from: https://doi.org/10.1016/j.biochi.2018.11.019. Online.

Wen, Z., Liao, W., and Chen, S., 2005. Production of cellulase/β-glucosidase by the mixed fungi culture Trichoderma reesei and Aspergillus phoenicis on dairy manure. *Process Biochemistry*, 40 (9), 3087–3094.

Xia, Y., Yang, L., and Xia, L., 2018a. High-level production of a fungal β -glucosidase with application potentials in the cost-effective production of Trichoderma reesei cellulase. *Process Biochemistry*, 70, 55–60. Available from: https://doi.org/10.1016/j.procbio.2018.03.031. Online.

Xia, Y., Yang, L., and Xia, L., 2018b. High-level production of a fungal β -glucosidase with application potentials in the cost-effective production of Trichoderma reesei cellulase. *Process Biochemistry*, 70, 55–60. Available from: https://doi.org/10.1016/j.procbio.2018.03.031. Online.

Xue, D. S., Chen, H. Y., Ren, Y. R., and Yao, S. J., 2012. Enhancing the activity and thermostability of thermostable β-glucosidase from a marine *Aspergillus niger* at high salinity. *Process Biochemistry*, 47 (4), 606–611.

Yanase, H., Yamamoto, K., Sato, D., and Okamoto, K., 2005. Ethanol production from cellobiose by Zymobacter palmae carrying the Ruminocuccus albus β-glucosidase gene. *Journal of Biotechnology*, 118 (1), 35–43.

Yang, J., Zhang, Y., and I-tasser, T., 2015. I-TASSER server : new development for

protein structure and function predictions, 43 (April), 174–181.

Yang, S., Hua, C., Yan, Q., Li, Y., and Jiang, Z., 2013. Biochemical properties of a novel glycoside hydrolase family 1 β-glucosidase (PtBglu1) from Paecilomyces thermophila expressed in Pichia pastoris. *Carbohydrate Polymers*, 92 (1), 784–791.

Yassin, M. A., Gad, A. A. M., Ghanem, A. F., and Rehim, M. H. A., 2019. Green synthesis of cellulose nanofibers using immobilized cellulase. *Carbohydrate Polymers*, 205 (October 2018), 255–260. Available from: https://doi.org/10.1016/j.carbpol.2018.10.040. Online.

Yeoman, C. J., Han, Y., Dodd, D., Schroeder, C. M., Mackie, R. I., and Cann, I. K. O., 2010. Thermostable enzymes as biocatalysts in the biofuel industry. *Advances in applied microbiology*, 70, 1-55.

You, A., Be, M. A. Y., and In, I., 2018. Review : Engineering of thermostable enzymes for industrial applications. APL Bioeng. 2, 011501 (2018); https://doi.org/10.1063/1.4997367

Zhang, J., Wang, D., Pan, J., Wang, J., Zhao, H., Li, Q., and Zhou, X., 2014. Efficient resveratrol production by immobilized β-glucosidase on cross-linked chitosan microsphere modified by l-lysine. *Journal of Molecular Catalysis B: Enzymatic*, 104, 29–34.

Zhang, J., Zhong, Y., Zhao, X., and Wang, T., 2010. Development of the cellulolytic fungus Trichoderma reesei strain with enhanced β-glucosidase and filter paper activity using strong artifical cellobiohydrolase 1 promoter. *Bioresource Technology*, 101 (24), 9815–9818.

Zheng, W., Chen, X., Xue, Y., Hu, J., Gao, M., and Fai, Y., 2017. The influence of soluble polysaccharides derived from rice straw upon cellulase production by Trichoderma reesei. *Process Biochemistry*, 61, 130–136. Available from: http://dx.doi.org/10.1016/j.procbio.2017.06.007. Online.

Zhou, Q., Xu, J., Kou, Y., Lv, X., Zhang, X., Zhao, G., Zhang, W., Chen, G., and Liu, W., 2012. Differential Involvement of NL-Glucosidases from *Hypocrea jecorina* in Rapid Induction of Cellulase Genes by Cellulose and Cellobiose, 11 (11), 1371–

1381.

Zhuang, X., Wang, W., Yu, Q., Qi, W., Wang, Q., Tan, X., Zhou, G., and Yuan, Z., 2016. Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products. *Bioresource Technology*, 199, 68-75.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF A THERMOPHILIC BACTERIAL STRAIN

3.1. Introduction

Microbial cellulases have gained considerable interest in recent years in the bioethanol industry for their use in the conversion of biomass to glucose (Harnpicharnchai et al. 2009). Currently, fungal cellulases are the most widely used in the industry, as fungal strains secrete cellulolytic enzymes of higher activity than bacterial strains. *Trichoderma reesei* is currently the preferred producer of cellulases (Zhang et al. 2010, Sumiyo et al. 2013, Bernardes et al. 2019). However, *T. reesei* cellulases have relatively low levels of β-glucosidase which is often insufficient for the complete hydrolysis of cellulose to glucose (Wen et al. 2005, Ahamed and Vermette 2008, Harnpicharnchai et al. 2009, Xia et al. 2018b). Thus, β-glucosidase is usually added to commercial cellulase preparations so as to enhance its total cellulolytic activity (Sánchez and Cardona 2008, Harnpicharnchai et al. 2009, Florindo et al. 2018).

There is an increasing need to find thermostable β-glucosidase as most commercial β-glucosidases of fungal origin have low stability at high temperatures (Chan et al. 2016). This is a concern as the saccharification step in biofuel production requires high temperatures. The use of thermostable enzymes for the hydrolysis of cellulosic biomass offers several advantages that include; improved hydrolysis rates, reduced enzyme loading, higher mass-transfer rates leading to better substrate solubility, lowered risk of contamination as well as increased flexibility on process design which in turn lowers production costs (Rastogi et al. 2010, Bhalla et al. 2013b).

Thermophilic microorganisms are organisms that grow at temperatures between 50 - 80˚C. Thermophiles are adapted to survive at high temperatures by producing proteins that are thermostable and resist denaturation (Kumar and Nussinov 2001). Some thermophiles produce proteins called chaperons that help stabilize thermostable proteins, by refolding the proteins to their original state after denaturisation (Haki and Rakshit 2003). Thermophilic organisms are generally fast growing and they can tolerate extreme temperature conditions (Goh and Kahar

2013).Thermophilic organisms are a good source of thermostable enzymes and have been isolated from a wide range of habitats, including compost heap, soil, wastewater (Rastogi et al. 2010) as well as hot springs and geysers (Wang et al. 2010, Okumus 2015). Hot springs are defined as a pool of water that has freely seeped through an opening in the earth's surface while geysers are characterised as water that periodically erupts through the earth's surface, spewing hot water and steam. The enzymes of thermophilic bacteria are adapted to function at high temperatures that are required for the growth of these organisms.

The need to produce environmentally friendly and cost effective bioethanol from lignocellulosic biomass together with the low levels of β-glucosidase in the leading industrial cellulases produced by *T. reseei* have created a need to find new sources of β-glucosidases. The saccharification step in production of bioethanol from lignocellulosic biomass usually has an optimal temperature of between 50˚C and 60˚C (Sánchez and Cardona 2008, Khattak et al. 2013). An ideal β-glucosidase for use with commercial cellulose preparations would have to be stable at temperatures above 50˚C.

The genus *Anoxybacillus* is comprised of the following species; *Anoxybacillus pushchinoensis*, *Anoxybacillys flavithermus, Anoxybacillus gonesis*, *Anoxybacillus contaminans*, *Anoxybacillus voinovskieensis*, *Anoxybacillus ayderensis* and *Anoxybacillus kestanbolensis* (Poli et al. 2006). *Anoxybacillus sp* are gram positive spore-forming thermophilic organisms found in various habitats and have been successfully isolated from hot springs (Zhang et al. 2014). The thermophilic nature of the genus makes it ideal as a source of thermostable and high biological activity enzymes for application in biotechnology (Kikani and Singh 2012, Lim et al. 2015). The *Anoxybacillus* genus is known to produce a number of biochemically important thermostable enzymes. These enzymes include the polyester poly-3-hydroxybutyrate esterase, fructose-1,6-bisphosphate aldolase, xylose isomerase, alpha amylase, l-arabinose isomerase and l-arabinofuranosidase (Zhang et al. 2014, Lim et al. 2015). Thermophilic microbes and their enzymes are highly adaptable to a wide pH range making them ideal for various bioprocesses (Bhalla et al. 2013a).

This study aimed to isolate and characterise thermophilic bacteria capable of producing thermostable β-glucosidases. A hot geyser was chosen to increase the likelihood of isolating a bacterial strain capable of producing thermostable enzymes.

3.2. Materials and methods

3.2.1. Maintenance of cultures

Water, biomass and soil samples were collected from a hot geyser in the Zambezi Valley, Zimbabwe and eleven thermophilic cultures were obtained from the three samples. Organisms were cultured on nutrient agar plates at 50˚C for 24 hours. Stock cultures were prepared on nutrient agar slants. Plate cultures were sub-cultured every three weeks and the stock cultures were sub-cultured every three months. All cultures were stored at 4˚C until needed.

3.2.2. Screening for β-glucosidase producing organisms

Bacterial cultures were grown on M9 minimal medium with 5% cellobiose and 0.5% glucose as carbon source. The cultures were incubated at 60˚C for 72 hours on an orbital shaker at 200 rpm. Samples (1 ml) were withdrawn every 24 hours and the absorbance of the culture was read at 605 nm in 96-well microtiter plates, using a Beckman coulter DTX 800 multimode detector (USA). β-glucosidase activity was determined as described in section 3.2.3.1 using the culture supernatant as the source of crude enzyme.

3.2.3. Enzyme assays

3.2.3.1 β-glucosidase assay

β-glucosidase activity was determined using 25 µl crude enzyme, 25 µl of 10 mM pNPG in 200 mM phosphate buffer (pH 7) in a final volume of 100 µl. The reaction mixture was incubated at 60˚C for 10 minutes after which 100 µl 1 M Na_2CO_3 was added to stop the reaction. The release of *p*-nitrophenol was monitored at 405 nm in 96-well microtiter plates using a Beckman Coulter DTX 800 multimode detector, microplate reader. One unit of β-glucosidase activity corresponds to the release of 1 µm *p*-nitrophenol per minute, under assay conditions.

3.2.3.2. Effect of temperature and pH on bacterial growth

Bacterial cultures were grown on M9 minimal broth medium (see section 3.2.2.) and incubated at temperatures of 50, 60, 65, 70˚C for 72 hours to assess their ability to grow at high temperatures. Optimum pH for growth was determined by growing

bacterial isolates in medium with pH adjusted from 3 up to 9. Samples (1ml) were withdrawn every 24 hours and the absorbance was measured at 605 nm in 96-well microtiter plates using a Beckman Coulter DTX 800 multimode detector, microplate reader.

3.2.4. Identification of β-glucosidase producing isolates

The eight β-glucosidase producing isolates were identified through 16S ribosomal subunit sequencing by Inqaba Biotechnological Industries, Pretoria. All sequences were aligned against a representative selection of prokaryote sequences from protein databases through the Blast search on the NCBI website.

Based on the sequencing results, specific biochemical tests were performed to check if the isolates were novel strains. The bacterial isolates were grown on M9 medium with starch, tributyrin, casein and gelatin as sole carbon sources, to detect the hydrolysing capabilities of the strains on these substrates. The organisms were also grown on media containing 0.5% glucose, fructose, sucrose, mannose, lactose, cellobiose or avicel. Growth in the presence of up to 5% $NaCl_2$, up to 3% ethanol and the catalase tests were used to aid in identification. The biochemical test results were compared to bacterial control strains.

3.2.5. Gram stain and spore formation test

The Gram stain was performed on all isolates and the Schaeffer-fulton spore formation test was used to determine whether the isolates were capable of producing spores (Bartholomew and Mittwer 1883).

3.3. Results

A thermophilic *Anoxybacillus* sp. that produces a thermostable β-glucosidase was isolated and characterised. Strain identification was achieved through biochemical test and sequencing of the 16S rRNA region (Veysi Okumus 2015).

3.3.1. Screening for β-glucosidase activity

Eleven bacterial strains were isolated from water and soil samples collected from a hot geyser at the Zambezi Valley, Zimbabwe (Figure 3.1). However, only eight of the eleven isolates (KTS2, KTG1, KTG2, KTBR, KT67, KTC5, KTWO and KTC2) were capable of producing β-glucosidase when cultured on cellobiose as the main carbon source. The strain designated KTC2 was the best enzyme producer at 60˚C and pH 8 after 48 hours of incubation, followed by KTG1 (Figure 3.2). The strain producing the highest *β*-glucosidase activity was chosen for further study. All β-glucosidase positive strains were capable of growth at temperatures 40, 50, 60, 65 and 70˚C.

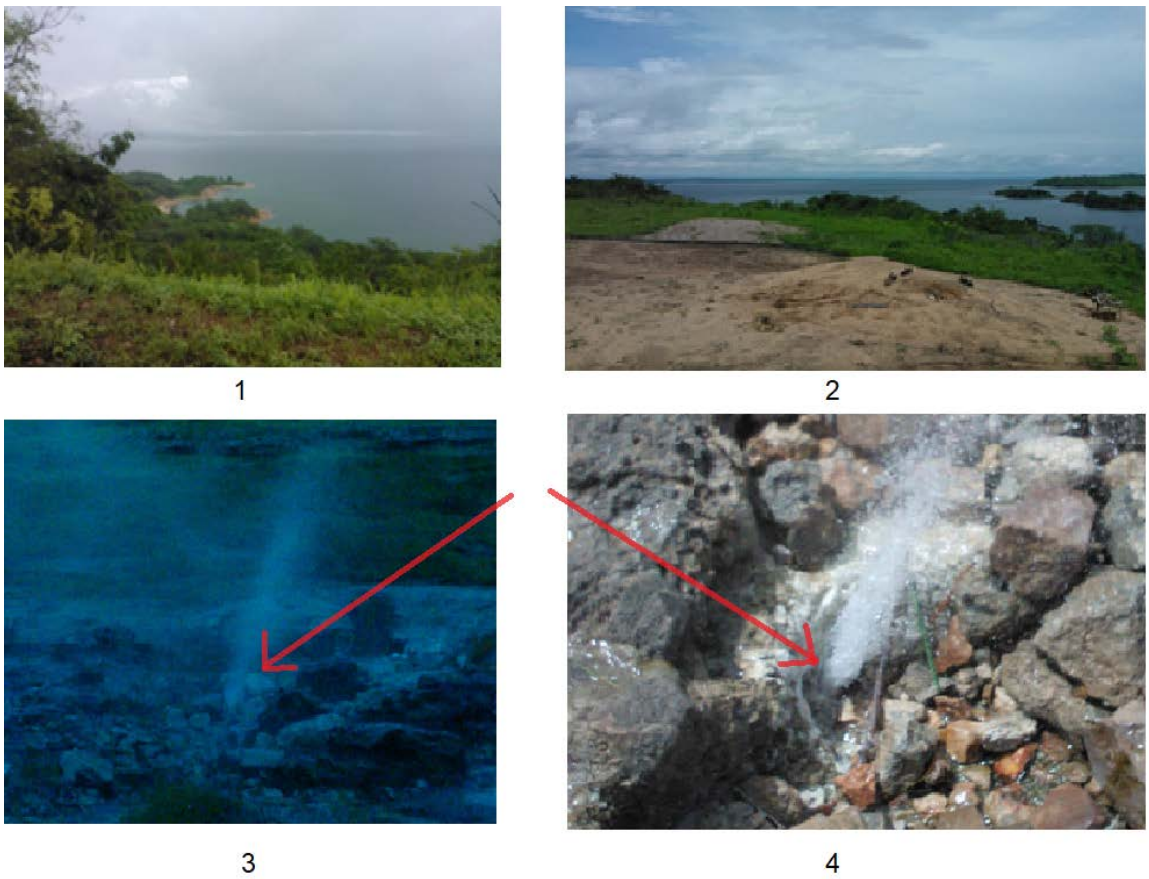


Figure 3.1. The hot geyser (3 and 4) in the Zambezi Valley (1 and 2) in Zimbabwe where the water and soil samples were collected.

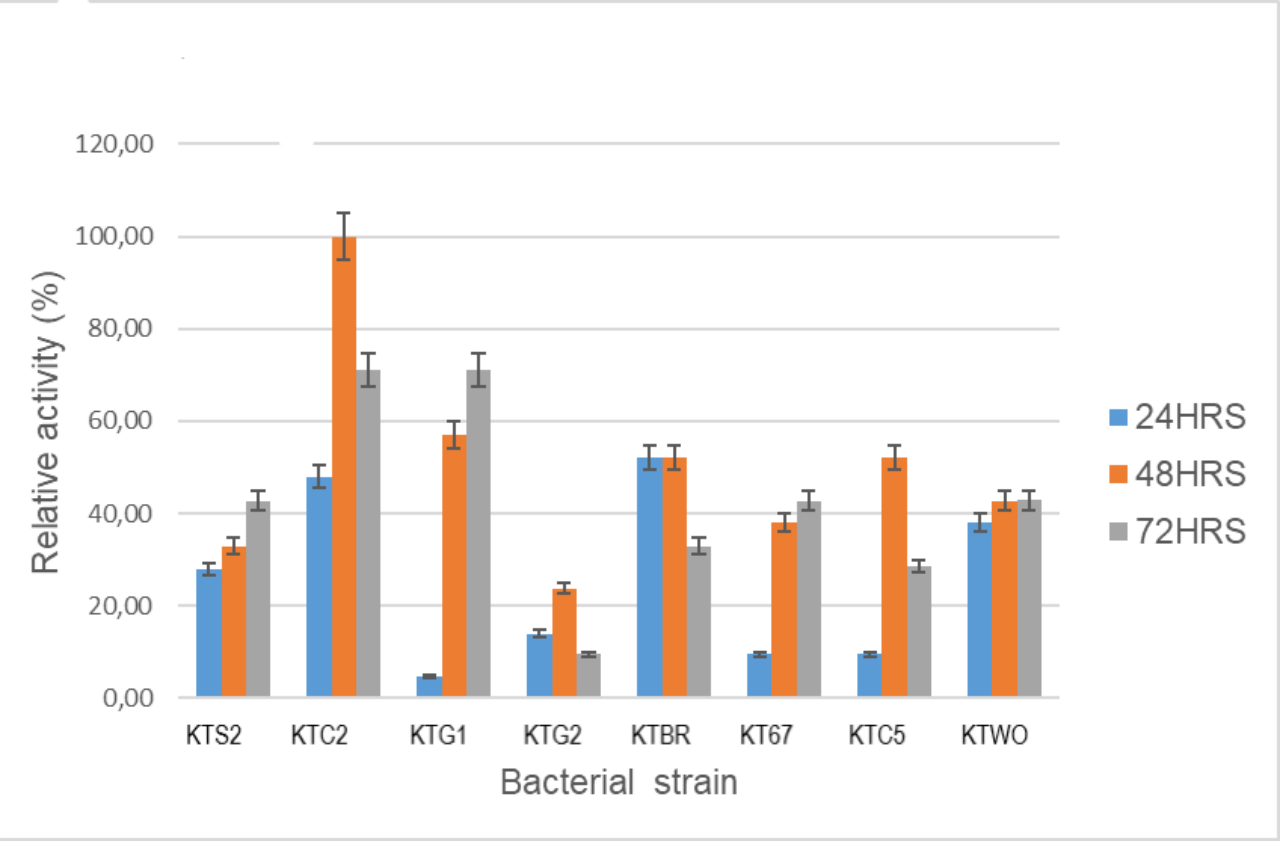


Figure 3.2. The *β*-glucosidase activity of isolates from a hot geyser at the Zambezi Valley, Zimbabwe. The isolates were grown at 60˚C for 72 hrs on M9 medium with cellobiose as the main carbon source. The results are given as a mean of three independent repeats.

