Bioprospecting for β-glucosidases and β-xylosidases from non-*Saccharomyces* yeast

by

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DECLARATION

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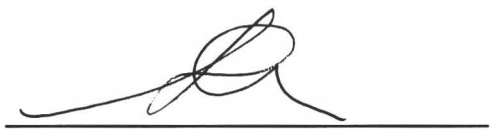

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SUMMARY

The argument of whether to use food for biofuel (bioethanol) production prompted the search for an alternative non-food biomass, such as lignocellulose, as feedstock for bioethanol production. However, a hindrance in producing bioethanol from lignocellulose on an industrial scale is the cost associated with hydrolysing the lignocellulose to its respective sugar monomers. Improving enzyme production and enhancement of enzyme cocktails for efficient lignocellulose hydrolysis is, therefore, a necessary prerequisite.

In this study, a yeast culture collection from the Wine and Fermentation Technology Division (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa), isolated from fruit from various regions in South Africa, was screened for β-glucosidase and β-xylosidase enzyme activities. β-glucosidases catalyse the hydrolysis of cellobiose and by doing so prevents end-product inhibition of cellobiohydrolases and endoglucanases during cellulose degradation. Similarly, β-xylosidases hydrolyse xylobiose and prevents end-product inhibition of endoxylanases during hemicellulose degradation. After initially screening 2180 non-*Saccharomyces* yeasts, two yeast isolates were selected that could potentially serve as enzyme source for lignocellulose hydrolysis; one as a producer of a β-glucosidase and another as a β-xylosidase producer. The yeasts were identified as a β-glucosidase producing *Rhodotorula slooffiae*-like yeast isolate 131B2 and a β-xylosidase producing *Aureobasidium pullulans* isolate 23B25, respectively.

The production of β-glucosidase by *Rhodotorula slooffiae*-like yeast isolate 131B2 and of β-xylosidase by *Aureobasidium pullulans* isolate 23B25 was optimised using response surface methodology according to a central composite design. Subsequently, the crude and partially purified enzymes were characterised based on molecular mass, pH optima and stability, temperature optima and stability and inhibition by lignocellulose hydrolysis end-products, such as glucose, xylose and ethanol. The crude β-glucosidase from *Rhodotorula slooffiae*-like yeast isolate 131B2 was also compared to the commercial *Aspergillus niger* β-glucosidase preparation (Novozyme 188) based on the characteristics mentioned above and as β-glucosidase supplement during Avicel (microcrystalline cellulose) hydrolysis by the commercial cellulase preparation (Celluclast). The crude β-xylosidase by *Aureobasidium pullulans* isolate 23B25 could not be compared to a commercial β-xylosidase as none was available at the time of the study. During the study, the crude β-glucosidase 131B2 and β-xylosidase 23B25 showed potential as lignocellulose hydrolytic enzymes. Attempts were made to obtain the β-glucosidase and β-xylosidase genes from the respective yeast isolates using PCR-based approaches and by constructing cDNA libraries. However, cloning the β-glucosidase and β-xylosidase genes using these methods proved after several attempts to be unsuccessful, although, during this section of the study valuable information was obtained about the obstacles involved with using these approaches when the desired gene sequence is unknown and novel.

OPSOMMING

Die debat oor die toepaslikheid van voedsel vir bio-brandstofproduksie (bio-etanol), het daartoe gelei dat alternatiewe nie-voedsel grondstowwe, soos lignosellulose, as voermateriaal vir bio-ethanol ondersoek word. Die koste geassosieer met die hidrolise van lignosellulose na die onderskeie suiker monomere belemmer industriële-skaal toepassing van lignosellulose vir bio-etanolproduksie. Verbeterde ensiemproduksie en verhoogde doeltreffendheid van ensiemmengsels vir lignosellulose hidrolise is dus 'n noodsaaklik voorvereiste.

In hierdie studie is 'n giskultuurversameling geisoleer vanaf vrugte van verskillende streke in Suid-Afrika deur die Wyn en Fermentasie Tegnologie Afdeling (ARC Infruitec-Nietvoorbij, Stellenbosch, Suid-Afrika) vir β-glukosidase en β-xilosidase ensiemaktiwiteite gesif. β-glukosidases wat die hidrolise van sellobiose kataliseer voorkom eindprodukinhibisie van sellobiohidrolases en endoglukanases tydens sellulose afbraak. β-xilosidases, op hul beurt, hydroliseer xilobiose en voorkom eindprodukinhibisie van endoxilanases tydens hemisellulose afbraak. Na afloop van die aanvanklike sifting van 2180 nie-*Saccharomyces* giste, is twee giste wat potensiëel as 'n ensiembron vir lignosellulose hidrolise kan dien geselekteer; een vir β-glukosidase en 'n ander vir β-xilosidase produksie. Die giste is as 'n β-glukosidase-produserende *Rhodotorula slooffiae*-agtige gisras 131B2 en 'n β-xilosidase-produserende *Aureobasidium pullulans* gisras 23B25 onderskeidelik geïdentifiseer.

Die *Rhodotorula slooffiae*-agtige gisras 131B2 se produksie van β-glukosidase en die *Aureobasidium pullulans* gisras 23B25 produksie van β-xylosidase was geoptimiseer met behulp van "response surface methodology" volgens 'n "central composite design". Daarna was die gedeeltelik-gesuiwerde kru-ensieme volgens molekulêre massa, pH optima en stabiliteit, temperatuur optima en stabiliteit, en inhibisie deur lignocelluloses hidrolise end-produkte soos glukose, xylose en etanol, gekarakteriseer. Die kru β-glukosidase van die *Rhodotorula slooffiae*-agtige gisras 131B2 is ook met die kommersiële *Aspergillus niger* β-glukosidase (Novozyme 188) volgens die eienskappe vroeër genoem vergelyk en as β-glukosidase aanvulling tydens die kommersiële sellulase (Celluclast) se hidrolise van Avicel (mikrokristalline sellulose). Die kru β-xylosidase van die *Aureobasidium pullulans* gisras 23B25 kon nie vergelyk word met 'n kommersiële β-xylosidase nie, aangesien daar nie een beskikbaar was tydens die studie nie. Gedurende die studie het altwee, die kru β-glukosidase 131B2 en β-xylosidase 23B25, potensiaal getoon as lignosellulose hidrolitiese ensieme. Pogings was aangewend om die β-glukosidase en β-xilosidase gene vanuit die onderskeie gis isolate met behulp van PKR-gebaseerde tegnieke en die opstel van cDNA biblioteke te kloneer. Hierdie klonering strategieë was egter na verskeie pogings onsuksesvol, maar waardevolle inligting oor die struikelblokke betrokke by die gebruik van hierdie benaderings wanneer die gewenste geen se DNS basispaarvolgorde onbekend en uniek is, was verkry.

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

General Introduction and Project Aims

1.1 Introduction

Research in biofuels was initiated approximately 40 years ago by fluctuations in crude oil prices, political instability in oil producing countries, a growing demand for energy and the reduction in global energy resources, such as fossil fuels (Antoni *et al.*, 2007; Banerjee *et al.*, 2010; Fukuda *et al.*, 2009; Sánchez & Cardona, 2008). In addition, it is generally accepted that an increased use of fossil fuels results in an increase in greenhouse gas (GHG) emissions contributing to global warming. One of the proposed biofuels is bioethanol, which is currently mostly derived from sucrose-containing (sugarcane and sugar beet) and starch-containing (mostly corn) feedstock materials; referred to as first-generation biofuels (Tan *et al.*, 2008). However, the use of land to cultivate these crops for fuel competes with their use as food and compromises environmental preservation (Antoni *et al.*, 2007; Balat *et al.*, 2008; Gottschalk *et al.*, 2010).

Lignocellulose for industrial bioethanol production, known as second-generation biofuels, poses tremendous potential as alternative feedstock material that is abundantly available (e.g. agricultural and forest residues, industrial and municipal wastes) and has a high carbohydrate content (Linoj Kumar & Sameer, 2006; Lynd *et al.*, 1991; Tan *et al.*, 2008). For example, the agroindustry of Brazil alone produces 597 million tons of residues per year from corn, sugarcane, rice, cassava, wheat, citrus, coconut and grass (Ferreira-Leitão *et al.*, 2010). Although lignocellulosic bioethanol production has been extensively studied and despite having considerable advantages over first-generation bioethanol production, lignocellulosic bioethanol still has low ethanol yields and high production costs (Banerjee *et al.*, 2010). The process to release the fermentable sugars from lignocellulosic biomass contributes up to 40 to 45% of the total production costs which require three steps: (1) biomass pre-treatment, (2) enzyme production and (3) enzymatic hydrolysis (Aden, 2002; Wooley *et al.*, 1999; Yang & Wyman, 2008; Zhu *et al.*, 2008). The use of enzymatic hydrolysis of the pre-treated biomass instead of acid and alkaline hydrolysis already reduces the production cost as it is conducted at milder conditions (pH 4.8 and temperature 45 to 50°C) and eliminates problems with corrosion of production equipment (Sun, 2002). It also produces better yields than acid-catalysed hydrolysis (Pan *et al.*, 2005). However, the cost of enzymes and the need for excessive enzyme dosages for pretreated biomass hydrolysis still prohibits the industrial implementation of this technology (Himmel *et al.*, 2007).

The main enzymes required for lignocellulosic hydrolysis are cellulases (consisting of endoglucanases, cellobiohydrolases and β-glucosidases) and hemicellulases (endoxylanases, β-xylosidases and several other accessory enzymes) (Saha, 2003; Zhang & Lynd, 2004). Currently, cellulases are mainly produced from fungi like *Trichoderma reesei* and bacteria such as *Cellulomonas fimi,* with production costs of 15 to 20 cents per

gallon ethanol (Banerjee *et al.*, 2010; Schubert, 2006). This amount is higher than for amylases used in starch-to-ethanol production, which is 2 to 4 cents per gallon ethanol. Finding enzymes with higher catalytic performance will reduce the cost of the process since lower enzyme loading will be required.

To obtain new lignocellulosic enzymes various approaches are available including: (a) screening new microorganisms for enzymes with superior activity compared to current enzymes, (b) screening for enzymes that can act synergistically with existing enzymes to enhance the process, and (c) using a suite of omics approaches to obtain novel gene sequences encoding cellulases and hemicellulases (Agbogbo & Wenger, 2007; Cherry & Fidantsef, 2003; Li *et al.*, 2011; Margolles-Clark *et al.*, 1996; Olsson *et al.*, 2007; Rasmussen *et al.*, 2006; Rey *et al.*, 2004; Sørensen *et al.*, 2007). The approach taken in this study was to screen a yeast culture collection for enzymes with superior activity, compared to known enzymes, and to characterise the enzymes for potential application as hydrolytic enzymes for bioethanol production. Attempts was made to obtain the genes of the enzymes displaying potential as a hydrolytic enzyme for bioethanol production, and to clone and heterologously express the gene in *Saccharomyces cerevisiae* with the purpose to contribute to the construction of a consolidated bioprocessing (CBP) microorganism.

1.2 Project aims and objectives

The principle aims of this study were to screen a collection of non-*Saccharomyce*s yeasts isolated from South Africa's natural biodiversity for β-glucosidase and β-xylosidase enzymes, to characterize novel β-glucosidase and β-xylosidase enzymes and, if possible, clone and express the respective enzyme genes in *Saccharomyces cerevisiae*.

The objectives identified to meet these aims are:

- To screen the non-*Saccharomyce*s yeast culture collection of the Wine and Fermentation Technology Division (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa) for β-glucosidases and β-xylosidases.
- To characterise the production and biochemical properties of a potential β-glucosidase and β-xylosidase to determine its potential application in bioethanol production.
- Use PCR and cDNA library approaches to clone the respective gene encoding for a β-glucosidase and β-xylosidase with the aim to heterologously express in *Saccharomyces cerevisiae*.

The dissertation is organised as a number of chapters covering the current literature on β-glucosidases and β-xylosidases and their application in lignocellulosic bioethanol production (Chapter 2), followed by the different objectives stated above (Chapter 3 and 4, and Addendum C).

Chapter 3 entailed the screening of a non-*Saccharomyces cerevisiae* yeast culture collection from the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa) for

β-glucosidase activity. A yeast isolate was selected for displaying the highest β-glucosidase activity compared to the other non-*Saccharomyces* yeast screened and was identified. A carbon and nitrogen source, and the appropriate pH and temperature for culturing were selected to improve the β-glucosidase production by the selected yeast. The crude and partially purified β-glucosidase produced by the selected yeast was preliminarily characterised and the crude β-glucosidase was compared to the commercial *Aspergillus niger* β-glucosidase preparation (Novozyme 188) based on pH optima and stability, temperature optima and stability, inhibition by glucose and ethanol, and as β-glucosidase supplement during hydrolysis of microcrystalline cellulose (Avicel) by a commercial cellulases preparation (Celluclast).

Chapter 4 involved the screening of the same non-*Sacchoromyces cerevisiae* yeast culture collection screened in Chapter 3, but for β-xylosidase activity instead of β-glucosidase activity. A yeast isolate displaying the highest β-xylosidase activity compared to the other non-*Saccharomyces* yeast screened was also selected and identified. The β-xylosidase production by the selected yeast was improved by selecting a carbon and nitrogen source and an appropriate pH and temperature for culturing the yeast. The crude and partially purified β-xylosidase produced by the selected yeast was preliminarily characterised, but a comparison with a commercial β-xylosidase preparation was not performed as none was available at the time of this study.

During the preliminarily characterisation of the selected β-glucosidase and β-xylosidase, both enzymes showed to have potential as hydrolytic enzymes during lignocellulosic bioethanol production. Attempts were made to obtain β-glucosidase and β-xylosidase genes using a PCR and cDNA library construction approach. Both approaches were unsuccessful in obtaining the gene and are discussed in **Addendum C**. However, improvements of the cDNA library strategy were made which might be valuable information for future attempts in constructing cDNA libraries.

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CHAPTER 2

LITERATURE REVIEW

β-glucosidases and β-xylosidases and their application in lignocellulosic bioethanol production

β-glucosidases and β-xylosidases and their application in lignocellulosic bioethanol production

2.1 The composition of lignocellulose

Lignocellulose, a renewable organic material, forms the major structural component of all plants cells (Dashtban, 2009). Lignocellulosic biomass usually consists of cellulose, hemicellulose and lignin (Fig 2.1) (Arın & Demirbas, 2004; Demirbas, 2005). The ratio of the constituents varies from plant species to plant species, but generally hardwoods would consist of 18-25% lignin, 45-55% cellulose, and 24-40% hemicellulose, whereas, softwoods consist of 25-35% lignin, 45-50% cellulose and 25-50% hemicellulose (Betts *et al.*, 1991).

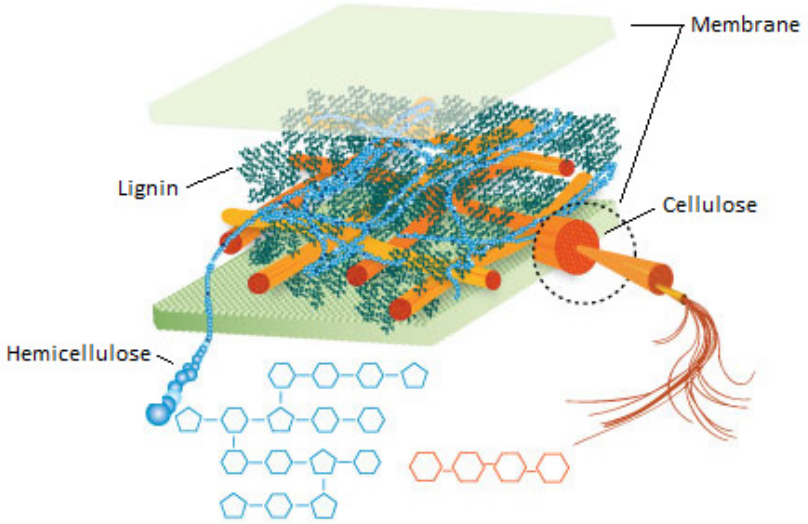

Fig 2.1 Schematic representation of lignocellulose (adapted from Ceres (2012)).

Following cellulose, the hemicellulosic fraction of lignocellulose has the greatest potential of being a source of fermentable sugars for bioethanol production (Chandel *et al.*, 2007; Kumar *et al.*, 2008). Lignocellulosic waste is continually produced by various industries and can include forestry, pulp and paper, agriculture, food, municipal and animal wastes (Champagne, 2007; Kalogo *et al.*, 2006; Kwoun Kim *et al.*, 2004; Pokhrel & Viraraghavan, 2005; Wen *et al.*, 2004). The carbohydrate fraction of

lignocellulose, which is predominately cellulose and hemicellulose, can be broken down or hydrolysed to fermentable monomeric sugars which in turn can be utilised for xylitol, 2, 3-butanediol or ethanol production through microbial fermentation (Chandel *et al.*, 2010). The only impediment to utilising the available carbohydrates is the recalcitrance of the lignocellulosic biomass (Chandel & Singh, 2011).

2.1.1 The composition of cellulose and hemicellulose

Cellulose is a homopolysaccharide consisting of D-glucose subunits linked together by β-1,4-glucosidic bonds (Fig 2.2) (Kumar *et al.*, 2009). Cellulose is usually in crystalline form and in small unorganised cellulose chains, known as the amorphous regions (Wilson & Walker, 1991). The crystalline structures are formed when adjacent cellulose chains are coupled by hydrogen bonds, hydrophobic interactions and Van der Waal's forces resulting in the recalcitrance of cellulose (Percival Zhang *et al.*, 2006; Wilson & Walker, 1991).

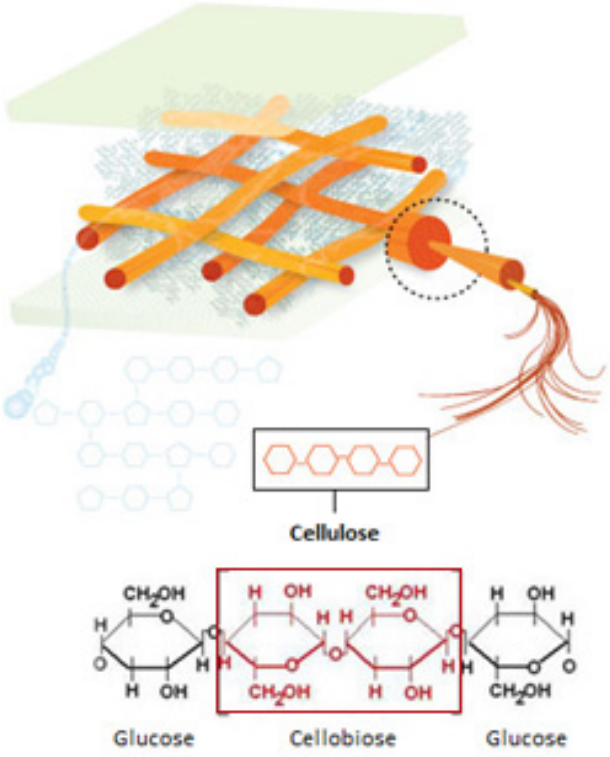

Fig 2.2 Schematic representation of cellulose (adapted from Ceres (2012) and Kumar *et al.* (2008)).

Hemicellulose is a heteropolysaccharide that consists of various monosaccharides which include pentoses (D-xylose, D-arabinose), hexoses (D-galactose, D-glucose, D-mannose) and sugar acids (4-*O*-methyl glucuronic acid and galacturonic acid) (Mohan *et al.*, 2006). Xylan is the major component of hemicellulose and consist of a β-1,4-linked xylopyranosyl backbone with side chain residues that varies from plant to plant (Fig 2.3) (Dodd & Cann, 2009). Xylans are classified into different groups based on these side chains, and can include homoxylans, arabinoxylans, glucuronoxylans, and arabinoglucuronoxylans.

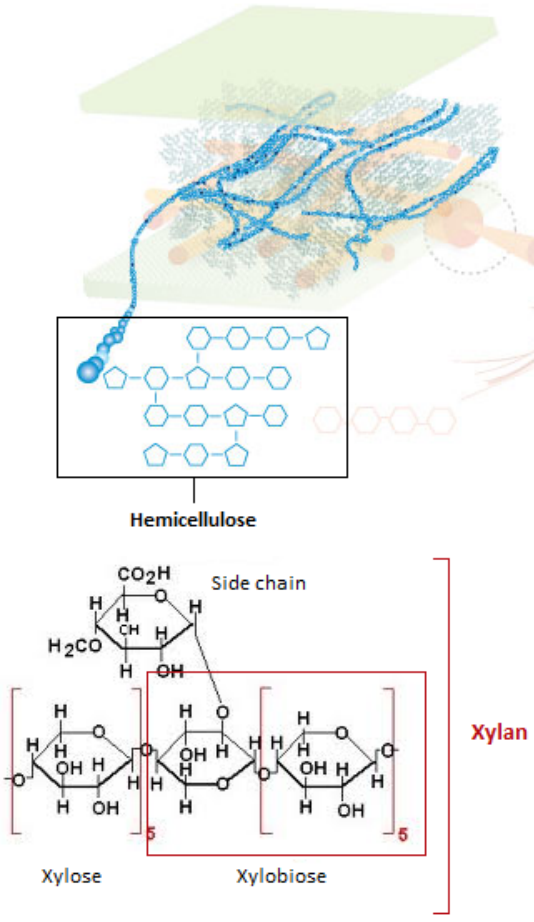

Fig 2.3 Schematic representation of hemicellulose (adapted from Ceres (2012) and Kumar *et al.* (2008)).

Rare homoxylans, which form part of cell walls in red seaweeds, consist of β-1,4 and β-1,3-linked xylose units (Painter, 1983). Arabinoxylans are mostly found in cereal grains such as wheat, rye, barley, oat, rice, sorghum, pagnola grass, bamboo shoots and in rye grass (Polizeli *et al.*, 2005). It consists of a β-1,4-linked xylopyranose backbone with arabinose residues linked to the *O*-2 or *O*-3 of xylose (Dodd & Cann, 2009). The arabinose may substitute the xylose backbone singly or doubly and the arabinose residues

may have additional linkages to the phenolic compound, ferulic acid, which may form covalent crosslinks to lignin or ferulic acid groups in other arabinoxylan chains (Dodd & Cann, 2009). Glucuronoxylans, found in hardwoods, herbs and woody plants (Ebringerova & Heinze, 2000; Timell, 1964) have 4-*O*-methyl-α-D-glucuronic acid residues linked to the *O*-2 position of xylose on the xylan backbone (Dodd & Cann, 2009). The hemicellulose mostly found in grasses (softwood) are arabinoglucuronoxylans which consist of arabinofuranosyl, 4-*O*-methyl derivative and acetyl side chains linked to the β-1,4-linked xylopyranose backbone (Dodd & Cann, 2009).

2.2 Approaches to improve lignocellulose bioethanol technology

The conversion of lignocellulose to bioethanol requires four processing steps of which the first three is bio-related and the last a chemical engineering process that will not be discussed in great depth (Dashtban, 2009). Initially, pretreatment of the lignocellulosic substrate is needed to reduce the recalcitrance of the crystalline cellulose fibers, followed by depolymerisation (hydrolysis) of the cellulose and hemicellulose fractions to its monomers and finally the conversion of monomeric sugars to bioethanol during a fermentation process (Fig 2.4). The fourth chemical engineering process step involves separation and purification of the product.

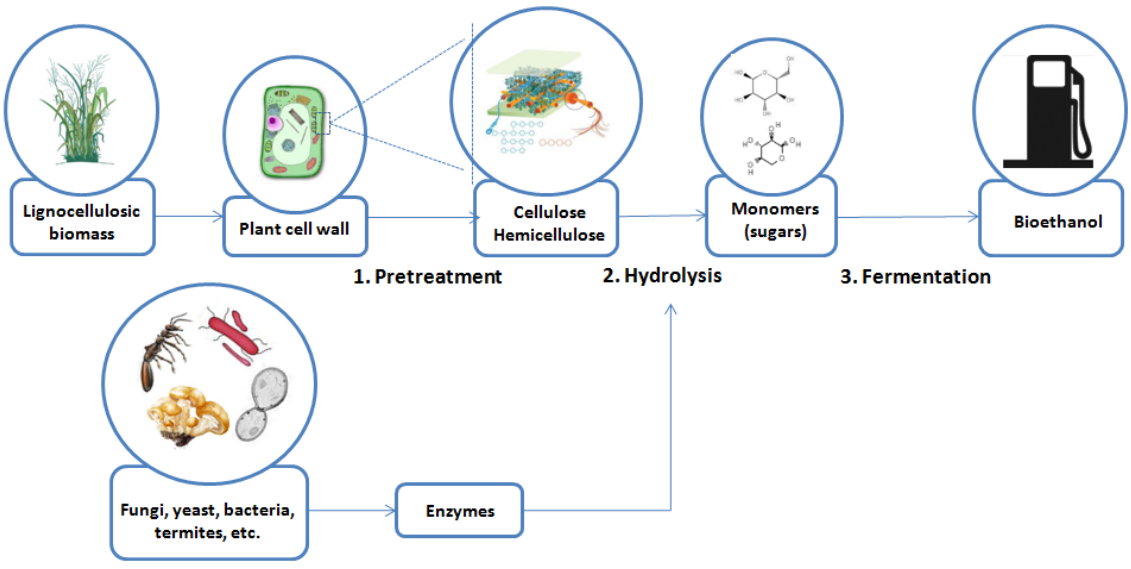

Fig 2.4 Schematic representation of the lignocellulosic biomass-to-bioethanol production process steps (adapted from Sanderson (2011)).

2.2.1 Pretreatment of lignocellulose biomass

The chemical and structural features of the plant used as lignocellulosic biomass are the main hindrances to hydrolysis. These include: (1) the plant's epidermal tissue, especially the cuticle and epicuticular waxes (Himmel *et al.*, 2007); (2) the vascular bundles' arrangement and density; (3) the sclerenchymatous (thick wall) tissue; (4) the high level of lignification (Cosgrove, 2005); and (5) the cell-wall component's complexity and structural heterogeneity, for instance the microfibrils and matrix polymers (Iiyama *et al.*, 1994). Pretreatment is therefore employed to alter lignocellulose, creating accessible cellulose for enzyme hydrolysis (Hendricks & Zeema, 2009; Yang & Wyman, 2008). The pretreatment process increases the surface area for enzymatic action and reduces the crystallinity of cellulose (i.e. partial depolymerisation of celluloses, hemicellulose and/or solubilisation of the lignin fraction and/or modification of the lignin structure). Pretreatment methods can be categorized into three groups: physical (e.g. physical ball milling) or physiochemical (e.g. steam explosion), chemical (e.g. acid/alkaline hydrolysis) and biological treatment by using microorganisms (Taherzadeh & Karimi, 2008).