3.3.2. Strain identification

3.3.2.1. Biochemical tests

The results of biochemical tests performed for strain characterisation are shown in Table 3.1. All isolated strains except for strain KTBR were motile. The strains were all able to grow in the presence of monosaccharides. The specific biochemical tests performed were chosen based on the strain identities obtained from 16S rDNA sequencing (Table 3.3).

Table 3.1. Biochemical tests for the characterisation of the bacterial isolates.

	KTS2	KTC2	KTG1	KTG2	KTBR	KT67	KTC5	KTWO
Motility	+	+	+	+	-	+	+	+
Catalase	+	+/-	-	+	+	+	+	+/-
Lipid hydrolysis	+	+	+	+	+	+	+	+
Casein hydrolysis	-	-	-	-	-	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+
Gelatin hydrolysis	-	+	+	+	+	-	-	-
NaCl 0.1 %	+	+	+	+	+	+	+	+
NaCl 2-5 %	+	+	+	+	+	+	+	+
0.5% Cellobiose	+	+	+	+	+	+	+	+
0.5% Avicel	+	+	+	+	+	+	+	+
0.5% Glucose	+	+	+	+	+	+	+	+
0.5% Fructose	+	+	+	+	+	+	+	+
0.5% Sucrose	+	+	+	+	+	+	+	+
0.5% Mannose	+	+	+	+	+	+	+	+
0.5% Lactose	+	+	+	+	+	+	+	+

+ Positive reaction
- Negative reaction

3.3.2.2. Gram reaction and spore formation

All isolates producing β-glucosidase were Gram positive and rod shaped. The spore formation test revealed that *Anoxybacillus* sp. KTC2, *Anoxybacillus beppuensis* KTS2, *Anoxybacillus beppuensis* KTG1, *Anoxybacillus flavithermus* KTG2 and *Paenibaccillus* sp. KTC5 are spore forming while *Anoxybacillus* sp. KT67, and *Anoxybacillus sp* KTWO, are not. The un-identified strain KTBR was also non-spore forming.

3.3.2.3. 16S rDNA sequencing and strain identification

Identification of isolates were achieved through sequencing of the 16S rDNA region. Bacteria identified to species level included; *Anoxybacillus beppuensis* KTS2, *Anoxybacillus beppuensis* KTG1 and *Anoxybacillus flavithermus* KTG2. Strain KTBR could not be identified through 16S rDNA sequencing.

Following the isolation, identification and characterisation of the *β*-glucosidase producing strains, the highest β-glucosidase producing strain, *Anoxybacillus* sp *KTC2,* was chosen for further investigation*.* A comparison of *Anoxybacillus* sp. KTC2 with published *Anoxybacillus* species is documented (Table 3.3).

Table 3.2 Characteristics of *Anoxybacillus sp* KTC2 and related species (Poli et al. 2006, Terme 2009)

	Anoxybacillus sp. KTC2	*A. flavithermus* DSM 2641	*A. amololyticus* MR3C	*A. gonensis* NCIMB 13933	*A. pushchinoensis* DSM 12423
Cell morphology	**Rod**	Rod	Rod	Rod	Rod
Gram stain	**+**	+	+	+	+
Relation to Oxygen	**Facultative Aerobe**	Facultative Aerobe	Facultative aerobe	Facultative Aerobe	Anaerobe
Temp range	**30-70**	30-72	45-65	40-70	37-66
Optimum temp	**60**	60-65	61	55-60	62
pH range	**7.5-9**	5.5-9	5-6.5	6-10	8-10.5
Optimum pH	**8**	7	5.6	7.5-8	9.5-9.7
Motility	**+**	-	+	+	+
NaCl (3%, w/v)	**+ (5%)**	-	-	+	+
Nitrate reduction	**ND**	+	-	-	+
Hydrolysis of					
Gelatine	**+**	-	-	+	-
Starch	**+**	+	+	+	-
Casein	**-**	+	ND	ND	-
Oxidase utilisation of					
Glucose	**+**	-	+	+	+
Sucrose	**+**	-	ND	+	+
Mannose	**+**	+	ND	-	ND
Xylose	**+**	-	-	+	ND
Avicel	**+**	ND	ND	ND	ND
Cellobiose	**+**	ND	ND	ND	ND

ND Not determined

\+ Positive reaction

\- Negative reaction

3.3. Discussion and conclusion

Thermophiles and hyperthermophiles are common in high temperature environments making places such as hot spring and geysers ideal environments for the isolation of thermophilic microorganism. Thermophiles are potential sources of thermostable enzymes, as the enzymes produced by these organisms are adapted to their physical environment. *Geobacillus*, *Meiothermus* and *Anoxybacillus* are amongst some of the most commonly isolated genera in hot springs and geothermal geysers. A hot geyser in the Zambezi valley, Zimbabwe, was identified as a possible source of thermophilic bacteria due to its high temperatures. Thermostable enzymes are of great importance in biotechnological processes such as biofuel production (Patel et al. 2019). A bacterial strain that produces high levels of β-glucosidase activity was isolated and characterised. This strain was chosen from 8 isolates capable of β-glucosidase production.

The Gram positive spore forming motile bacterium *Anoxybacillus* sp*. K*TC2 grew optimally at 60˚C, making it a thermophilic organism. By definition, the optimal temperature of thermophiles is above 55˚C, while hyperthermophiles grow optimally at temperatures above 80˚C. In addition to growth at high temperatures, thermophilic bacteria also have the ability to grow and thrive under extreme environments such as extreme pH and high salt concentrations. *Anoxybacillus* sp*. K*TC2 was able to grow in the presence of 5% NaCl, classifying it as a moderate halophile. Moderate halophiles are organisms that can grow at salt concentrations of 4.7 to 20%. *Anoxybacillus* sp*.* KTC2 grew optimally at pH 8 classifying it as an alkaliphile. It is an alkali tolerant thermophile making it suitable for many industrial applications, including the production of industrially important thermostable enzymes.

Anoxybacillus sp*.* KTC2 was able to hydrolyse lipids, starch and gelatine, as well as the ability to utilise glucose, fructose, sucrose, mannose, lactose, and Avicel as carbon sources. The ability to hydrolyse different polymeric substrates highlight the potential of *Anoxybacillus sp* as a source of industrially important thermostable enzymes. This is evident from the number of thermophilic species already studied. *Sp*ecies such as *A. flavithermus*, *A. beppuensis* and *A. amylolyticus* have been extensively studied for their ability to produce thermostable amylases with potential for use in the starch industry (Poli et al. 2006, Kikani and Singh 2012, Okumus

2015). Chan et al. 2016, recently published a study on the isolation and characterisation of a glucose-tolerant β-glucosidases from an *Anoxybacillus sp.*

Phylogenetic analysis based on the 16S rDNA gene sequence indicated that the strain belonged to the genus *Anoxybacillus.* The *Anoxybacillus* strain showed a 98% sequence similarity to *Anoxybacillus flavithermus* AK1, *Anoxybacillus flavithermus* KW and *Anoxybacillus* sp. JS40 and 97% similarity to *Anoxybacillus pushchinoensis*. However, the physiological and biochemical characteristics of the isolate suggest that the *Anoxybacillus sp KTC2* isolated may be a novel strain.

In conclusion, the thermophilic bacterial strain, *Anoxybacillus* sp*. KTC2* isolated from a hot geyser has potential as a source of thermostable industrial enzymes. The bacterial strain potentially produces two industrially important enzymes, namely a β-glucosidase and amylase, based on the organisms' ability to hydrolase starch. The β-glucosidase, an inducible enzyme, was produced in the presence of the inducer cellobiose. The β-glucosidase will be characterised and its efficiency as part of a commercial cellulase enzyme cocktail for lignocellulosic biomass saccharification will be investigated. While the *Anoxybacillus* sp*.* KTC2's ability to produce more thermostable industrial enzymes may be explored in the future.

3.4 References

Ahamed, A. and Vermette, P., 2008. Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor. *Biochemical Engineering Journal*, 42 (1), 41–46.

Bartholomew, J. W. and Mittwer, T. O. D., 1883. municipal hospital, 1–29.

Bernardes, A., Pellegrini, V. O. A., Curtolo, F., Camilo, C. M., Mello, B. L., and Johns, M. A., 2019. Carbohydrate binding modules enhance cellulose enzymatic hydrolysis by increasing access of cellulases to the substrate. *Carbohydrate Polymers*, 21 (December 2018), 57–68. Available from: https://doi.org/10.1016/j.carbpol.2019.01.108. Online.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., and Sani, R. K., 2013a. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, 128, 751–759. Available from: http://dx.doi.org/10.1016/j.biortech.2012.10.145. Online.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., and Sani, R. K., 2013b. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, 128, 751-759.

Chan, C. S., Sin, L. L., Chan, K. G., Shamsir, M. S., Manan, F. A., Sani, R. K., and Goh, K. M., 2016. Characterisation of a glucose-tolerant β-glucosidase from Anoxybacillus sp. DT3-1. *Biotechnology for Biofuels*, 9 (1), 1–11.

Florindo, R. N., Souza V. P., Mutti, H. S., Camilo, C., Manzine L. R., and Marana, S. R., 2018. Structural insight into beta-glucosidase transglycosylation based on biochemical, structural and computational analysis of two GH1 enzymes from Tricoderma hararzium. *New Biotechnology*, 40 (0), 218–227.

Goh, K. M. and Kahar, U. M., 2013. Recent discoveries and applications of Anoxybacillus. Applied Microbiology and Biotechnology, 97, 1475–1488.

Haki, G. D. and Rakshit, S. K., 2003. Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, 89 (1), 17-34.

Harnpicharnchai, P., Champreda, V., Sornlake, W., and Eurwilaichitr, L., 2009. A thermotolerant β-glucosidase isolated from an endophytic fungi, Periconia sp., with a possible use for biomass conversion to sugars. *Protein Expression and Purification*, 67 (2), 61–69.

Khattak, W. A., Khan, T., Ha, J. H., Ul-Islam, M., Kang, M. K., and Park, J. K., 2013. Enhanced production of bioethanol from waste of beer fermentation broth at high temperature through consecutive batch strategy by simultaneous saccharification and fermentation. *Enzyme and Microbial Technology*, 53 (5), 322–330.

Kikani, B. A. and Singh, S. P., 2012. The stability and thermodynamic parameters of a very thermostable and calcium-independent α-amylase from a newly isolated bacterium, Anoxybacillus beppuensis TSSC-1. *Process Biochemistry*, 47 (12), 1791–1798. Available from: http://dx.doi.org/10.1016/j.procbio.2012.06.005. Online.

Kumar, S. and Nussinov, R., 2001. How do thermophilic proteins deal with heat ?. *Cellular and Molecular Life Sciences*, 58, 1216–1233.

Lewis Liu, Z., Weber, S. A., Cotta, M. A., and Li, S. Z., 2012. A new β-glucosidase producing yeast for lower-cost cellulosic ethanol production from xylose-extracted corncob residues by simultaneous saccharification and fermentation. *Bioresource Technology*, 104, 410–416.

Lim, Y. L., Chan, K. G., Ee, R., Belduz, A. O., Canakci, S., Kahar, U. M., Yaakop, A. S., and Goh, K. M., 2015. Complete genome of the potential thermozyme producer Anoxybacillus gonensis G2Tisolated from the Gönen hot springs in Turkey. *Journal of Biotechnology* , 212, 65–66. Available from: http://dx.doi.org/10.1016/j.jbiotec.2015.08.007. Online

Patel, A. K., Singhania, R. R., Sim, S. J., and Pandey, A., 2019. Thermostable cellulases : Current status and perspectives. *Bioresource Technology*, 279, 385–392.

Poli, A., Esposito, E., Lama, L., Orlando, P., Nicolaus, G., de Appolonia, F., Gambacorta, A., and Nicolaus, B., 2006. Anoxybacillus amylolyticus sp. nov., a thermophilic amylase producing bacterium isolated from Mount Rittmann

(Antarctica). *Systematic and Applied Microbiology*, 29 (4), 300–307.

Rastogi, G., Bhalla, A., Adhikari, A., Bischoff, K. M., Hughes, S. R., Christopher, L. P., and Sani, R. K., 2010. Characterisation of thermostable cellulases produced by Bacillus and Geobacillus strains. *Bioresource Technology*, 101 (22), 8798–8806.

Sánchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*.

Sánchez, Ó. J. and Cardona, C. A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*, 99 (13), 5270-5296).

Sumiyo, K. S., Wakayama, J., Tsukamoto, K., Hayashi, N., Miyazaki, Y., Ohmori, H., Tajima, K., and Yokoyama, H., 2013. Degradation of microcrystalline cellulose and non-pretreated plant biomass by a cell-free extracellular cellulase/hemicellulase system from the extreme thermophilic bacterium *Caldicellulosiruptor bescii*. *Seibutsu-kogaku Kaishi*, 115 (1), 64–70.

Veysi Okumus, S. O., 2015. Isolation of a Novel Thermophilic Anoxybacillus flavithermus SO-13, Production, Characterisation and Industrial Applications of its Thermostable alpha-Amylase. *Journal of Bioprocessing & Biotechniques*, 05 (07). Available from: https://www.omicsonline.org/open-access/isolation-of-a-novel-thermophilic-anoxybacillus-flavithermus-so13-production-characterisation-and-industrial-applications-of-itsthermostable-amylase-2155-9821-1000237.php?aid=56297. Online

Wang, Y., Wang, X., Tang, R., Yu, S., Zheng, B., and Feng, Y., 2010. A novel thermostable cellulase from Fervidobacterium nodosum. *Journal of Molecular Catalysis B: Enzymatic*, 66 (3–4), 294–301.

Wen, Z., Liao, W., and Chen, S., 2005. Production of cellulase/β-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. *Process Biochemistry*, 40 (9), 3087–3094.

Xia, Y., Yang, L., and Xia, L., 2018a. High-level production of a fungal β -glucosidase with application potentials in the cost-e ff ective production of Trichoderma

reesei cellulase. *Process Biochemistry*, 70 (April), 55–60. Available from: https://doi.org/10.1016/j.procbio.2018.03.031. Online.

Xia, Y., Yang, L., and Xia, L., 2018b. High-level production of a fungal β -glucosidase with application potentials in the cost-e ff ective production of Trichoderma reesei cellulase. *Process Biochemistry*, 70, 55–60. Available from: https://doi.org/10.1016/j.procbio.2018.03.031. Online.

Zhang, J., Wang, D., Pan, J., Wang, J., Zhao, H., Li, Q., and Zhou, X., 2014. Efficient resveratrol production by immobilized β-glucosidase on cross-linked chitosan microsphere modified by l-lysine. *Journal of Molecular Catalysis B: Enzymatic*, 104, 29–34.

Zhang, J., Zhong, Y., Zhao, X., and Wang, T., 2010. Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced β-glucosidase and filter paper activity using strong artifical cellobiohydrolase 1 promoter. *Bioresource Technology*, 101 (24), 9815–9818.

CHAPTER 4

ENZYME PRODUCTION, CHARACTERISATION AND AVICEL SACCHARIFICATION

4.1. Introduction

Enzyme hydrolysis is an essential step in bioethanol production from lignocellulosic biomass. Enzymatic saccharification is preferred over acid based approaches due to moderate hydrolysis conditions, higher conversion efficiency, absence of substrate loss due to chemical modification and no production of toxic chemicals (Almeida et al. 2019). However, cellulase production is still the major cost component in lignocellulosic bioethanol production (Berlin et al. 2005, Wen et al. 2005, Brijwani et al. 2010, Joo et al. 2010, Kalyani et al. 2013, Pamella Santa-Rosa et al. 2017, Alves et al. 2018). The high cost is due to the long fermentation times and low product yields. The current cost of cellulases does not allow for cost effective bioethanol production (Garvey et al. 2013, Alves et al. 2018), hence, the increased interest in finding or development of strains that can produce large quantities of enzymes on inexpensive substrates. Some leading manufacturers have tried to lower the cost of enzymes by using crude enzyme cocktails instead of purified enzymes. However, further cost reduction is needed for bioethanol production from lignocellulosic biomass to be economically feasible (Berlin et al. 2005).

Fungi are known to produce higher levels of extracellular cellulases than bacteria. The strains used for commercial cellulase production include; *Trichoderma, Aspergillus, Penicillium, and Fusarium* (Brijwani et al. 2010, Kalyani et al. 2013). *Trichoderma reesei* is however, still the leading cellulase producer (Tengerdy and Szakacs 2003, Brijwani et al. 2010, Garvey et al. 2013, Almeida et al. 2019, Ndlovu and Wyk 2019). Most commercial cellulase preparations, including those produced by *T. reseei*, have high activities of endo- and exoglucanases, but β-glucosidase activity is often rate limiting (Wen et al. 2005, Brijwani et al. 2010, Manickam et al. 2018). Suboptimal β-glucosidase activities in commercial cellulase preparations negatively affects the enzymatic hydrolysis of biomass. In addition, substrate loading, enzyme loading and reaction conditions influence enzymatic hydrolysis of biomass (Manickam et al. 2018).

Thermostability is defined by the capacity of an enzyme to retain its active structural conformation at high temperature for a period of time (Bhalla et al. 2013). A number of thermostable enzymes are currently used in industrial processes (You et al. 2018). Saccharification experiments conducted at high temperatures decrease the risk of contamination (Oguro et al. 2019), reduce enzyme loading and offer shorter hydrolysis times. Reduced enzyme loading is key to reducing overall process costs in bioethanol production from lignocellulosic biomass (Manickam et al. 2018).

Adaptability of thermophilic organisms and their enzymes to high temperatures and wide pH range render them ideal for extremophilic bioprocessing. Thermoacidophilic and thermoalkaliphilic enzymes could provide benefits during the saccharification step. Lignocellulosic biomass is usually either acidic or alkaline after pre-treatment and requires neutralization before saccharification. Saccharification using thermoacidophilic or thermoalkaliphic enzymes could eliminate the need for neutralization which will result in reduced bioethanol production cost (Bhalla et al. 2013a).

Utilization of lignocellulosic biomass for ethanol production is largely dependent on efficient pre-treatment and saccharification. An ideal saccharification process needs to produce sufficient concentrations of fermentable sugars for conversion to biofuel (Sukumaran et al. 2005). Product yields can be affected by factors such as; the degree of access cellulases have to cellulose, the stability of the enzymes during saccharification and the enzyme inhibitors produced during pre-treatment (Oleson and Schwartz 2019). The isolation of thermostable β-glucosidases with a wide pH range will be useful in developing an enzyme cocktail of commercial importance. Being the rate limiting factor in enzymatic hydrolysis of cellulose, β-glucosidases play an important role in the saccharification step (Ichikawa et al. 2019).

A thermophilic Gram positive bacterial strain *Anoxybacillus* sp. KTC2, that produces a β-glucosidase enzyme was isolated from a hot geyser and characterized. The main aim of this study was to isolate and characterize the β-glucosidase produced by *Anoxybacillus* sp. KTC2. The saccharification efficiency of the β-glucosidase enzyme as part of a commercial enzyme cocktail was investigated.

4.2. Materials and methods

4.2.1. Enzyme production and characterisation

4.2.1.1. β-glucosidase activity determination

β-glucosidase activity was determined using 25 µl crude enzyme, 25 µl of 10 mM pNPG in 200 mM phosphate buffer (pH 7) in a final volume of 100µl. The reaction was carried out at 60˚C for 10 minutes before 100 µl Na_2CO_3 was added to stop the reaction. The release of *p*-nitrophenol was monitored at 405 nm in 96-well microtiter plates using a Beckman Coulter DTX 800 multimode detector, microplate reader. One unit of β-glucosidase activity corresponds to the release of 1 µm *p*-nitrophenol per minute, under the conditions of the assay.

4.2.1.2. Effect of substrate on β-glucosidase production

The bacterial strain *Anoxybacillus* sp*.* KTC2 was grown on M9 minimal medium with 5% cellobiose and 0.5% glucose as carbon sources. The culture was incubated at 60˚C for 144 hours on an orbital shaker at 200 rpm. The culture supernatant was harvested and used as a source of crude enzyme. A sample (1 ml) was collected every 24 hours and β-glucosidase activity determined. In addition to growth on cellobiose as the main carbon source, *Anoxybacillus* sp*.* KTC2 was grown on three nutrient sources, to determine the best medium for enzyme production. The bacterium was grown on M9 medium with Avicel as a carbon source, nutrient broth and nutrient broth with 2% cellobiose.

4.2.1.3. Effect of cellobiose concentration on enzyme production

Since cellobiose was the best inducer for β-glucosidase activity, different concentrations were used to determine the optimum concentration. *Anoxybacillus* sp. KTC2 was cultured on 1-5% cellobiose at 60˚C over 72 hours. Crude enzyme was harvested and enzyme activity determined.

4.2.1.4. Effects of temperature and pH on enzyme production

The optimum temperature and pH for β-glucosidase production was determined by growing the bacterial strain on M9 minimal medium with cellobiose as the main carbon source over a temperature range of 30-80°C and pH 3-5 citrate buffer, pH 6 acetate buffer and pH 6.5-9 phosphate buffer.

4.2.1.5. Optimum temperature and temperature stability

The optimum temperature of the β-glucosidase was determined from 50 to 60°C, while the enzyme's thermo-stability was determined by incubating the crude enzyme at 30 - 80°C for 24 hours before measuring the residual β-glucosidase activity.

4.2.1.6. Optimum pH and pH stability

The effect of pH on β-glucosidase activity was determined by conducting enzyme activity assays over a pH range of 4 - 9 using different buffers. pH stability was determined by incubating crude enzyme in buffers ranging from pH 4-5 citrate buffer, pH 6 acetate buffer and pH 6.5-9 phosphate buffer for 24 hours.

4.2.2. Partial purification of the β-glucosidase

An appropriately concentrated crude protein sample was dialysed overnight using seamless tubing from Sigma Chemical Co; in 10 mM phosphate buffer, pH 7. The sample was run on a Q-sepharose column, calibrated with 10 mM phosphate buffer. Protein elution was done with 10mM phosphate buffer containing 0.5 M NaCl. The active fractions were pooled together, concentrated through ultrafiltration, supplemented with 1 M NaCl and loaded onto the butyl sepharose 4 fast flow column. The butyl sepharose 4 fast flow column was calibrated with 10mM phosphate buffer and proteins eluted with 30% isopropanol in 10mM phosphate buffer. The purity of the protein was checked on SDS-PAGE.

4.2.3. SDS-PAGE and Zymography

The purity and estimated molecular mass of the protein was determined by SDS-PAGE while zymography was performed to detect the active band. A 12% denaturing SDS-PAGE was run for molecular mass determination and to check the purity of the sample after each purification step. The SDS-PAGE gel ran for

zymography, was incubated in 40% isopropanol in 10 mM phosphate buffer for 20 minutes to renature the enzyme. The zymography reaction was carried out at 50˚C, using 0.1% esculin as substrate and 0.03% ferric iron for colour detection. The reaction was stopped by immersing the gel in a 10% glucose solution.

4.2.4. Protein sequencing

4.2.4.1. Isoelectric focusing and 2D gel electrophoresis

The *Anoxybacillus* sp. KTC2 crude enzyme extract was precipitated with TCA-acetone. A 12 µl sample of the TCA-acetone precipitated crude enzyme extract was mixed with 132 µl of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 2 M DTT, 6% ampholytes and a trace of bromophenol blue). The enzyme extract was used to rehydrated an immobilised 7 cm pH gradient, 4-7 ReadyStrip™ (Bio-Rad) IPG strip overnight at room temperature. Following isoelectric focusing, the IPG strip was equilibrated for 15 minutes with equilibration buffer 1 (20 ml 0.375 M Tris-HCl (pH 8.8), 6M urea, 30% glycerol, 2% SDS, 2% w/v DTT) and DTT was substituted with 1.5% iodoacetamide in equilibration buffer 2 for alkylation of proteins. The IPG strip was then run on 12% SDS-PAGE gel and stained with Coomassie Brilliant blue R250. The protein spots on the SDS-PAGE gel were manually excised and stored at -80˚C until further processing.

4.2.4.2 Trypsin digestion and peptide sequencing

Protein spots were washed with water for 10 minutes and cut into 1 mm cubes and transferred to a 0.5 µl microfuge tube. The gel pieces were destained by adding approximately 30 µl of a freshly prepared 1:1 (v/v) mixture of $K_3[Fe(CN)_6]$ and $Na_2S_2O_3$ dissolved in water. The mixture was incubated for 30 minutes at room temperature. Following this incubation, the gel pieces were washed with water and 50mM NH_4HCO_3/acetonitrile (ACN) 1:1 (v/v) for 15 minutes and the solution discarded. The gel pieces were soaked in acetonitrile and left to shrink and stick together. The acetonitrile solution was discarded, and the gel pieces were dried in a vacuum centrifuge. The gel pieces were reduced and alkylated by swelling them in 100 µl of freshly prepared 10 mM dithiothreitol (DTT)/50 mM NH_4HCO_3 solution and incubated for 45 minutes at 56˚C. The tubes were chilled at room temperature and excess liquid discarded and replaced quickly with roughly the same volume of freshly

prepared 55 mM iodoacetamide (IAA) in 50 mM NH_4HCO_3. The mixture was incubated for 30 minutes at room temperature in the dark. The iodoacetamide solution was removed and the gel pieces were washed with 50 mM NH_4HCO_3/acetonitrile (ACN) 1:1 (v/v), one or two changes each, 15 minutes per change. The solution was removed, and enough acetonitrile was added to cover the gel pieces. The gel pieces were left to shrink and dried down in a vacuum centrifuge.

For in-gel digestion, freshly prepared enzyme solution (25 mM NH_4HCO_3 with 5 ng/µl trypsin) was added to cover the gel pieces and incubated at 37˚C for 30 minutes. The excess enzyme solution was removed and enough 25 mM NH_4HCO_3 was added to keep the gel wet. The mixture was incubated at 37˚C overnight. The peptides were extracted by adding extraction buffer [30% ACN, 70% (0.1% Trifluoroacetic acid in water)] enough to cover the gel pieces completely.

4.2.5. Saccharification

Saccharification experiments were conducted to check the saccharification efficiency of the thermostable β-glucosidase enzyme from *Anoxybacillus* sp. KTC2. Enzyme cocktails for Avicel saccharification were created by mixing an appropriate dilution of commercial enzyme Celluclast^{TM} with different concentrations of the *Anoxybacillus* sp. KTC2 β-glucosidase.

4.2.5.1 .*Anoxybacillus sp* KTC2 β-glucosidase production

The β-glucosidase used for Avicel saccharification was produced under optimal conditions, on M9 minimal medium with 5% cellobiose and 0.5% glucose as carbon sources. The culture was incubated at 60˚C for 48 hours with orbital shaking at 200 rpm. The culture supernatant from multiple batches was harvested pooled together and stored at -20˚C for later use.

4.2.5.2. Preparation of β-glucosidase for saccharification

Three litres of stored crude enzyme was divided into smaller volumes and lyophilised. The powdered crude enzyme was stored at -20˚C until needed. A gram of the lyophilized supernatant was dissolved in 5 ml distilled water. Insoluble residue was removed through centrifugation at 13000 rpm for 5 minutes. The supernatant was transferred to a new centrifuge tube and used as source of crude β-glucosidase

for saccharification experiments. The concentrated enzyme was diluted 10X, 100X and 200X to create three concentrations (BGL1, BGL2 and BGL3) for use in enzyme mixtures with Celluclast™. Celluclast™ was mixed with β-glucosidase dilutions in a ratio of 3:1 (3 parts Celluclast™ to 1 part BGL1,2 or 3).

The enzyme combinations of Celluclast™ and *Anoxybacillus* sp. KTC2 β-glucosidase were Celluclast™: BGL1 (Cocktail 1), Celluclast™: BGL2 (Cocktail 2) and Celluclast™: BGL3 (Cocktail 3). The filter paper assay for Cocktail 2 showed the highest total cellulase activity. Based on the activity profiles obtained, Cocktails 2, Cocktail 3 and an appropriate dilution of; Celluclast™, were used for Avicel saccharification. The experiments were performed in triplicates.

4.2.5.3. β-glucosidase assay

The initial β-glucosidase activity of Celluclast™ and the crude *Anoxybacillus* sp. KTC2 β-glucosidase was determined. The activity was determined as described in section 4.2.1.1. The initial β-glucosidase activity of the cocktails was not measured as the total cellulase activity would be measured through the filter paper assay (4.2.5.2). The focus of the study was on the saccharification efficiency of the commercial enzyme when supplemented with an external β-glucosidase.

4.2.5.4. Filter paper assay

Total cellulase activity of Celluclast™ and *Anoxybacillus* sp. KTC2 β-glucosidase were determined using a modified method by Ghose (1987). Two hundred and fifty microliters (250 µl) of enzyme (Celluclast™, *Anoxybacillus* sp. KTC2 β-glucosidase, Cocktail 1, 2 and 3) was mixed with 500 µl of 50 mM acetate buffer pH 5 in a test tube. A filter paper strip (Whatman No 1) measuring 0.5 x 3 cm was placed in a test tube that contained enzyme and buffer. Test tubes were incubated for an hour at 50˚C in a water bath. Following incubation, 1.5 ml of DNS solution was added. The reaction mixtures were boiled in a water bath followed by cooling in cold water and addition of 10 ml distilled water. Once the pulp had settled, 100 µl was transferred into a microplate and colour formation was analysed at 540 nm using the Beckman Coulter DTX 800 multimode detector, microplate reader. Released reducing sugars were determined using glucose standards. Filter paper activity (FPU) is defined as

0.37 divided by the amount of enzyme required to liberate 2.0 mg of glucose from filter paper strip (≈ 50 mg) in 1h.

4.2.5.5. Saccharification of Avicel cellulose

To check the saccharification efficiency of the thermostable β-glucosidase on cellulose, Avicel saccharification was performed according to method described by (Kim et al. 2011) with minor modifications. Experiments were conducted in 30ml reaction volumes. Reactions consisted of 2% Avicel, 3 ml enzyme, 2% sodium azide and 50 mM acetate buffer pH 5. Saccharification experiments were performed at 50˚C in a shaking water bath at 150 rpm in 50ml Erlen Meyer flasks. Sampling was done at 0, 24, 48, 72, and 96 hours. Samples were boiled at 100˚C for 10 minutes and then stored at -20˚C until analyses through the high performance liquid chromatography (HPLC).

4.2.5.6. Analysis of sugars from the Avicel hydrolysis mixture

The Avicel hydrolysate samples were analysed using the Shimadzu HPLC in an Aminex HPX-87C column (300 x 7.8 mm, Ca^{2+}, particle 9 µm) (Biorad, Hercules, CA, USA). The Aminex HPX-87C was fitted with an Aminex Resin Microguard, Carbo C colunm (Biorad, Richmond CA, USA). The detection of eluents was done using an RID 10A refractive index detector (Shimadzu, Kyoto, Japan). The samples were filtered through a 0.2 µm syringe filter. Glucose, maltose, mannose, fructose, were used as standards to determine the retention time of sugars in the samples separated on the column. The Aminex HPX-87C column temperature was maintained at 85°C. degassed deionised distilled water at a flow rate of 0.6 ml/min was used as the mobile phase for the Aminex HPX-87C column. The initial glucose present was subtracted from the final glucose obtained at each sampling time. Peack detection and integration were done using LC solutions software from Shimadzu (Tokyo, Japan). Peak height was used for analysis of the results using Sigmaplot
Data analysis and graphing.

4.3. Results

4.3.1. Enzyme production and characterisation

The saccharification efficiency of a commercial cellulase preparation in combination with the *Anoxybacillus* sp. KTC2 *β*-glucosidase was evaluated.

4.3.1.1. β-glucosidase production on different substrates

The β-glucosidase was initially produced on M9 medium with 5% cellobiose as the main carbon source. Different medium compositions were evaluated to determine the best substrate components for optimal enzyme production. Nutrient broth with 2% cellobiose was found to be the best for enzyme production over 48 hours of incubation while M9 salts with Avicel and nutrient broth produced the lowest activity.

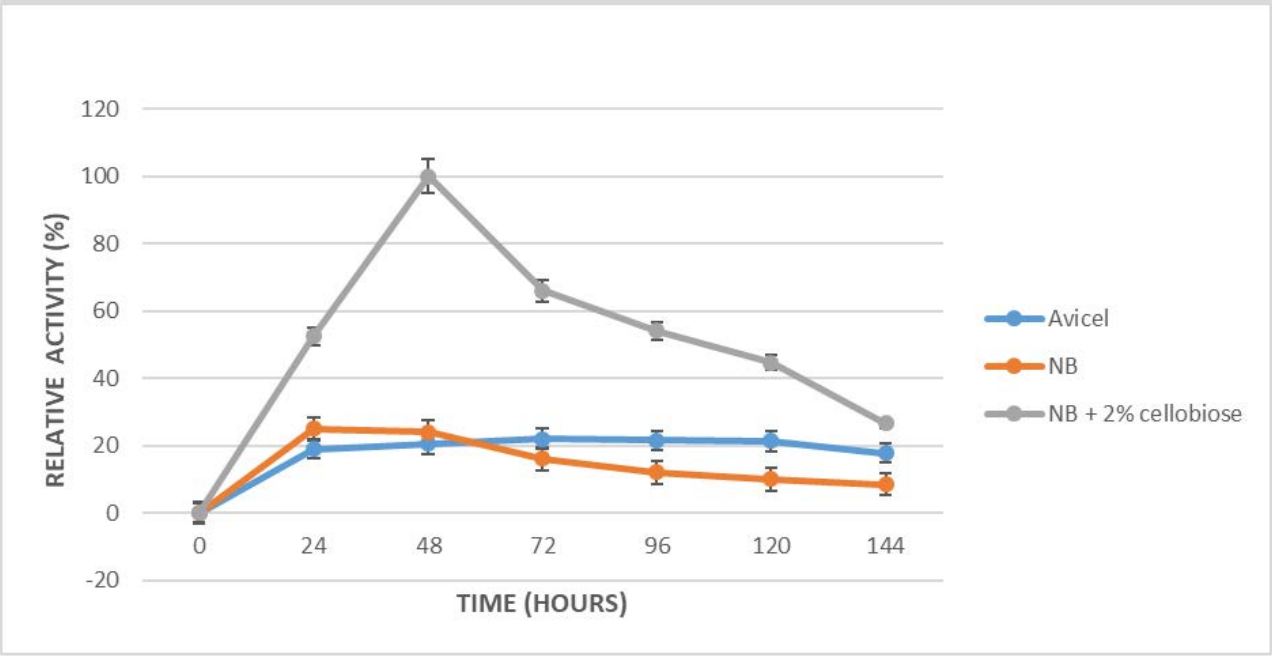


Figure 4.1. β-glucosidase enzyme production on M9 medium with Avicel, Nutrient broth and Nutrient broth (NB) plus 2% Cellobiose over time of incubation. Results are a mean of three determinations.

4.3.1.2. Effect of cellobiose concentration on β-glucosidase production

Anoxybacillus sp. KTC2 was grown on cellobiose as the main carbon source at a concentration range of 1-5% over 72 hours to determine the effect of varying cellobiose concentration on enzyme production. The highest β-glucosidase activity was observed at 5% cellobiose after 48 hours' inoculation at 60˚C (Figure 4.2). This was followed by 4% cellobiose at 24 hours' incubation.

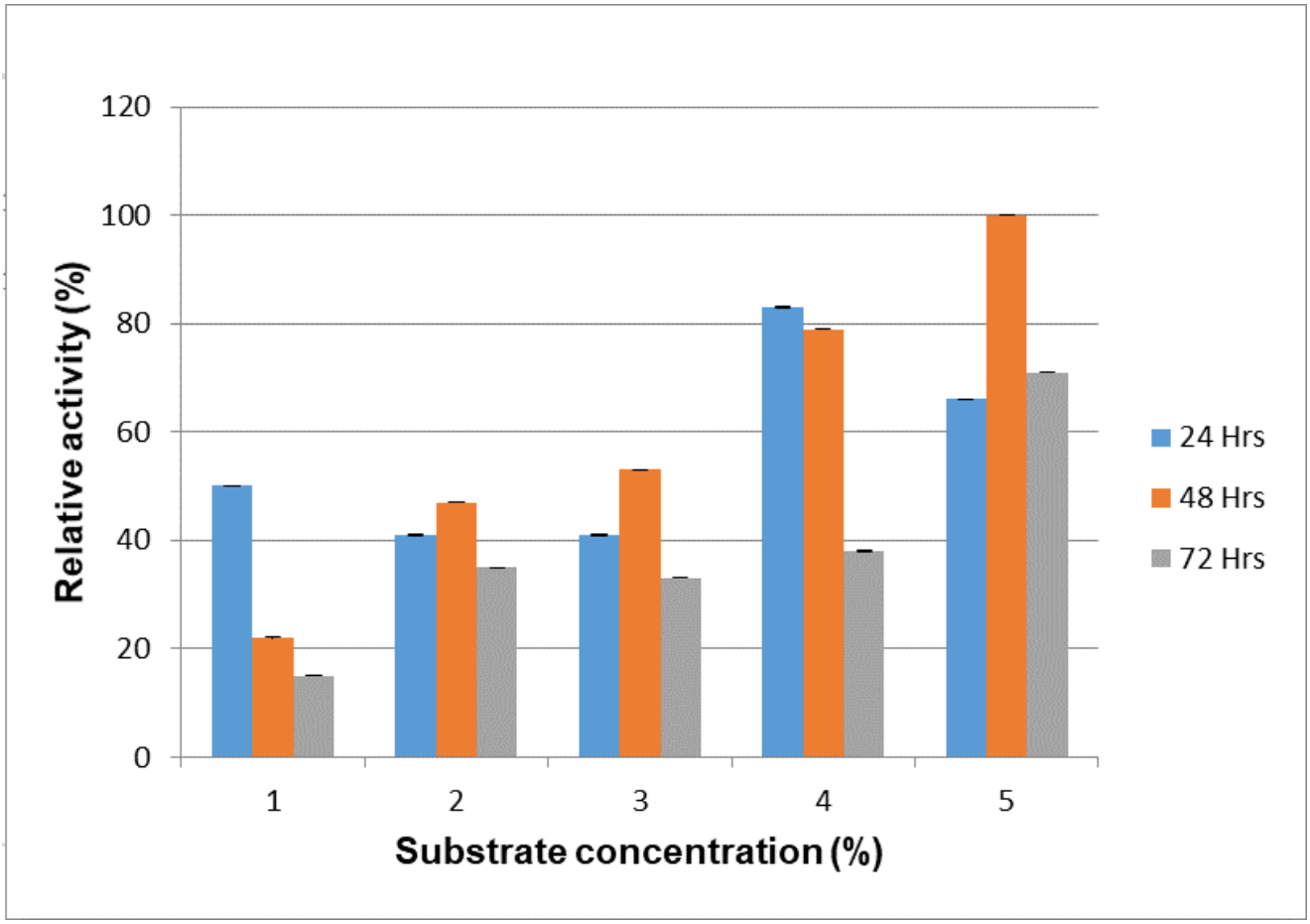


Figure 4.2. Enzyme production on cellobiose over time of incubation at 60˚C. Results are a mean of three determinations ± standard deviation.