2.2.2 Hydrolysis of pretreated lignocellulose and the fermentation of the sugar monomers

There are different process configurations considered for the conversion of pretreated lignocellulolytic biomass to bioethanol, which are: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) (Fig. 2.5) (Bisaria & Ghose, 1981; Boyle *et al.*, 1997; Hari Krishna *et al.*, 1998; Lynd *et al.*, 2005; McAloon *et al.*, 2000).

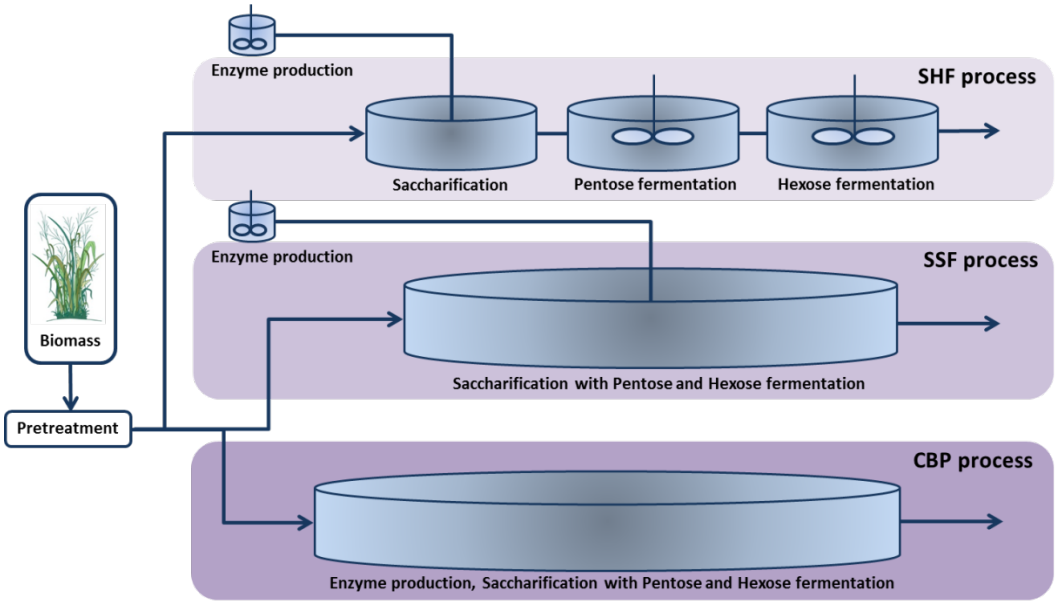

Fig 2.5 Diagram illustrating the different process configurations for the conversion of pretreated biomass to bioethanol for lignocellulose-to-ethanol processing (adapted from Fukuda *et al.* (2009)).

In SHF the hydrolysis and fermentation step takes place in separate processing tanks (Fig 2.5) (Dashtban, 2009). The advantage of SHF is that the processes can be optimised individually, i.e. the hydrolysis can be at its optimal temperature of 45 to 50°C and the fermentation at 30°C. The disadvantage is that enzyme inhibiting end-products, such as cellobiose and glucose, can accumulate during hydrolysis. Eventually, this process will become costly, because additional enzymes, such as β-glucosidases, need to be added to prevent the end-product inhibition (Philippidis *et al.*, 1993).

In the case of SSF, the pretreated biomass is simultaneously saccharified and fermented to bioethanol by the microorganism (Fig 2.5) (Stenberg *et al.*, 2000). The advantage of this process is that additional β-glucosidase is not required, but the disadvantage is that the process is at suboptimal conditions for both the hydrolysis and fermentation steps. The temperature optimum for the fungal cellulases used in SSF has an optimum hydrolysis temperature of 50°C, whereas the yeast *Saccharomyces* isolates commonly used for fermentation requires a temperature of 35°C (Kádár *et al.*, 2004).

CBP aims to combine all the bioconversion steps into one process in a single reactor by using one or more microorganism(s) (Fig 2.5) (Dashtban, 2009). By having the production of cellulases/hemicellulases, cellulose/hemicellulose hydrolysis and fermentation of the 5- and 6-carbon sugars in one step, the cost of bioconversion of lignocellulolytic biomass to ethanol can be drastically reduced (Lynd *et al.*, 2005). No natural microorganism has all the desired features for a CBP, although a number exhibits some of them. These microorganisms are either natively cellulolytic/hemicellulolytic microorganisms with a superior hydrolytic capability, but unable to ferment the available sugars to ethanol or are microorganisms that produce superior ethanol yields, but lack a cellulolytic/hemicellulolytic system (Lynd *et al.*, 2002; Lynd *et al.*, 2005). The most considered characteristics of a potential CBP microorganism are inhibitor tolerance (la Grange *et al.*, 2010), simultaneous utilisation of sugars, GRAS status, minimal nutrient supplementation and low pH and high temperature tolerance (van Zyl *et al.*, 2007; Zaldivar *et al.*, 2001).

2.3 Enzymes involved in lignocellulose hydrolysis for bioethanol production

2.3.1 Cellulase for cellulose hydrolysis

The enzymes involved in cellulose hydrolysis are known as cellulases, which include a mixture of hydrolytic enzymes (Fig 2.6) (Dashtban, 2009). These enzymes are known as endoglucanases (Enzyme Commision (EC) 3.2.1.4; endo-1,4-β-D-glucanase), exoglucanases (EC 3.2.1.91; cellobiohydrolase I and II) and β-glucosidases (EC 3.2.1.21) (Zhang & Lynd, 2004).

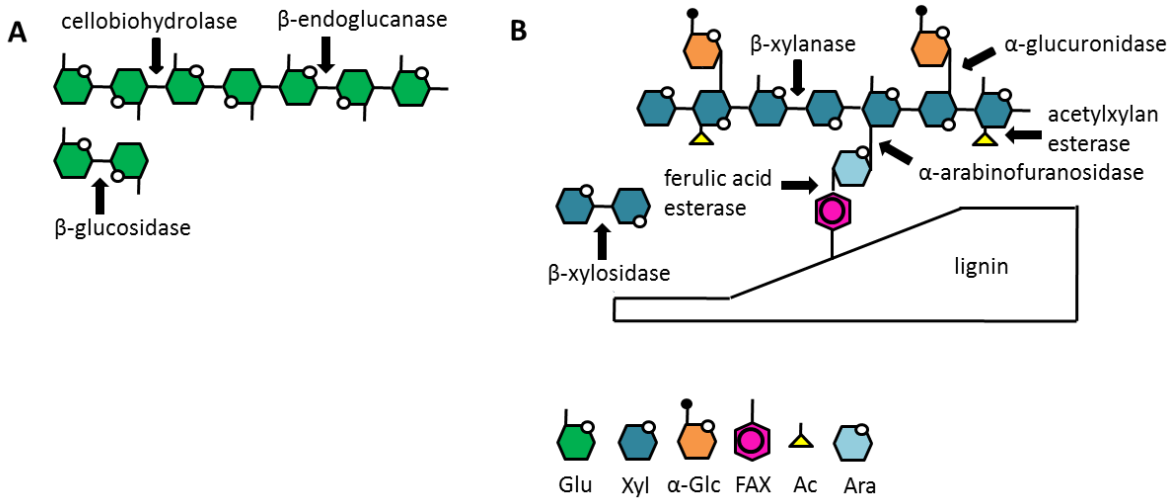

Fig 2.6 An illustration representing the (A) cellulose and (B) hemicellulose fraction of lignocellulose and their corresponding hydrolytic enzymes. The arrows indicate the bonds targeted for hydrolysis (adapted from Olsson *et al.* (2007), Polizeli *et al.* (2005), and Reiter (2002))

Amorphous cellulose can be hydrolysed by endoglucanases, which cleave internal β-1,4-glucosidic bonds producing cellobiose and cello-oligosaccharides. Crystalline cellulose requires all three types of enzymes for complete hydrolysis. Endoglucanases hydrolyse the cellulose chain randomly where there are accessible intra-molecular β-1,4-glucosidic bonds and produces reducing and non-reducing ends for cellobiohydrolases (Fujita *et al.*, 2004). Cellobiohydrolases I and II (CBHI and CBHII) hydrolyse the inaccessible crystalline cellulose regions, especially CBHI, and the cellulose chain ends producing mainly cellobiose as end-product (Chanzy *et al.*, 1983; Irwin *et al.*, 1993). The remaining cellobiose and cello-oligosaccharides are then hydrolysed by β-glucosidases to produce glucose. β-Glucosidases degrade cellulose synergistically with endoglucanases and exoglucanases and catalyse the final step in

cellulose degradation; preventing the cellobiose-mediated inhibition of cellobiohydrolases and endoglucanases (Workman & Day, 1982).

2.3.2 Other applications of cellulases

In addition to being relevant in the bioconversion of biomass, cellulases also has an industrial application in the textile, paper, animal feed and food industry (Xia & Cen, 1999). In the textile industry, cellulases are used for biostoning of denim garmets (Belghith *et al.*, 2001; Bhat & Bhat, 1997; Olson, 1990; Olson & Stanley, 1991), to create the faded look of denim and to improve the fabric finish (Cortez *et al.*, 2001; Galante *et al.*, 1998a). In the pulp and paper industry, cellulases are used to modify the coarse mechanical pulp (Akhtar, 1994; Bedford *et al.*, 2003), to adjust the hand sheet strength properties of paper, to de-ink recycled fibers (Pere *et al.*, 1995), improving the drainage of paper mills (Prasad *et al.*, 1992), to remove inks, coats and toners from paper (Pere *et al.*, 1996), to prepare biodegradable cardboard (Buchert *et al.*, 1998) and in the manufacturing of soft paper (Hsu & Lakhani, 2002; Salkinoja-Salonen, 1990). In the food and beverage industry, cellulases are used to extract and clarify fruit and vegetable juices (Galante *et al.*, 1998a), for fruit nectars and puree production and for the extraction of olive oil (Galante *et al.*, 1998a). For example, in the beer and wine industry cellulases are added to improve the malting of barley (Barbesgaard *et al.*, 1984) and the aroma of wines (Galante *et al.*, 1998b). In the production of food colourants, cellulases are employed for the extraction of carotenoids (Pajunen, 1986) and in animal feed production it is used to improve the nutritional quality and digestibility of the feed (Cinar, 2005; Graham & Balnave, 1995; Lewis *et al.*, 1996).

2.3.3 Hemicellulase (xylanases) for hemicellulose hydrolysis

Xylan is the main component of hemicellulose and the side chain residues of xylan differ from plant-to-plant. Xylan hydrolysis to its respective monomers requires a combination of enzymes which include endoxylanases (EC 3.2.1.8; endo-1,4-β-xylanase), β-xylosidases (EC 3.2.1.37; xylan 1,4-β-xylosidase) and several accessory enzymes (debranching enzymes and esterases), such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and ferulic acid esterase (Fig 2.6) (Saha, 2003a). The debranching enzymes and esterases remove the acetyl and phenyl side groups of the chain (Wong *et al.*, 1988). Endoxylanases hydrolyse the interior β-1,4-xylose linkages of the xylan backbone (Saha, 2003a). β-Xylosidases, in turn, hydrolyses xylo-oligosaccharides and xylobiose producing xylose. Endoxylanases do not completely hydrolyse xylan, making β-xylosidases an important enzyme in the hydrolysis of xylan to

xylose and in the prevention of end-product inhibition of endoxylanases (Poutanen & Puls, 1988; Zhang *et al.*, 2007). Although the xylan structure is more complex than cellulose and requires several enzymes for complete hydrolysis, it is less crystalline and thus more susceptible to enzymatic attack (Gilbert & Hazlewood, 1993).

2.3.4 Other applications of hemicellulases (xylanases)

Xylanases also has other applications such as in the textile industry where it is added to process plant fibres to reduce bleaching (Polizeli *et al.*, 2005). The pulp and paper industry uses xylanases to aid the bleaching step of cellulose. In the food and beverage industry, xylanases are added to improve the kneading of the dough, the crumb formation (Polizeli *et al.*, 2005) and to increase the volume, water absorption and resistance to fermentation of the dough during bread production (Camacho & Aguilar, 2003; Harbak & Thygesen, 2002; Maat *et al.*, 1992). Xylanases are added to cereal grain to reduce the viscosity during beer brewing (Dervilly-Pinel *et al.*, 2001; Dervilly *et al.*, 2002), are used to clarify wine (Biely, 1985; Wong *et al.*, 1988) and for extracting coffee, plant oils and starches (Biely, 1991; Wong *et al.*, 1988). Xylanase, with a combination of other enzymes, are used in the neutraceutical industry as a dietary supplement and to improve digestion (Polizeli *et al.*, 2005). Xylanases are also used for making artificial sweeteners, such as xylitol (Parajó *et al.*, 1998). In animal feed production, xylanases are added to feed to reduce the viscosity of feed and to improve the digestibility of the nutrients.

2.4 Characteristics of β-glucosidases

2.4.1 Classification of β-glucosidases

Every enzyme is supplemented with an Enzyme Commision (EC) number which is a classification approach to categorise enzymes based on the chemical reactions they catalyse, therefore, different enzymes that catalyse the same reaction will receive the same EC number. Some enzymes can catalyse reactions that fall in more than one category and thus receive more than one EC number. The EC number of β-glucosidase is EC 3.2.1.21 where EC 3 is for hydrolases, EC 3.2 is for glycosylases, EC 3.2.1 is for glycosidases, *O*- and *S*-glycosyl hydrolysing enzymes and EC 3.2.1.21 which only refers to β-glucosidases (obtained from http://www.chem.qmul.ac.uk/iubmb/enzyme/).

Another classification method is the grouping of glycosidase hydrolases based on similarity of their catalytic module. Glycosyl hydrolases are grouped into families based on primary sequence homology

of their catalytic modules, for example GH7 is for glycoside hydrolase family 7. Families can be further grouped into clans (eg. GH-A) based on fold similarities or grouped into subfamilies (eg. GH13_1) based on a common ancestor that are uniform in molecular function. A database for information and updated classification of carbohydrate active enzymes is available on the Carbohydrate-Active Enzymes (CAZy) server (http://www.cazy.org/).

β-glucosidases are found in GH groups 1, 3, 5, 9, 30 and 116 (http://www.cazy.org/). β-glucosidases from these groups are obtained from various sources, for example the β-glucosidases from GH1 are derived from archaea, bacteria, plants, fungi and insects. The β-glucosidase that from part of GH1 and GH30 are also members of the GH-A clan because they have a $(\alpha/\beta)_8$ TIM barrel fold with two key active sites, which is glutamic acids, about 200 residues apart in the sequence and located at the C-terminal of β-strands 4 (acid/base) and 7 (nucleophile) (Henrissat *et al.*, 1995). Synonyms of β-glucosidase include: gentiobiase, cellobiase, emulsin, elaterase, aryl-β-glucosidase, β-D-glucosidase, β-glucoside glucohydrolase, arbutinase, amygdalinase, *p*-nitrophenyl β-glucosidase, primeverosidase, amygdalase, limarase, salicilinase, β-1,6-glucosidase and esculinase (Edenberg *et al.*, 1985; http://www.enzyme-database.org).

2.4.2 Mode of action of β-glucosidases during hydrolysis

The β-glucosidase reaction mechanism can be either retaining (Fig 2.7) or inverting (Fig 2.8). Retaining β-glucosidases obtain hydrolysis with a net retention of configuration with a two step double-displacement mechanism, called the classical Koshland retaining mechanism, which was initially outlined by Koshland in 1953, but was later described by (Gebler *et al.*, 1992).

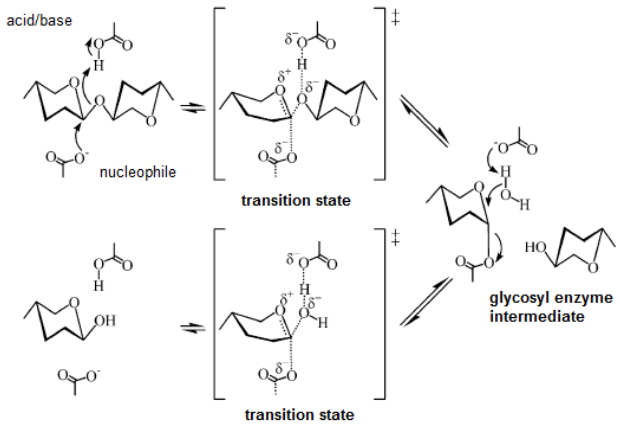

Fig 2.7 The retaining reaction mechanism of enzymes such as β-glucosidase (Rye & Withers, 2000; http://www.cazy.org/)

The mechanism involves a covalent glycosyl-enzyme intermediate and each step requires an oxocarbenium ion-like transition state (Fig 2.7). The reaction requires an acid/base and nucleophile provided by two amino acid side chains, usually glutamate or aspartate. Initially, one residue acts as nucleophile, attacking the anomeric centre which displaces the aglycon and produces the glycosyl-enzyme intermediate. Simultaneously, the acid catalyst enacted by the other residue, protonates the glycosidic oxygen as the bond cleaves. Eventually, the glycosyl enzyme is hydrolysed by water while the other residue, now enacting the base catalyst, deprotonates the water molecule as the enzyme hydrolyses the substrate.

Inverting β-glucosidases hydrolyse with a net inversion of configuration with a direct-displacement of the leaving group with water (Fig 2.8) (McCarter & Stephen Withers, 1994). Inverting β-glucosidase also requires an oxocarbenium ion-like transition state. Similarly, as with the retention mechanism the inversion reaction occurs with an acid/base obtained from two amino acid side chains, usually glumate or aspartate.

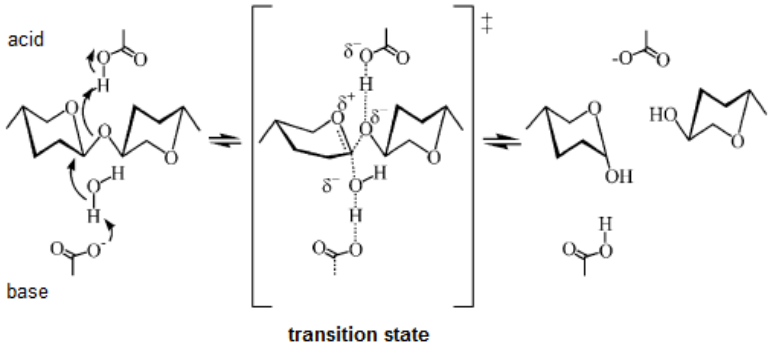

Fig 2.8 The inverting reaction mechanism of enzymes such as β-glucosidase (Rye & Withers, 2000; http://www.cazy.org/)

2.4.3 Structure, localization and substrate specificity of yeast β-glucosidase

Compared to the other cellulolytic enzymes, β-glucosidases show the most variability with regards to their structure and localization. For instance, a β-glucosidase can be located extracellularly and have a structure that is monomeric with a molecular mass around 43 kDa e.g. in *Candida peltata* (Saha & Bothast, 1996), intracellular and dimeric with a molecular mass of 110 kDa e.g. in *Candida cacaoi* (Drider *et al.*, 1993), cell-wall associated and dimeric with a molecular mass of 144 kDa e.g. in *Rhodotorula minuta* (Onishi & Tanaka, 1996) or intracellular and tetrameric with four 70 kDa units e.g. in *Pichia pastoris* (Turan & Zheng, 2005) (Table 2.1).

Table 2.1. Selected properties of isolated β-glucosidase from yeast.

Yeast	Location	Structure	Molecular mass (kDa)	K_M* (mM)	pH optimum	Temperature optima (°C)	References
Aureobasium pullulans					4.0-4.5	80	Leite *et al.* (2007)
Candida cacaoi	Intracellular	dimeric	220	0.44	5.5	60	Drider *et al*, (1993)
Candida molischiana	extracellular		330		3.5	30	Janbon *et al.* (1995)
Candida pelliculosa	intracellular	tetrameric	360	0.50	6.5	50	Kohchi *et al.* (1985)
Candida peltata	extracellular	monomeric	43	2.30	5.0	50	Saha & Bothast (1996)
Candida sake	intracellular	tetrameric	220	6.9	4.25	52	Gueguen *et al.* (2001)
Candida wickerhamii	intracellular	dimeric	180	0.28	6.0-6.5	35	Skory *et al.* (1996)
Candida wickerhamii	extracellular	dimeric	198	4.17	4.0-5.0	35	Freer, (1985)
Debaryomyces vanrijiae				0.77	5.0	40	Belancic *et al.* (2003)
Debaryomyces hansenii	intracellular		95		7.0	25	Yanai & Sato (1999)
Hanseniaspora uvarum				0.61	5.0	20	Barbagallo *et al.* (2004)
Hanseniaspora vineae	intracellular	tetrameric	295	0.10	6.0-6.5	55	Vasserot *et al.* (1989)
Kluyveromyces fragilis[¤]		tetrameric	320	0.27	5.8	55	Leclerc *et al.* (1987)
Metschnikowia pulcherrima	intracellular	oligomeric	400	1.50	4.5	50	Gonzalez-Pombo *et al.* (2008)
Pichia anomala				0.47	5.5	20	Barbagallo *et al.* (2004)
Pichia etchellsii[¥]		tetrameric	200	0.88	6.5	50	Pandey & Mishra (1997)
Pichia etchellsii		dimeric	186	0.33	6.0	50	Wallecha & Mishra (2003)
Pichia etchellsii		tetrameric	340	0.33	6.0	50	Wallecha & Mishra (2003)
Pichia etchellsii[¥]		dimeric	176				Bhatia *et al.* (2005)
Pichia pastoris	intracellular	tetrameric	275	0.12	7.3	40	Turan & Zheng (2005)
Rhodotorula minuta	cell associated	dimeric	144	1.2	4.7-5.2	70	Onishi & Tanaka (1996)
Saccharomyces cerevisiae	intracellular	dimeric	313	0.095	6.8	45	Woodward & Wiseman (1982)
Saccharomyces cerevisiae				2.50	4.0-4.5	20	Barbagallo *et al.* (2004)
Saccharomyces cerevisiae	extracellular	monomeric	100	11.98[§]	4.0	40	Villena *et al*, (2007)
Saccharomycopsis fibuligera			220		5.0	37	Gundllapalli *et al.* (2007)
Sporobolomyces singularis	cell associated	dimeric	146	1.96	3.5		Ishikawa *et al*, (2005)

*Substrate: *p*-nitrophenyl-β-D-glucopyranoside.

[§]Substrate: cellobiose.

[¥]Recombinant gene expressed in *Escherichia coli.*

[¤]Recombinant protein expressed in *Saccharomyces cerevisiae*.

Yeast β-glucosidases are generally glycosylated, but the sugar unit content is variable, for example the glycoprotein of *Candida molischiana* has 60% (w/w) carbohydrate content (Janbon *et al.*, 1995) and in *Candida wickerhamii* it has 30.5% (w/w) carbohydrate content (Freer, 1985). The glycosylation of the β-glucosidases does not necessarily influence the activity of the enzyme, as was found with the *Pichia etchellsii* β-glucosidase expressed by the recombinant *E. coli* (Pandey & Mishra, 1997).

β-Glucosidases can also be categorized into three groups based on their substrate specificity, namely aryl-β-glucosidase that hydrolyse aryl β-glucoside linkages as found with *p*-nitrophenyl-β-D-glucosides, cellobiases which hydrolyses β-1,4-glucosidic linkages such as cellobiose and short chain cellodextrins, and thirdly broad substrate specificity β-glucosidase which displays activity on all the substrates mentioned above (Pandey & Mishra, 1997). *Saccharomyces cerevisiae*'s β-glucosidase has a high affinity for alkyl and aryl-β-D-glucosides, but not for cellobiose and is therefore classified as an aryl-β-glucosidase (Duerkson & Halvorson, 1958), whereas *Aspergillus oryzae* produces a β-glucosidase that is more active on cellobiose than on *p*-nitrophenyl-β-D-glucoside and thus is classified as a cellobiase.

Commonly, β-glucosidases with broad substrate specificity are found among cellulolytic microorganisms (Chen *et al.*, 1994; Gueguen *et al.*, 1995; Paavilainen *et al.*, 1993; Painbeni *et al.*, 1992; Saha Badal *et al.*, 1996; Schmid & Wandrey, 1987; Woodward & Wiseman, 1982). Yeast β-glucosidases with broad substrate specificity that have been characterised are from *Debaryomyces vanrijjiae* (Belancic *et al.*, 2003)*, Pichia etchellsii* (Wallecha & Mishra, 2003)*, Saccharomycopsis fibuligera* (Machida *et al.*, 1988) and *Hanseniaspora vinea* (Vasserot *et al.*, 1989), among others. Other examples of a broad spectrum β-glucosidases are from *Aureobasidium pullulans* ATCC 20524 which was able to hydrolyse oligosaccharides such as gentiobiose, cellobiose and glucosides such as salicin, methyl-β-glucoside and *p*-nitrophenyl-β-glucoside (Hayashi *et al.*, 1999) and *Candida peltata* which was able to hydrolyse cellobiose, cellooligosaccharides and *p*-nitrophenyl-β-glucopyranoside (*p*NPG) and had minimal β-xylosidase, L-arabinofuranosidase, β-glucuronidase and *p*-nitrophenyl-β-D-galactoside activity (Saha & Bothast, 1996).

In addition to having broad substrate specificity, one of the β-glucosidases from *Pichia etchellsii* displayed bifunctional activity by having the ability to also hydrolyse aryl-linked xylosides (Wallecha & Mishra, 2003). Other β-glucosidases with the ability to hydrolyse aryl-linked xylosides was found in

Candida peltata (Saha & Bothast, 1996) and the bacterium *Agrobacterium tumefaciens* (Watt *et al.*, 1998). Transglucosylation, to produce gluco-oligosaccharides (Glc-OS) from cellobiose, and transgalactosylation, to produce galacto-oligosaccharides (Gal-OS) from lactose, by β-glucosidase have also been described (Onishi & Tanaka, 1996). Examples are the β-glucosidase from *Rhodotorula minuta* IFO 879 (Onishi & Tanaka, 1996) and from *Pichia etchellsii* (Pandey & Mishra, 1997).

2.4.4 Characteristics of β-glucosidase considered for bioethanol production

When investigating β-glucosidase for use in industrial processes certain aspects, other than its substrate specificity, are considered such as (1) glucose inhibition of the enzyme, which reduces the hydrolysis of cellulose drastically since accumulated glucose inhibits cellobiose hydrolysis and thus results in cellobiose inhibition of other cellulases (Bissett & Sternberg, 1978), (2) ethanol inhibition of the enzyme, (3) β-glucosidase inactivation by temperature, and (4) tolerance to low or high pH levels.

2.4.4.1 *Tolerance to glucose and ethanol inhibition*

The tolerance of β-glucosidase to its end product (glucose) is important for an efficient industrial bioethanol process since it speeds up the hydrolysis of cellulose by being less affected by feedback inhibition (Wright *et al.*, 1992). The effect of glucose on β-glucosidases, however, varies e.g. activity of the β-glucosidase from *Debarymyces vanrijiae* decreased by 78% of its activity at optimal conditions with the addition of 450 mM glucose (Belancic *et al.*, 2003) and the β-glucosidase of *Candida wickerhamii* lost 42% of its activity with the addition of 100 mM glucose (Skory *et al*., 1996).

Ethanol tolerance, as with glucose, is a desired trait for a β-glucosidase for industrial bioethanol processes, especially for a CBP process. Reports have been made of the positive and negative effects of ethanol on β-glucosidases. For instance, the *Debaryomyces vanrijiae* β-glucosidase was inhibited by 20 to 40% after exposure to 10 to 15% (v/v) ethanol and completely inhibited by 30% (v/v) ethanol (Belancic *et al.*, 2003). However, the activity of β-glucosidase from *Dekkera intermedia* (Blondin *et al.*, 1983), *Candida molischiana* (Gonde *et al.*, 1985) and *Debaryomyces hansenii* (Yanai & Sato, 1999) were shown to be enhanced by low ethanol concentrations. This activation is thought to be due to glucosyltransferase activity of the β-glucosidase (Pemberton *et al.*, 1980). It has been proposed that the enzyme uses alcohols as the acceptor molecule for glucose instead of water when hydrolysing *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), therefore elevating the reaction rates (Pemberton *et al.*, 1980; Saha *et al.*, 1994).

2.4.4.2 *Temperature and pH optimum and stability*

The optimal pH and temperature range of yeast β-glucosidases that have been characterised are at pH 3.5 to 7.3 and 20 to 80°C, repectively (Table 2.1). Most fungal-derived cellulases and β-glucosidases has a maximum enzyme activity at pH 4.0 to 5.0 and 50°C, but optimal conditions for these enzyme depend on hydrolysis time and its source (Tengborg *et al.*, 2001). Belancic *et al.* (2003) reported that the β-glucosidase from *Debaryomyces vanrijiae* had its maximum activity at 40°C but when the temperature was dropped to 20°C, which is the optimal temperature of fermentation during wine making, the enzyme only retained 38% of its maximum activity. At 45°C the β-glucosidase remained stable and at 70°C the enzyme was completely inactivated. Similar results were obtained for pH where the β-glucosidase had a pH optimum at 5.0, remained stable at a pH range of 4.0 to 5.5 with residual activities higher than 89%. However, the β-glucosidase lost up to 80% of its initial activity when the pH dropped to 3.15. At a higher pH of 5.6 to 7.0 the β-glucosidase retained 42 to 52% of its initial activity. Gunata *et al.* (1993) reported similar results for β-glucosidase enzymes from *Saccharomyces cerevisiae* and *Candida wickerhamii*.

2.5 Characteristics of β-xylosidases

2.5.1 Classification of β-xylosidases

β-Xylosidases (EC 3.2.1.37) are placed in the glucoside hydrolase family 3, 30, 39, 43, 52, 54 or 116. GH30 and GH39 is part of the GH-A clan and GH43 of the GH-F clan. Enzymes from the GH-F clan displays a propeller structure that is based on a five-fold repeat of blades composed of four stranded β-sheets (Nurizzo *et al.*, 2002). Synonyms of β-xylosidase are: xylobiase, exo-1,4-β-xylosidase, β-D-xylopyranosidase, exo-1,4-xylosidase, exo-1,4-β-D-xylosidase and 1,4-β-D-xylan xylohydrolase (http://www.enzyme-database.org).

2.5.2 Mode of action of β-xylosidase during hydrolysis

Similar to β-glucosidases, the β-xylosidase reaction mechanism can either be retaining or inverting. β-Xylosidases from the GH3, 30, 39, 52, 116 use a retaining mechanism while those of GH43 have an inverting mechanism (http://www.cazy.org/).

2.5.3 Structure, localization and substrate specificity of yeast β-xylosidases

Structure variability exists within the β-xylosidases, as with β-glucosidases (Table 2.2). For instance, the intracellular β-xylosidase expressed by *Candida utilis* was shown to be monomeric with a molecular mass of 120 kDa (Yanai & Sato, 2001), the extracellular β-xylosidase from *Aureobasidium pullulans* is dimeric with subunits having a molecular mass of 121 kDa (Dobberstein & Emeis, 1991) and cell-wall associated β-xylosidase of *Aureobasidium pullulans* is dimeric with a molecular mass of 88.5 kDa (Ohta *et al.*, 2010) (Table 2). β-xylosidases are also glycosylated, for instance the β-xylosidase from *Trichoderma reesei* has a carbohydrate content of 7.5% (w/w) (Poutanen & Puls, 1988) and *Trichoderma viride* 4.5% (w/w) (Matsuo & Yasui, 1988).

In addition to having activity on *p*-nitrophenyl-β-D-xylopyranoside, the β-xylosidases of some microorganisms are bifunctional as they also display α-L-arabinofuranosidase activity (Yan *et al.*, 2008). Examples of β-xylosidases with bifunctional activity from fungi are: *Paecilomyces thermophila* (Yan *et al.*, 2008), *Trichoderma reesei* (Herrmann *et al.*, 1997); *Cochliobolu carbonum* (Ransom & Walton, 1997); *Aspergillus versicolor* (de Vargas Andrade *et al.*, 2004); *Sporotrichum thermophile* (Katapodis *et al.*, 2006) and *Aspergillus awamori* (Eneyskaya *et al.*, 2007). Some fungi can produce multifunctional β-xylosidases displaying low β-glucosidase activity and α-L-arabinofuranosidase activity, examples are the β-xylosidases from *Aspergillus versicolor* (de Vargas Andrade *et al.*, 2004), *Scytalidium thermophilum* (Zanoelo *et al.*, 2004) and *Aureobasidium pullulans* (Hayashi *et al.*, 2001).

β-Xylosidases have been reported with transxylosylation activity, which occur when the xylobiose substrate is used as an acceptor and xylooligosaccharides are synthesised (Win *et al.*, 1988). *p*-Nitrophenyl-β-D-xylooligosaccharides, with a chain length between DP 2 to 7, was produced from *p*-nitrophenyl-β-D-xylopyranoside by *Aspergillus awamori* during transxylosidation (Eneyskaya *et al.*, 2003). Alkyl β-xylosides have also been produced by β-xylosidases from *Aspergillus niger* and *Trichoderma viride* from the substrates xylobiose or methyl-β-D-xyloside with an alcohol as the acceptor during the reaction (Drouet *et al.*, 1994; Shinoyama *et al.*, 1988).

Table 2.2 Selected properties of isolated β-xylosidase from yeast

Yeast	Location	Structure	Molecular mass (kDa)	K_M (mM)	pH optimum	Temperature optima (°C)	References
Aureobasidium pullulans	extracellular	dimeric	224	0.43†	4.5	80	Dobberstein & Emeis (1991)
Aureobasidium pullulans	cell wall associated	dimeric	88.5	3.5*	3.5	70	Ohta *et al.* (2010)
Candida utilis	intracellular	monomeric	120		6.0	40	Yanai & Sato (2001)
Pichia stipitis	extracellular			1.6§	5.0	45	Basaran & Ozcan (2008)

†Substrate: *p*-nitrophenyl-β-D-xyloside.
*Substrate: xylobiose.
§Substrate: *p*-nitrophenyl-β-D-xylopyranoside.

2.5.4 Characteristics of β-xylosidase considered for bioethanol production

Similar to β-glucosidases, for the use of β-xylosidases in industrial bioethanol processes certain aspects should be considered, such as: (1) xylose inhibition of the enzyme, (2) ethanol inhibition of the enzyme, and (3) β-xylosidase inactivation by temperature and tolerance to low or high pH levels.

2.5.4.1 *Tolerance to xylose and ethanol inhibition*

For efficient conversion of xylobiose and xylooligosaccharides to xylose, it is essential for the β-xylosidase to be tolerant to feedback inhibition from xylose (Zanoelo *et al.*, 2004). β-Xylosidases that are highly inhibited by xylose, ranging from 2 to 10 mM xylose, have been described for *Trichoderma reesei* (Herrmann *et al.*, 1997; Ximenes *et al.*, 1996) and *Fusarium proliferatum* (Saha, 2003). The β-xylosidase from *Pichia stipitis* was completely inhibited by 33.3 mM xylose (Basaran & Ozcan, 2008). Xylose tolerant β-xylosidase includes those characterised in fungi, *Paecilomyces thermophila* (Yan *et al.*, 2008), *Fusarium verticillioides* (Saha, 2001), *F. proliferatum* (Saha, 2003) and *Humicola grisea* var. *thermoidea* (de Almeida *et al.*, 1995). The β-xylosidase with the highest xylose tolerance was from *Scytalidium thermophilum*, which was not inhibited by 200 mM xylose (Zanoelo *et al.*, 2004).

In contrast to having a negative effect on β-glucosidases, ethanol in general seems to improve the β-xylosidase activities of most microorganisms. Yanai & Sato (2001) reported that an ethanol concentration of 10% (v/v) stimulated the enzyme activity of *Candida utilis* by 57%. Another β-xylosidase that displayed a similar response was from *Pichia anomala* (Manzanares *et al.*, 1999). However, the β-xylosidase from the fungus, *Sporotrichum thermophile*, was inhibited by 10% (v/v) ethanol, displaying only 70% of its original activity (Katapodis *et al.*, 2006).

2.5.4.2 *Temperature and pH optimum and stability*

The enzyme activity of most fungal β-xylosidases is optimal at an acidic pH range of between 4.0 and 6.0 (de Vargas Andrade *et al.*, 2004; Eneyskaya *et al.*, 2007; Herrmann *et al.*, 1997; Matsuo *et al.*, 1998; Rizzatti *et al.*, 2001; Saha, 2003; Sulistyo *et al.*, 1995; Ximenes *et al.*, 1996). Yeast β-xylosidases seems to display a similar trend, with pH optima between 3.5 and 6.0 (Table 2.2). However, an alkaline pH optimum has been observed for the *Penicillium herquei* (Ito *et al.*, 2003) and *Humicola grisea* var. *thermoidea* β-xylosidases (Iembo *et al.*, 2002). A change in pH can have an adverse effect on the enzyme activity, for instance *Hanseniaspora uvarum* β-xylosidase had an optimal pH at 4.5 to 5.0, but lost 20 to 30% of its activity at a pH of 3.0 to 3.8 (Basaran & Ozcan, 2008). The β-xylosidase from *Pichia anomala* had a pH optimum of 6.5 to 7.0 and lost up to 70% of its activity at a pH of 3.0 to 3.8 (Manzanares *et al.*, 1999). In contrast, *Pichia stipitis* β-xylosidase has an optimal pH between 4.8 and 5.0, but still remained stable at a pH between 3.5 and 7.2 (Basaran & Ozcan, 2008).

Overall, β-xylosidases have an optimal temperature between 40 and 80°C, but most is at 60°C (Polizeli *et al.*, 2005). The yeast β-xylosidases characterised so far displayed a similar temperature optimum, with a temperature range between 40 and 80°C (Table 2.2). Similar to pH, temperature is important for optimal activity, for example the β-xylosidase from *Pichia stipitis* has an optimal temperature of 45°C, but lost up to 20% of its activity at 50°C (Basaran & Ozcan, 2008). *Aureobasidium pullulans* produced a β-xylosidase which was optimal at 80°C and lost 27% of its activity at 70°C after 60 min (Dobberstein & Emeis, 1991) and the β-xylosidase from *Pichia anomala* has a temperature optimum at 40 to 45°C and lost 95% of its activity at 25°C (Manzanares *et al.*, 1999).

2.6 Bioprospecting for enzymes with industrial bioethanol application

A simple definition given for bioprospecting is "the systematic search of genes, natural compounds, designs and whole organisms in wildlife with a potential for product development" (Mateo *et al.*, 2001). This approach has been employed to obtain improved industrial significant enzymes, such as cellulases and hemicellulases, by utilising classical and non-classical methods.

2.6.1 Classical methods to obtain new enzymes

2.6.1.1 *Screening based on growth/no-growth and other enzyme activity detection methods*

Classical methods of screening microorganisms can be based on growth/no growth on a selective medium. For instance Anuradha *et al.* (2009) screened bacteria isolated from soil for cellulolytic activity on CMC-Blue, carboxymethyl cellulose combined with a dye. Bacteria producing cellulases were able to grow on the CMC as carbon source and produced a clearing zone due to the release of the dye during hydrolysis of CMC-Blue. Laitila *et al.* (2006) screened yeast isolated from a malting process for cellulase and xylanase activity by culturing the yeast on carboxymethyl cellulose (CMC) and xylan. Yeast producing cellulases and xylanases were detected by staining the plates with Congo red after growth and rinsing with sodium chloride. Hydrolysis of the CMC or xylan by the microorganism is detected by a clearing zone where the Congo red could not bind to the CMC/xylan substrate.

A substrate such as CMC screens for cellulases, which include endoglucanases, exoglucanases and β-glucosidase. If an individual cellulase is desired, such as β-glucosidases, other substrates can be used which is specific for the enzyme during screening e.g. cellobiose is a substrate that can only be hydrolysed by β-glucosidases, but not by endoglucanases and exoglucanases (Zhang & Lynd, 2004). Another screening method of β-glucosidases is the use of synthetic substrates where a colour/fluorescent compound is bound to glucose. Hydrolysis of the substrate by β-glucosidase will lead to the release of the coloured/fluorescent compound which can be quantitatively measured using spectrophotometry. Examples are *p*-nitrophenyl-β-

D-1,4-glucopyranoside (Deshpande *et al.*, 1984; Strobel & Russell, 1987), β-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside (Polacheck *et al.*, 1987) and 4-methylumbelliferyl-β-D-glucopyranoside (Setlow *et al.*, 2004).

2.6.1.2 *Bioinformatic analysis of sequenced genome*

Genome studies of conventional cultured microorganisms can be used to identify genes encoding glycoside hydrolases and to obtain useful information about the enzymes. The complete genome of various cellulose and hemicellulose degraders such as *Cellulomonas* (Abt *et al.*, 2010) and *Trichoderma reesei* (Martinez *et al.*, 2008) and xylose/glucose-to-ethanol fermenters such as *Pichia stipitis* (Jeffries *et al.*, 2007) and *Zymomonas mobilis* (Seo *et al.*, 2005) have been sequenced. Many novel genes have been assigned as putative enzymes based on sequence similarity or homology with known enzymes (Kwoun Kim *et al.*, 2004). This approach can be achieved for example by amplifying a part of the enzyme coding gene by PCR using degenerate consensus primers and then applying genome-walking PCR in order to clone the complete gene (Bell *et al.*, 2002). For example, the cellobiohydrolase 1 gene (*cbh1*) was obtained from *Penicillium chrysogenum* FS010 chromosomal DNA by using degenerate primers with homology to other *cbh1* genes available (Hou *et al.*, 2007).

2.6.2 Non-classical methods to obtain new enzymes (-omics approaches)

Metagenomics are the function-based or sequence-based analysis of uncultured microbial genomes in order to study the genetic potential of a specific habitat (Riesenfeld *et al.*, 2004). With metagenomics the total microbial DNA is isolated, the DNA fragmented, the fragments ligated with a suitable vector and expressed in a suitable host (Fig. 2.9) (Pottkamper *et al.*, 2009). This technology has become a useful tool to obtain novel enzymes with biotechnological application.

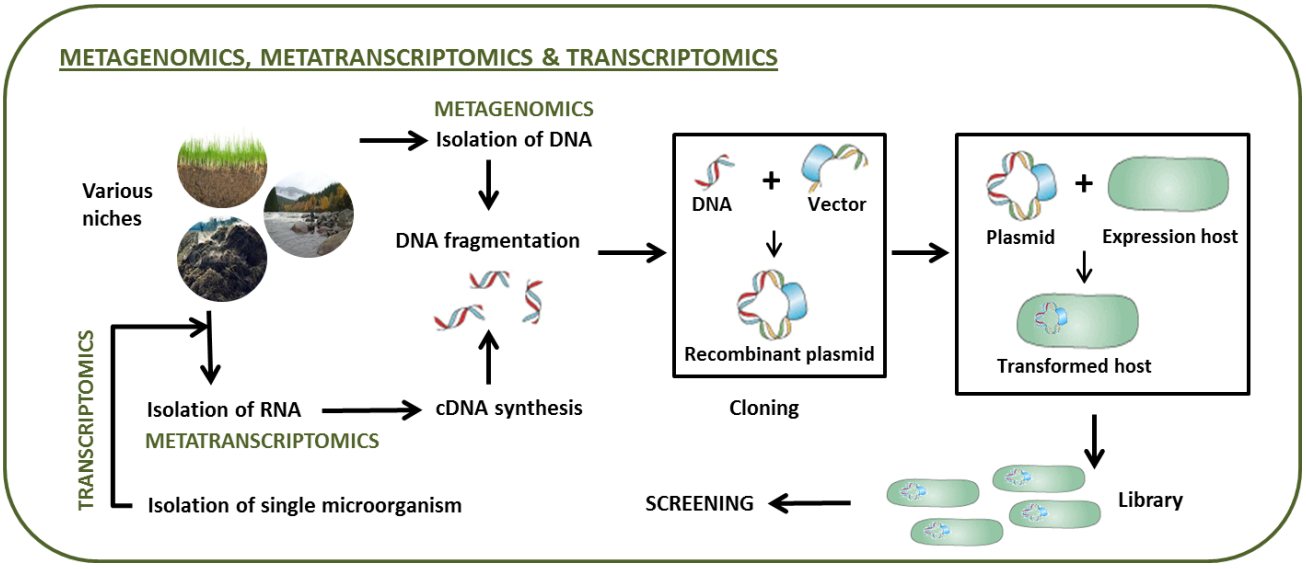

Fig. 2.9 Diagram illustrating the metagenomics, metatranscriptomic and transcriptomic approach.

Li *et al.* (2011) used the metagenomic approach to obtain novel glycoside hydrolases from decaying wood. They initially cloned the metagenomic DNA library into plasmid and fosmid libraries for paired-end Sanger sequencing. The libraries were then directly sequenced by using 454 pyrosequencing. The resulting metagenome were prospected for novel glycoside hydrolases by using tiled blastx to search against the CAZy database. About 4 000 glycoside hydrolases homologs were identified. Alongside the sequence-based analysis of the metagenome, a shotgun expression library was constructed and screened for functional glycoside hydrolases. Two enzymes displayed activity on *p*-nitrophenyl-α-L-arabinofuranoside, one enzyme on *p*-nitrophenyl-β-D-glucopyranoside and one enzyme showed activity against *p*-nitrophenyl-β-D-xylopyranoside.

Another technique derived from metagenomics is metatranscriptomics which involves isolating messenger RNA (mRNA) from the environment instead of genomic DNA (Fig 2.9). This technique allows the identification of expressed biological activity of the environment at that specific time (Simon & Daniel, 2009). Metatranscriptomics and transcriptomics involve constructing a complementary DNA (cDNA) library from the isolated mRNA (Grant *et al.*, 2006). The cDNA library is introduced into an appropriate host and screened for the desired activity. Constructing a library from mRNA poses two challenges, firstly separating the mRNA from the other RNA species is difficult and, secondly, mRNA is extremely unstable (Margesin *et al.*, 2008). cDNA is, however, preferred because eukaryotic genes contains introns which hinders their heterologous expression in yeast due to proper splicing of the introns (Moreau *et al.*, 1992). In a study conducted by Bailly *et al.* (2007) 400 clones sufficient for sequencing were produced, and by Poretsky *et al.* (2005) 119 clones sufficient for sequencing were produced, when cDNA libraries were constructed from RNA isolated from the environment. The sequences obtained in both studies, however, were not homologous to any of the protein sequences deposited in the public databases. This result made the researchers conclude that the constructed cDNA library might contain novel proteins and demonstrated the capacity of this technique to discover novel enzymes with possible environmental or industrial potential.

Transcriptomics is a similar approach to metatranscriptomics, but the total RNA is isolated from a known microorganism instead of a diverse unknown microbial population (Fig. 2.9). An example is a study by Hong *et al.* (2003) where the endo-β-D-1,4-glucanase of *Thermoascus aurantiacus* was obtained by cloning the fungus cDNA library into a λ phage, amplifying the library in *E. coli* and screening for the enzyme by using a *Aspergillus niger* endo-β-D-1,4-glucanase as probe .

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CHAPTER 3

RESULTS CHAPTER

Screening and preliminarily characterisation of a β-glucosidase enzyme from non-*Saccharomyces* yeasts

Screening and preliminarily characterisation of a β-glucosidase enzyme from non-*Saccharomyces* yeasts

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3.1 Abstract

This study focused on the discovery of novel β-glucosidases from yeast isolates isolated from various South African vineyards as well as indigenous and exotic fruit orchards. The initial screen of 2180 non-*Saccharomyces* yeast on cellobiose as sole carbon source identified 261 yeasts with β-glucosidase activity. The yeast isolate that exhibited the highest volumetric β-glucosidase activity when cultured in media containing triticale bran (a wheat/rye hybrid) as carbon source was identified as a *Rhodotorula slooffiae*-like yeast based on the D1/D2 domain and ITS region of ribosomal DNA. Response surface methodology according to a central composite design was used to improve the β-glucosidase production by the *Rhodotorula slooffiae*-like yeast for characterising and comparing the crude β-glucosidase 131B2 to the commercially available Novozyme 188 β-glucosidase. The β-glucosidase fraction was also partially purified using size exclusion chromatography. The crude β-glucosidase 131B2 had a pH optimum range of pH 3 to 5, which was broader than the Novozyme 188 pH range of 4 to 5. The crude β-glucosidase 131B2 also had a temperature stability of 72 hours at 60°C, whereas Novozyme 188 only was stable for 1 hour at 60°C. Furthermore, the crude β-glucosidase 131B2 was less inhibited by 50 mM glucose and 30% v/v ethanol than Novozyme 188. The partially purified β-glucosidase 131B2 had similar pH and temperature characteristics than the crude β-glucosidase 131B2, with the exception of being optimal only at pH 4 and being stable for only 24 hours at pH 3 to 5. The partially purified β-glucosidase 131B2 was more inhibited by glucose; losing 59% activity in 50 mM glucose whereas the crude β-glucosidase 131B2 lost only 37% activity. The effect of ethanol on the partially purified β-glucosidase also differed from the crude β-glucosidase 131B2 by having a 3 fold improvement in 10% (v/v) ethanol. The hydrolytic activity on microcrystalline cellulose (Avicel) of the crude β-glucosidase 131B2 and Novozyme 188 was also compared when added as a β-glucosidase supplement to a commercial cellulase preparation (Celluclast); the two β-glucosidases performed comparatively.

3.2 Introduction

The availability and low cost of lignocellulose makes it a promising source of biomass for bioethanol production (Balat *et al.*, 2008). However, a major impediment for the development of lignocellulosic bioethanol production on an industrial scale is the cost of biomass hydrolysis. Most strategies for the conversion of lignocellulose to ethanol involve enzyme-catalysed depolymerisation of the plant polysaccharides, but due to the recalcitrance of lignocellulose, large quantities of enzymes are required for complete hydrolysis of lignocellulose to its sugar monomers (e.g. glucose and xylose) adding to the cost of bioethanol production (Olsson *et al.*, 2007).

Lignocellulose mainly consist of three components: cellulose (30-45% w/w), hemicellulose (25-45% w/w), and lignin (15-30% w/w) (Betts *et al.*, 1992). Cellulose is a linear polymer of glucose linked by β-1,4-glycosidic bonds, which form numerous intra- and inter-molecular hydrogen bonds (Béguin & Aubert, 1994). These bonds account for the formation of the rigid, insoluble microfibrils, known as crystalline cellulose. These cellulosic microfibrils are usually embedded in a matrix of hemicellulose and lignin. The enzymes involved in the depolymerisation of cellulose are cellobiohydrolases (CBH), endoglucanases (EG) and β-glucosidases (BG) (Beguin, 1990). Amorphous cellulose is hydrolysed by endoglucanase yielding cellobiose and cello-oligosaccharides (Zhang & Lynd, 2004). Cellobiohydrolase hydrolyses the crystalline region of the cellulose, yielding similar end-products as endoglucanase. The β-glucosidase, in turn, converts the cellobiose and cello-oligosaccharides to glucose and is crucial for the final step in the hydrolysis of cellulose to glucose and in preventing end-product (i.e. cellobiose) inhibition of cellobiohydrolase and endoglucanase activities (Poutanen & Plus, 1988; Workman & Day, 1982; Zhang *et al.*, 2007).