4.3.1.3. Effects of growth temperature on enzyme production

The effect of temperature on enzyme production was determined by growing *Anoxybacillus* sp. KTC2 at 50˚C and 60˚C for 72 hours. The optimum temperature for β-glucosidase production was 60˚C after 48 hours. At 50˚C, maximum β-glucosidase activity was observed after 24 hours incubation.

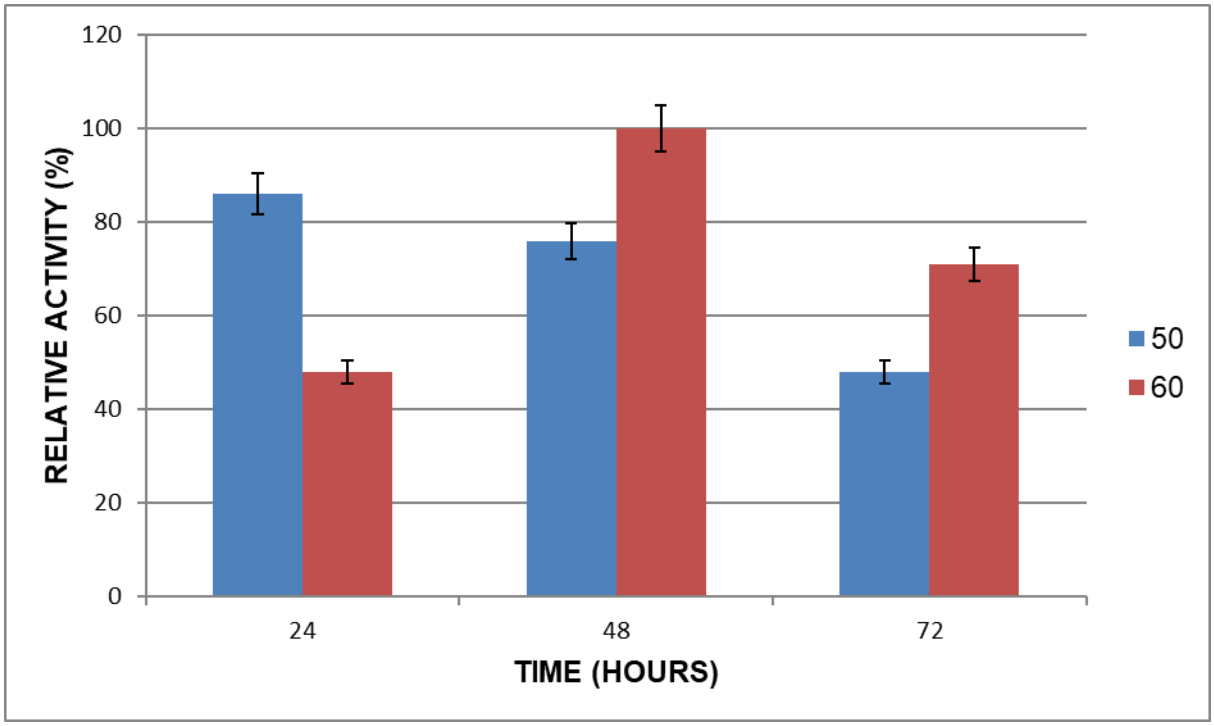


Figure 4.3. Effect of different growth temperatures on β-glucosidase production over time of incubation. Results are a mean of three determinations ± standard deviation.

4.3.1.4. Effects of pH on enzyme production

Growth of *Anoxybacillys sp* KTC2 over a pH range of 7.5 - 9 showed pH 8 as the optimum growth pH for β-glucosidase production, followed by pH 7.5 over 24 hours of incubation (Figure 4.4).

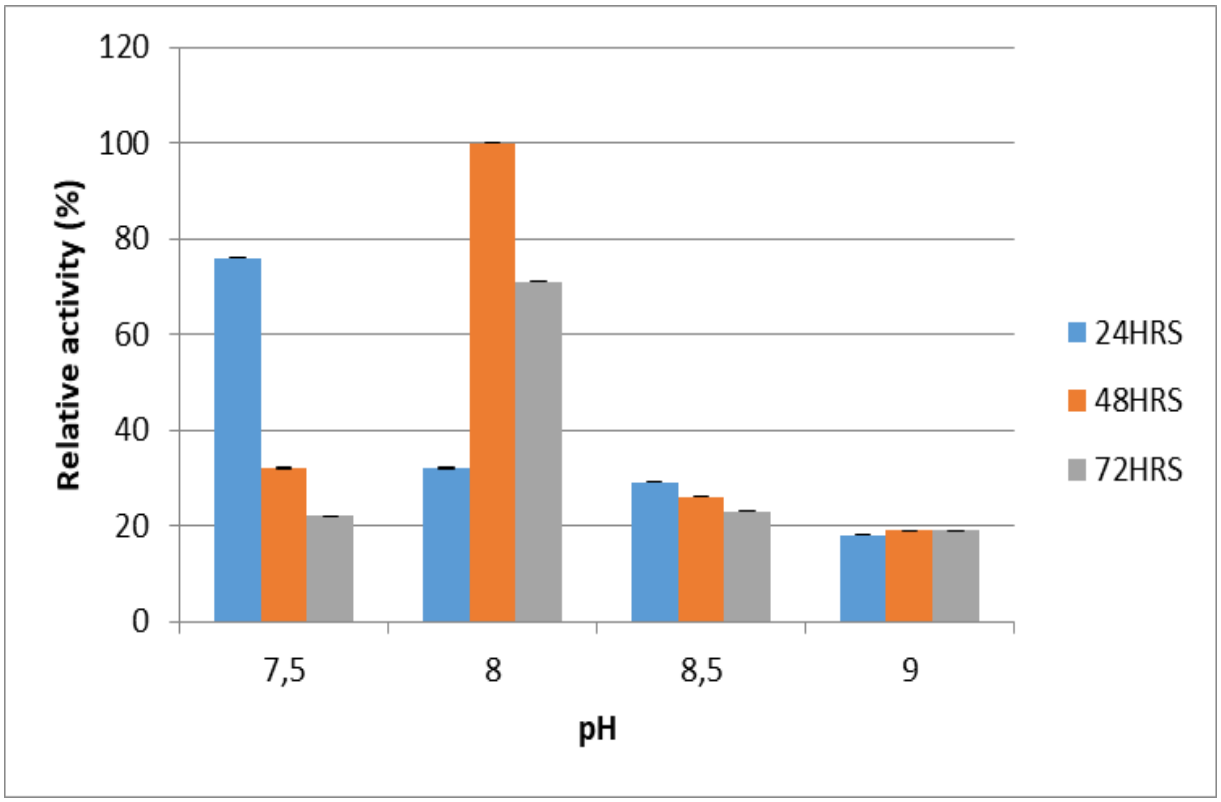


Figure 4.4. β-glucosidase enzyme production pH profile over time of incubation. Results are a mean of three determinations ± standard deviation.

4.3.1.5. Enzyme optimal activity and thermal stability

The enzyme was optimally active at 60˚C with over 80% activity observed at 70˚C (Figure 4.5). The β-glucosidase enzyme showed 55% residual activity after 24 hours of incubation at 60˚C (Figure 4.6).

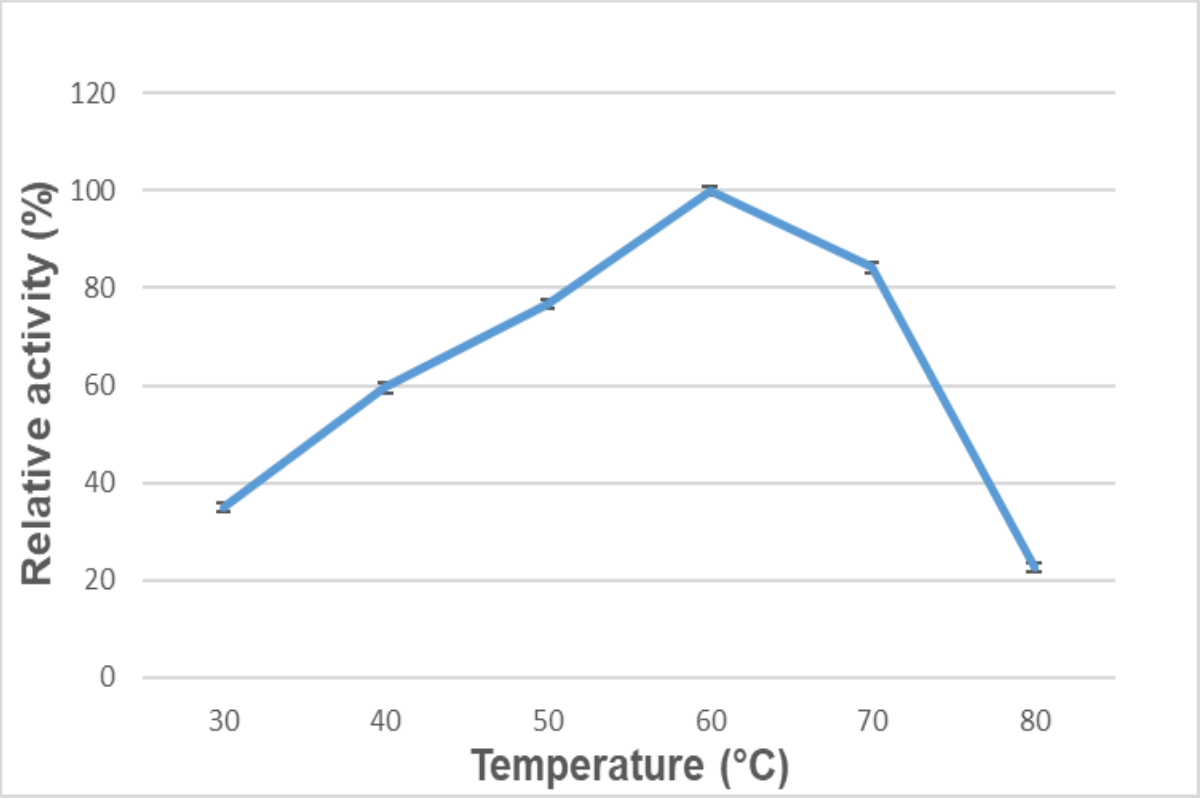


Figure 4.5. The effects of temperature on enzyme activity. Enzyme activity assays was performed at temperatures 30 - 80˚C. Results are a mean of three determinants ± standard deviation.

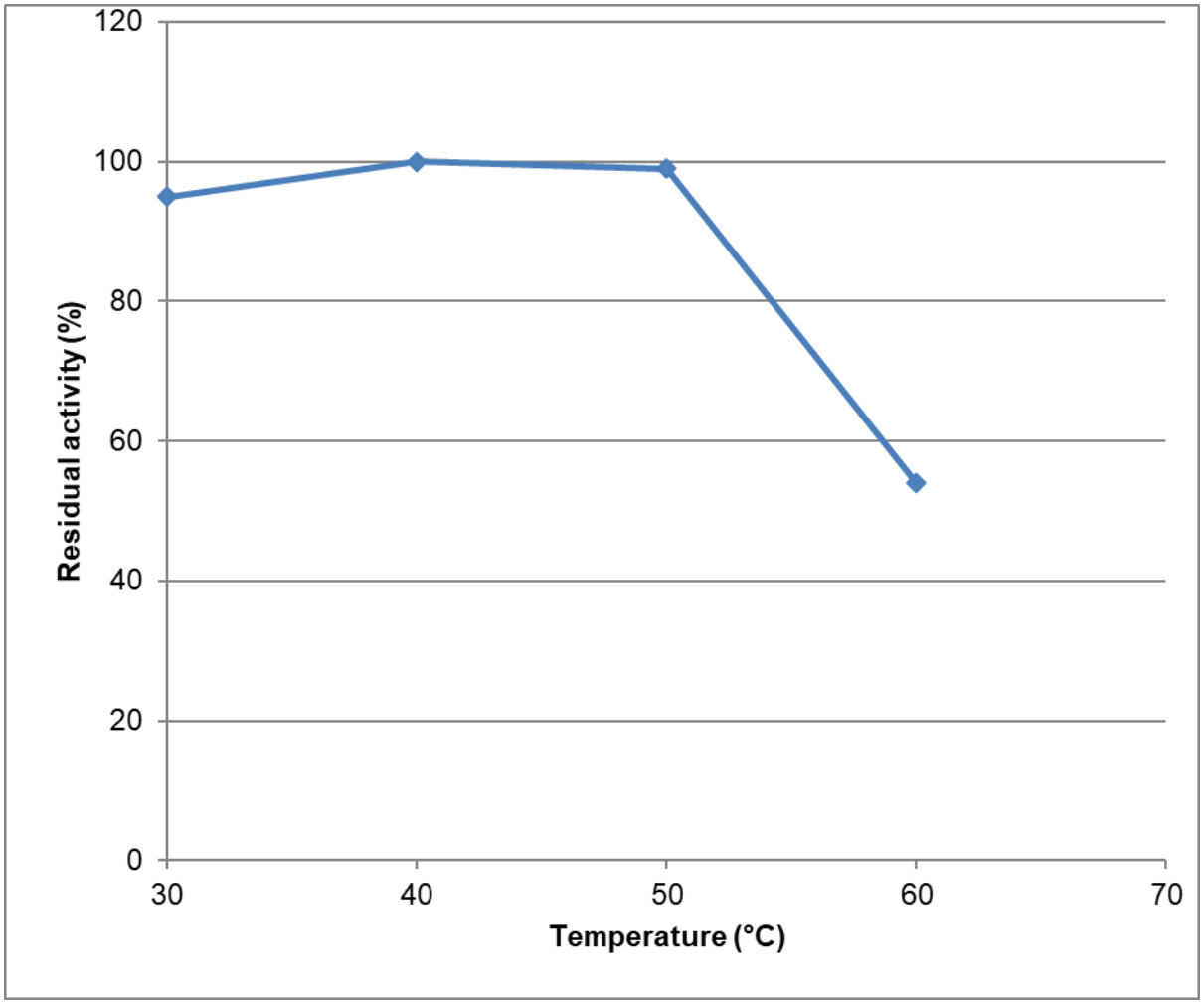


Figure 4.6. Thermal stability of the *Anoxybacillus* sp. KTC2 β-glucosidase was investigated by incubating the enzyme at temperatures 30 - 60˚C for 24 hours' prior the determining the residual β-glucosidase activity. Results are a mean of three determinations ± standard deviation.

4.3.1.6. Optimum pH and pH stability

The optimum pH for enzyme activity was determined by performing enzyme activities over a pH of 3 - 10. The β-glucosidase was active over a wide pH range with more that 60% of the maximum activity between pH 4.5-7.5. The optimum pH for activity was pH 7 and 92% activity at pH 5.5. (Figure 4.7).

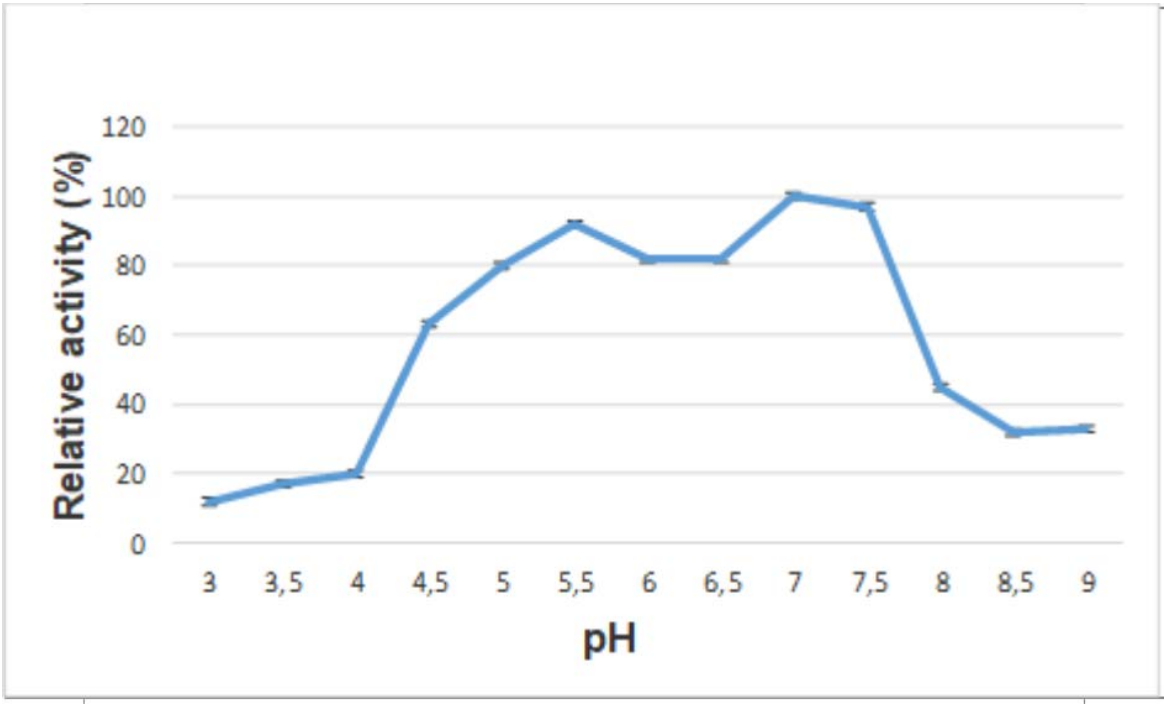


Figure 4.7. The effect of pH on β-glucosidase activity, showing a wide pH range (5 - 7.5). Results are a mean of three determinations ± standard deviation.

Enzyme stability was determined by incubating the crude enzyme in different pH buffers for 24 hours at 60˚C before determining residual activity. The enzyme retained 100% activity at pH 5 and 7, with more than 80% activity at pH 6.5, 7 and 8 after 24 hours incubation.

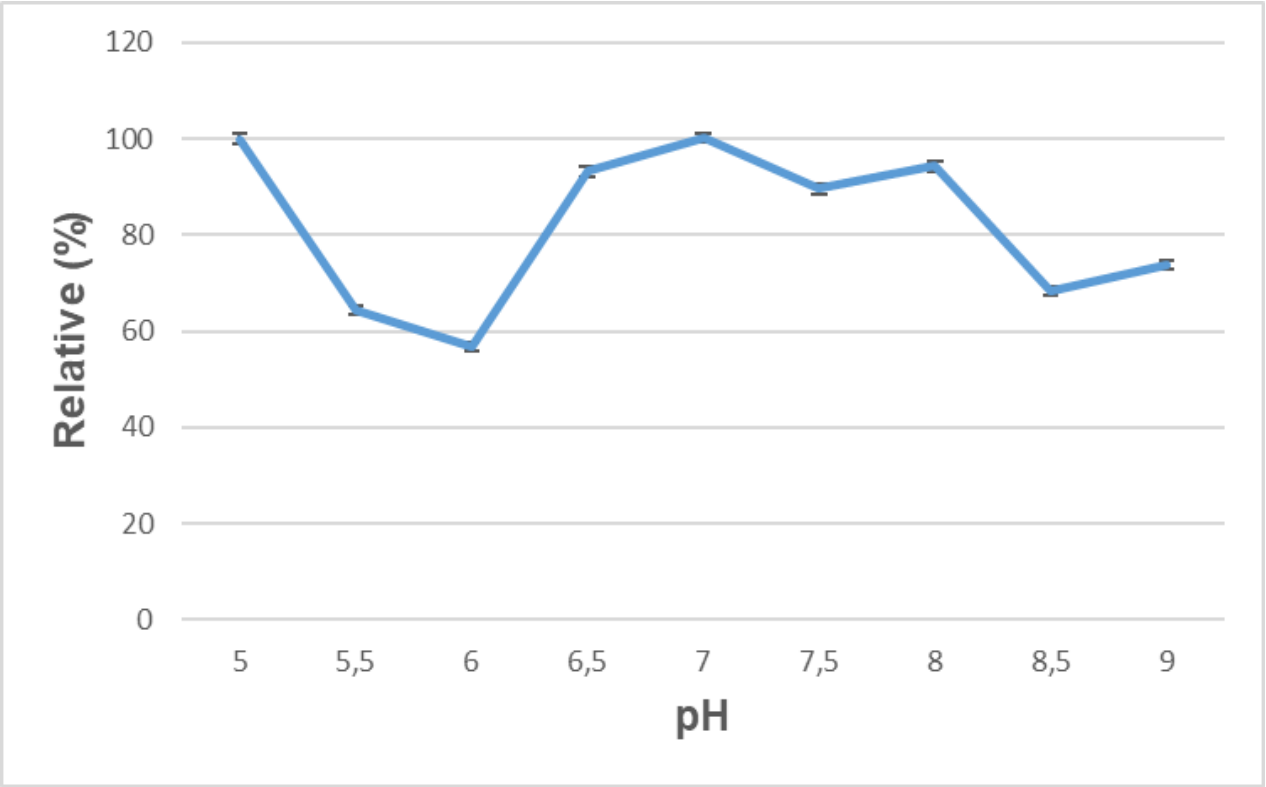


Figure 4.8. The effects of pH on enzyme stability after incubation in different pH buffers for 24 hours. Results are a mean of three determinations ± standard deviation.