Several research efforts have focused on the discovery of hydrolytic enzymes from the natural environment, such as the study performed by Rosi *et al.* (1994) where 317 yeast isolates representing 20 wine yeast species were screened for β-glucosidase activity. The study identified yeast genera such as *Zygosaccharomyces, Saccharomycodes, Hansenula, Dekkera, Metschnikowia, Kluyveromyces, Kloeckera, Hanseniaspora, Debaryomyces, Candida* and *Schizosaccharomyces* possessing β-glucosidase activity. In another study, Strauss *et al.* (2001) screened 245 non-*Saccharomyces* yeast isolates (representing 21 species) from South Africa's vineyards and grape juice for lignocellulose hydrolytic enzymes and identified yeast isolates from the genera *Candida* and *Kloeckera.*

Plant material is rich in lignocellulose and the microorganisms associated with plants potentially produce enzymes for lignocellulose hydrolysis (Rosi *et al.*, 1994; Strauss *et al.*, 2001; Swangkeaw *et al.*, 2011). In this study, a large collection of non-*Saccharomyces* yeast isolates isolated from fruit were screened for a

β-glucosidase with potential as a hydrolytic enzyme for lignocellulosic bioethanol production. Two-thousand one-hunderd and eighty non-*Saccharomyces* yeast isolates from the yeast culture collection of the ARC Infruitec-Nietvoorbij Wine and Fermentation Technology Division (Stellenbosch, South Africa) were screened and a yeast isolate was selected and identified which displayed higher β-glucosidase activity compared to the other non-*Saccharomyces* yeast isolates screened. The β-glucosidase production by the selected yeast was improved by selecting a carbon source, nitrogen source, and the optimal pH and temperature for culturing. The crude and partially purified β-glucosidase was preliminarily characterised and the crude β-glucosidase was compared to the commercial β-glucosidase (Novozyme 188) based on pH optima and stability, temperature optima and stability, inhibition by glucose and ethanol, and as β-glucosidase supplement during the hydrolysis of microcrystalline cellulose, Avicel, by a commercial cellulases preparation (Celluclast).

3.3 Materials and Methods

3.3.1. Yeast isolates and culturing conditions

The 2180 non-*Saccharomyces* yeast isolates used in this study were obtained from the curated yeast culture collection of the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa (Table 3.1). The yeast isolates were previously isolated from different fruit types, i.e. various grape cultivars, Kei apple (*Dovyalis caffra*), stem fruit (*Englerophytum magalismontanum*), Jaboticaba (*Myrciaria* spp.) and Mobola plum (*Parinari curatellifolia*), from different regions in South Africa. The Kei apple, stem fruit and Mobola plum are indigenous to South Africa and the Jaboticaba is a locally grown exotic fruit. The positive control for β-glucosidase activity was *Saccharomyces cerevisiae* BGL, a recombinant *S. cerevisiae* Y294 isolate over-expressing the *Saccharomycopsis fibuligera BGL1* gene (Van Rooyen *et al.*, 2005). The negative control for β-glucosidase activity was *Saccharomyces cerevisiae* VIN17. The yeast were stored as glycerol freeze cultures, and were cultured and maintained on solid yeast peptone dextrose (YPD) medium for subsequent experiments.

To evaluate the effect of glucose in the culturing medium on the production of β-glucosidase by the yeast, the supernatant of the cultures were assessed for β-glucosidase activity (as described in section 3.3.2) after culturing the yeast in 50 ml YPD (containing glucose as sole carbon source) for 3 days at 30°C with shaking at 100 rpm. Other yeast known to produce β-glucosidase were also included: *Rhodotorula minuta* (Onishi & Tanaka, 1996), *Candida wickerhamii* (Skory *et al.*, 1996), *C. pelliculosa* (Kohchi *et al.*, 1985), and *Kluyveromyces fragilis* (Leclerc *et al.*, 1987). Related species were also included: an unknown *Rhodotorula* sp., *R. toruloides*, *R. mucilaginosa*, *C. shehatae* and *K. lactis.* Yeasts were obtained from the Department of Microbiology (Stellenbosch University) culture collection.

Table 3.1 Yeast isolates used for screening.

Culture Collection	Culture year	Number of yeast
Constantia vineyard	1997	60
Madeba vineyard	1997	51
Opstal vineyard	1997	59
Rustenburg vineyard	1997	60
Chardonnay grapes	1997	125
Rustenburg vineyard	1998	30
Opstal vineyard	1998	30
Madeba vineyard	1998	30
Constantia vineyard	1998	30
Isolates from various areas	1999	50
Springfield vineyard	2000	30
Van Loveren vineyards	2000	30
Zandvliet vineyards	2000	30
Madeba rail vineyards	2000	30
Rustenburg vineyard	2000	30
Constantia vineyards	2000	30
Opstal vineyards	2000	30
Madeba vineyards	2000	30
Shiraz	2002	240
Madeba vineyard	2002	52
Isolates from various areas	2002	28
Shiraz	2003	196
Shiraz	2004	160
Grapes (Algulhas)	2007	405
Jaboticaba (Nelspruit)	2007	7
Mobola plum (Nelspruit)	2007	3
Kei Apple (Nelspruit)	2007	162
Kei Apple & Stem fruit (Nelspruit)	2007	75
Stem fruit (Nelspruit)	2007	87
Total		**2180**

3.3.2 The screening of non-*Saccharomyces* yeast for β-glucosidase activity

Initially, the isolates were screened for β-glucosidase activity in 200 µl cellobiose synthetic media containing 0.68% (w/v) Yeast Nitrogen Base (YNB) with amino acids (Difco laboratories, Detroit, MI, USA) and 1% (w/v) filter-sterilised cellobiose (Sigma-Aldrich, Steinheim, Germany), with or without 2% (w/v) agar (Biolab, Merck, Gauteng, SA) [referred to as YNB-cellobiose medium hereafter]. The cultures were inoculated for 3 days at 30°C in sterile flat-bottom 96-well microtitre plates (Greiner Bio-one, Frickenhausen, Germany). Growth in YNB-cellobiose medium was confirmed by re-culturing the yeast onto solid YNB-cellobiose medium. Growth curve analysis were also performed of the yeast in 200 µl liquid YNB-cellobiose medium and growth monitored for 7 days by measuring the optical density (OD) at 600 nm with an xMark™ Microplate Spectrophotometer (BioRAD, Richmond, CA, USA).

Yeast selected in the previous screening step was cultured in 20 ml triticale medium (1% triticale bran and 0.68% YNB) for 3 days at 30°C and β-glucosidase activity measured by incubating the appropriate volume of supernatant with 0.005 M of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma-Aldrich) in 0.05 M citrate buffer (pH 5.0) at 50˚C for 10 min (Den Haan *et al.*, 2007). The reaction was stopped by adding 1 M Na_2CO_3. The ratio for buffer : substrate : cells : Na_2CO_3 was 89 : 1 : 10 : 100. The *p*-nitrophenol (*p*NP) released from the *p*NPG was detected at an absorbance of 405 nm with a xMark™ Microplate Spectrophotometer (BioRAD). Enzymatic activity was expressed in international units (IU) which is defined as the amount of µmole *p*NP released per minute and a range of 0 mM – 0.25 mM *p*NP was used to construct a standard curve. Most of the results were not normalised but expressed as volumetric β-glucosidase activity because of the insoluble characteristic of triticale bran which influences the accuracy of determining the dry cell weight (DCW) or total protein content. In results that were expressed as normalised β-glucosidase activity the DCW was determined by pelleting a 2 ml culture at 12000 rpm for 2 min and then drying the pellet overnight at 65°C. The assays were conducted in triplicate and measurements were taken over 5 days.

3.3.3 Identification of the non-*Saccharomyces* yeast selected during the screening for β-glucosidase activity

Genomic DNA (gDNA) was isolated from the yeast isolate displaying superior β-glucosidase activity during the screening step, using the protocol described by Ausubel *et al.* (1994). The D1/D2 domain of the 26S subunit of the ribosomal DNA was amplified using polymerase chain reaction (PCR) with primers LR3 and F63 from Integrated DNA Technologies (IDT, Coraville, IA) (Table 3.2). Further confirmation of the yeast identity was conducted by amplifying the internal transcribed space (ITS) region with primers ITS1 and ITS4 from IDT (Table 3.2). All amplifications were performed using Kapa Taq Ready Mix PCR kit (Kapa Bioscience, South Africa). The reactions were carried out in a final volume of 50 µl, containing 2.5 µl gDNA (1 ng/µl), 2.5 µl primer (final concentration of 12 µM), 25 µl Kapa Taq Ready Mix and the cycling conditions were 95°C for 5 min for pre-denaturation, 30 cycles of 95°C for 45 sec denaturation, 55°C for 1 min, 72°C for 1 min for extension and a final extension step of 72°C for 5 min. PCR products were purified from agarose gels using the High Pure PCR Purification kit (Roche, Mannheim, Germany) and ligated into the pGEM®-T Easy Vector System according to the manufacturer's instructions (Promega, Madison, WI, USA).

Table 3.2 Primers used in polymerase chain reaction (PCR)

Application	Primer	Direction	Sequence
D1/D2 domain of 26 rDNA region	F63	Forward	5'-GCA TAT ACA ATA AGC GGA GGA AAA C-3'
	LR3	Reverse	5'-GGT TGT TTC AAG ACG G-3'
ITS region	ITS1	Forward	5'-TCC GTA GGT GAA CCT GCG G-3'
	ITS4	Reverse	5'-TCC TCC GCT TAT TGA TAT GC-3'
pGEM®-T insert confirmation	T7	Forward	5´-TAA TAC GAC TCA CTA TAG GG-3´
	SP6	Reverse	5´-TAT TTA GGT GAC ACT ATA G-3´

The transformed chemically component *E. coli* DH5α was plated onto LB/Amp/Xgal/IPTG solid medium (100 µg/ml Amp, 80 µg/ml Xgal, 0.5 mM IPTG) and grown overnight at 37°C. The presence of the insert in the plasmid was confirmed by preparing small-scale plasmid-DNA isolations (Del Sal et al., 1988) and amplifying the insert using universal primers T7 and SP6 (IDT) (Table 3.2). The reaction and cycling conditions were similar as described above. The sequence of the insert was determined using the dideoxy chain termination method, using the 3730*xl* DNA Analyzer (Applied Biosytems Inc., Forster City, CA, USA). Seqman™ II (DNA-STAR Inc., WI, USA) was used to assemble the sequence contigs and subsequent sequence homology searches were conducted using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Relevant sequences of type isolates were obtained from Centraalbureau voor Schimmelcultures (CBS) Database (http://www.cbs.knaw.nl). A database was constructed from the homologous sequences and aligned in CLC Genomics Workbench 4.7.1 (CLC Bio, Aarhus, Denmark) and distance analysis was used to infer the phylogenetic relationships for the taxa and a single tree was obtained by using neighbour-joining analysis. *Trichosporon aquatile* (AF075520) was chosen as outgroup and the confidence levels of the nodes were determined using bootstrap analysis with 1000 replicates. A bootstrap value of 95% or above was considered definitive.

3.3.4 Optimisation of the yeast culturing conditions for β-glucosidase production

3.3.4.1 Selection of a substrate for effective β-glucosidase induction

Various substrates as carbon sources were selected for β-glucosidase induction which included: Avicel (Sigma-Aldrich), cheese whey, molasses, sawdust, toilet paper, wheat bran and triticale bran. Avicel, wheat bran (Maes & Delcour, 2002), triticale bran (García-Aparicio *et al.*, 2011) and toilet papter (Champagne & Li, 2009) were selected as carbon source because of its cellulose content. Sawdust, molasses and cheese whey were selected as it has been previously used as carbon source for enzyme production (Chinedu *et al.*, 2007; Okafor *et al.*, 2007; Jung *et al.*, 2010; Rose & van Zyl, 2002; Youssef, 2011). Approximately 1 x 10^8 cells yeast cells were inoculated into 2% substrate with 0.68% (w/v) YNB and incubated for 3 days at 30°C and 100 rpm. The volumetric β-glucosidase activity was determined as described in section 3.3.2.

3.3.4.2 Determining the optimal growth conditions for β-glucosidase production

Enzyme production is determined by parameters such as pH and temperature. The effect of these parameters on the β-glucosidase production of selected yeast isolates was assessed by experimental design with the attempt of improving its production. A central composite design (CCD) was created and data analysed using the statistical software package Statistica 7.1 (Statsoft Inc., Tulsa, USA) and Design Expert® version 8.0.4 (Stat-Ease Inc., Minneapolis, MN, USA). Each independent variable (temperature, pH) was evaluated at two levels (high, low) in a CCD with 2 centre points and 2 star points, which resulted in a total of 10 different assays that were performed in a random order (Table 3.3). The temperature range selected was between 20 to 40°C and the pH range between 3 to 6. Culturing was performed in 250 ml volumetric

flasks containing medium with 2% triticale bran with 0.1 M glycine-HCl buffer (pH 2.4 to 3.6), 0.05 M citrate buffer (pH 3.0 to pH 6.2) or potassium phosphate buffer (pH 5.7 to 8.0). The dependent variable was enzyme activity, which was expressed as volumetric β-glucosidase activity (IU/ml) and was determined as described in section 3.3.2.

An analysis of variance (ANOVA) was used to study interactions and main effects of the two independent variables (pH and temperature) on the enzyme production at a confidence level of 95% ($p>0.05$). Response Surface Methodology (RSM) was applied to generate a regression model to predict the effect of combined variables on responses thereby finding the optimal pH and temperature for enzyme production by the yeast isolate.

Table 3.3 The 2^2 factorial central composite experimental design for the optimal β-glucosidase production by the selected yeast isolate when evaluating temperature and pH.

Standard run	Temperature	pH
Factorial points		
1	20	3
2	20	6
3	40	3
4	40	6
Star points (Temperature, pH)		
5	15.9	4.5
6	44.1	4.5
7	30	2.4
8	30	6.6
Central points		
9 (C)	30	4.5
10 (C)	30	4.5

3.3.4.3 Selection of nitrogen source for optimal growth conditions for β-glucosidase production

The effect of different nitrogen sources on the β-glucosidase production levels by the selected yeast isolate. The nitrogen source selection included: yeast nitrogen base (YNB; 2% (m/v); Sigma-Aldrich), potassium nitrate (KNO_3; 2% (m/v); Saarchem); ammonium nitrate (NH_4NO_3; 2% (m/v); Saarchem), ammonium chloride (NH_4Cl; 2% (m/v); Saarchem), ammonium sulfate $(NH_4)_2SO_4$; 2% (m/v); Saarchem), yeast peptone (1% (m/v) and 2% (m/v); YP; Biolab) and cheese whey (2% (m/v)). Triticale bran (2% (m/v)) was used as carbon source and the culture grown at the pH and temperature selected in section 3.3.4.3. The volumetric β-glucosidase activity was determined as described in section 3.3.2.

3.3.5 Characterisation of crude and partially purified extracellular β-glucosidase

3.3.5.1 Preparation of crude and partially purified extracellular β-glucosidase

Yeast isolates were grown in 50 ml triticale medium containing (2% (w/v) triticale bran and 2% (w/v) $(NH_4)_2SO_4$ for β-glucosidase induction, and 50 ml glucose medium containing (2% (w/v) glucose and 2% (w/v) $(NH_4)_2SO_4$) for no induction for 5 days (120 hours) at 30°C. Cells were removed by centrifuging the culture at 15000 rpm for 7 min and 4°C, and the supernatant used as the crude extracellular β-glucosidase. The concentrated crude β-glucosidase was prepared by ultrafiltration (Amicon, Ultrafiltration cell 8200) with a Millipore Polyethersulfone (PES) membrane (30 kDa MWCO).

Partial purification of the crude protein was based on size exclusion using chromatography (SEC) on an AKTA FPLC system (Amersham Biosciences, Piscataway, NJ) with a Superose column. The mobile phase was 0.1 M citrate-phosphate buffer (pH 5), the flow rate was 0.5 ml/min and a wavelength of 280 nm was used to separate the protein. The sample was filtered with Membrane Solutions MS® Nylon syringe filters (0.22µm pore size) before loading.

3.3.5.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Zymogram analysis

The crude and partially purified extracellular protein was subjected to SDS-PAGE using the BIO-RAD electrophoresis system. Twenty µl of the protein samples were loaded onto a discontinuous polyacrylamide gel (5% (v/v) stacking gel and 8% (v/v) separating gel) with 0.2% (w/v) 4-methylumbelliferyl β-D-glucopyranoside (MUG; Sigma-Aldrich). Electrophoresis was conducted at 120 Volts for approximately 1.5 hours. The protein marker used was a prestained Spectra™Multicolor High Range Protein Ladder (ranges from 43 to 300 kDa; MBI Fermentas, Lithuania). The samples were prepared in a SDS-PAGE treatment buffer (containing 12% (w/v) SDS, 30% (w/v) glycerol, 0.05% (w/v) comassie blue G-250 and 150 mM Tris-HCl, pH 7) with boiling for 5 min at 60°C without reducing agent. Prior to silver staining, the zymogram was developed by washing the gel with 2.5% (v/v) Triton X-100 for 30 min and incubating the gel at 50°C for 30 min. β-glucosidase activity present in the gel hydrolyses the MUG substrate, releasing 4-methylumbelliferyl which was visualised by fluorescence under UV illumination. Staining of the gel was performed using a silver staining method (Sasse & Gallagher, 2001).

3.3.5.3 Determination of the pH optima and stability of β-glucosidase

The pH optima of the crude and partially purified β-glucosidase were determined by using 0.05 M citrate buffer (pH 3.0 to 6.0) and 0.1 M phosphate buffer (pH 7.0). The pH stability of the enzyme was performed by pre-incubating the enzyme in different buffers (pH 3.0 to 7.0) for 0.5, 1, 2, 4, 6, 24, 48 and 72h at 50°C. The volumetric β-glucosidase activity was determined as described in section 3.3.2. The crude β-glucosidase was compared to the *Aspergillus niger* β-glucosidase preparation (Novozyme 188; Sigma-Aldrich) and each experiment was performed twice in triplicate to confirm repeatability.

3.3.5.4 Determination of the temperature optima and stability of β-glucosidase

The temperature optima of the crude and partially purified β-glucosidase were evaluated by performing the activity assay at different temperatures (30 to 80°C with 10°C intervals) on the substrate *p*NPG. The temperature stability of the enzyme was determined by pre-incubating the enzyme for 0.5, 1, 2, 4, 6, 24, 48 and 72h at temperature 30 to 80°C. The crude β-glucosidase was compared to Novozyme 188 and each experiment was performed twice in triplicate to confirm repeatability.

3.3.5.5 Effect of glucose and ethanol on β-glucosidase activity

The effect of glucose and ethanol on β-glucosidase activity was evaluated by performing the activity assay as described earlier on the substrate *p*NPG with an addition of glucose (final concentration of 0 mM, 10 mM, 50 mM, 100 mM, 200 mM and 450 mM) and ethanol (final concentration of 0, 5, 10, 15, 20, 30% (v/v)). A comparison of the effect of glucose and ethanol between the crude β-glucosidase and Novozyme 188 was also performed. Each experiment was performed twice in triplicate to confirm repeatability.

3.3.6 Comparison of crude β-glucosidase and a commercial β-glucosidase (Novozyme 188) as supplements during Avicel hydrolysis by Celluclast

3.3.6.1 Enzymes used during the hydrolysis of Avicel and the preparation of the crude β-glucosidase

The enzymes used were a commercial *Trichoderma reesei* cellulase preparation (Celluclast; Sigma-Aldrich) and *Aspergillus niger* β-glucosidase preparation (Novozyme 188; Sigma-Aldrich). The Celluclast had the following characteristics: 123.33 filter-paper units (FPU)/ml and 4.19 IU/ml β-glucosidase. The Novozyme 188 had a β-glucosidase activity of 35.13 IU/ml. The FPU was determined by using the filter paper assay as prescribed by the National Renewable Energy Laboratory (Denver, CO, USA) (Adney & Baker, 1996) and the activity assay of β-glucosidase was performed as described in section 3.3.2.

The crude β-glucosidase was prepared by inoculating 400 ml buffered triticale media, containing 2% (w/v) triticale bran, 2% (w/v) $(NH_4)_2SO_4$ and citrate buffer pH 4.5, with 1 x 10^8 cells and cultured for 120 hours at 30°C. The supernatant was obtained by centrifuging the culture at 10 000 rpm for 15 min at 4°C and concentrated by ultrafiltration (Amicon, Ultrafiltration cell 8200) with a Millipore Polyethersulfone (PES) membrane (30 kDa MWCO).

3.3.6.2 A comparison of the crude β-glucosidase with Novozyme 188 during hydrolysis of avicel

The crude β-glucosidase (0.3 U/ml) and Novozyme 188 (0.3 U/ml) was added to 33 g/L Avicel solution (prepared in 0.5 M citrate buffer at pH 5), at a final volume of 40 ml. The mixture was incubated at 50°C at 100 rpm. An aliquot of 1 ml was routinely taken at various intervals and analysed by High Performance Liquid Chromatography (HPLC). Glucose concentrations was measured by HPLC (Finnigan Surveyor™ HPLC

system, Thermo Electron, San Jose, CA, USA) with a Rezex RHM column in combination with a guard column (7.8 mm x 300 mm; Phenomenex, USA) and operated at 60°C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The sample run time was 25 min. Samples were diluted with MilliQ ultra-pure water, and diluted samples and standard solutions were filtered with Membrane Solutions MS® Nylon syringe filters (0.22µm pore size) into HPLC vials before running analysis.

3.4 Results

3.4.1 Screening non-*Saccharomyces* yeasts for β-glucosidase activity

In the initial screening of the yeast isolates on liquid YNB-cellobiose medium, 1272 (58%) of the yeast isolates tested showed β-glucosidase activity by being able to hydrolyse the cellobiose (sole carbon source) to glucose, which could be utilised by the yeast to sustain growth. Further evaluation of the growth of the selected yeast isolates on solid YNB-cellobiose resulted in the identification of 261 (12%) yeast isolates showing β-glucosidase activity. These yeast isolates were assessed for superior and prolonged growth over seven days on liquid YNB-cellobiose medium and four yeast were selected, which were isolate 126A6 isolated from Jaboticaba (*Myrciaria* spp.), isolate 131B2 isolated from Stem fruit (*Englerophytum magalismontanu*), and isolates 45A27W and 45A27R isolated from Rustenburg vineyards. The β-glucosidase activity of the four yeast isolates was further evaluated on *p*NPG as substrate to select one yeast isolate displaying the highest β-glucosidase activity (Fig 3.1).

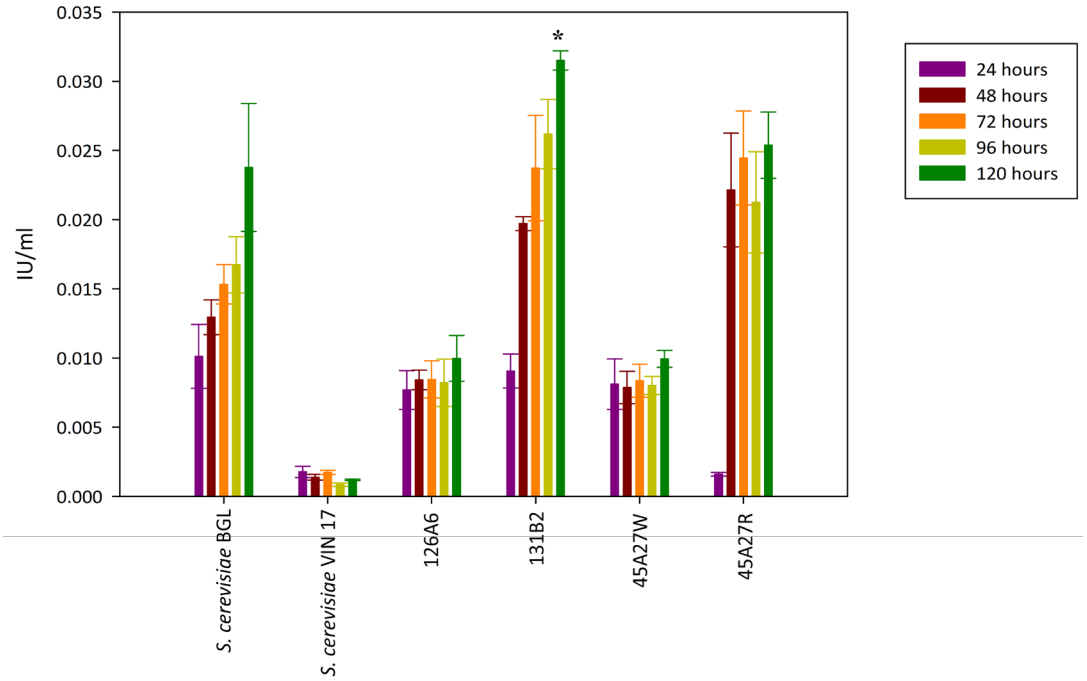

Fig. 3.1 Volumetric β-glucosidase activity of the non-*Saccharomyces* yeast isolates when cultured on 1% triticale bran and 0.68% YNB for 5 days (120 hours).

The negative control, *Saccharomyces cerevisiae* VIN17, produced low levels of β-glucosidase activity as was expected. Yeast isolates 126A6 and 45A27W produced similar levels of β-glucosidase activity, which were 0.01 ± 0.002 IU/ml and 0.01 ± 0.006 IU/ml, respectively; approximately 8 fold higher than the *S. cerevisiae* VIN17 (0.001 ± 0.0001 IU/ml) after 120 hours of culturing. Yeast isolates 131B2 and 45A27R showed the highest β-glucosidase activity compared to the other yeast isolates screened, which were 0.03 ± 0.0007 IU/ml and 0.03 ± 0.0024 IU/ml, respectively, but yeast isolate 131B2 was chosen for having a 1.4 fold higher β-glucosidase activity, after 120 hours of culturing, than the positive control *Saccharomyces cerevisiae* BGL, a recombinant *S. cerevisiae* Y294 isolate over-expressing the *Saccharomycopsis fibuligera BGL1* gene (van Rooyen *et al.*, 2005). The volumetric β-glucosidase activity of yeast isolate 131B2 after 120 hours was 0.03 ± 0.0007 IU/ml and *Saccharomyces cerevisiae* BGL was 0.02 ± 0.0046 IU/ml.

3.4.2 Identification of the non-*Saccharomyces* yeast selected during the screening for β-glucosidase activity

A preliminarily identification of the four yeast isolates, displaying β-glucosidase activity, was performed based on the large subunit (LSU) rDNA D1/D2 domain. The yeast isolates 126A6 and 45A27W were identified as *Candida* spp. and yeast isolates 45A27R and 131B2 as *Rhodotorula* spp. Yeast isolate 131B2 was selected during the screening for showing the highest volumetric β-glucosidase activity compared to the other yeast isolates, therefore further analyses were performed for identifying this yeast isolate. Isolate 131B2 showed sequence identity to *Rhodotorula slooffiae* with 98% in the D1/D2 domain and 99% in the ITS region, but the neighbour-joining tree showed that the yeast isolate formed a clade with *Rhodotorula slooffiae* with a bootstrap value of 73.6%, which is too low (less than 95%) to identify the yeast 131B2 as a *Rhodotorula slooffiae* (Fig. 3.2).

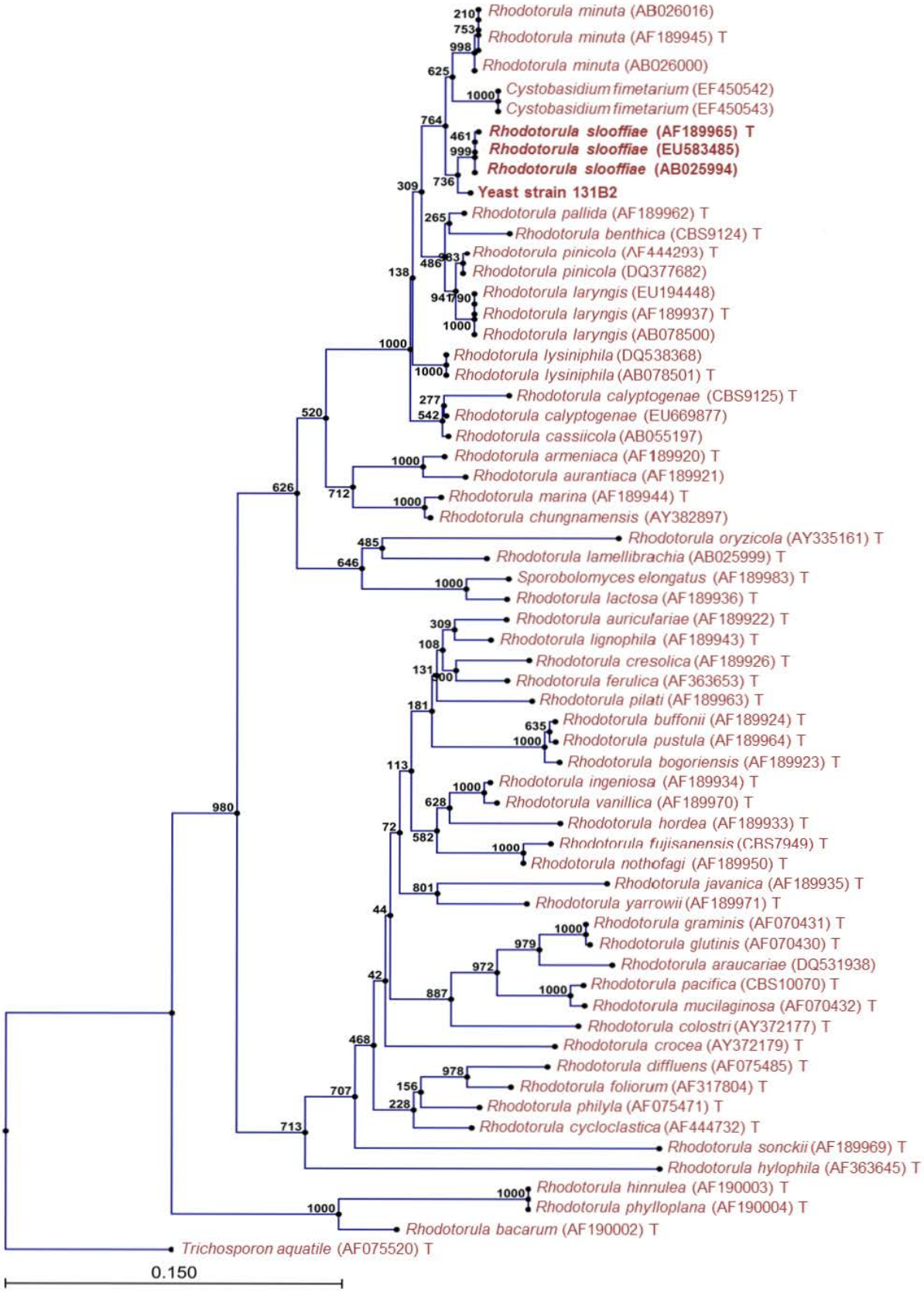

Fig. 3.2 Neighbour-joining tree of non-*Saccharomyces* yeast isolate 131B2 and related species based on the LSU rDNA D1/D2 domain. Numerals represent values from a 1000 replicate bootstrap sampling. Sequences retrieved from CBS/GenBank database under the accession numbers indicated. T, type isolate.

3.4.3 Optimisation of the yeast culturing conditions for β-glucosidase production

The cultivation conditions for *Rhodotorula slooffiae*-like yeast 131B2 were optimised to increase the concentration of the crude β-glucosidase in the supernatant with the aim to compare the characteristics of the enzyme as lignocellulose hydrolysis supplement with the commercial β-glucosidase, Novozyme 188. Initially, the carbon source to induce production of the β-glucosidase by the *Rhodotorula slooffiae*-like yeast 131B2 was evaluated. The carbon sources selected based on availability were Avicel (microcrystalline cellulose), wheat bran, toilet paper, cheese whey, molasses, sawdust and triticale bran. The β-glucosidase production by *Rhodotorula slooffiae*-like yeast isolate 131B2 appeared to be induced effectively by the wheat bran and triticale bran as carbon source (Fig. 3.3). The induction of the β-glucosidase production by wheat bran and triticale bran was not significantly different and thus triticale bran was selected for further analyses. The β-glucosidase activity of the supernatant produced by the *Rhodotorula slooffiae*-like yeast 131B2 was approximately 0.0064 (± 0.000761) IU/ml when cultured in 2 % (w/v) triticale bran for 3 days at 30°C.

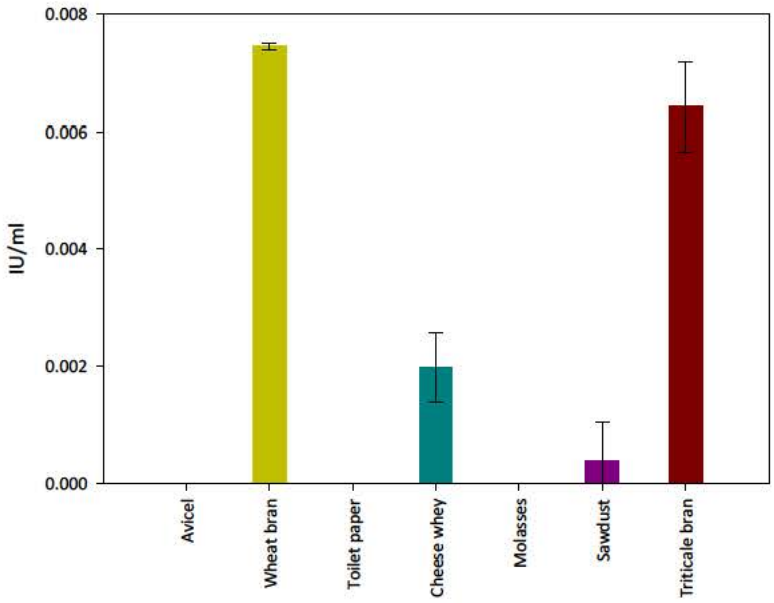

Fig. 3.3 Volumetric β-glucosidase activity of the supernatant produced by *Rhodotorula slooffiae*-like isolate 131B2 when cultured in various carbon sources.

Two variables (temperature and pH) were considered; therefore the conditions that significantly affect the β-glucosidase production by the *Rhodotorula slooffiae*-like yeast isolate 131B2 were identified statistically. The temperature range of 20°C to 40°C was selected as *Rhodotorula* spp. were reported to be mesophilic (Alchihab *et al.*, 2009; Srivastava & Smith, 1979) and the pH range of pH 3 to 6 was selected because *Rhodotorula* spp. are capable of growing within this range (Latha *et al.*, 2005; Spotholz *et al.*, 1956). The β-glucosidase production was expressed as volumetric β-glucosidase activity (Table 3.4).

Table 3.4 The 2^2 factorial central composite experimental design for the optimal β-glucosidase production by the selected yeast isolate when evaluating temperature and pH.

Standard run	Temperature	pH	Activity (IU/ml)
Factorial points			
1	20	3	0.018692
2	20	6	0.001649
3	40	3	0
4	40	6	0
Star points (Temperature, pH)			
5	15.9	4.5	0.023845
6	44.1	4.5	0
7	30	2.4	0
8	30	6.6	0
Centre points			
9 (C)	30	4.5	0.024708
10 (C)	30	4.5	0.018969

The ANOVA results as calculated with the use of the Design-Expert® software are presented in Table 3.5. The model predicting the production of β-glucosidase by *Rhodotorula slooffiae*-like yeast was statistically significant with a p-value of 0.0197 at 120 hours of cultivation. The lack of fit was non-significant (p-value of 0.56 was obtained), the R^2 value was 0.93 and the adjusted was 0.89, which implies that the model fits the data and was able to explain 93% of the experimental data. The production of β-glucosidase by *Rhodotorula slooffiae*-like yeast was primarily influenced by temperature with a p-value of 0.012 and a quadratic influence by pH with a p-value of 0.006 was also observed. The results can be observed in the standardized Pareto chart (Fig. 3.4 and Table 3.5). Therefore it is expected that the model will have a curvature and that an optimal value can be reached with pH and temperature (Fig. 3.5).

Table 3.5 Analysis of variance (ANOVA) for β-glucosidase production as dependent variable of the two factors, temperature and pH, during the 120 hours of culturing in 2% triticale bran.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value (Probability factor)	
Model	0.00104	5	0.00021	10.69557	0.01970	*significant*
A – Temperature	0.00037	1	0.00037	18.96116	0.01210	*significant*
B – pH	0.00004	1	0.00004	1.86614	0.24370	
A^2 – Temperature	0.00012	1	0.00012	6.28678	0.06620	
B^2 – pH	0.00057	1	0.00057	29.05181	0.00570	*significant*
AB	0.00007	1	0.00007	3.72996	0.1256	
Residual sum of squares	0.00008	4	0.00002	-	-	*not significant*
Lack of fit	0.00006	3	0.00002	1.24293	0.56420	
Pure error	0.00002	1	0.00002	-	-	
Total	0.00112	9	-	-	-	

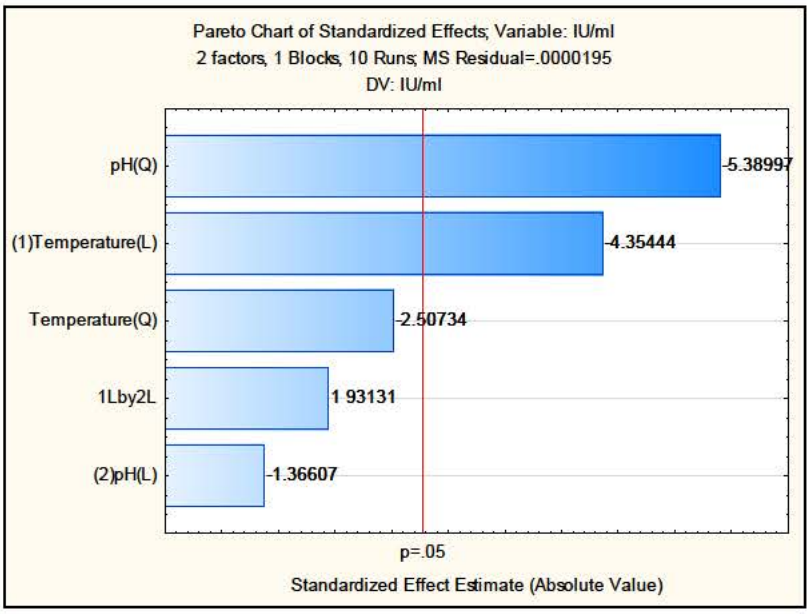

Fig. 3.4 A standardized Pareto chart indicating the influenced of pH and temperature of the *Rhodotorula slooffiae*-like yeast isolate 131B2 β-glucosidase production.

The optimal culturing temperature and pH for the culturing medium was estimated at 120 hours using a central composite design (CCD) and the model predicted that between the temperature (16°C to 28°C) and pH (3.5 to 4.5) a β-glucosidase activity of > 0.02 IU/ml will be obtained (Fig. 3.5). The model was confirmed by culturing the *Rhodotorula slooffiae*-like yeast isolate 131B2 at pH 4.5 and 28°C for 120 hours; the β-glucosidase activity obtained was 0.02 ± 0.001 IU/ml.

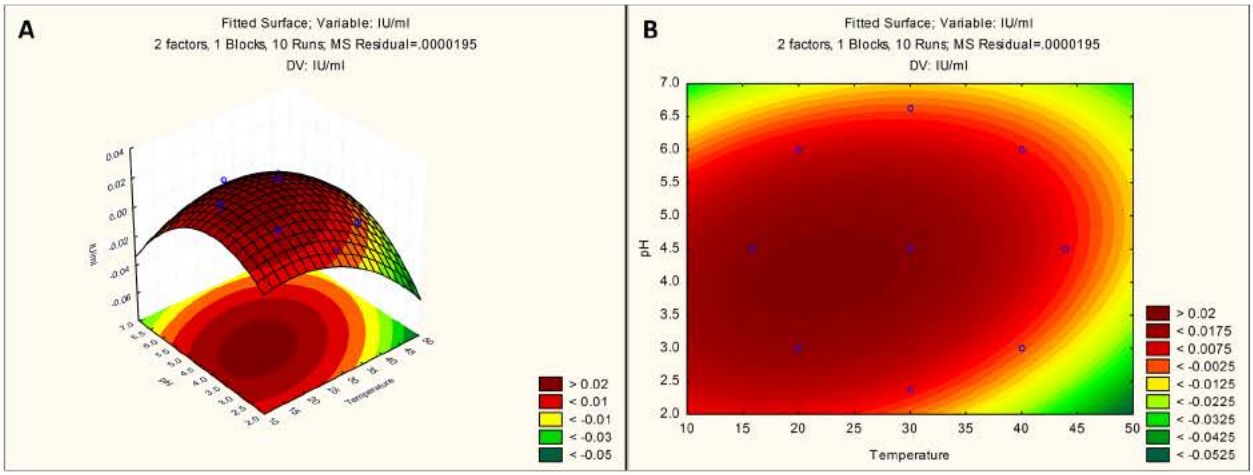

Fig. 3.5 (A) Response surface plot and (B) contour plot for the optimal pH and temperature for effective β-glucosidase production by the *Rhodotorula slooffiae*-like isolate 131B2; results were expressed in volumetric β-glucosidase activity (IU/ml).

The nitrogen source for the maximum production of β-glucosidase by *Rhodotorula slooffiae*-like isolate 131B2 was evaluated and yeast nitrogen base (YNB), potassium nitrate, ammonium nitrate, ammonium

chloride, ammonium sulfate, yeast peptone and cheese whey were selected as nitrogen sources. β-glucosidase production by *Rhodotorula slooffiae*-like isolate 131B2 was the highest when ammonium sulphate was used (Fig 3.6). The YNB used also contained ammonium sulphate, but 1.54 times less than when 2% (w/v) ammonium sulphate was added. After multiplying this factor (1.54) with the β-glucosidase activity obtained when using YNB as nitrogen source, the volumetric β-glucosidase activity value for YNB (0.0127 IU/ml ± 0.001604) was theoretically similar to the activity obtained for ammonium sulphate (0.0204 IU/ml ± 0.004167). Therefore, it can be assumed that the *Rhodotorula slooffiae*-like isolate 131B2 does not require the additional components to ammonium sulphate present in YNB to sustain growth and that the yeast might be obtaining the required vitamins and trace elements for growth from the triticale bran in the medium. Ammonium sulphate was eventually selected and the β-glucosidase activity of the supernatant produced by the *Rhodotorula slooffiae*-like yeast 131B2 was improved to approximately 0.0204 (± 0.004167) IU/ml, when cultured in 2% (w/v) triticale bran and 2% (w/v) $(NH_4)_2SO_4$ buffered at pH 4.5 for 120 hours (5 days) at 30°C.

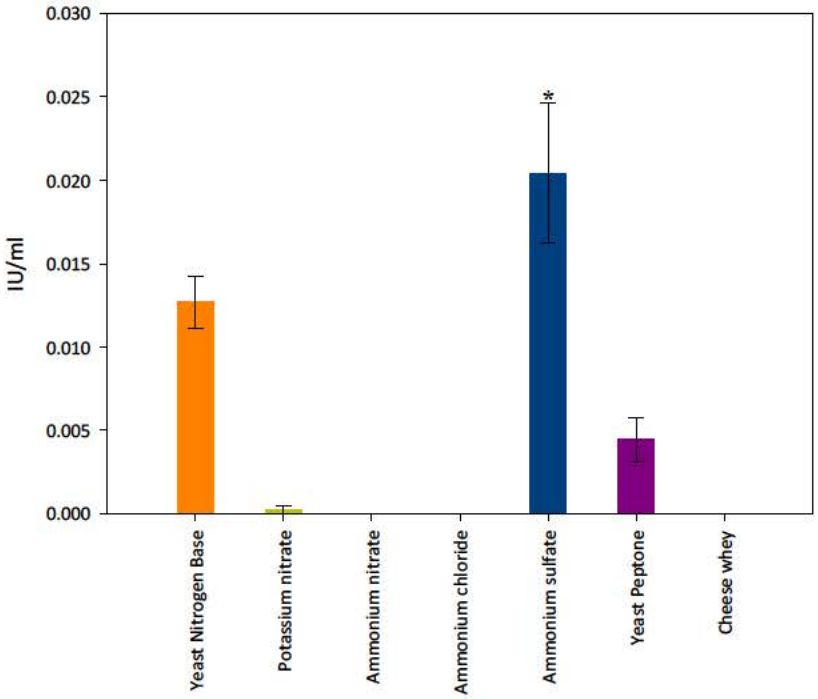

Fig. 3.6 Volumetric β-glucosidase activity of the supernatant produced by *Rhodotorula slooffiae*-like isolate 131B2 when cultured in various nitrogen sources.

3.4.4 Characterisation of the crude and partially purified extracellular β-glucosidase

3.4.4.1 SDS-PAGE and Zymogram

A zymogram and SDS-PAGE was performed to determine the molecular mass of the β-glucosidase produced by the *Rhodotorula slooffiae*-like yeast stain 131B2. The zymogram showed that the *Rhodotorula slooffiae*-like yeast stain 131B2 produced a β-glucosidase when cultivated in glucose and triticale containing media, as indicated by the fluorescent bands. These fluorescent bands had a molecular weight (M_r) of approximately 130 kDa (Fig. 3.7 A and B; Lane 1 to 3). This correlates with the band observed in the SDS-PAGE of the concentrated crude β-glucosidase (Fig. 3.7 A; Lane 3) and was confirmed by the SDS-PAGE and zymogram analysis of the partially purified β-glucosidase 131B2 (Fig. 3.7 C and D; Lane 4). However, it was essential to retain the activity of the β-glucosidase to determine the approximate M_r therefore the enzyme was not denatured (heated at 60°C and no reducing agent added) and the M_r of the denatured protein might differ from the 130 kDa observed.

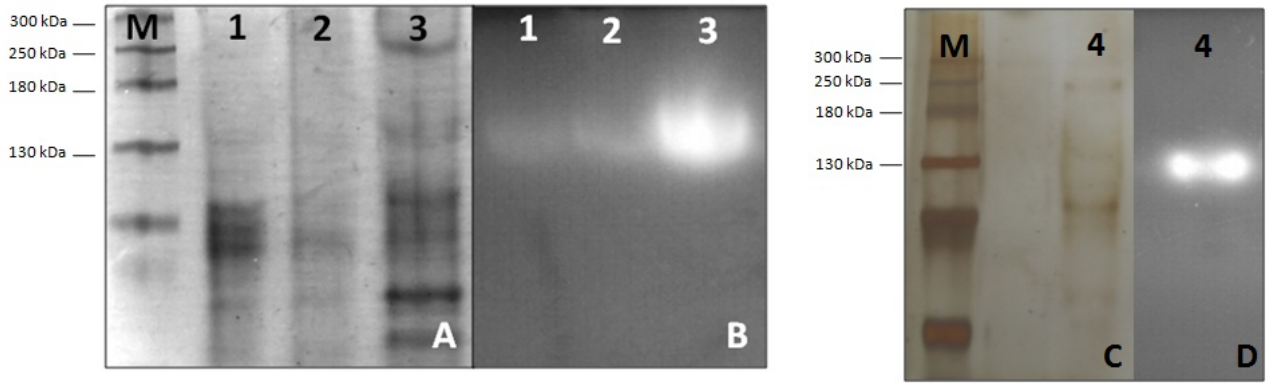

Fig 3.7 SDS-PAGE gel (A and C) and zymogram (B and D) of (1, 2 and 3) crude and (4) partially purified extracellular β-glucosidase produced by *Rhodotorula slooffiae*-like stain 131B2. β-glucosidase were analysed from cultures grown in (1) glucose and (2) triticale bran and (3 and 4) concentrated β-glucosidase from culture grown in triticale bran. M: molecular marker (Spectra™ Multicolor High Range Protein Ladder).

β-glucosidase activity was detected in the zymogram when the *Rhodotorula slooffiae*-like yeast was cultured with glucose as sole carbon source (Fig. 3.7 A and B; Lane 1). To evaluate if the *Rhodotorula slooffiae*-like yeast generally produces β-glucosidase in the presence of glucose, the yeast and other yeasts known to produce β-glucosidase were evaluated for β-glucosidase production after being cultured in YPD medium. Of the selected yeast, only *Rhodotorula slooffiae*-like yeast isolate 131B2 displayed β-glucosidase activity after being cultured in YPD (glucose as carbon source) (Fig. 3.8).

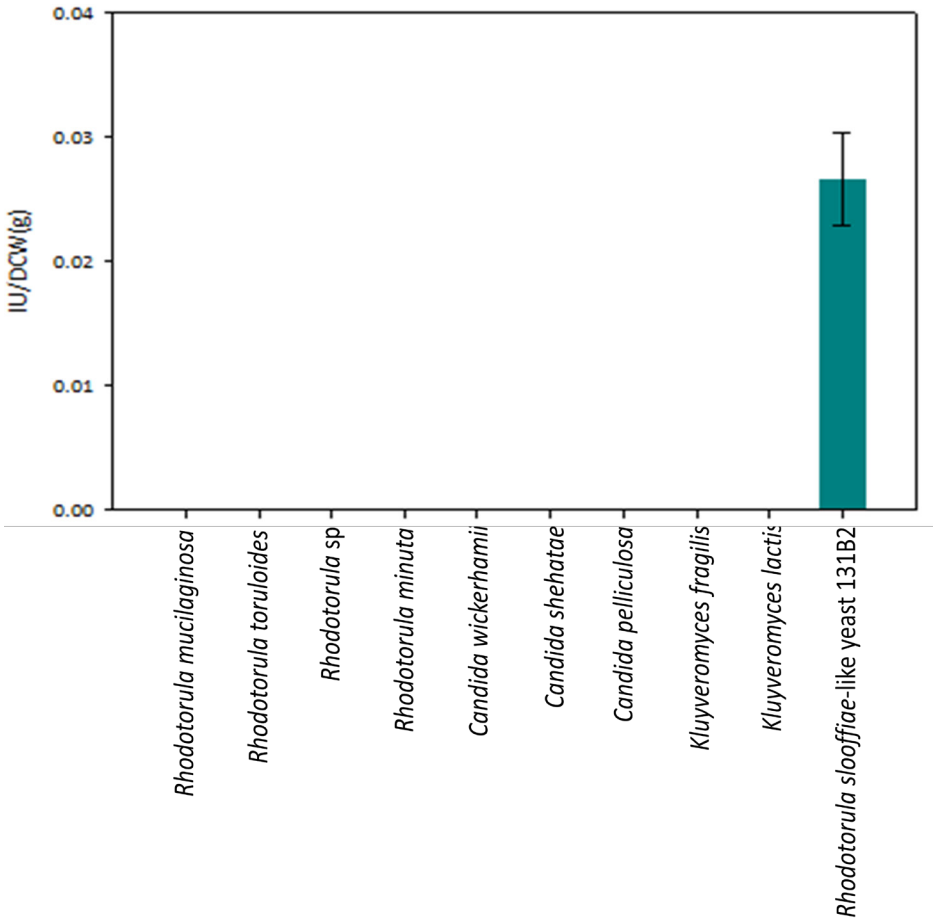

Fig. 3.8 The effect of glucose in the culturing medium on the production of β-glucosidase activity by yeast isolates known to produce β-glucosidase.

3.3.4.2 Determination of the pH optima and stability of β-glucosidase

The pH optima and stability of the crude β-glucosidase was evaluated to determine in which lignocelluloses hydrolysis process this enzyme might be used. The pH optima of crude β-glucosidase 131B2 were between 3 and 5 (Fig. 3.9 A) suggesting that the enzyme prefers acidic conditions. The crude β-glucosidase 131B2 had a broader acidic pH range as Novozyme 188 only had a pH optima of between 4 and 5. At pH 3 the crude β-glucosidase 131B2 displayed no activity reduction, but the Novozyme 188 had 56% less activity than at pH5. A reduced activity of only 0.19% was observed for the crude β-glucosidase 131B2 at pH 6 than pH5, but 70% less activity was observed for the Novozyme 188. At pH 7 the crude β-glucosidase 131B2 had 50% less activity, whereas the Novozyme 188 lost most of its activity. The stability of the crude β-glucosidase 131B2 and Novozyme 188 was observed for all pH levels at 72 hours of incubation (Table 3.6 or Addendum A).

The partially purified β-glucosidase 131B2 was evaluated to determine whether components present in the medium effects the characteristics of the enzyme. The partially purified β-glucosidase 131B2 had a pH

optimum of 4. The activity of the partially purified β-glucosidase 131B2 was similar at pH 3 and pH 5. When comparing the β-glucosidase activity at pH 3 and 5 with pH 4, only 22% less activity was observed at pH 3 and 24% less at pH 5 (Fig. 3.9). However, the stability of the partially purified β-glucosidase 131B2 was only for 24 hours at pH 3, 4 and 5 (Table 3.6 or Addendum A), which is in contrast with the pH stability of the crude β-glucosidase 131B2.