4.3.2. Enzyme purification and size determination

4.3.2.1. β-glucosidase purification

Partial purification of the β-glucosidase was achieved through sequential chromatography using Q-sephrose and butyl-sepharose fast flow 4B (Table 4.1**)**. The presence of the *Anoxybacillus* sp. TKC2 β-glucosidase was visualised through SDS-PAGE and active β-glucosidase band determined through zymography (Figure 4.9). An active enzyme was recovered after partial purification. The β-glucosidase concentration was too low to be visualised on SDS-PAGE with Coomassie staining.

Table 4.1. Partial purification of the *Anoxybacillus* sp. TKC2 β-glucosidase.

Enzyme	Volume (ml)	Activity (U/ml)	Total activity	Yield	Protein (mg/ml)	Specific activity (U/mg)	Purification fold
Crude	50	15.2	760	100	4.4	3.5	1
Q-Sepharose	69	1.4	97	12.8	0.107	13.1	3.5
Butyl Seph	15	0.9	14	14.4	0.002	450	34.4

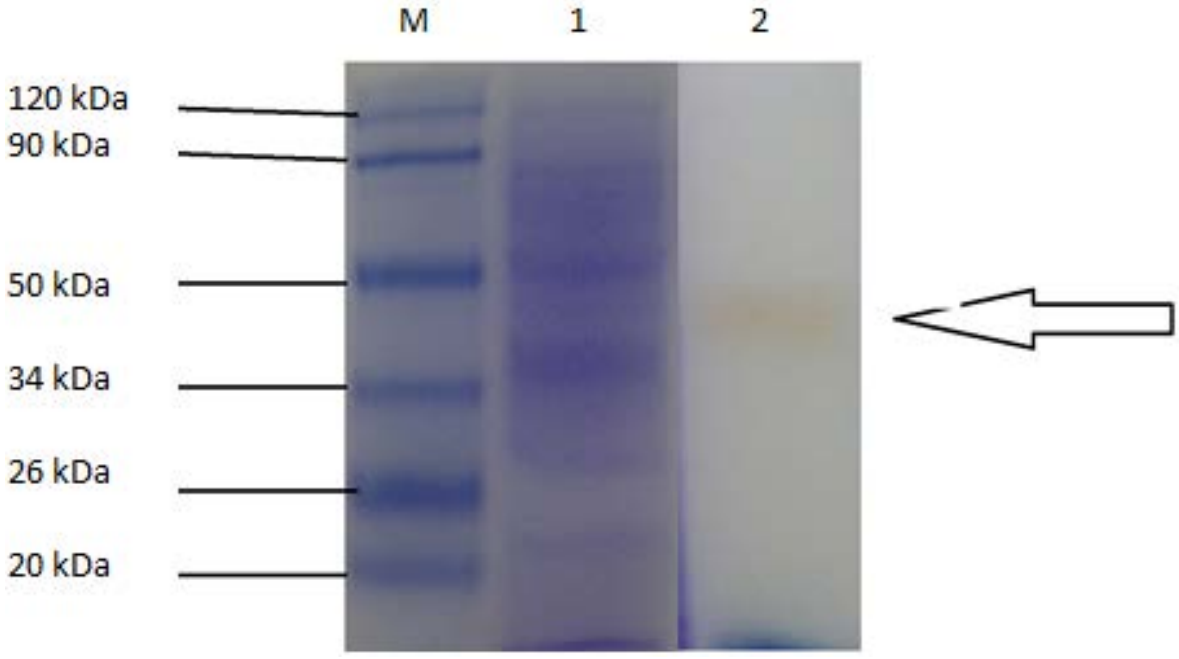
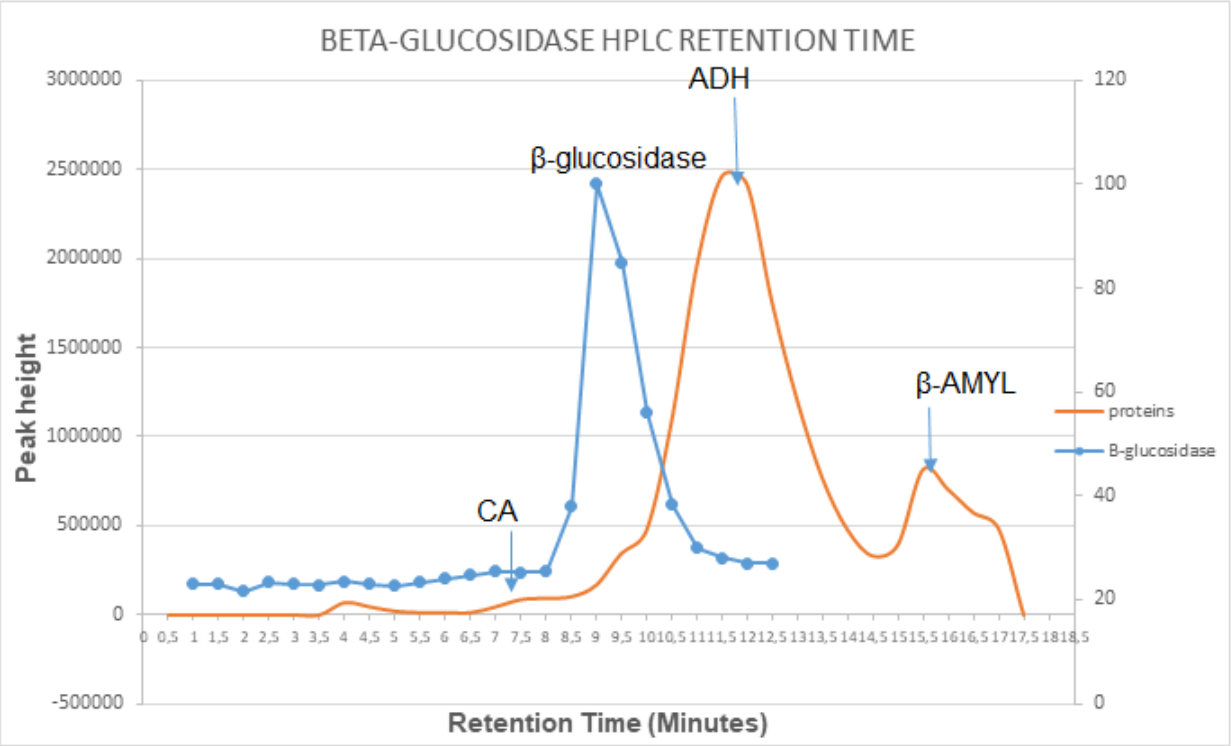


Figure 4.9. SDS-PAGE and zymography analysis of the *Anoxybacillus* TKC2 β-glucosidase, showing a single band of about 45 kDa. Lane 1: molecular weight marker, Lane 2: crude enzyme, Lane 3: zymogram/activity staining.

4.3.2.2 High-performance liquid chromatography

The β-glucosidase enzyme was eluted as a single sharp peak on HPLC against known standards (Figure 4.10).



CA- Carbonic anhydrase 29 kDa
ADH- Alcohol dehydrigenase 150 kDa
β-AMYL- β-Amylase 200 kDa

Figure 4.10. β-glucosidase HPLC elution profile. β-glucosidase eluted as a single sharp band with a retention time of 9 min. Enzyme standards used for calibration are indicated.

4.3.3. MALDI TOF MS protein sequencing

Two dimensional analysis of the *Anoxybacillus sp* KTC2 proteome was done to separate proteins based on their isoelectric point (Figure 4.11). The crude enzyme was used for isoelectric focusing (IEF) as partial purification resulted in very low concentrations of the protein and the bacterium has a large proteome which could not be separated through one dimension only. Protein spots between 40 and 50 kDa were picked based on the estimated protein size obtained through zymography.

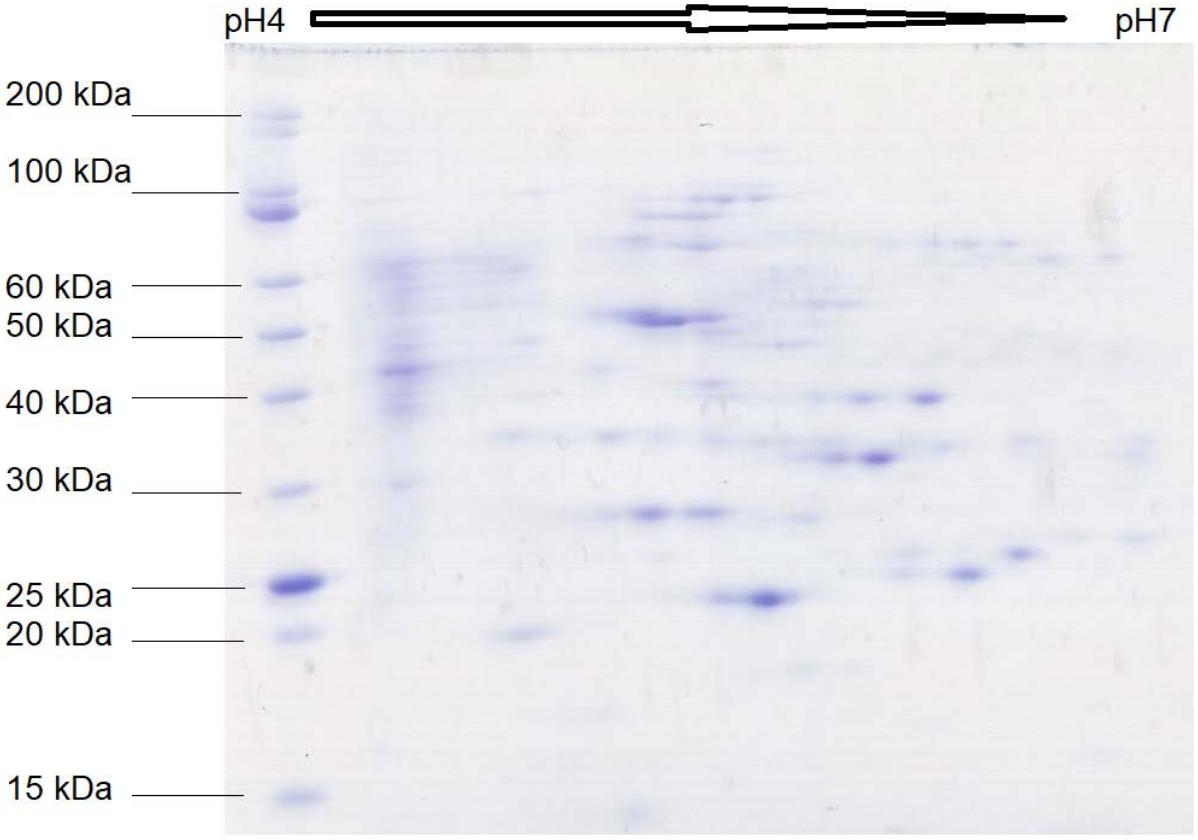


Figure 4.11. 2D gel analyses of the *Anoxybacillus* sp. KTC2 proteome.

Protein spots were excised and trypsin-digested for peptide sequencing through Matric Assisted Laser Desorption Ionisation Time of Flight analysis (MALDI-TOF). Peptide sequences obtained are listed in Table 4.2. A BLAST search of these peptides identified the enzyme as a β-glucosidase. The enzyme belongs to the *Anoxybacillus* genus, with the highest sequence similarity to a β-glucosidase from *Anoxybacillus flavithermus WK1.*

Table 4.2. LC-MS/MS analysis of β-glucosidase protein after trypsin digestion.

Peptide sequences of the *Anoxybacillus* sp. KTC2 β-glucosidase
VANDDNGDIAIDHYNR
DGEIGITLNLTPGYAVDLKDER
EGDLQTIQQPIDFFGFNYYSTATLK
RFGIVYVDYETLER

The peptides obtained through MALDI TOF MS were aligned to *A. flavithermus WK1* β-glucosidase protein sequence to determine the positions of the peptides (Figure 4.12). Sequence alignment showed a high sequence homology between the two β-glucosidases and the high degree of sequence conservation between bglB family enzymes.

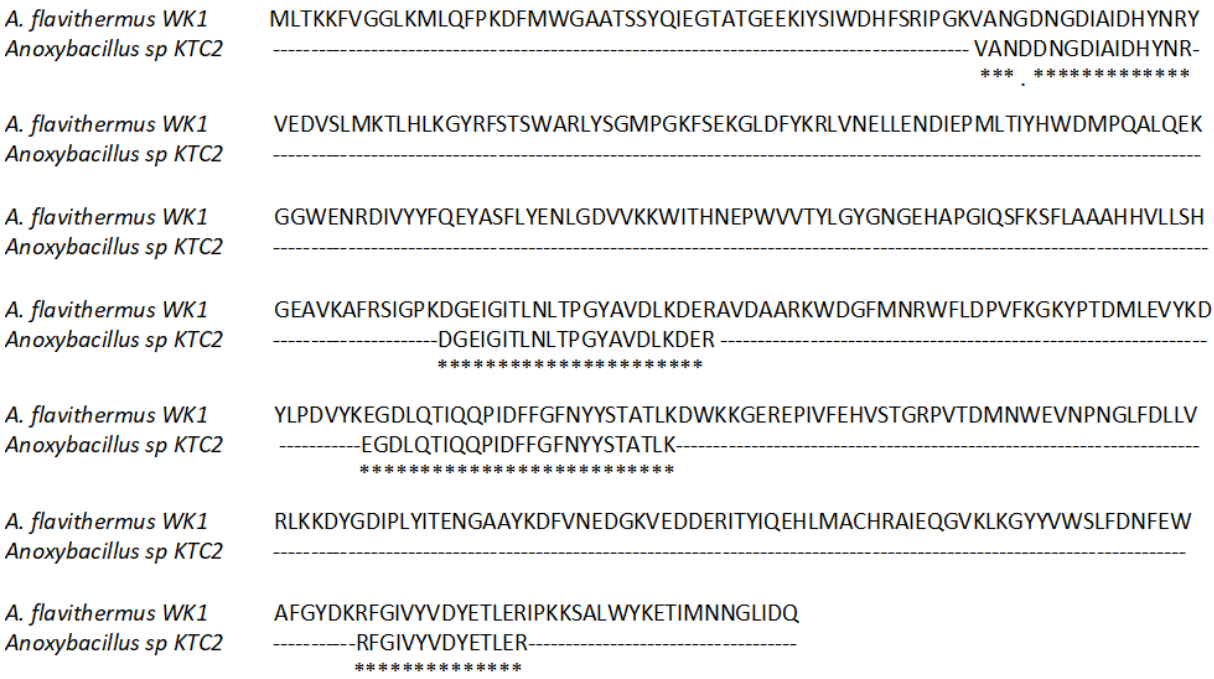


Figure. 4.12. Sequence alignment of *Anoxybacillus flavithermus* WK1 β-glucosidase and the *Anoxybacillus* sp. KTC2 peptides. The figure shows the positions of the sequenced peptides; identical amino acids are depicted with asterisks.

4.3.4. Saccharification of Avicel cellulose

4.3.4.1. Initial total cellulase and β-glucosidase activity

The initial Celluclast™ and β-glucosidase activities were determined. Celluclast™ had an initial total cellulase activity of 58.73 FPU with 158 U/ml β-glucosidase activity. The total cellulase activity for the crude *Anoxybacillus* KTC2 β-glucosidase was not determined as the glucose produced was less than 2 mg/ml. The β-glucosidase activity was 302 U/ml. The initial total cellulase activity of Cocktail 2 and Cocktail 3 was 74.0 and 71.15 FPU, respectively (Table 4.3).

Table 4.3. The initial total cellulase and β-glucosidase activities of the individual enzymes and cocktails.

	Total cellulase (FPU)	β-glucosidase (U/ml)
Celluclast™	58.73	158
Crude KTC2 β-glucosidase	-	302
Cocktail 2	74.0	-
Cocktail 3	71.15	-

Key: - Not determined

4.3.4.3. Saccharification of pure Avicel cellulose

Celluclast^{TM}, Cocktail 2 and Cocktail 3 were used to hydrolyse pure Avicel cellulose. Cocktail 3 had the highest glucose production at 7.2% above the glucose produced by the commercial enzyme Celluclast^{TM} after 96 hours incubation (Figure 4.13).

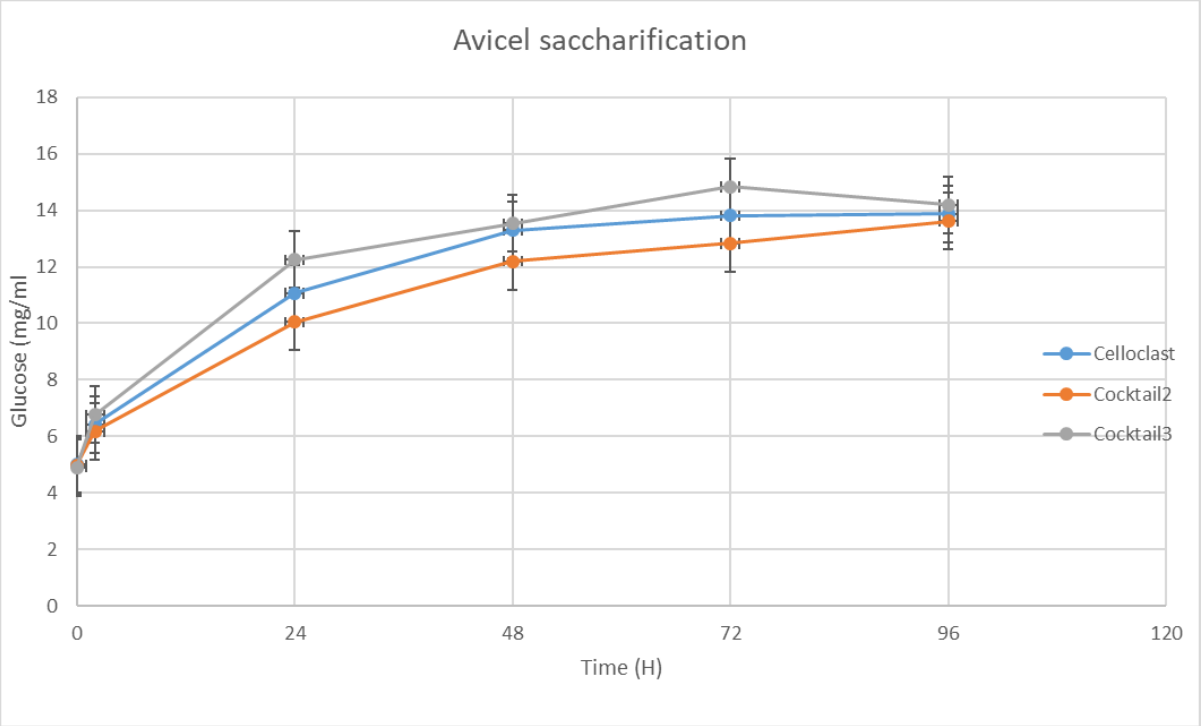


Figure 4.13. Saccharification of Avicel by Celluclast^{TM}, and two Celluclast^{TM}-crude *Anoxybacillus* sp. KTC2 β-glucosidase cocktails over 96 hours. Results are a mean of three determinations.