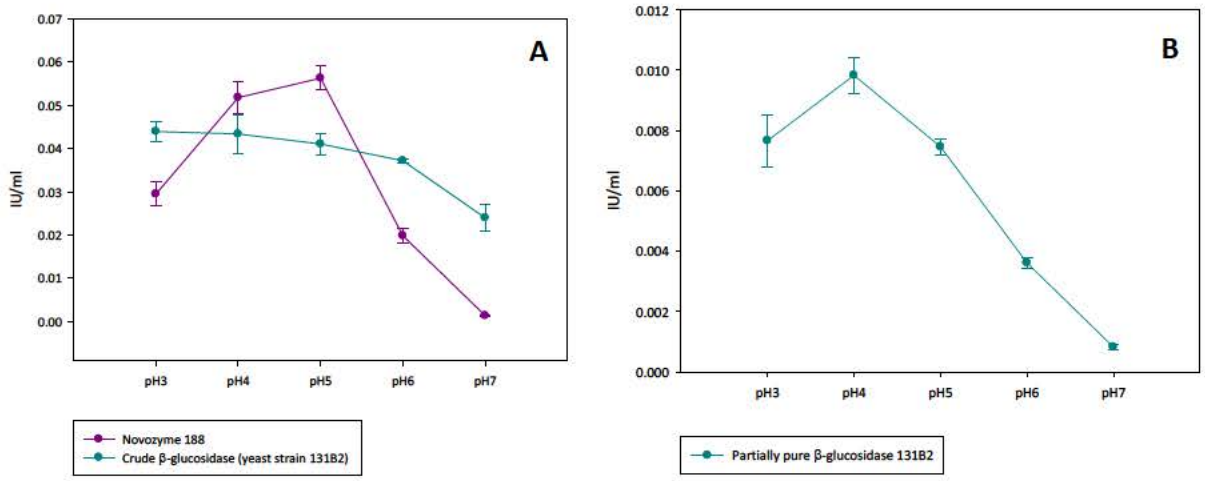

Fig. 3.9 Volumetric β-glucosidase activity of (A) crude β-glucosidase 131B2 and Novozyme 188, and (B) of partially purified β-glucosidase 131B2 to determine the pH optima at pH 3 to 7.

Table 3.6 pH stability of the crude and partial purified β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2, and Novozyme 188. Refer to Addendum A for graphs.

Condition	Time of stability (hours)		
	Yeast isolate 131B2	Yeast isolate 131B2	Novozyme 188
	Crude	Partial purified	Crude
pH 3	72	24	72
pH 4	72	24	72
pH 5	72	24	72
pH 6	72	N/A	72
pH 7	72	N/A	72

3.3.4.3 Determination of the temperature optima and stability of β-glucosidase

The temperature optima and stability of the β-glucosidase was evaluated to determine its use during lignocelluloses hydrolysis. The activity of the crude β-glucosidase 131B2 appeared to be thermophilic by having its optimal temperature between 60°C and 70°C (Fig. 3.10). The Novozyme 188 had a temperature optimum only at 60°C. At 30, 40, 70 and 80°C the crude β-glucosidase 131B2 showed significantly higher activity than Novozyme 188. The activity of the crude β-glucosidase 131B2 improved by a factor of 1.7 at 70°C which could be the high temperature aiding the hydrolysis of the substrate.

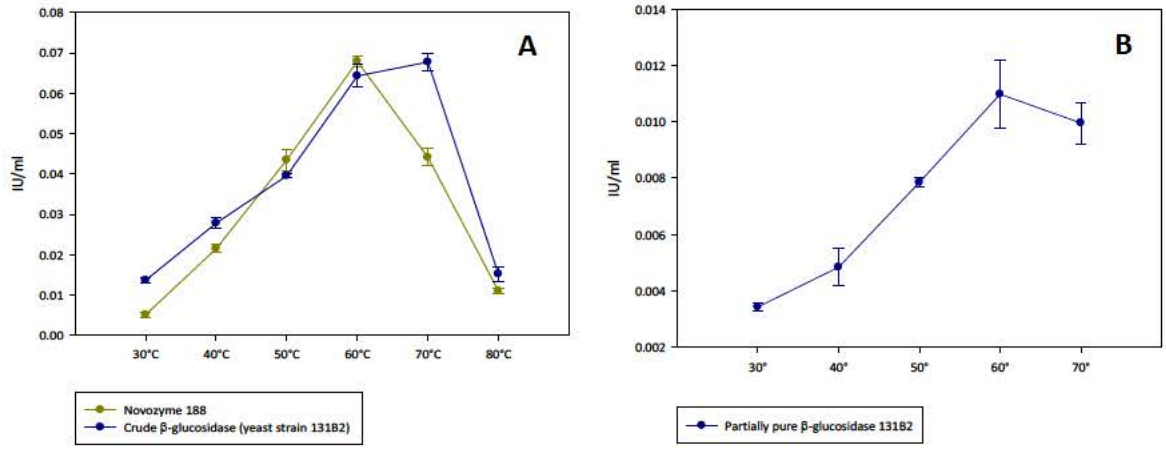

Fig. 3.10 Volumetric β-glucosidase activity of (A) crude β-glucosidase 131B2 and Novozyme 188, and (B) of partially purified β-glucosidase 131B2 to determine the temperature optima at 30 to 80°C.

Both enzymes were stable at 30, 40 and 50°C for 72 hours (Table 3.7 or Addendum A). At 60°C the crude β-glucosidase 131B2 retained stability for 72 hours (Fig. 3.11), but the Novozyme 188 only had activity for 1 hour. At 70°C the crude β-glucosidase 131B2 only had activity for 1 hour and Novozyme 188 for 30 min. β-glucosidase activities for both enzymes were lost after 30 min incubation at 80°C. The partially purified β-glucosidase 131B2 only had a temperature optimum at 60°C and similar to the crude β-glucosidase 131B2 remained stable at 50°C and 60°C for 72 hours (Fig. 3.11, Table 3.7 or Addendum A).

Table 3.7 Temperature stability of the crude and partial purified β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2, and Novozyme 188. Refer to Addendum A for graphs.

Condition	Time of stability (hours)		
	Yeast isolate 131B2	Yeast isolate 131B2	Novozyme 188
	Crude	Partial purified	Crude
30°C	72	N/A	72
40°C	72	N/A	72
50°C	72	72	72
60°C	**72**	**72**	**1**
70°C	1	0.5	0.5
80°C	0	0	0

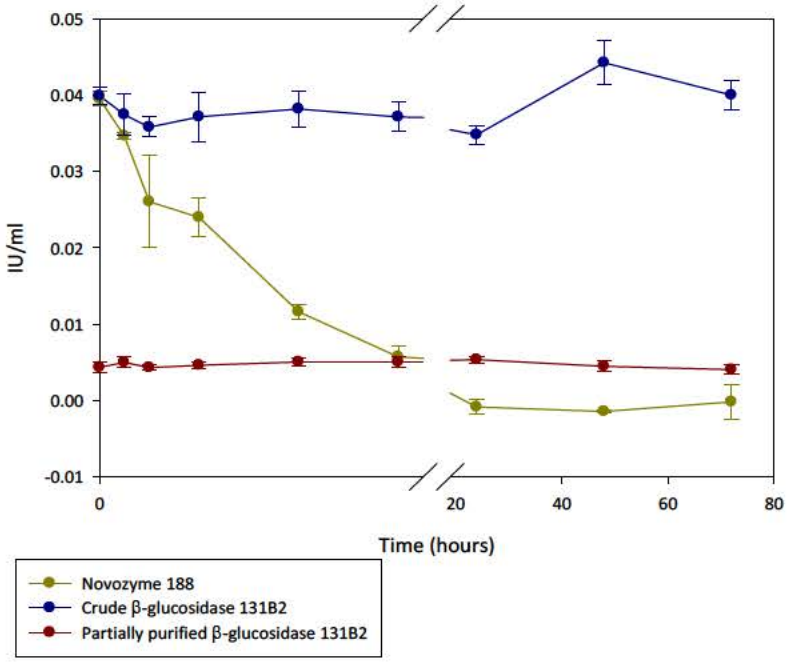

Fig. 3.11 Temperature stability of the crude and partially purified β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 60°C for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

3.3.4.4 Effect of glucose and ethanol on β-glucosidase activity

The effects of end-products of lignocelluloses hydrolysis and fermentation, such as glucose and ethanol, on the β-glucosidase were evaluated. The crude β-glucosidase 131B2 appeared to be less sensitive to product inhibition than the Novozyme 188 for glucose concentrations ranging from 50 mM to 450 mM (Fig. 3.12 A). At 50 mM glucose the crude β-glucosidase 131B2 was inhibited by 37% but Novozyme 188 had 70% activity reduction.

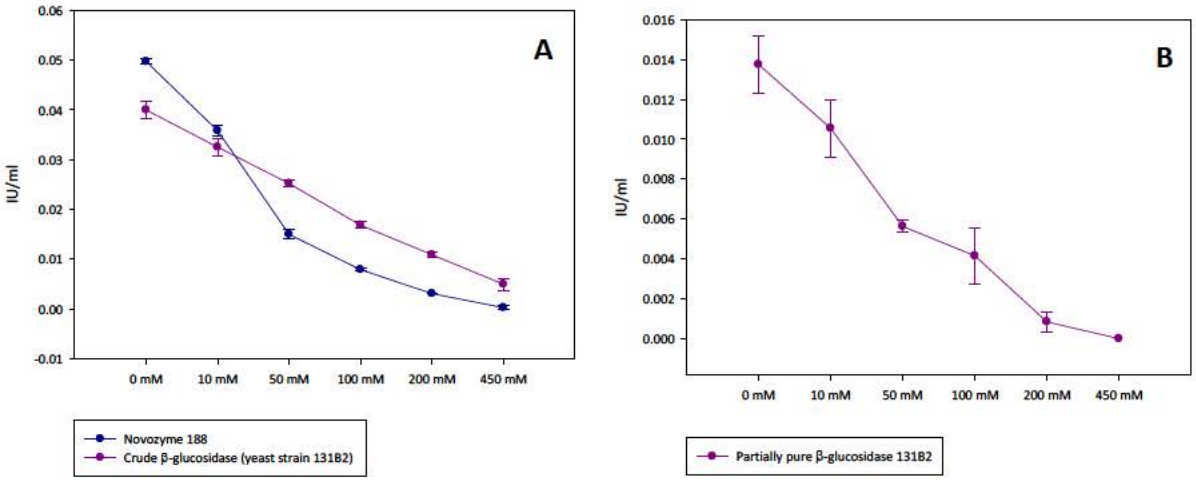

Fig. 3.12 The effect of glucose on the hydrolytic activity of (A) the crude β-glucosidase 131B2 and Novozyme 188, and (B) the partially purified β-glucosidase 131B2.

Ethanol inhibition was similar for both enzymes but at 20 to 40% (v/v) ethanol the crude β-glucosidase 131B2 outperformed the Novozyme 188 by having 19% higher activity at 20% (v/v) ethanol, 48% higher activity at 30% (v/v) ethanol and 29% at 40% (v/v) ethanol (Fig. 3.13 A). At 30% v/v ethanol crude β-glucosidase 131B2 reduced in activity by 20% whereas Novozyme 188 had 55% activity reduction.

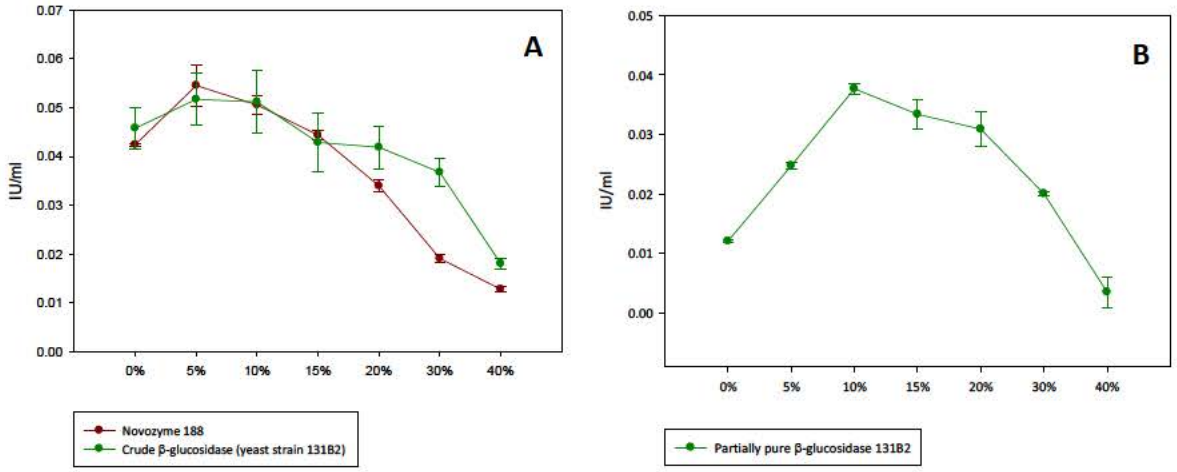

Fig. 3.13 The effect of ethanol on the hydrolytic activity of (A) the crude β-glucosidase 131B2 and Novozyme 188, (B) the partially purified β-glucosidase 131B2.

The partially purified β-glucosidase was more sensitive to 50 mM glucose than the crude β-glucosidase, having 59% less activity (Fig. 3.12 B). This is still lower than the 70% activity reduction by the Novozyme 188. In contrast with the crude β-glucosidase, the partially purified β-glucosidase appeared to be improved by ethanol, having an approximately 3 fold improvement at 10% (v/v) ethanol, 15% (v/v) ethanol and 20% (v/v) ethanol, and 2 fold at 30% (v/v) ethanol (Fig. 3.13 B).

3.4.5 Comparison of crude β-glucosidase and a commercial β-glucosidase (Novozyme 188) as supplements during Avicel hydrolysis by Celluclast

The objective was to compare the crude β-glucosidase 131B2 and Novozyme 188 as β-glucosidase supplement during the hydrolysis of Avicel by Celluclast. The enzyme units added was as follows: 61.67 FPU/ml endoglucanase for Celluclast, 0.3 IU/ml β-glucosidase for Novozyme 188 and 0.3 IU/ml β-glucosidase for crude β-glucosidase 131B2. The glucose released after 120 hours for the Novozyme 188/Celluclast combination was 17.51 ±0.91 g/L (0.10 M glucose), crude β-glucosidase 131B2/Celluclast combination 16.85 ± 0.22 g/L (0.10 M glucose) and Celluclast 13.62 ± 0.22 g/L (0.08 M glucose) (Fig. 3.14). Low levels of β-glucosidase was added, therefore only a slight increase in

glucose production was detected for the Novozyme 188/Celluclast combination and the crude β-glucosidase 131B2/Celluclast combination when compared to Celluclast. A statistically significant difference between the glucose released during Avicel hydrolysis could not be observed for the Novozyme 188/Celluclast and crude β-glucosidase 131B2/Celluclast combination after 96 and 120 hours, therefore it can be concluded that the two β-glucosidases performs similarly as supplements during the hydrolysis of Avicel by Celluclast. At 24, 48 and 72 hours the glucose released by the crude β-glucosidase 131B2/Celluclast combination was less than the Novozyme 188/Celluclast, but at 96 and 120 hours the Novozyme 188/Celluclast and crude β-glucosidase 131B2/Celluclast combinations released similar levels of glucose. This suggests that the crude β-glucosidase 131B2 was slow to hydrolyse the available cellobiose, the Novozyme 188 started losing stability or the crude β-glucosidase 131B2 might have been less inhibited than Novozyme 188 by the glucose present.

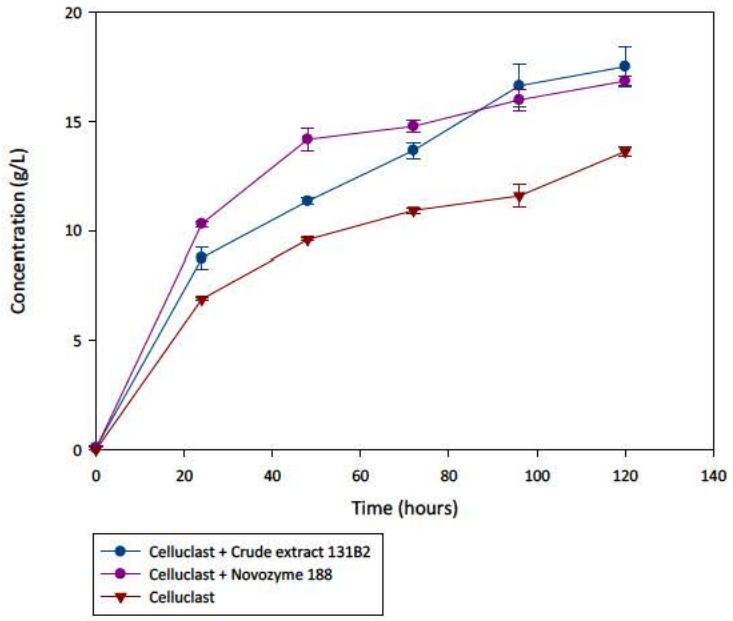

Fig. 3.14 Comparison of the hydrolysis of Avicel between crude β-glucosidase 131B2 and Novozyme 188.

3.5 Discussion

The objective of this study was to search the non-*Saccharomyces* yeast culture collection of the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa) for yeast displaying β-glucosidase activity. A total of 2180 yeast were initially screened on cellobiose as carbon source of which 1272 showed to have β-glucosidase activity. The total of 1272 yeast were further reduced to 261 yeast, of which four yeast isolates were selected for further screening on the substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) to eventually select one yeast isolate displaying superior β-glucosidase activity. The four yeast isolates selected was isolate 126A6 isolated from the exotic fruit, Jaboticaba, isolate 131B2 isolated from the indigenous stem fruit, and isolate 45A27W and 45A27R isolated

from the Rustenburg vineyards. The four yeast isolates were preliminarily identified based on the rDNA D1/D2 domain as *Candida* spp. (isolate 126A6 and 45A27W) and as *Rhodotorula* spp. (isolate 131B2 and 45A27).

The yeast isolate selected for displaying superior β-glucosidase activity, compared to the positive control (*Saccharomyces cerevisiae* BGL) and the other yeast isolates, was isolate 131B2. Further identification of the yeast isolate 131B2 based on the ITS region resulted in a 99% homology with *Rhodotorula slooffiae*, and the neighbour-joining tree constructed showed that the isolate formed a clade with only a bootstrap value of 73.6%. As a bootstrap value of 95% or higher is considered as conclusive, definate identification could not be obtained. A morphological and biochemical comparison with *Rhodotorula slooffiae* type isolates was required for identification. An additional housekeeping gene, such as β-tubulin, can also be used to clarify the identity of unknown yeasts, however the β-tubulin gene sequence database is not as extensive as that of the D1/D2 domain of the rDNA. *Rhodotorula slooffiae* was first described in 1962 in Hungary (Novák & Vörös-Felkai, 1962) and until recently it was considered a synonym of *R. minuta* (Fell & Statzell-Tallman, 2000). *Rhodotorula* spp. are broadly distributed in nature, where they are generally found on green leaves, fruits and buds (Buck, 2002; Heidenreich *et al.*, 1997). The yeast can assimilate ethanol, D-glucose, D-xylose and cellobiose (delayed and weakly), but is unable to ferment glucose (http://www.cbs.knaw.nl).

Prior to the characterisation of the crude β-glucosidase 131B2, the culturing medium of the *Rhodotorula slooffiae*-like yeast was optimised to improve the β-glucosidase production. The culturing conditions that showed to improve the β-glucosidase production by *Rhodotorula slooffiae*-like yeast isolate 131B2 was an incubation temperature between 16°C to 28°C and optimal pH between 3.5 to 4.5. An increase of 3.2 fold was obtained from culturing *Rhodotorula slooffiae*-like yeast isolate 131B2 in 2 % (w/v) triticale bran for 3 days at 30°C, where 0.0064 (± 0.000761) IU/ml β-glucosidase activity was observed, to 0.0204 (± 0.004167) IU/ml β-glucosidase activity when culturing in 2% triticale, 2% $(NH_4)_2SO_4$ for 5 days (120 hours) at 30°C and pH 4. A further increase of β-glucosidase production might be obtained if other culturing parameters are included, for instance β-glucosidase production by *Debaryomyces pseudopolymorphus* UCLM-NS7A was optimised by 12.5 fold from 0.02 IU/ml (cultured in cellobiose) to 0.25 IU/ml when addition culturing conditions such as the addition of NaCl, $MgSO_4$, yeast extract, ethanol and Tween 80, and the change from small-schale shaker flask to lab-fermenter were included (Barbosa *et al.*, 2010).

During the preliminarily characterisation of the β-glucosidase 131B2, the zymogram showed a fluorescent band, indicating β-glucosidase activity, when the *Rhodotorula slooffiae*-like yeast isolate 131B2 was cultured in glucose and triticale bran containing media. The fluorescent band showed that the β-glucosidase 131B2 has a molecular mass of approximately 130 kDa, which was similar to the 144 kDa

reported for the dimeric *Rhodotorula minuta* isolate IFO879 β-glucosidase (Onishi & Tanaka, 1996). A fluorescent band was not expected for the *Rhodotorula slooffiae*-like yeast isolate 131B2 when cultured in glucose. To evaluate if glucose generally induces β-glucosidase production, the *Rhodotorula slooffiae*-like yeast isolate 131B2 and other yeast known to produce β-glucosidase were evaluated for β-glucosidase production after being cultured in YPD medium. *Rhodotorula slooffiae*-like yeast isolate 131B2 was the only yeast that produced β-glucosidase when grown in glucose as carbon source (Fig 3.7).

Crude β-glucosidase 131B2 was eventually compared to the commercial *Aspergillus niger* β-glucosidase preparation (Novozyme 188) to determine whether it has application as a hydrolytic enzyme. The characteristics evaluated were pH optima and stability, temperature optima and stability, and inhibition by glucose and ethanol. The results obtained showed that the crude β-glucosidase 131B2 had a broader pH optima range of pH 3 to 5 compare to Novozyme 188, which was optimal at pH 4 to 5. Both β-glucosidase preparations were stable at pH 3 to 7. Crude β-glucosidase 131B2 also had a broader temperature optima range of 60°C and 70°C, whereas Novozyme 188 only had a temperature optimum at 60°C. The crude β-glucosidase 131B2 displayed exceptional temperature stability by being stable for 72 hours at 60°C whereas the Novozyme 188 lost activity after 1 hour. Crude β-glucosidase 131B2 was also less inhibited by glucose and ethanol. For instance 50 mM glucose inhibited crude β-glucosidase 131B2 by 37%, but Novozyme 188 was inhibited by 70%. Ethanol at a concentration of 30% (v/v) reduced the crude β-glucosidase 131B2 by 20%, whereas Novozyme 188 had 55% less activity. Crude 131B2 β-glucosidase was also compared to Novozyme 188 as a β-glucosidase supplement to the commercial *Trichoderma reesei* cellulase preparation (Celluclast) during the hydrolysis of synthetic microcrystalline cellulose (Avicel). The enzymes performed similarly after 96 and 120 hours of the hydrolysis process of Avicel, indicating that the enzyme will perform equally to Novozyme 188 at hydrolysis conditions of 50°C and pH 5.

The culturing medium and various components produced by the *Rhodotorula slooffiae*-like yeast isolate 131B2 could have an impact on the characteristics of an enzyme, therefore the crude β-glucosidase 131B2 was partially purified using size exclusion chromatography and characterised. The partially purified and crude β-glucosidase 131B2 had similar characteristics, but partially purified β-glucosidase 131B2 was less stable at pH 3 to 5, having a loss of activity after 24 hours. Temperature had a minimal effect on the stability of the β-glucosidase 131B2 as the partially purified β-glucosidase 131B2 retained its temperature stability of 72 hours at 60°C. A glucose concentration of 50 mM inhibited the partially purified β-glucosidase 131B2 by 59% whereas the crude purified β-glucosidase 131B2 was only inhibited by 37%. However, the Novozyme 188 was still more inhibited than the partially purified β-glucosidase 131B2 having 70% less activity. Ethanol, in contrast with the crude β-glucosidase 131B2 and Novozyme 188, increased the partially purified β-glucosidase 131B2, e.g. at 10% (v/v) ethanol a 3 fold increase in activity was observed. The β-glucosidase activity of yeast *Dekkera intermedia* (Blondin *et al.*, 1983),

Candida molischiana (Gonde *et al.*, 1985) and *Debaryomyces hansenii* (Yanai & Sato, 1999) also showed to be enhanced by the presence of low ethanol concentrations, which is thought to be for glucosyltransferase activity by the β-glucosidase (Pemberton *et al.*, 1980). Reports have also been made that β-glucosidase uses alcohols as an acceptor molecule for glucose instead of water when hydrolysing *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG); elevating the reaction rates (Pemberton *et al.*, 1980; Saha *et al.*, 1994). The differences observed between the crude and partially purified β-glucosidase 131B2 suggest that the component within the supernatant, whether produced by the yeast or from the culturing medium, do have an impact on the characteristics of the enzyme and the enzyme might have different characteristics when the yeast is cultured in a different medium.

3.6 Conclusion

Enzymatic hydrolysis of biomass usually takes place for about 24 to 72 hrs at 50°C (Singhania *et al.*, 2011) and fungal cellulolytic enzymes frequently used in bioethanol processes are active at a low pH of 4 to 5 (Hahn-Hägerdal *et al.*, 2006). These characteristics provide a benchmark of attributes an enzyme should have when searching for a new enzyme for bioethanol production. The crude β-glucosidase 131B2 showed potential by being optimal and stable for 72 hours at pH 3 to 5 and at 60°C. The partially purified β-glucosidase 131B2 was also stable for 72 hours at 60°C. Novozyme 188 was only optimal and stable at pH 4 to 5 for 72 hours and at 60°C for 1 hour. The crude and partially purified β-glucosidase 131B2 was also less inhibited by 50 mM glucose and 30% (v/v) ethanol than Novozyme 188. During hydrolysis of Avicel at pH 5 and 50°C, the crude β-glucosidase 131B2 and Novozyme 188 performed similarly as β-glucosidase supplement to Celluclast. In this study, the crude and partially purified β-glucosidase 131B2 showed to have potential as a hydrolytic enzyme for bioethanol production; making this enzyme promising for attempts to clone and heterologously express the gene in *Saccharomyces cerevisiae* to improve the production levels of the enzyme and simplify the purification and characterisation of the enzyme. Refer to Addendum C for attempts made in this study to obtain the β-glucosidase gene of the *Rhodotorula slooffiae*-like yeast isolate 131B2.

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CHAPTER 4

RESULTS CHAPTER

Screening and preliminarily characterisation of a β-xylosidase enzyme from non-*Saccharomyces* yeasts

Screening and preliminarily characterisation of a β-xylosidase enzyme from non-*Saccharomyces* yeasts

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4.1 Abstract

Hydrolysis of lignocellulose for bioethanol production is still suboptimal and various approaches have been taken to address the problem, which includes searching for superior enzymes that could replace currently-used enzymes or be used to augment existing enzyme cocktails. The aim of this study was to screen and characterise yeast producing superior β-xylosidase activity, from a fruit-sourced yeast culture collection. The culture collection comprised of non-*Saccharomyces* yeasts isolated primarily from various vineyards and fruit. A total of 2180 yeasts were initially screened on Remezol Brilliant Blue (RBB)-xylan-containing plates for xylanase activity, of which 70 showed clearing zones. These 70 yeast isolates were subjected to a secondary quantitative screening on *p*-nitrophenyl-β-D-xylopyranoside for β-xylosidase activity. One yeast isolate was selected for its superior β-xylosidase production compared to the other non-*Saccharomyces* yeast screened and was identified as an *Aureobasidium pullulans* isolate. Response surface methodology according to a central composite design was used to improve the β-xylosidase production by the *Aureobasidium pullulans* isolate 23B25 for characterisation of the enzyme. The crude β-xylosidase was also partially purified using size exclusion and characterised. The temperature and pH optima and stability was determined, and the effect of glucose, xylose and ethanol on the enzyme's activity was evaluated. The crude β-xylosidase had a pH optima and stability of 6 hours at pH 2 to 3. The temperature optima of the crude β-xylosidase was at 80°C and 90°C, but was only stable for 30 min at 80°C and non-stable at 90°C. However, a temperature stability of 72 hours at 30° to 70°C was observed. The crude β-xylosidase was uninhibited by glucose and an improved activity at 200 mM and 450 mM glucose was observed. It was inhibited by 10 mM xylose and 30% (v/v) ethanol. The partially purified β-xylosidase had similar characteristics as the crude β-xylosidase with the exception of being stable at 60°C for 72 hours, but only for 30 min at 70°C. It was less inhibited by xylose than the crude β-xylosidase; losing activity at 50 mM xylose instead of 10 mM xylose. The partially purified β-xylosidase was more inhibited by ethanol, losing activity at 20% (v/v) ethanol.

4.2 Introduction

The use of lignocellulose biomass for bioethanol production is considered a viable alternative to the controversial food-based feedstocks, such as sugarcane and maize. However, the high dosages of enzyme required to hydrolyse pretreated lignocellulosic biomass and the cost associated with enzyme production makes second generation bioethanol production not yet economically viable (Himmel *et al.*, 2007). To improve the hydrolysis of lignocellulose, research has focused on improving the feedstock hydrolysis step by improving the specific enzymes involved and optimizing enzyme cocktail efficiencies (Margeot *et al.*, 2009). Major research focused on the hydrolysis of cellulose to glucose, but the realization of benefits associated with utilizing both hexoses (e.g. glucose) and pentoses (e.g. xylose) during the fermentation phase in terms of increased theoretical bioethanol yields has driven research to also improve hemicellulose hydrolysis (Merino *et al.*, 2007).

Hemicellulose is a heteropolysaccharide consisting of a variety of monosaccharides such as xylose, arabinose, glucose, mannose and galactose (Mohan *et al.*, 2006). However, the main component of hemicellulose is xylan (Dodd & Cann, 2009) . The xylan backbone is not as recalcitrant to enzymatic attack as crystalline cellulose, but carries acetyl, methylglucuronosyl, and arabinofuranosyl side-chains in varying proportions, which needs to be removed by a range of accessory enzymes for the complete hydrolysis of xylan (Puls & Poutane, 1989). The major xylan backbone-hydrolysing enzymes include endoxylanases and β-xylosidases (Saha, 2003). Endoxylanases hydrolyse the xylan backbone, producing xylo-oligosaccharides, which are in turn hydrolysed to xylose by β-xylosidases (Zhang *et al.*, 2007). β-xylosidases are crucial for the final hydrolysis step of xylan to monomers (xylose) and in preventing end-product (i.e. xylobiose) inhibition of endoxylanase (Poutanen & Plus, 1988; Workman & Day, 1982; Zhang *et al.*, 2007).

Research efforts have been directed towards the natural environment for the discovery of new enzymes as the consensus are that the natural environment harbours various undiscoved microbes with high levels of metabolic diversity (Banerjee *et al.*, 2010). One method to obtain new enzyme from the natural environment is to screen culturable microorganism for their activity or presence. Various researchers have screened yeasts isolated from natural habitats for hydrolytic enzymes, such as Manzanares *et al.* (1999) that screened yeasts isolated from grapes and wine, and Jiménez *et al.* (1991) that screened yeasts isolated from decaying wood for β-xylosidase activity

In this study, a large collection of non-*Saccharomyces* yeast isolates, isolated from different fruit types was screened for β-xylosidase activity. During the screening of 2180 yeast isolates, a single yeast was selected that had the highest β-xylosidase activity compared to the other non-*Saccharomyces* yeast screened. The selected yeast was identified, its β-xylosidase production increased by improving the culturing conditions of the selected yeast, and the β-xylosidase characterised.

4.3 Materials and Methods

4.3.1. Yeast isolates and the culturing of thereof

The 2180 non-*Saccharomyces* yeast isolates used in this study were obtained from the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa. The yeast isolates were previously isolated from different fruit types, i.e. various grape cultivars, Kei apple (*Dovyalis caffra*), stem fruit (*Englerophytum magalismontanum*), Jaboticaba (*Myrciaria* spp.) and Mobola plum fruits (*Parinari curatellifolia*), from different regions in South Africa (Table 4.1). The Kei apple, stem fruit and Mobola plum are indigenous to South Africa and the Jaboticaba is a locally grown exotic fruit. The positive control for β-xylosidase activity was the *Aureobasidium pullulans* NRRL Y2311, known to produce β-xylosidase (Myburgh *et al.*, 1991), and the negative control was *Saccharomyces cerevisiae* VIN17. All yeast isolates were stored as glycerol freeze cultures, and were cultured and maintained on solid yeast peptone dextrose (YPD) medium for subsequent experiments.

Table 4.1 Yeast isolates used for screening.

Culture Collection	Culture year	Number of yeast
Constantia vineyard	1997	60
Madeba vineyard	1997	51
Opstal vineyard	1997	59
Rustenburg vineyard	1997	60
Chardonnay grapes	1997	125
Rustenburg vineyard	1998	30
Opstal vineyard	1998	30
Madeba vineyard	1998	30
Constantia vineyard	1998	30
Isolates from various areas	1999	50
Springfield vineyard	2000	30
Van Loveren vineyards	2000	30
Zandvliet vineyards	2000	30
Madeba rail vineyards	2000	30
Rustenburg vineyard	2000	30
Constantia vineyards	2000	30
Opstal vineyards	2000	30
Madeba vineyards	2000	30
Shiraz	2002	240
Madeba vineyard	2002	52
Isolates from various areas	2002	28
Shiraz	2003	196
Shiraz	2004	160
Grapes (Algulhas)	2007	405
Jaboticaba (Nelspruit)	2007	7
Mobola plum (Nelspruit)	2007	3
Kei Apple (Nelspruit)	2007	162
Kei Apple & Stem fruit (Nelspruit)	2007	75
Stem fruit (Nelspruit)	2007	87
Total		**2180**

4.3.2 The screening of the non-*Saccharomyces* yeast for β-xylosidase activity

4.3.2.1 Screening for xylanase activity

Yeasts were screened for xylanase activity on solid Remazol Brillant Blue (RBB)-xylan medium; containing 0.2% RBB-xylan, 1% glucose (Saarchem, Merck, Gauteng, SA), 0.68% yeast nitrogen base (YNB; Sigma-Aldrich) and 2% agar bacteriological (Biolab, Merck, Gauteng, SA) (Farkas *et al.*, 1985). β-Xylanase cleaves the RBB-xylan into a colourless product, therefore yeast producing xylanase produce a clearing zone, which can be viewed against diffuse white light. As mentioned in section 4.3.1, the yeast cultures were maintained on YPD. These cultures were re-cultured onto solid RBB-xylan medium and incubated for 3 to 4 days at 30˚C.

For the preparation of Remazol Brilliant Blue (RBB)-xylan, 28 g of birchwood xylan (Roth, Karlsruhe, Germany) were dissolved in 625 ml distilled water and heated to 40 to 60°C before adding 25 g of RBB (Sigma-Aldrich, Steinheim, Germany) while stirring (Biely *et al.*, 1985; Biely *et al.*, 1988). The mixture was removed from the heat and 125 ml sodium acetate (0.4 M; aqueous solution) were added. Two-hundred-and-fifty millilitres of 6% w/v sodium hydroxide were added and the mixture stirred for 1 hr at 28°C. The coupled RBB-xylan was precipitated with 2 volumes of ethanol (approximately 100%) and the solution centrifuged at 5000 rpm for 5 min at 28°C. The supernatant was discarded and the pellet re-suspended with ethanol. Washing of the pellet was performed with ethanol-0.05 M sodium acetate solution (2:1 (v/v)) until the supernatant was colourless. The RBB-xylan pellet was dissolved in 1 L 0.05 M sodium acetate and precipitated with 1 L of ethanol. Another wash of the RBB-xylan was performed with ethanol and the resulting pellet desalted with ethanol-water solution (4:1 (v/v)). The final RBB-xylan pellet was re-dissolved in 50 ml distilled water and lyophilised using a Virtis freeze dryer (SP Industries, Gardiner, NY, USA).

4.3.2.2 Screening for β-xylosidase activity

Selected xylanase-positive yeast isolates obtained in the previous screening step, the *Aureobasidium pullulans* NRRL Y2311 β-xylosidase-producer and *S. cerevisiae* VIN17 negative control was cultured in 20 ml triticale medium (containing 1% triticale bran and 0.68% YNB) in 100 ml Erlenmeyer flasks for 3 days at 30°C with shaking at 100 rpm. Supernatant β-xylosidase enzyme activity was measured by incubating the appropriate volume of supernatant with 0.005 M of *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX, Sigma-Aldrich, Steinheim, Germany) in 0.05 M citrate buffer (pH 5.0) at 50˚C for 10 min (den Haan *et al.*, 2007). The reaction was stopped by adding 1 M Na_2CO_3. The ratio for buffer : substrate : cells : Na_2CO_3 was 89 : 1 : 10 : 100 and all the reactions were performed in 1.5 ml eppendorf tubes. The *p*-nitrophenol (*p*NP) released from the *p*NPG was detected as absorbance at 405 nm with a xMark™ Microplate Spectrophotometer (BioRAD). Enzymatic activity was expressed in international units (IU)/ml which is defined as the amount of µmole *p*NP released per minute and a concentration range of 0 mM – 0.25 mM *p*NP was used to obtain a standard curve.

4.3.3 Identification of the non-*Saccharomyces* yeast isolates

Genomic DNA was isolated using the protocol described by Ausubel *et al.* (2007) from the yeast isolate displaying superior β-xylosidase activity during the screening step. The D1/D2 domain of the 26S subunit of the ribosomal DNA was amplified using polymerase chain reaction (PCR) with primers LR3 and F63 from Integrated DNA Technologies (IDT, Coraville, IA; Table 4.2). Further confirmation of the yeast identity was conducted by amplifying the internal transcribed space (ITS) region with primers ITS1 and ITS4 from IDT (Table 4.2). All amplifications were performed using the Kapa Taq Ready Mix PCR kit (Kapa Biosystems, South Africa). The reactions were carried out in a final volume of 50 µl, containing 2.5 µl gDNA, 2.5 µl primer (20 µM), 25 µl Kapa Taq Ready Mix and the cycling conditions were 95°C for 5 min for pre-denaturation, 30 cycles of 95°C for 45 sec denaturation, 55°C for 1 min, 72°C for 1 min for extension and a final extension step of 72°C for 5 min. PCR products were purified after 0.8% agarose gel electrophoresis using the High Pure PCR Purification kit (Roche, Mannheim, Germany) and ligated into the pGEM®-T Easy Vector System according to the manufacturer's instructions (Promega, Madison, WI, USA).

Table 4.2 Primers used in polymerase chain reaction (PCR)

Application	Primer	Direction	Sequence
D1/D2 domain of 26 rDNA region	F63	Forward	5'-GCA TAT ACA ATA AGC GGA GGA AAA C-3'
	LR3	Reverse	5'-GGT TGT TTC AAG ACG G-3'
ITS region	ITS1	Forward	5'-TCC GTA GGT GAA CCT GCG G-3'
	ITS4	Reverse	5'-TCC TCC GCT TAT TGA TAT GC-3'
pGEM®-T insert confirmation	T7	Forward	5´-TAA TAC GAC TCA CTA TAG GG-3´
	SP6	Reverse	5´-TAT TTA GGT GAC ACT ATA G-3´

The pGEM clones were transformed into chemically component *E. coli* DH5α and was plated onto LB/Amp/Xgal/IPTG solid medium (100 µg/ml Amp, 80 µg/ml Xgal, 0.5 mM IPTG) and grown overnight at 37°C. The presence of the insert in the plasmid was confirmed by preparing small-scale plasmid-DNA isolations (Del Sal *et al.*, 1988) followed by PCR amplifying for insert detection using universal primers T7 and SP6 (IDT; Table 4.1). The forward and reverse sequence of the insert was determined using the dideoxy chain termination method, using the 3730*xl* DNA Analyzer (Applied Biosytems Inc., Forster City, CA, USA). Seqman™ II (DNA-STAR Inc., WI, USA) was used to assemble the sequence reads and subsequent consensus sequence homology searches were conducted using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

4.3.4 Optimisation of the culturing conditions for β-xylosidase production by isolate 23B25

4.3.4.2 Evaluation of pH on the morphology and β-xylosidase activity

The pH range for β-xylosidase enzyme production by yeast isolate 23B25 was determined by culturing the yeast at pH 1.5 to 2.0 (hydrochloric-potassium chloride buffer), pH 3.0 to 6.0 (citrate buffer) and pH 7.0 (potassium phosphate buffer), in yeast dextrose medium (0.02% yeast extract, 0.5% $(NH_4)_2SO_4$, 2% glucose and 0.05% $MgSO_4.7H_2O$) or triticale medium (1% triticale bran and 0.68% yeast nitrogen base). The culture was grown for 3 days at 30°C with shaking at 100 rpm. Volumetric β-xylosidase activity assays were performed as described in section 4.3.2.2. The cell morphology was observed at the various pH conditions and photographed under the light microscope (Nikon Eclipse E800) connected to a camera (Nikon DSFi 1).

4.3.4.3 Selection of a carbon source for effective β-xylosidase induction

A range of available carbon sources were selected for β-xylosidase induction by yeast isolate 23B25, including: cheese whey, molasses, sawdust, toilet paper, wheat bran and triticale bran. Wheat bran (Maes & Delcour, 2002) and triticale bran (García-Aparicio *et al.*, 2011) were selected as carbon source as both contain celluloses and hemicelluloses. Toilet paper was chosen because it contains celluloses (Champagne & Li, 2009) and might contain residual xylan. Sawdust, molasses and cheese whey has been previously used as carbon source for enzyme production (Chinedu *et al.*, 2007; Okafor *et al.*, 2007; Jung *et al.*, 2010; Rose & van Zyl, 2002; Youssef, 2011) and was therefore selected as carbon source in this study. Approximately 10×10^7 yeast cells were inoculated into 50 ml medium containing 2% substrate with 0.68% (w/v) YNB for 3 days at 30°C and 100 rpm. Volumetric β-xylosidase activity was determined as described in section 4.3.2.2.

4.3.4.4 Determining the optimal growth conditions for β-xylosidase production

Parameters, such as pH and temperature, have an impact on the production of an enzyme by yeasts. These effects were assessed for the production of β-xylosidase by *Aureobasidium pullulans* 23B25 using an experimental design in order to enhance its production. Statistical software packages, such as Statistica 7.1 (Statsoft Inc., Tulsa, USA) and Design Expert® version 8.0.4 (Stat-Ease Inc., Minneapolis, MN, USA), were used to create a central composite design (CCD) and to perform data analysis. Each independent variable (temperature, pH) was evaluated at two levels (high, low) in a CCD with 2 centre points and 2 star points, which resulted in a total of 10 different assays that were performed in a random order. The temperature ranges selected were between 20 to 40°C and 15°C and 35°C. The pH ranges selected were between 3 to 6 and 2 and 7. Culturing was performed in 250 ml volumetric flasks containing medium with 2% triticale bran with 0.1 M glycine-HCl buffer (pH 2.4 to 3.6), 0.05 M citrate buffer (pH 3.0 to pH 6.2) or potassium phosphate buffer (pH 5.7 to 8.0). The dependent variable was enzyme activity expressed as volumetric β-xylosidase activity (IU/ml) and was determined as described in section 3.3.2.

Interactions and main effects of the two independent variables (pH and temperature) on the enzyme production at a confidence level of 95% ($p>0.05$) was evaluated using an analysis of variance (ANOVA). A regression model was generated using a response surface methodology to predict the effect of the combined variables on the responses thereby predicting the optimal pH and temperature for β-xylosidase production.

4.3.4.5 Selection of nitrogen source for optimal growth conditions for β-xylosidase activity

A range of nitrogen sources was evaluated to determine optimal β-xylosidase production by the yeast isolate 23B25. The nitrogen source selection included: yeast nitrogen base (YNB; 2% (m/v); Sigma-Aldrich), potassium nitrate (KNO_3; 2% (m/v); Saarchem); ammonium nitrate (NH_4NO_3; 2% (m/v); Saarchem), ammonium chloride (NH_4Cl; 2% (m/v); Saarchem), ammonium sulfate $(NH_4)_2SO_4$; 2% (m/v); Saarchem), yeast peptone (1% (m/v) and 2% (m/v); YP; Biolab) and cheese whey (2% (m/v)). Triticale bran (2% (m/v)) was used as carbon source and the culture grown at the pH 4.5 and 30°C with shaking at 100 rpm. The volumetric β-xylosidase activity was determined as described in section 4.3.4.4.

4.3.5 Characterisation of crude and partially purified extracellular β-xylosidase produced by yeast isolate 23B25

4.3.5.1 Preparation of crude and partially purified extracellular β-xylosidase

Yeast isolate 23B25 was cultured in 50 ml triticale medium, containing 2% (w/v) triticale bran and 2% (w/v) $(NH_4)_2SO_4$, for β-xylosidase induction and 50 ml glucose medium, containing 2% (w/v) glucose, for no induction for 144 hours at 30°C with shaking at 100 rpm. Cells were removed by centrifugation at 15000 rpm, 7 min, 4°C and the supernatant used as the crude extracellular β-xylosidase. Concentrated crude β-xylosidase was prepared by ultrafiltration (Amicon, Ultrafiltration cell 8200) with a Millipore Polyethersulfone (PES) membrane (30 kDa MWCO).

Partial purification of the β-xylosidase protein was based on size exclusion using chromatography (SEC) on an AKTA FPLC system (Amersham Biosciences, Piscataway, NJ) with a Superose column. The mobile phase was 0.1 M citrate-phosphate buffer (pH 5), the flow rate was 0.5 ml/min and a wavelength of 280 nm was used for separating the proteins. The sample was filtered with Membrane Solutions MS® Nylon syringe filters (0.22µm pore size) before loading.

4.3.5.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Zymogram analysis

The crude and partially purified extracellular β-xylosidase was subjected to SDS-PAGE using the BIO-RAD electrophoresis system. Twenty µl of protein samples were loaded onto a discontinuous polyacrylamide gel (5% (v/v) stacking gel and 8% (v/v) separating gel) with 0.2% (w/v) 4-methylumbelliferyl β-D-xylopyranoside (MUX; Sigma-Aldrich- Aldriech). Electrophoresis was conducted at 120 Volts for

approximately 1.5 hours. The protein marker used was a prestained Spectra™Multicolor High Range Protein Ladder (ranges from 43-300 kDa; MBI Fermentas, Lithuania). The samples were prepared in a SDS-PAGE treatment buffer (containing 12% (w/v) SDS, 30% (w/v) glycerol, 0.05% (w/v) comassie blue G-250 and 150 mM Tris-HCl, pH 7) with boiling for 5 min at 70°C without reducing agent. Prior to silver staining, the zymogram was developed by washing the gel with 2.5% (v/v) Triton X-100 for 30 min and incubating the gel at 50°C for 30 min. β -xylosidase present in the gel hydrolyses the MUX, releasing 4-methylumbelliferyl which was visualised by fluorescence under UV illumination. Staining of the gel was performed using a silver staining method (Sasse & Gallagher, 2001).

4.3.5.3 Determination of the pH optima and stability of β-xylosidase

The pH optimum of the crude and partially purified β-xylosidase produced by yeast isolate 23B25 was determined by varying the pH of the assay reaction buffers. This was achieved by using 0.05 M hydrochloric acid-potassium chloride buffer (pH 2), 0.05 M citrate buffer (pH 3-6) and 0.1 M phosphate buffer (pH 7). The pH stability of the enzyme was determined by pre-incubating the enzyme in different buffers (pH 2 to 8) for 0.5, 1, 2, 4, 6, 24, 48 and 72h at 50°C followed by volumetric β-xylosidase activity analysis as described in section 4.3.2.2. Each experiment was performed twice in triplicate to confirm repeatability.

4.3.5.4 Determination of the temperature optima and stability of β-xylosidase

The temperature optimum of the crude and partially purified β-xylosidase was evaluated by performing the activity assay at different temperatures (20 to 90°C at 10°C intervals) on the substrate *p*NPX. The temperature stability of the β-xylosidase was determined by pre-incubating the enzyme for 0.5, 1, 2, 4, 6, 24, 48 and 72h at temperature 20 to 90°C followed by volumetric β-xylosidase activity analysis as described in section 4.3.2.2. Each experiment was performed twice in triplicate to confirm repeatability.

4.3.5.5 Effect of glucose, xylose and ethanol on β-xylosidase activity

The effects of glucose, xylose and ethanol on the crude and partially purified β-xylosidase activity were evaluated by performing the activity assay as described earlier on the substrate *p*NPX with an addition of glucose, xylose and ethanol. Glucose and xylose was added at a final concentration of 0 mM, 10 mM, 50 mM, 100 mM, 200 mM and 450 mM, and ethanol at a final concentration of 0, 5, 10, 15, 20, 30% v/v. Each experiment was performed twice in triplicate to confirm repeatability.

4.4 Results

4.4.1 Screening and identification of β-xylosidase yeast isolate

There is a general consensus that microorganisms growing on xylan as sole carbon source, induces a entire set of xylan-active hydrolytic enzymes (specifically endoxylanases, β-xylosidases and some accessory enzymes) to enable complete hydrolysis of the substrate (Basaran & Ozcan, 2008). Due to the exorbitant cost of the β-xylosidase-specific substrate, xylobiose, yeast isolates with a xylan active phenotype was first identified and used as a smaller subset of the yeast culture collection for β-xylosidase detection. During the initial screening of the 2180 yeast isolates on RBB-xylan, 70 displayed xylanases activity. These 70 yeast isolates were subjected to the secondary screening on *p*-nitrophenyl-β-D-xylopyranoside, to specifically determine β-xylosidase activity and 60 yeast isolates showed β-xylosidase activity (data not shown). Five yeast isolates with similar or greater β-xylosidase activity compared to the positive control, *A. pullulans* NRRL Y2311-1 β-xylosidase-producer, were selected for further analysis (Fig. 4.1). At 4 days (96 hours) of culturing on triticale bran yeast isolates 129A12, 130A19 and 132B28 produced the same levels of β-xylosidase to the *Aureobasidium pullulans* NRRL Y2311-1. However, yeast isolate 23B24 and 23B25 showed respectively an approximately 2 fold and 3 fold higher β-xylosidase activity than the *A. pullulans* NRRL Y 2311-1 at 120 hours (5 days) of culturing in triticale bran. The negative control, *Saccharomyces cerevisiae* VIN17, showed insignificantly low levels of β-xylosidase activity as was expected.

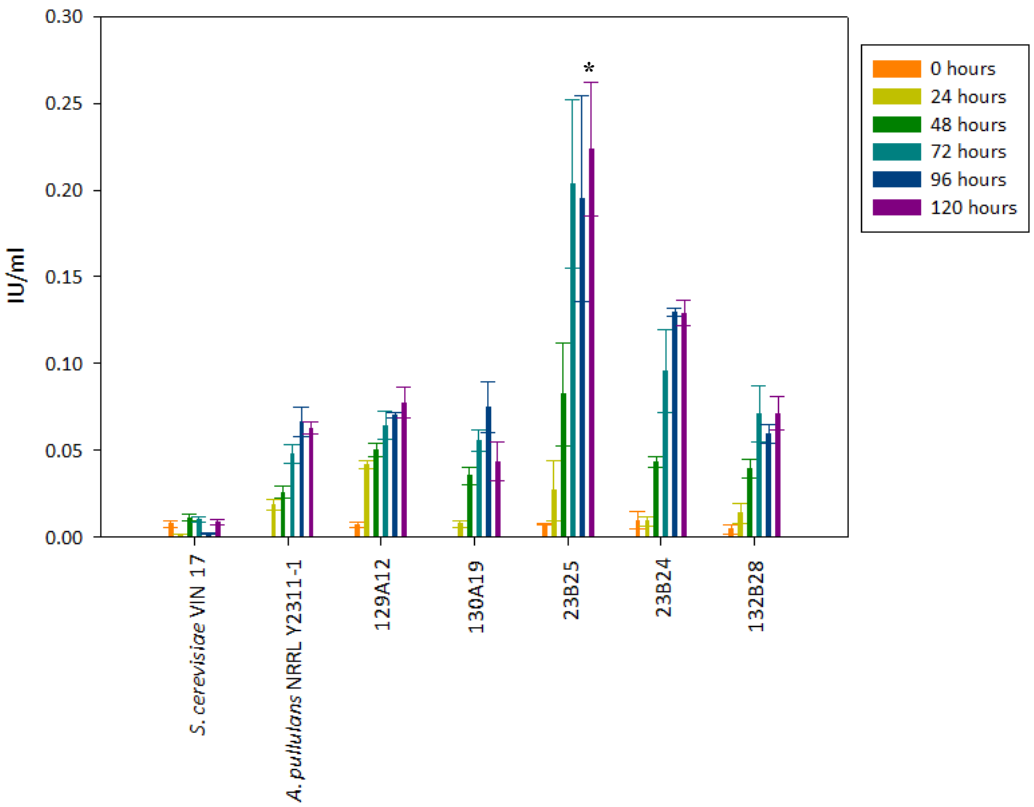

Fig. 4.1 Volumetric β-xylosidase activity of the non-*Saccharomyces* yeast isolates when cultured on 1% triticale bran and 0.68% YNB for 5 days (120 hours).