4.4. Discussion and conclusion

Lignocellulosic biomass is widely studied for its potential as a feedstock for bioethanol production. However, utilization of lignocellulosic biomass for ethanol production is dependent on efficient saccharification of lignocellulose with resultant high glucose yields. Industrial processes that use lignocellulosic biomass currently use fungal cellulase preparations supplemented with external β-glucosidases to improve the saccharification efficiency of the commercial cellulase preparations.

A thermostable β-glucosidase isolated from the bacterium, *Anoxybacillus* sp. KTC2 was optimally produced on M9 medium with 5% celolbiose as the main carbon source, at 60˚C and pH 7. Enzyme production on different growth medium showed cellobiose as the preferred carbon source for maximal enzyme production. Optimal enzyme production was observed with nutrient agar with 5% cellobiose. This is due to the inducible nature of β-glucosidases. Inducible enzymes are produced in the presence of an inducer and cellobiose was the prefered subtrate as it is a good inducer for β-glucosidases.

The *Anoxybacillus* sp. KTC2 β-glucosidase had an optimum temperature for activity of 60˚C with 80% activity at 70˚C, classifying the enzyme as a thermostable enzyme. The optimal activity of the *Anoxybacillus* sp. KTC2 β-glucosidase was comparable to those from *C. thermocellum*, *M. biospora*, *T. brockii* and *T. fusca* which also exhibited optimal activity at 60°C. β-glucosidases from *C. thermocellum and T. fusca* were unstable and rapidly inactivated at 60 and 65°C while *M. biospora β-glucosidase retained almost 70°C activity at 60° after 48 hours incubation* (Hong et al. 2009). The *Anoxybacillus* sp. KTC2 β-glucosidase retained 55% activity after 24-hour incubation at 60˚C with almost 100% activity after 24-hours at 50˚C. The high residual activity at 50˚C after 24 hours is noted as a crucial characteristic, as this is the saccharification temperature of most industrial cellulases. β-glucosidases with higher activity and thermostability than that of *Anoxybacillus* sp. KTC2 β-glucosidase have been reported (Hong et al. 2009, Bai et al. 2013) however, these β-glucosidases are mostly recombinant enzymes that exhibit improved activity and stability. Thermostable enzymes isolated mainly from thermophilic organisms, have found a number of commercial applications due to their overall inherent stability, reduced risk of contamination and increased reaction rates (Haki and Rakshit 2003).

The β-glucosidase enzyme exhibited a wide pH range of between 5.0 and 7.5. This too is of note for industrial enzymes, the wide pH range exhibited by the β-glucosidase enzyme allows for its addition to a variety of industrial cellulase preparations. Additionally, the enzyme was 100% stable at its optimal pH of 7 and at pH 5 after 24-hour incubation. The wide pH range of the *Anoxybacillus* sp. is a common characteristic of thermostable enzymes used in industrial application. Thermostable enzymes are highly adaptable to a wide pH range making them ideal for various bioprocesses (Bhalla et al. 2013)

The *Anoxybacillus* sp. KTC2 β-glucosidase was partially purified through sequential chromatographic steps on Q-sepharose followed by Butyl sepharose 4 fast flow. The enzyme was partially purified to a 34,4% purification fold, resulting in an increase in specific enzyme activity from 3.5 U/mg crude protein, to 450 U/mg protein, while the protein concentration reduced from 4.4 mg/ml to 0.002 mg/ml. The activity of the enzyme increased with subsequent purification steps. The increase in activity may be due to a slight increase in the β-glucosidase enzyme concentration per µl used in the reaction, or the reduction in impurities found in the crude enzyme. The partially purified β-glucosidase is highly active but the concentration was too low to be visualised on SDS-PAGE gel. The high activity observed even with very low concentrations may be due to the enzyme's high specificity. Thermostable enzymes are highly specific, resulting in increased reaction rates.

The β-glucosidase protein size was estimated to be approximately 45 kDa through zymography. Furthermore, the presence of only one active β-glucosidase was visually observed.

Protein analysis through HPLC revealed that the *Anoxybacillus* sp. KTC2 β-glucosidase is a monomeric protein and confirmed the presence of a single β-glucosidase. The protein eluted as a single sharp peak. Peptide sequencing through MALDI TOF further confirmed the active protein as a β-glucosidase from the genus, *Anoxybacillus*. A BLAST search and sequence alignment displayed a high sequence homology between the *Anoxybacillus* sp. KTC2 β-glucosidase and β-glucosidases from the genus *Anoxybacillus*, as well as the high degree of sequence conservation between GH1 family enzymes.

Addition of an external β-glucosidase to commercial cellulase preparations is known to improve the saccharification efficiency of the cocktail. An improvement in the saccharification efficiency of Celluclast^{TM} was observed with the addition of diluted crude *Anoxybacillus* sp. KTC2 β-glucosidase. Enzyme Cocktail 3 showed a 7.2% increase in saccharification efficiency from that observed for the commercial enzyme, containing 25% more Celluclast^{TM}. β-glucosidases function to catalyse the final step in cellulose hydrolysis and remove the inhibitory effect of cellobiose on endo-glucanase and cellobiohydrolase (Florindo *et al*., 2018), resulting in improved cellulose hydrolysis and glucose production. Cocktail 2 had a lower saccharification efficiency than both Celluclast^{TM} alone and Cocktail 3. Cocktail 2 had a higher concentration of the crude β-glucosidase, but produced less glucose. The lower cellulose hydrolysis efficiency observed in Cocktail 2 is believed to be due to the presence of more inhibitory components from the growth mediun, which are more diluted in Cocktail 3.

In addition to increasing Celluclast^{TM} saccharification effieciency, the high activity at low concetrations observed, makes the *Anoxybacillus* sp. KTC2 β-glucosidase a good candidate for bioethanol production, as reduced enzyme loading is one way to reduce the production cost of bioethanol. The high price associated with enzyme production is still a bottleneck in the utilization of lignocellulosic biomass as feedstock.

4.5. References

Almeida, M. N. De, Falkoski, D. L., Guimarães, V. M., and Rezende, S. T. De, 2019. Industrial Crops & Products Study of gamba grass as carbon source for cellulase production by Fusarium verticillioides and its application on sugarcane bagasse sacchari fi cation. *Industrial Crops & Products*, 133, 33–43. Available from: https://doi.org/10.1016/j.indcrop.2019.03.008. Online.

Alves, L. D. F., Meleiro, L. P., Silva, R. N., Westmann, C. A., Guazzaroni, M., and Hickey, W. J., 2018. Novel Ethanol- and 5-Hydroxymethyl Retrieved From a Brazilian Secondary Atlantic Forest Soil Metagenome. *Frontiers in Microbiology*, 9 , 1–17.

Bai, A., Zhao, X., Jin, Y., Yang, G., and Feng, Y., 2013. A novel thermophilic β-glucosidase from Caldicellulosiruptor bescii: Characterisation and its synergistic catalysis with other cellulases. *Journal of Molecular Catalysis B: Enzymatic*, 85–86, 248–256.

Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Markov, A., Skomarovsky, A., Okunev, O., Gusakov, A., Maximenko, V., Gregg, D., Sinitsyn, A., and Saddler, J., 2005. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates - Evidence for the role of accessory enzymes. *Enzyme and Microbial Technology*, 37 (2), 175–184.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M., and Sani, R. K., 2013a. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology*, 128, 751–759. Available from: http://dx.doi.org/10.1016/j.biortech.2012.10.145. Online.

Brijwani, K., Oberoi, H. S., and Vadlani, P. V., 2010. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochemistry*, 45 (1), 120–128.

Garvey, M., Klose, H., Fischer, R., Lambertz, C., and Commandeur, U., 2013. Cellulases for biomass degradation: Comparing recombinant cellulase expression platforms. *Trends in Biotechnology*, 31 (10), 581-593.

Ghose, T. K., 1987. Measurement of cellular activities. *Pure & Applied. Chem*, 59,

257–268.

Haki, G. D. and Rakshit, S. K., 2003. Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, 89 (1), 17-34.

Hong, M. R., Kim, Y. S., Park, C. S., Lee, J. K., Kim, Y. S., and Oh, D. K., 2009. Characterisation of a recombinant β-glucosidase from the thermophilic bacterium Caldicellulosiruptor saccharolyticus. *Journal of Bioscience and Bioengineering*, 108 (1), 36–40.

Ichikawa, S., Ichihara, M., Ito, T., Isozaki, K., Kosugi, A., and Karita, S., 2019. Glucose production from cellulose through biological simultaneous enzyme production and saccharification using recombinant bacteria expressing the β-glucosidase gene. *Journal of Bioscience and Bioengineering*, 127 (3), 340–344. Available from: https://doi.org/10.1016/j.jbiosc.2018.08.008. Online.

Joo, A. R., Jeya, M., Lee, K. M., Lee, K. M., Moon, H. J., Kim, Y. S., and Lee, J. K., 2010. Production and characterisation of β-1,4-glucosidase from a strain of Penicillium pinophilum. *Process Biochemistry*, 45 (6), 851–858.

Kalyani, D., Lee, K. M., Kim, T. S., Li, J., Dhiman, S. S., Kang, Y. C., and Lee, J. K., 2013. Microbial consortia for saccharification of woody biomass and ethanol fermentation. *Fuel*, 107, 815–822.

Kim, Y., Mosier, N. S., Ladisch, M. R., Ramesh Pallapolu, V., Lee, Y. Y., Garlock, R., Balan, V., Dale, B. E., Donohoe, B. S., Vinzant, T. B., Elander, R. T., Falls, M., Sierra, R., Holtzapple, M. T., Shi, J., Ebrik, M. A., Redmond, T., Yang, B., Wyman, C. E., and Warner, R. E., 2011. Comparative study on enzymatic digestibility of switchgrass varieties and harvests processed by leading pretreatment technologies. *Bioresource Technology*, 102 (24), 11089–11096.

Manickam, N. K., Rajarathinam, R., and Muthuvelu, K. S., 2018. Bioresource Technology New insight into the e ff ect of fungal mycelia present in the bio-pretreated paddy straw on their enzymatic saccharification and optimization of process parameter. Bioresource Technology, 267, 291–302.

Ndlovu, T. M. and Wyk, J. P. H. Van, 2019. Relative sacchari fi cation of waste paper

during successive treatment with garden snail (Cornu aspersum) cellulase. *Sustainable Chemistry and Pharmacy*, 11 (December 2018), 54–60. Available from: https://doi.org/10.1016/j.scp.2018.12.003. Online.

Oguro, Y., Nakamura, A., and Kurahashi, A., 2019. Effect of temperature on sacchari fication and oligosaccharide production efficiency in koji amazake. *Journal of Bioscience and Bioengineering*, 127 (5), 570–574.

Oleson, K. R. and Schwartz, D. T., 2019. Bioresource Technology Reports Douglas-fir extractives inhibit saccharification. *Bioresource Technology Reports*, 5, 335–338. Available from: https://doi.org/10.1016/j.biteb.2018.10.001. Online.

Pamella Santa-Rosa., Anita Souza, Rosemary Roque, Andrade Filho, Spartaco Edmar, Adolfo Mota, C. N.-S., 2017. Production of β-glucosidase and CMCase thermostables by Penicillium sp. LMI01 isolated in the Amazon region. *Electronic journal of biotechnology*, 31 (31. 10.1016/j.ejbt.2017.11.005.).

Sukumaran, R. K., Singhania, R. R., and Pandey, A., 2005. Microbial cellulases Production , applications and challenges. *Journal of Scientific and Industrial Research*, 64, 832–844.

Tengerdy, R. P. and Szakacs, G., 2003. Bioconversion of lignocellulose in solid substrate fermentation. *Biochemical Engineering Journal*, 13 (2-3), 169-179.

Wen, Z., Liao, W., and Chen, S., 2005. Production of cellulase/β-glucosidase by the mixed fungi culture Trichoderma reesei and Aspergillus phoenicis on dairy manure. *Process Biochemistry*, 40 (9), 3087–3094.

You, A., Be, M. A. Y., and In, I., 2018. Review : Engineering of thermostable enzymes for industrial applications, *APL Bioengineering*. 2, 011501; https://doi.org/10.1063/1.4997367.

CHAPTER 5

SEQUENCING OF THE *Anoxybacillus* sp. KTC2 β-GLUCOSIDASE GENE

5.1. Introduction

The high cost of cellulosic enzymes is driving research to find ways to improve enzyme production and organisms to reduce the cost of production. These approaches range from modifying cellulase producing strains to optimizing parameters involved in enzyme production. Improvement in cellulase expression has the potential to reduce the cost involved in biofuel production (Garvey et al. 2013, Li et al. 2019). Cellulases play a major role in biofuel production as they are responsible for enzymatic hydrolysis of plant biomass to fermentable sugars. However, the use of these cellulases is currently not economically viable due to the high costs associated with enzyme production and the quality of cellulase enzymes currently available (Srivastava et al. 2018).

The cellulolytic fungus, *Tricoderma reesei* is still the leading producer for industrial cellulases (Li et al. 2019). However, the *T. reesei enzyme* cocktail widely used in industrial preparations has one major limitation of a lack of sufficient activities of the β-glucosidase, for complete hydrolysis of lignocellulosic biomass (Xia et al. 2018a). Many research groups are working on the development of heterologous systems to improve enzyme production yields. However, *T. reesei* remains the preferred cellulase producer because of its high production yields and the high costs involved in the development and use of genetically modified organisms (GMO's) (Garvey et al. 2013, Srivastava et al. 2018). These challenges drive the need to explore alternative methods to improve cellulase enzyme production.

Current methods to improve cellulase cocktails for the complete hydrolysis of lignocellulosic biomass for biofuel production involves the use of externally produced β-glucosidases (Florindo et al. 2018 , Wei et al. 2019). There are many reports in literature on studies to find new organisms capable of producing highly active extracellular β-glucosidases. This is due to the vital role these enzymes play in cellulose hydrolysis. β-glucosidases are responsible for the final step in cellulose

hydrolysis, converting cellobiose to two glucose molecules and relieving product inhibition effect of cellobiose on the endoglucanases and cellobiohydrolases (Mei et al. 2016, Srivastava et al. 2018).

The ideal β-glucosidases for industrial use should be active at high temperatures, be thermostable, exhibit high catalytic efficiency and be tolerant to inhibitors that result from pre-treatment (Alves et al. 2018). Thermostable enzymes from thermophilic organisms are important due to their characteristic high stability under different environmental stress, high specific activity, long shelf life, activity at high temperatures resulting in higher substrate solubility and higher diffusion rates. These are important traits for industrial enzymes and may ultimately assist in lowering the cost of enzymes in bioethanol production, through improved hydrolysis rates, product yields and reduced energy costs. Less cooling required after pre-treatment (Ay et al. 2011, Diogo et al. 2015, Srivastava et al. 2018). Recombinant enzyme expression systems for β-glucosidase production may help to improve enzyme yields.

Escherichia coli is the most commonly used expression system for recombinant proteins. Cellulases from extreme environments have been expressed and characterised through the use of *E. coli* (Garvey et al. 2013). A number of recombinant β-glucosidases have been expressed in *E. coli* and are well-studied.

The main aim of this study was to sequence the *Anoxybacillus* sp. *K*TC2 thermostable β-glucosidases gene (GLU), to gain sequence information for further characterisation of the gene.

5.2. Materials and methods

5.2.1. Genomic DNA extraction and sequencing

Genomic DNA was isolated using the Quick-DNATM Fungal/Microbial Miniprep Kit (Zymo Research, USA), following the manufacturer's instructions and separated on a 0.8% agarose gel to assess the quality of the DNA. The extracted DNA was sent to Inqaba Biotechnology Industries for whole genome sequencing. All subsequent sequence analysis was performed in silico.

5.2.2 Cloning of the β-glucosidase gene

Gene specific primers were designed to amplify the β-glucosidases gene from the genome of *Anoxybacillus* sp. KTC2 (Table 5.1). Restriction sites were added to the primers, to facilitate directional cloning into the pTYB21 expression vector. Genomic DNA isolated as stated in section 5.2.1. was used as template for gene amplification.

Table 5.1. Primers used for PCR amplification of the β-glucosidase gene from the genome of *Anoxybacillus* sp. KTC2.

Primer name	Sequence (5'-3')	Restriction site
BGLfor1	5'-CG**CATATG**GGCATAGTTTATGATTCGATTTCCG-3'	*Nde*I
BGLrev1	5'-GC**GTCGAC**CCATCACCTCCTTCATTTATATCG-3'	*Sal*I

Restriction sites introduced are indicated in bold.

5.2.3 Recombinant strain construction

Gene amplification through PCR was performed according to the manufacturers specification using the Q5 High fidelity master mix (New England Biolabs, USA). The PCR amplicon was separated on a 0.8% agarose. DNA was recovered and purified using the Zymoclean^{TM} Gel DNA Recovery Kit (USA), according to the manufactures instructions. The expression vector pTYB21 and amplified GLU gene were digested with restriction enzymes; *Nde*I and *Sal*I (Table 5.3), according to the manufacturer's instruction. The digested GLU fragment was ligated into the

corresponding sites on the pTYB21 expression vector to create pTYB21-GLU (Figure 5.1).

The β-glucosidase gene was cloned into an *E. coli* expression N-terminal fusion vector designed for in-frame insertion of a target gene into the polylinker downstream of the intein tag. The intein tag can be used for purification of the recombinant protein. The recombinant vector was propagated in *E. coli* T7. Recombinant *E. coli* cells were cultured at 37˚C in Luria Bertani medium (LB medium) supplemented with 50 µg/ml ampicillin. Plasmid DNA isolation was done according to the manufacturers instruction using the Ziggy^{TM} Plasmid Miniprep Kit. The presence of an insert was confirmed through recombinant vector sequencing.

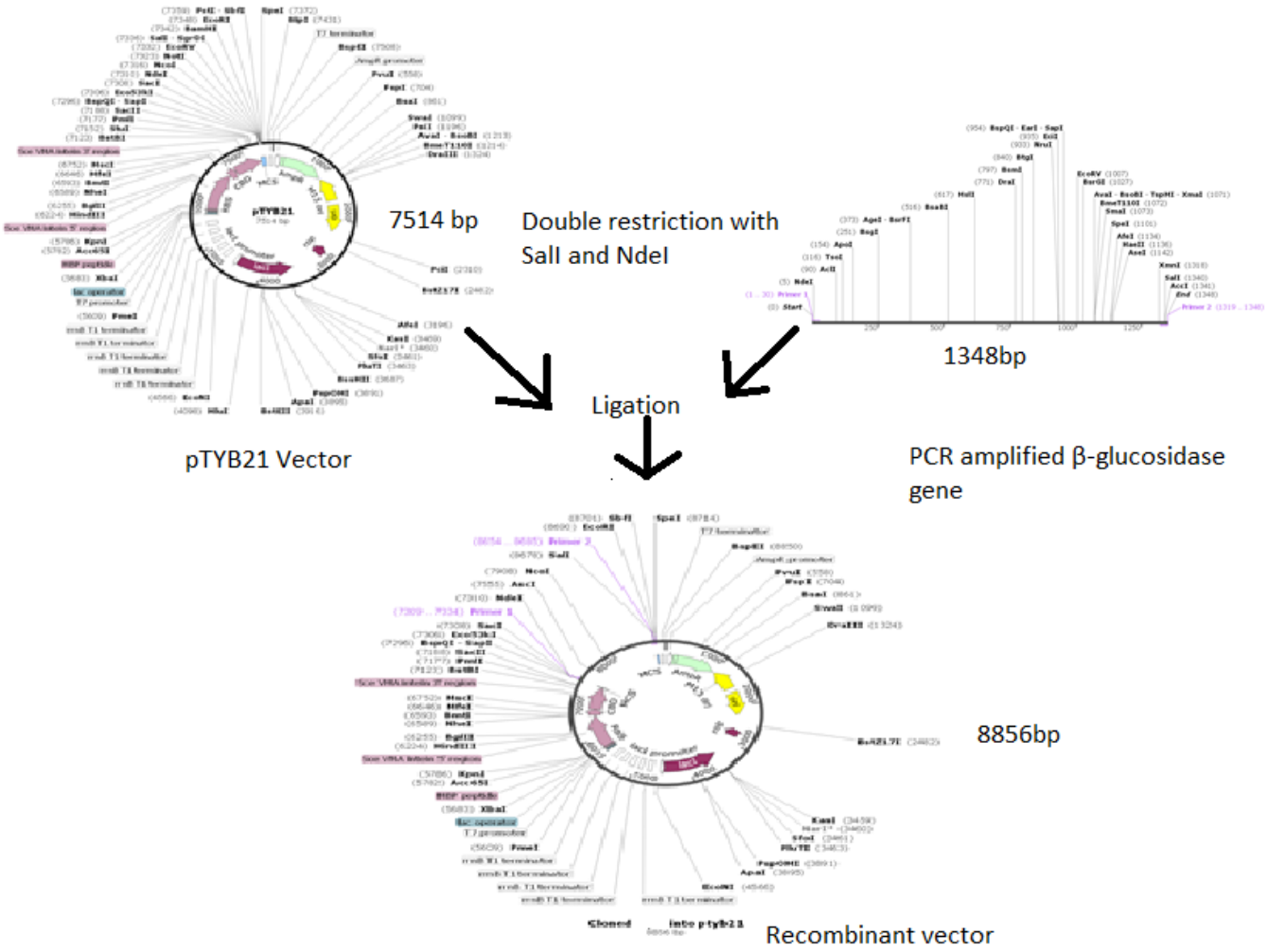


Figure 5.1. An illustration of the pTYB21-BGL construction.

5.3. Results

5.3.1. Whole genome sequencing

Whole genome sequencing of *Anoxybacillus* sp. KTC2 revealed the presence of only one gene coding for a β-glucosidase. The open reading frame (ORF) finder was used to locate the 1362 bp putative complete β-glucosidase gene (https://www.ncbi.nlm.nih.gov/orffinder/). The gene codes for a 453 amino acid protein with a molecular mass of 53 kDa and a theoretical pI of 5.44. The deduced amino acid sequence was analysed using BlastP against non-redundant databases. Protein alignments were performed using the ClustalW alignment tool (http://www.ebi.ac.uk./tools/clustalw2/index.html).

Sequence alignment of the deduced amino acid sequence to similar proteins revealed that the β-glucosidase belongs to the Glycoside Hydrolase family 1 proteins (GH1), with high sequence homology between the sequences (Figure 5.2).

```
Anoxybacillus sp            EDVALMKALHLKAYRFSTSWARLYSETPGKFNEKGLDFYKRLV 50
ACL38401             ------VEDVSLMKTLHLKGYRFSTSWARLYSGMPGKFSEKGLDFYKRLV 44
ABV987405            ----------------IQSYRFSISWPRIFPQGTGQANLKGLDFYKRLI 33
ACJ34717.1           -------------GRLNMKAYRFSTSWARCMPDGVTP-NPDGIAFYSRLV 36
WP_025027279.1       DHYHRLDRDVALMAELGLRSYRFSVSWSRVQPGGHGPVNQEGLDFYRRLV 50

Anoxybacillus sp     HELLENDIEPMLTIYHWDMPQALQEKGGWENRDIVHYFREYAEFLYKNLG 100
ACL38401             NELLENDIEPMLTIYHWDMPQALQEKGGWENRDIVYYFQEYASFLYENLG 94
ABV987405            DQLLKQGIEPMATIYHWDLPQALEDQGGWQNREMVDHFEQYASFLYEELG 83
ACJ34717.1           DELLAAGITPWLTLYHWDLPQALEDNGGWANRDTAYRFADYAALMHSVLG 86
WP_025027279.1       DQLLANGIEPWLTLYHWDLPQPLEDAGGWPTRDTSARFAEYTSLVAGALG 100

Anoxybacillus sp     DVVKKWITHNEPWVVTYLGYGNGEHAPGIQNFTSFLKAAHHVLLSHGEAV 150
ACL38401             DVVKKWITHNEPWVVTYLGYGNGEHAPGIQSFKSFLAAAHHVLLSHGEAV 144
ABV987405            DVVKKWITHNEPWVVAYLGYGNGEFAPGIKGFEEYLRAAHHVLLSHGKSV 133
ACJ34717.1           DRVRIWTTLNEPWCSAFLGYAAGIHAPGRQEPRAALAAAHHLLLGHGLAA 136
WP_025027279.1       DRVRYWTTLNEPWCSAFLGYGSGAHAPGRSDPADAVRAGHHLMLGHGLAV 150

Anoxybacillus sp     KAFREIGSKDGEIGITLNLTPGYAVDPQDEKAVDAARKWDGFMNRWFLDP 200
ACL38401             KAFRSIGPKDGEIGITLNLTPGYAVDLKDERAVDAARKWDGFMNRWFLDP 194
ABV987405            SAFREKGPKDGQIGITFNLNSTYAGSPSRE-DQEAARRYDGFLNRWYLDP 182
ACJ34717.1           AELRRRD-TEASLGITLNLTVSDPRDPGSESDRDAARRIDGQFNRIFLDP 185
WP_025027279.1       QALRSSARSDAEVGVTVNLYPVTP-ATDSPGDADAARRIDALANRFFLDP 199

Anoxybacillus sp     VFKGHYPQDMLEVY-KDYLPDVYREGDLQTIQQPIDFFGFNYYS------ 245
ACL38401             VFKGKYPTDMLEVY-KDYLPDVYKEGDLQTIQQPIDFFGFNYYS------ 237
ABV987405            VFKGEYPKDMLDLYLQKYNLDFVQDGDLKAISQPIDFLGINYYS------ 226
ACJ34717.1           LFRGEYPADVLADVAHLGMADLVQDGDLELIATPLDLLGVNYYHGESLTK 235
WP_025027279.1       LLRGAYPVDLMLDLERVADFGHVHEGDLDTIAAPLDLVGINYYS------ 243

Anoxybacillus sp     -----TATLKDWKKGER------------EPIVFEHVSTGRPVTDMNWEV 277
ACL38401             -----TATLKDWKKGER------------EPIVFEHVSTGRPVTDMNWEV 270
ABV987405            -----IMTALDESQAE-------------DVLKFQALKTGRPTTAMDWEI 258
ACJ34717.1           DLAGAQEQAAPETTSVPGQATRAVASPFVAADGARSVRRGLPVTGMGWEV 285
WP_025027279.1       ----RHVVAAPAAQAPP-QPYWRTPSCWPGSEHVRFVTRGVPVTDMDWEI 288

Anoxybacillus sp     NPNGLFDLLVRLKNDYG---DIPLYITENGAAYKDFINEQGKVEDDERVA 324
ACL38401             NPNGLFDLLVRLKKDYG---DIPLYITENGAAYKDFVNEDGKVEDDERIT 317
ABV987405            EPNGLFDLLVRINKEYCS--KTPIFITENGAAFEDQVVN-GEIMDDDRLD 305
ACJ34717.1           QPEGLRRLLNRLHTEYTGPAGIPIYITENGAAYDDVPDATGFVDDQDRLG 335
WP_025027279.1       DPPGLVETLQRVYEEYT---DLPLYVTENGSAFVDT-VVEGHVDDPDRVA 334

Anoxybacillus sp     YIQEHLAACHRAIEQGVNLRGYYVWSLFDNFEWAFGYDKRFGIVYVDYET 374
ACL38401             YIQEHLMACHRAIEQGVKLKGYYVWSLFDNFEWAFGYDKRFGIVYVDYET 367
ABV987405            YIKQHLQACHKGIEHGINVSGYYVWSLLDNFEWASGYEKRFGIVHVDFDS 355
ACJ34717.1           FFAAHLDAVHRAIADGVDVRGYLAWSLLDNFEWSFGYHQRFGMVRVDYCT 385
WP_025027279.1       YFDAHLRAAHQAITAGVPLRGYFAWSLMDNFEWAWGYTKRFGMIHIDYRS 384

Anoxybacillus sp     LERIPKKSALWYKETI---------------------- 395
ACL38401             LERIPKKSALWYKETIMNNGLIDQ-------------- 391
ABV987405            LKRTPKKSALWYKEVISNQGCFE--------------- 378
ACJ34717.1           QDRIPKASALWYSSVASGNALPAGSSPVPPPARGVVLSV 424
WP_025027279.1       QVRTLKSSGRWYAEVIRRNGLAAQ-------------- 408
```

Figure 5.2. Alignment of the deduced-amino acid sequence of the *Anoxybacillus* sp. KTC2 β-glucosidase and related GH1 enzymes, *Arthrobacter chlorophenolicus*, *Salinispora arenicola*, *Anoxybacillus flavithermus WK1* and *Bacillus mannanilyticus.* The conserved catalytic central in GH1 are shown in grey background with E as the catalytic amino acid.

Secondary structure prediction was done through web based tools, I-TASSER server. The crystal structure was modelled against the *Ruminiclostridium thermocellum* β-glucosidase A (Figure 5.3 B), a GH 1 family enzyme that hydrolyses terminal non-reducing β-D-glycosyl residues with the release of a β-D-glucose (Yang et al., 2015). Sequence alignment of the two enzymes (Figure 5.3 C) and active site prediction through I-TISSER showed the same catalytic amino acid observed for GH1 family enzymes (Figure 5.2). Based on the template model, the first catalytic amino acid at position 164 is predicted to be a proton donor while the catalytic amino acid at position 354 is predicted to be a nucleophile.

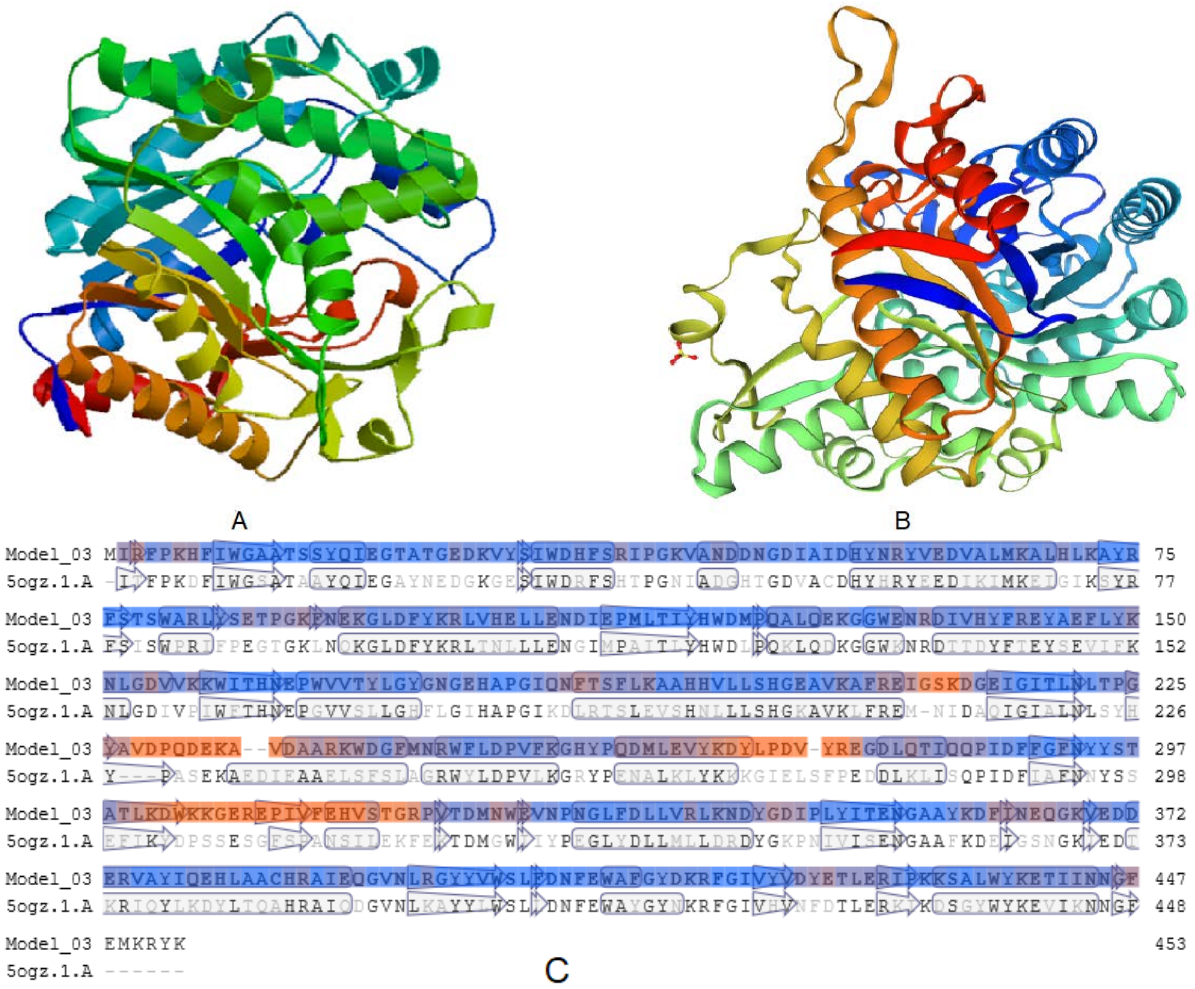


Figure 5.3. The predicted tertiary structure of the *Anoxybacillus* sp. KTC2 β-glucosidase (A), the secondary protein structure of β-glucosidase A from *Ruminiclostridium thermocellum* (B), and sequence alignment of the two protein sequences (C).

5.3.2 Cloning of the *Anoxybacillus* sp. KTC2 β-glucosidase gene

The β-glucosidase gene was amplified using gene specific primers BGLF1/BGLR1. DNA analysis of the PCR amplicon showed a single band of 1423 bp in size (Figure 5.4). This was the expected size range based on the β-glucosidase gene sequence obtained from whole genome sequencing (section 5.3.1).

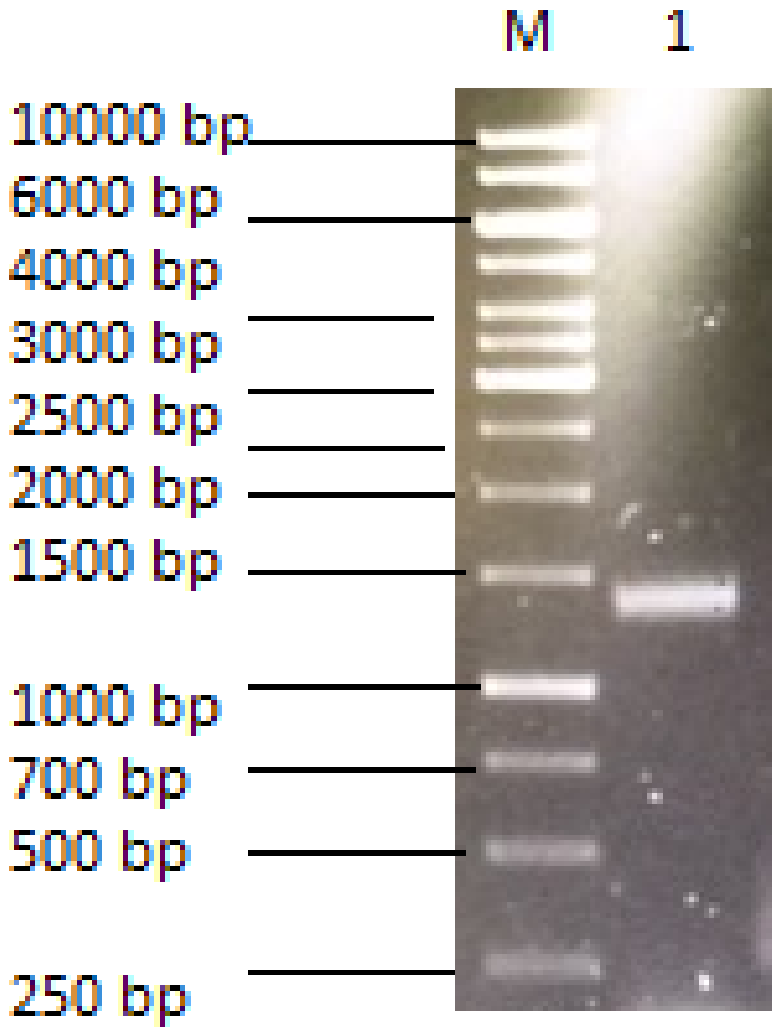


Figure 5.4. PCR amplification of the β-glucosidase gene amplicon. The gene was amplified from *Anoxybacillus* sp. KTC2 genomic DNA. Lane M, GeneRuler 1kb DNA Ladder, Lane 1, β-glucosidase gene.

The amplified gene was cloned into pTYB21 expression vector and transformed into *E. coli* T7 and the cells propagated on LB medium with ampicillin to maintain plasmid selection. The presence of the β-glucosidase gene was confirmed through sequencing (Figure 5.4).

```
Anoxybacillus sp. KTC2     MIRFPKHFIWGAATSSYQIEGTATGEDKVYSIWDHFSRIPGKVANDDNGD   50
Anobaxycillus sp. KTC2     ----------------------------------------------------------------------------------   0

Anoxybacillus sp. KTC2     IAIDHYNRYVEDVALMKALHLKAYRFSTSWARLYSETPGKFNEKGLDFYK   100
Anoxybacillus sp. KTC2     ------------------------MKALHLKAYRFSTSWARLYSETPGKFNEKGLDFYK   35
                                                    ***********************************

Anoxybacillus sp. KTC2     RLVHELLENDIEPMLTIYHWDMPQALQEKGGWENRDIVHYFREYAEFLYK   150
Anoxybacillus sp. KTC2     RLVHELLENDIEPMLTIYHWDMPQALQEKGGWENRDIVHYFREYAEFLYK   85
                           **************************************************

Anoxybacillus sp. KTC2     NLGDVVKKWITHNEPWVVTYLGYGNGEHAPGIQNFTSFLKAAHHVLLSHG  200
Anoxybacillus sp. KTC2     NLGDVVKKWITHNEPWVVTYLGYGNGEHAPGIQNFTSFLKAAHHVLLSHG  135
                           **************************************************

Anoxybacillus sp. KTC2     EAVKAFREIGSKDGEIGITLNLTPGYAVDPQDEKAVDAARKWDGFMNRWF  250
Anoxybacillus sp. KTC2     EAVKAFREIGSKDGEIGITLNLTPGYAVDPQDEKAVDAARKWDGFMNRWF  185
                           **************************************************

Anoxybacillus sp. KTC2     LDPVFKGHYPQDMLEVYKDYLPDVYREGDLQTIQQPIDFFGFNYYSTATL   300
Anoxybacillus sp. KTC2     LDPVFKGHYPQDMLEVYKDYLPDVYREGDLQTIQQPIDFFGFNYYSTATL   235
                           **************************************************

Anoxybacillus sp. KTC2     KDWKKGEREPIVFEHVSTGRPVTDMNWEVNPNGLFDLLVRLKNDYGDIPL  350
Anoxybacillus sp. KTC2     KDWKKGEREPIVFEHVSTGRPVTDMNWEVNPER-LVRLACSLKNDYVTFRI  285
                           **********************************   *   *   *****

Anoxybacillus sp. KTC2     YITENGAAYKDFIN-EQGKVEDDERVAYIQEHLAACHRAIEQ--GVNLRGYY   399
Anoxybacillus sp. KTC2     HYRNGAAYQDFYHEQGKVETNERXAVXTEHLASVSSCID-QXGXTX-------   330
                              ******  **   ******    ** *    ***          *  *

Anoxybacillus sp. KTC2     VWSLFDNFEWAFGYDKRFGIVYVDYETLERIPKKSALWYKETIINNGFEM   449
Anoxybacillus sp. KTC2     ----------------------------------------------------------------------------------   330

Anoxybacillus sp. KTC2     KRYK   453
Anoxybacillus sp. KTC2     ------   330
```

Figure 5.5. Sequence alignment of the deduced-amino acid sequence of the *Anoxybacillus* sp. KTC2 β-glucosidase from whole genome sequencing and the cloned gene (bold), showing the high sequence similarity between the two sequences.

5.4. Discussion and conclusion

Recombinant technology may help combat the high cost of cellulases through the use of expression systems such as *E. coli*. Overexpression of recombinant cellulases will lower the cost of enzymes in bioethanol production. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The enzymes can be produced by thermophilic microorganisms through either optimised fermentation or cloning of genes into fast-growing mesophiles such as *E. coli* (Haki and Rakshit 2003).

Sequence analyses of the translated protein revealed that, the β-glucosidase gene has high sequence homology to other GH1 family enzymes. The protein shared conserved regions with related GH1 enzymes as well as the catalytic amino acids. The β-glucosidase gene has the two TL/FNEP AND I/VTENG motifs that have glutamic residues involved in the catalysis of β-glucosidic bonds as consistent with GH1 family enzymes (Li. 2012, Bai et al. 2013). Additionally, the gene sequence shows E as the catalytic amino acid as observed in β-glucosidase genes from Caldiecellulosiruptor bescii and a β-glucosidase gene isolated from a metagenomic library (Li. 2012, Bai et al. 2013). Characterisation based on the sequence information is done using protein similarities from a structural point of view (Singhania et al. 2013). Glycosyl hydrolases are enzymes that hydrolyse the glycosidic bonds between carbohydrates or between carbohydrates and non-carbohydrate moieties. Although GH enzymes have similar catalytic functions, the different families have different 3D folds (Pozzo et al. 2010, Garvey et al. 2013, Florindo et al. 2018).

The tertiary structure of the *Anoxybacillus* sp KTC2 β-glucosidase was modelled against similar proteins in protein data bases though the I-TISSER server. The *Anoxybacillus* sp KTC2 β-glucosidase was modelled against a GH1 family enzyme, β-glucosidase A isolated from *Ruminiclostridium thermocellum*, with a confidence score (C score) of 1.32. The confidence score estimates the quality of the predicted model at a range of -5 to 2 (Roy et al. 2010, Yang et al. 2015). The ligand binding sites/catalytic sites of the β-glucosidase were confirmed at a C score of 0.97 (score range 0 - 1). The high C-score obtained confirms the amino acids at position 164 and 354 as catalytic sites for studied β-glucosidases. Furthermore, modelling against a

GH1 family enzyme with catalytic activity against cellulose confirms the activity of the current enzyme.

5.4. References

Alves, L. D. F., Meleiro, L. P., Silva, R. N., Westmann, C. A., Guazzaroni, M., and Hickey, W. J., 2018. Novel Ethanol- and 5-Hydroxymethyl Retrieved From a Brazilian Secondary Atlantic Forest Soil Metagenome. *Frontiers in Microbiology*, 9, 1–17.

Ay, F., Karaoglu, H., Inan, K., Canakci, S., and Belduz, A. O., 2011. Cloning, purification and characterisation of a thermostable carboxylesterase from Anoxybacillus sp. PDF1. *Protein Expression and Purification*, 80 (1), 74–79. Available from: http://dx.doi.org/10.1016/j.pep.2011.06.019. Online.

Bai, A., Zhao, X., Jin, Y., Yang, G., and Feng, Y., 2013. A novel thermophilic β-glucosidase from Caldicellulosiruptor bescii: Characterisation and its synergistic catalysis with other cellulases. *Journal of Molecular Catalysis B: Enzymatic*, 85–86, 248–256.

Diogo, A., Cota, J., Corre, T. L. R., Hoffmam, Z. B., Garcia, W., Oliveira, L. C., Prade, R. A., and Squina, F. M., 2015. Comparative analysis of three hyperthermophilic GH1 and GH3 family members with industrial potential. *New Biotechnology,* 32 (1), 13–20.

Florindo, R. N., Souza V. P., Mutti, H. S., Camilo, C., Manzine L. R., and Marana, S. R., 2018. Structural insight into beta-glucosidase transglycosylation based on biochemical, structural and computational analysis of two GH1 enzymes from Tricoderma hararzium. *New Biotechnology*, 40 (0), 218–227.

Garvey, M., Klose, H., Fischer, R., Lambertz, C., and Commandeur, U., 2013. Cellulases for biomass degradation: Comparing recombinant cellulase expression platforms. *Trends in Biotechnology*, 31 (10), 581-593

Haki, G. D. and Rakshit, S. K., 2003. Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*, 89 (1), 17-34.

Li, G., Jiang, Y., Fan, X. J., and Liu, Y. H., 2012. Molecular cloning and characterization of a novel β-glucosidase with high hydrolyzing ability for soybean isoflavone glycosides and glucose-tolerance from soil metagenomic library. *Bioresource Technology*, 123, 15–22.

Li, C., Li, D., Feng, J., Fan, X., Chen, S., Zhang, D., and He, R., 2019. Duckweed (Lemna minor) is a novel natural inducer of cellulase production in Trichoderma reesei. *Journal of Bioscience and Bioengineering*, 127 (4), 486–491. Available from: https://doi.org/10.1016/j.jbiosc.2018.09.017. Online.

Mei, H., Xia, D., Zhao, Q., Zhang, G., and Qiu, Z., 2016. Molecular cloning , expression , puri fi cation and characterisation of a novel cellulase gene (Bh-EGase I) in the beetle Batocera hors fi eldi. *Gene* 576 (1), 45–51. Available from: http://dx.doi.org/10.1016/j.gene.2015.09.057. Online.

Pozzo, T., Pasten, J. L., Karlsson, E. N., and Logan, D. T., 2010. Structural and Functional Analyses of β-Glucosidase 3B from Thermotoga neapolitana: A Thermostable Three-Domain Representative of Glycoside Hydrolase 3. *Journal of Molecular Biology*, 397 (3), 724–739.

Roy, A., Kucukural, A., and Zhang, Y., 2010. a unified platform for automated protein structure and function prediction. *I-TASSER*, 5 (4), 725–738.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., and Pandey, A., 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource Technology*, 127, 500-507

Wei, Y., Acquah, C., Obeng, E. M., Dullah, E. C., Jeevanandam, J., and Ongkudon, C. M., 2019. Biochimie Parametric study of immobilized cellulase-polymethacrylate particle for the hydrolysis of carboxymethyl cellulose. *Biochimie*, 157, 204–212. Available from: https://doi.org/10.1016/j.biochi.2018.11.019. Online.

Xia, Y., Yang, L., and Xia, L., 2018a. High-level production of a fungal β -glucosidase with application potentials in the cost-effective production of Trichoderma reesei cellulase. *Process Biochemistry,* 70, 55–60. Available from: https://doi.org/10.1016/j.procbio.2018.03.031. Online.

Yang, J., Zhang, Y., and I-tasser, T., 2015. New development for protein structure and function predictions. *I-TASSER server*, 43, 174–181.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1. Discussion and conclusion

The main aim of the study was to find a thermostable β-glucosidase enzyme, that could be added to commercial cellulase preparations, with low β-glucosidase activity. The addition of a β-glucosidase was to increase the saccharification efficiency of cellulase the cocktail, for use in bioethanol production. A hot geyser was chosen for the isolation of thermophilic cellulase-producing bacteria, to increase the likelihood of obtaining thermostable enzymes.

A thermophilic bacterial strain with potential as a source of industrial enzymes was isolated and characterised. The bacterium produced a thermostable β-glucosidase and may be a source of more industrially important enzymes. The observation is based on the bacteria's ability to hydrolyse substrates such as starch and lipids.

Phylogenetic analysis based on the 16S rDNA gene sequence indicated that the strain belonged to the genus *Anoxybacillus.* However, comparison of the physiological and biochemical characteristics with other *Anoxybacillus* strains*,* suggests that the strain may be novel. The bacterium was denoted as *Anoxybacillus* sp*. KTC2*.

Anoxybacillus sp. KTC2 was able to grow on cellobiose as the main carbon source. The strain produced very low amounts of both the endoglucanases and cellobiohydrolases which are responsible for the hydrolysis of the crystalline cellulose to produce cellobiose. This is consistent with literature that indicates that most bacterial species have an incomplete cellulase system, rendering them inefficient in cellulose hydrolysis (Kalyani et al. 2013).

The β-glucosidase enzyme has a potential application in biomass conversion to fermentable sugars; as part of a cellulose cocktail with commercial cellulases. This is evident from the increase in saccharification efficiency of the commercial cellulase preparation when mixed with the *Anoxybacillus* sp. KTC2 β-glucosidase. The

addition of external β-glucosidases to commercial cellulase preparations of fungal origin, is a commonly used and documented industrial practice (Florindo et al. 2018). The β-glucosidase enzyme has been proven to be the limiting factor in the enzymatic hydrolysis of cellulose by *T. reesei* cellulases (Florindo et al. 2018).

Anoxybacillus sp. KTC2 β-glucosidases exhibited the crucial characteristics required for an industrial enzyme and an enzyme that would be beneficial during the saccharification step in biofuel production. An ideal industrial β-glucosidase should be active over a wide pH range, should be stable at high temperatures and exhibit high catalytic efficiency (Diogo et al. 2015, Alves et al. 2018). All these characteristics were observed in the *Anoxybacillus* sp. KTC2 β-glucosidase. The enzyme had a pH range of 5.0-7.5, retained almost 100% residual activity after 24-hour incubation at 50˚C and had high catalytic activity as well as specificity towards cellobiose.

Sequence analyses of the deduced amino acid sequence revealed that the β-glucosidase enzyme belongs to the GH1 family enzymes. β-glucosidases have been classified into families GH1, GH2, GH3, GH5, GH9, GH30 and GH116. However, GH3 and GH5 are documented to be the most relevant β-glucosidases, for biotechnological applications. The β-glucosidase enzyme in this study shows great potential as an industrial enzyme although it belongs to the GH1 family.

In conclusion, the *Anoxybacillus* sp. KTC2 β-glucosidase has shown great potential as an industrial strain due to its high catalytic activity and thermostability.

6.2 FUTURE WORKS.

Protein expression studies will be conducted on the cloned *Anoxybacillus* sp. KTC2 β-glucosidase gene.

6.2. References

Alves, L. D. F., Meleiro, L. P., Silva, R. N., Westmann, C. A., Guazzaroni, M., and Hickey, W. J., 2018. Novel Ethanol- and 5-Hydroxymethyl Retrieved From a Brazilian Secondary Atlantic Forest Soil Metagenome. *Frontiers in Microbiology,* 9, 1–17.

Diogo, A., Cota, J., Corre, T. L. R., Hoffmam, Z. B., Garcia, W., Oliveira, L. C., Prade, R. A., and Squina, F. M., 2015. Comparative analysis of three hyperthermophilic GH1 and GH3 family members with industrial potential. *New Biotechnology,* 32 (1), 13–20.

Florindo, R. N., Souza V. P., Mutti, H. S., Camilo, C., Manzine L. R., and Marana, S. R., 2018. Structural insight into beta-glucosidase transglycosylation based on biochemical, structural and computational analysis of two GH1 enzymes from Tricoderma hararzium. *New Biotechnology*, 40, 218–227.

Kalyani, D., Lee, K. M., Kim, T. S., Li, J., Dhiman, S. S., Kang, Y. C., and Lee, J. K., 2013. Microbial consortia for saccharification of woody biomass and ethanol fermentation. *Fuel*, 107, 815–822.

APPENDIX

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ggcaaagtggcgaacgatgataacggcgatattgcgattgatcattataaccgctatgtg

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gatatgccgcaggcgctgcaggaaaaaggcggctgggaaaaccgcgatattgtgcattat

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acccataacgaaccgtgggtggtgacctatctgggctatggcaacggcgaacatgcgccg

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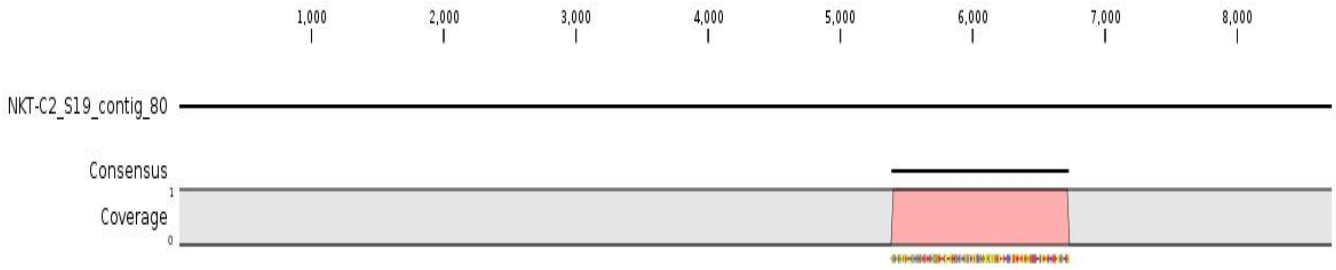

Figure 1. The β-glucosidase nucleotide sequence and an Illustration of the glucosidase gene position on contig 80.

```
M I R F P K H F I W G A A T S S Y Q I E G T A T G E D K V Y S I W D H F S R I P G K
V A N D D N G D I A I D H Y N R Y V E D V A L M K A L H L K A Y R F S T S W A R L Y
S E T P G K F N E K G L D F Y K R L V H E L L E N D I E P M L T I Y H W D M P Q A L
Q E K G G W E N R D I V H Y F R E Y A E F L Y K N L G D V V K K W I T H N E P W V V
T Y L G Y G N G E H A P G I Q N F T S F L K A A H H V L L S H G E A V K A F R E I G
S K D G E I G I T L N L T P G Y A V D P Q D E K A V D A A R K W D G F M N R W F L D
P V F K G H Y P Q D M L E V Y K D Y L P D V Y R E G D L Q T I Q Q P I D F F G F N Y
Y S T A T L K D W K K G E R E P I V F E H V S T G R P V T D M N W E V N P N G L F D
L L V R L K N D Y G D I P L Y I T E N G A A Y K D F I N E Q G K V E D D E R V A Y I
Q E H L A A C H R A I E Q G V N L R G Y Y V W S L F D N F E W A F G Y D K R F G I V
Y V D Y E T L E R I P K K S A L W Y K E T I I N N G F E M K R Y K
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Figure 2. Deduced amino acid sequence of the β-glucosidase enzyme from the bacterium *Anoxybacillus* sp KTC2.

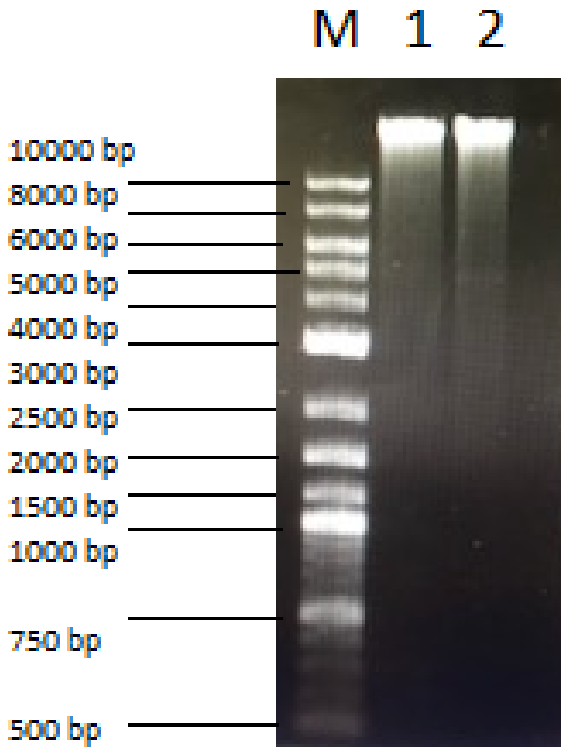

Figure 3. DNA analysis of the *Anoxybacillus sp* KTC2 genomic DNA. Lane 1 and 2: The extracted genomic DNA with a molecular weight larger than the 10000bp. Lane M: GeneRuler 1kb DNA Ladder.

The pTYB21 expression vector and the β-glucosidase Insert were double digested with restriction enzymes SalI and NdeI. Restriction analysis of the vector shows a linearized vector (Figure 5.4 A).

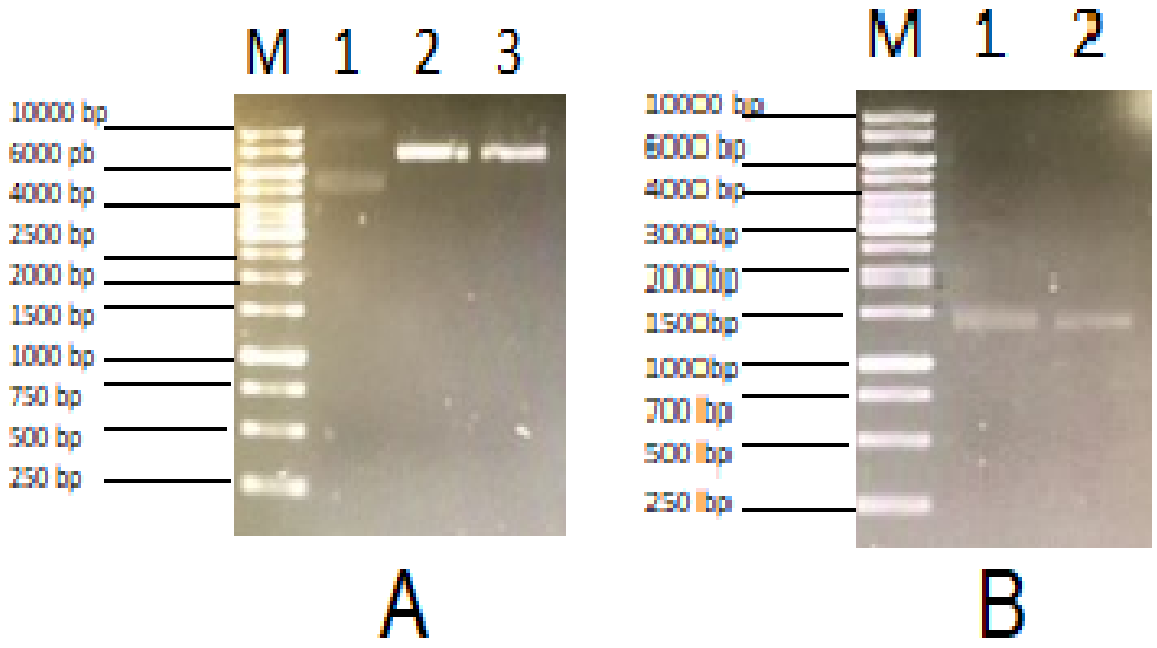

Figure 4: Restriction analysis of Ptyb21 expression vector (figure A). Lane 1 shows the unrestricted vector, showing both the nicked and supercoiled plasmid forms. Lane 2 and 3 show a linear double digested ptyb21 Vector. Figure B shows the double digested β-glucosidase PCR amplicon in both lane 1 and 2. Lane M: GeneRuler 1kb DNA Ladder