The five selected yeast isolates were preliminarily identified based on the D1/D2 region of the rDNA as *Aureobasidium pullulans* (isolates 129A12, 130A19, 23B25 and 23B24) and *Hanseniaspora* sp. (isolate 132B28). β-xylosidase activity has been reported for *A. pullulans* (Manzanares *et al.*, 1999; Iembo *et al.*, 2002) and *Hanseniaspora* spp. (Jiménez *et al.*, 1991; Capece *et al.*, 2005). Yeast isolates 129A12 and 130A19 originated from Kei apple (*Dovyalis caffra*), isolates 23B25 and 23B24 from Constantia vineyards and isolate 132B28 from stem fruit (*Englerophytum magalismontanum*). Yeast isolate 23B25 was chosen for further analysis after showing the highest β-xylosidase activity (0.22 IU/ml ± 0.04) over 5 days of growth on triticale bran compared to the other non-*Saccharomyces* yeast (Fig. 4.1). Further identified of yeast isolate 23B25 was performed based on the ITS region and a homology of 100% with *Aureobasidium pullulans* was obtained.

4.4.3 Optimisation of the *Aureobasidium pullulans* isolate 23B25 culturing conditions for β-xylosidase production

The production of the crude β-xylosidase of *Aureobasidium pullulans* isolate 23B25 was optimised to facilitate subsequent characterisation of the enzyme. Various media components and culture conditions were evaluated, including different carbon and nitrogen sources, and pH and temperature. Wheat bran and triticale bran appeared to induce β-xylosidase activity more than toilet paper, cheese whey, molasses and sawdust (Fig. 4.2). The difference in induction of the *A. pullulans* isolate 23B25 for β-xylosidase by wheat bran and triticale bran was not significant and triticale bran was selected for further analyses. The β-xylosidase activity of the yeast isolate was 0.044 (± 0.004) IU/ml when cultured on 2% triticale bran, 0.68% YNB for 3 days at 30°C while shaking at 100 rpm.

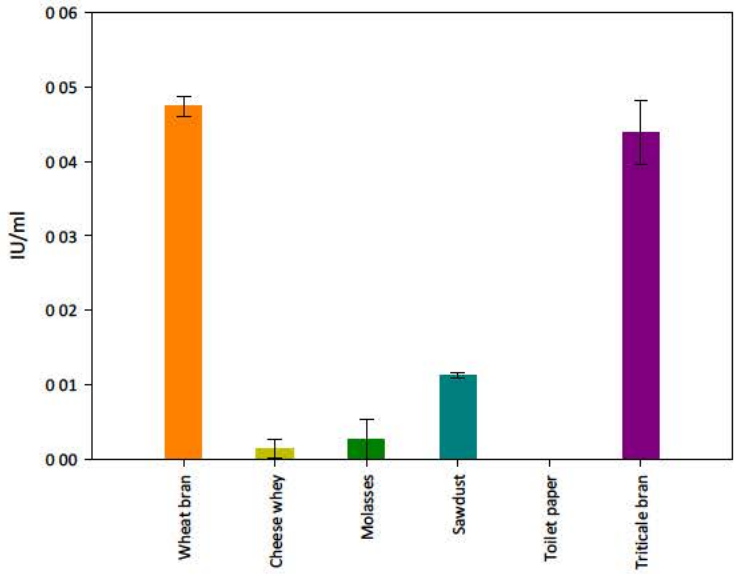

Fig. 4.2 Volumetric β-xylosidase of the supernatant produced by *Aureobasidium pullulans* 23B25 when cultured in various carbon sources.

Response surface methodology using a central composite design (CCD) was employed to determine the appropriate pH and temperature for culturing. The initial pH and temperature conditions chosen were pH 3 to 6 and 20°C to 40°C, which were selected because the *Aureobasidium pullulans* is a known mesophile (Leite *et al.*, 2008; Leite *et al.*, 2007) and is capable of growing at the selected pH levels (Li *et al.*, 2009). However, the model showed that no statistical significance could be observed for *Aureobasidium pullulans* 23B25 β-xylosidase production with the chosen pH and temperature combinations (data not shown) and another pH and temperature range had to be selected.

Li *et al.* (2009) reported that *A. pullulans* NG displayes different morphological states under different pH conditions, for examples an isolate evaluated by the authors showed meristimatic growth when cultured at pH 1.5. Meristematic growth is characterised by isodiametric cellular extension and indeterminate wall thickening by the organism during various kinds of stress (De Hoog, 1993). Li *et al.* (2009) also emphasized that the performance of *A. pullulans* under specific pH conditions varied between isolates and furthermore differential gene expression patterns might be present under the different morphological states. We set out to evaluate the morphology of the *A. pullulans* 23B25 isolate under different pH conditions and to correlate these findings with β-xylosidase expression. The *A. pullulans* 23B25 isolate was cultured in glucose and triticale containing medium buffered at pH 1.5 to 7. The cell morphology and β-xylosidase enzyme activity were monitored at these conditions.

The *Aureobasidium pullulans* 23B25 isolate displayed hyphal growth in yeast dextrose (YD) and triticale medium, buffered at pH 1.5 and 2 (Fig 4.3 A&G and B&H). Yeast-like morphology was observed for the triticale media at pH 3 to 7 (Fig. 4.3 C-F and I-L) and β-xylosidase activity was observed at pH 2 to 7 (Fig 4.4), therefore it seems that the yeast produces the enzyme preferencially in its yeast-like phase, although low β-xylosidase activity was also detected at pH 2 corresponding to hyphal morphology growth state of the isolate.

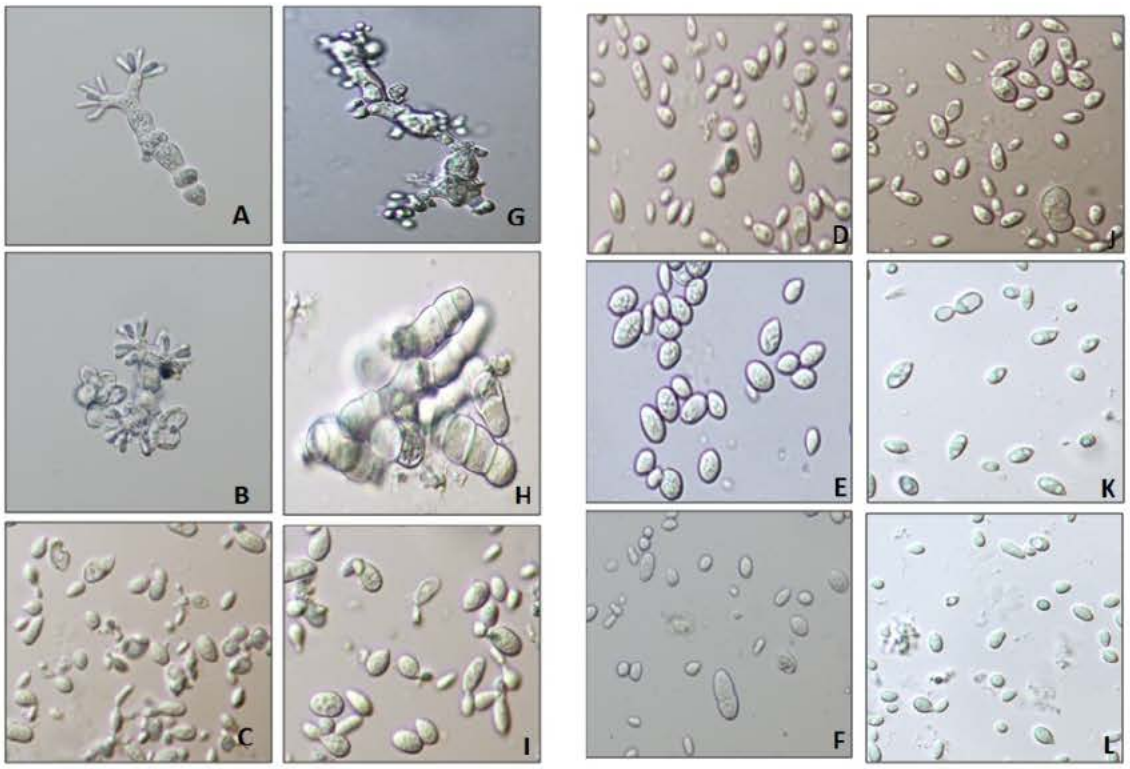

Fig. 4.3 Cell morphology when *Aureobasidium pullulans* isolate 23B25 was cultured in yeast dextrose (A-F) or triticale bran medium (G-L), buffered at various pH levels. *A. pullulans* isolate 23B25 was cultured at pH 1.5 (A and G), pH 2 (B and H), pH 3 (C and I), pH 4.5 (D and J), pH 6 (E and K) and pH 7 (F and L). Hyphal formation of *A. pullulans* at A, B, G and H, and yeast-like cell formation at C, D, E, F, I, J, K and L.

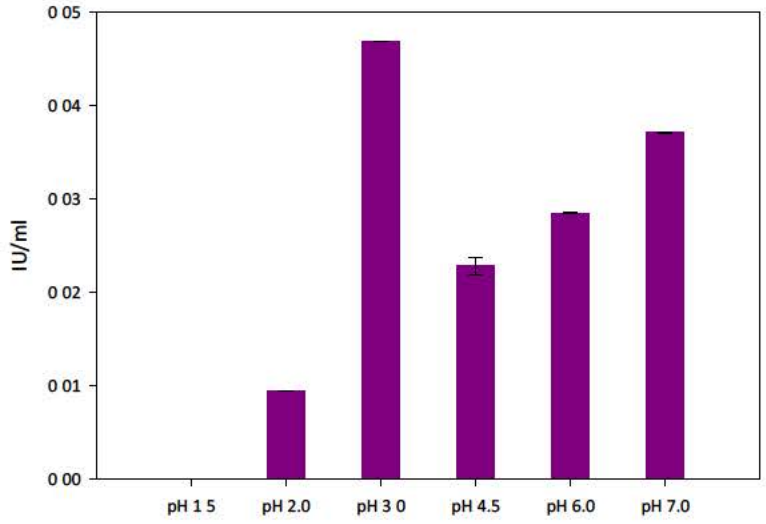

Fig. 4.4 Volumetric β-xylosidase activity of the *Aureobasidium pullulans* isolate 23B25 when cultured in triticale medium, buffered at various pH levels (pH 1.5, 2.0, 3.0, 4.5, 6 and pH 7).

As the *Aureobasidium pullulans* isolate 23B25 appear to be producing β-xylosidase between pH 2 and 7, the pH parameters chosen for the next CCD, to determine the optimal pH and temperature for culturing the *A.*

pullulans isolate 23B25 for β-xylosidase production, was pH 2 to 7 and 15°C to 35°C. However, the model showed that no statistical significance for *Aureobasidium pullulans* yeast isolate 23B25 β-xylosidase production could be observed at the selected pH and temperature range. *Aureobasidium pullulans* has the capacity to morphologically adapt to a variation of conditions, as was highlighted by Li *et al.* (2009), and using a response surface methodology to determine the optimal pH and temperature for β-xylosidase production might not be the optimal method to use. Instead, the classical microbiological approach of only changing one parameter at a time to determine the optimal β-xylosidase production could be taken. An arbitrary culturing condition was chosen for the subsequent experiments, which was pH 4.5 at 30°C for 144 hours.

Nitrogen sources for β-xylosidase production by *Aureobasidium pullulans* 23B25 was evaluated and yeast nitrogen base (YNB), potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, yeast peptone (YP) and cheese whey were selected as nitrogen sources. β-xylosidase production by *Aureobasidium pullulans* 23B25 was the highest when YNB, ammonium sulphate and YP was used (Fig 4.6) and ammonium sulphate was selected as nitrogen source because it was comparatively cheaper than YNB and YP. The β-xylosidase activity of the supernatant produced by the *Aureobasidium pullulans* was 0.062 IU/ml (± 0.014), when cultured for 144 hours at 30°C in 2% (w/v) triticale bran and 2% (w/v) $(NH_4)_2SO_4$ buffered at pH 4.5.

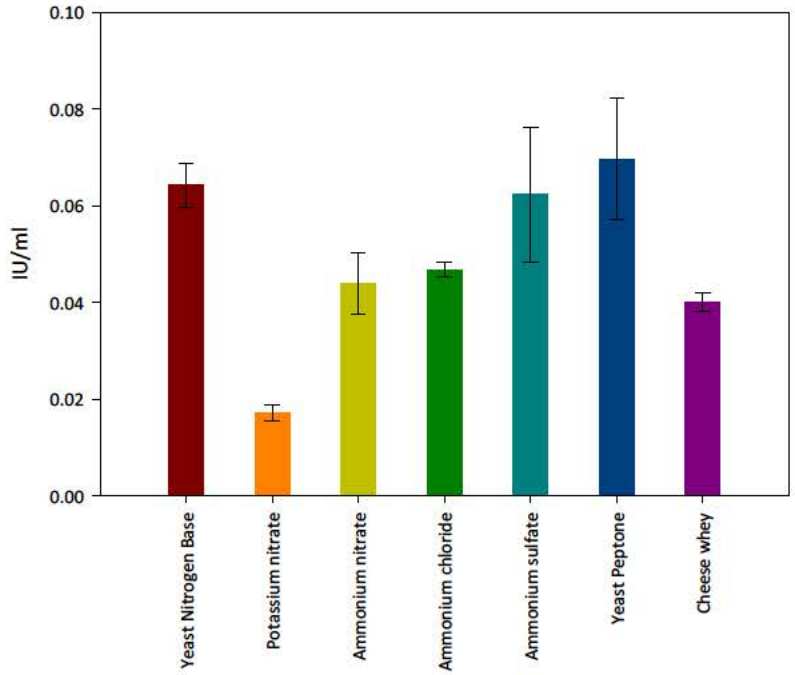

Fig. 4.6 Volumetric β-xylosidase activity of the *Aureobasdium pullulans* 23B25 when cultured on various nitrogen sources.

4.4.4 Characterisation of crude and partially purified extracellular β-xylosidase

4.4.4.1 SDS-PAGE and Zymogram analysis

A SDS-PAGE and zymogram was peformed of the crude and partially purified β-xylosidase to determine the molecular mass of the enzyme. The SDS-PAGE and zymogram showed a smear produced by the crude β-xylosidase that ranged between an approximate molecular mass of 150 and 300 kDa. The partially purified β-xylosidase produced a smear between 180 to 300 kDa (Fig. 4.7 C, D).

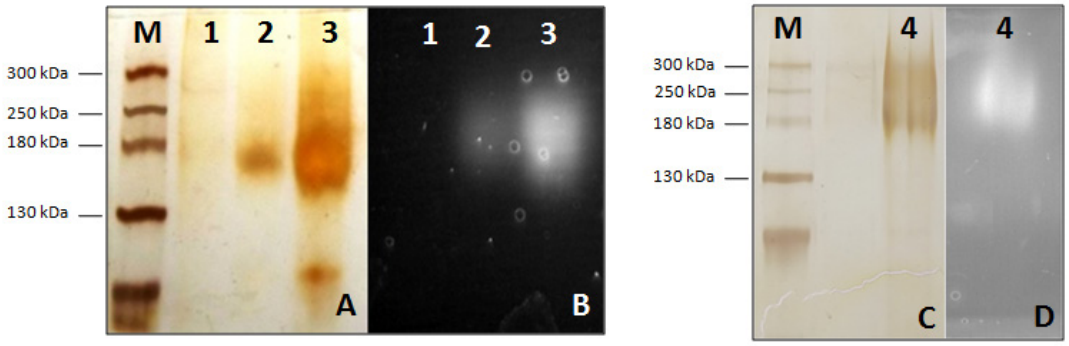

Fig 4.7 SDS-PAGE gel (A and C) and zymogram (B and D) performed to determine the molecular mass of the β-xylosidase 23B25. Crude β-xylosidase enzymes (A and B) are in lane 1 (cultured in glucose), 2 and 3 (cultured in triticale bran), with lane 3 representing the concentrated enzyme. Partially purified extracellular β-xylosidase (C and D) produced by *Aureobasidium pullulans* 23B25 in lane 4. M: molecular marker (Spectra™ Multicolor High Range Protein Ladder).

4.4.4.2 Determination of the pH optima and stability of β-xylosidase

The pH optima and stability of the β-xylosidase 23B25 was performed to determine its use in a lignocelluloses hydrolysis process. The crude β-xylosidase 23B25 had its pH optima at 2 and 3, while the partially purified β-xylosidase had a pH optima between 2 and 4 (Fig. 4.8) suggesting that the β-xylosidase prefers acidic conditions for optimal performance. The crude β-xylosidase 23B25 remained stable for 6 hours at pH 2 and pH 3; having approximately 29% less activity at pH 2 and at pH 3 approximately 24% than the initial enzyme activity (Table 4.3 and Addendum B). Although the crude β-xylosidase 23B25 has 41% less activity at pH 5 and 55% less at pH 6 compared to pH 3, the enzyme was stable for up to 72 hours at these pH levels. The partially purified β-xylosidase had pH optima between pH 2 to 4 and was stable at these pH levels for 6 hours.

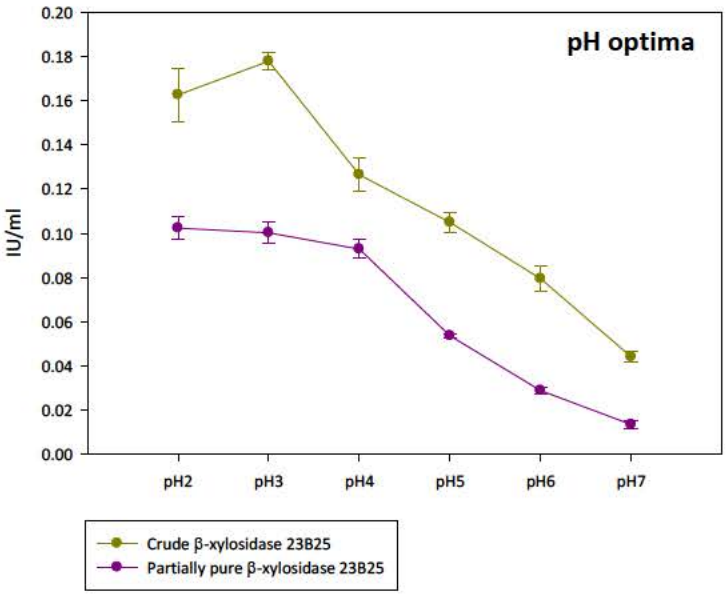

Fig. 4.8 Volumetric activity of crude and partially purified β-xylosidase 23B25 to determine the pH optima at pH 2 to 7.

Table 4.3 pH stability of the *Aureobasidium pullulans* (isolate 23B25) crude and partial purified β-xylosidase. Refer to Addendum B for graphs.

Condition	Time of stability (hours)	
	Crude	Partial purified
pH 2	6	6
pH 3	6	6
pH 4	**48**	**6**
pH 5	**72**	**6**
pH 6	72	N/A
pH 7	1	N/A

4.4.4.3 Determination of the temperature optima and stability of β-xylosidase

The temperature optima and stability was performed to determine at which lignocelluloses hydrolysis temperature the enzyme might be used. The crude β-xylosidase 23B25 had its optimal temperature between 80°C and 90°C, and the partially purified β-xylosidase had a temperature optimum at 80°C (Fig. 4.9). The crude β-xylosidase 23B25 still had activity at 40 to 70°C after 72 hours, but had no activity after 0.5 hours at 80°C (Table 4.4 or Addendum B). The partially purified β-xylosidase retained its stability after 72 hours at 30 to 60°C, but no activity after 0.5 hours at 70°C (Table 4.4 or Addendum B).

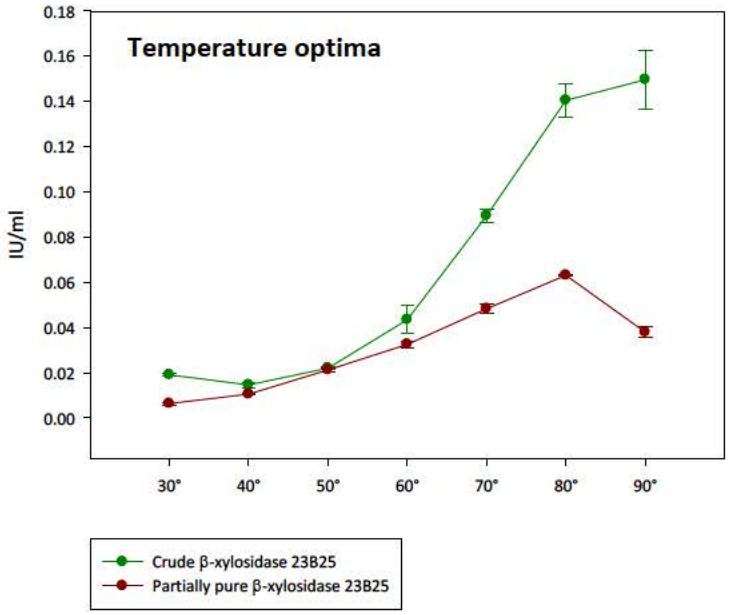

Fig. 4.9 Volumetric activity of crude and partially purified β-xylosidase 23B25 to determine the temperature optima at 30 to 90°C.

Table 4.4 Temperature stability of the *Aureobasidium pullulans* (isolate 23B25) crude and partial purified β-xylosidase. Refer to Addendum B for graphs.

Condition	Time of stability (hours)	
	Crude	Partial purified
30°C	72	72
40°C	72	72
50°C	72	72
60°C	**72**	**72**
70°C	**72**	**0.5**
80°C	0.5	0
90°C	0	N/A

4.4.4.4 Effect of glucose, xylose and ethanol on β-xylosidase activity

The effects of end-products of lignocelluloses hydrolysis and fermentation, such as glucose, xylose and ethanol, on the β-xylosidase were evaluated. The crude and partially purified β-xylosidase 23B25 was uninhibited by glucose (Fig. 4.10). A glucose concentration of 200 mM and 450 mM improved the activity of the crude β-xylosidase 23B25; increasing the activity by approximately 22% at 200 mM and approximately 21% at 450 mM. Xylose had an inhibitory effect on the crude β-xylosidase (Fig. 4.11), having 26% less activity at 10 mM xylose and 93% less activity at 450 mM xylose. The partially purified β-xylosidase 23B25 differed from the crude β-xylosidase by being uninhibited by 10 mM xylose, although at 50 mM xylose 57% activity was lost. The crude β-xylosidase had 20% less activity at 30% (v/v) ethanol and 61% less activity at 40% ethanol (Fig. 4.12). The partially purified β-xylosidase outperformed the crude β-xylosidase by losing activity starting at 20% (v/v) ethanol; having a reduction of 28% in activity.

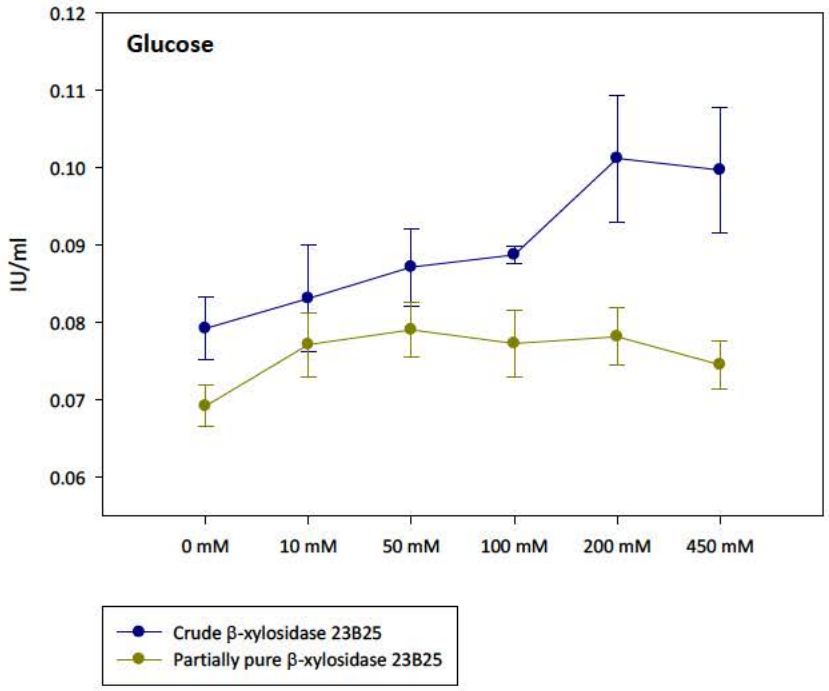

Fig. 4.10 The effect of glucose on the crude and partially purified β-xylosidase 23B25 activity.

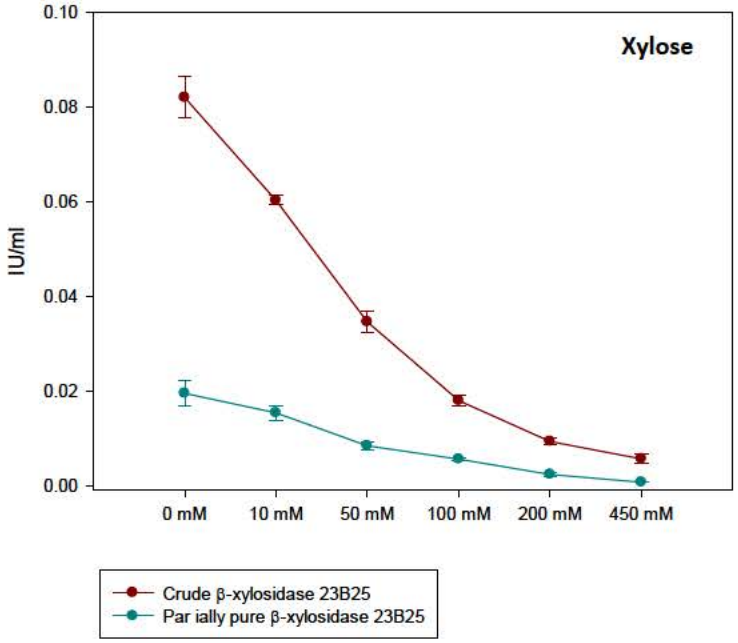

Fig. 4.11 The effect of xylose on the crude and partially purified β-xylosidase 23B25 activity.

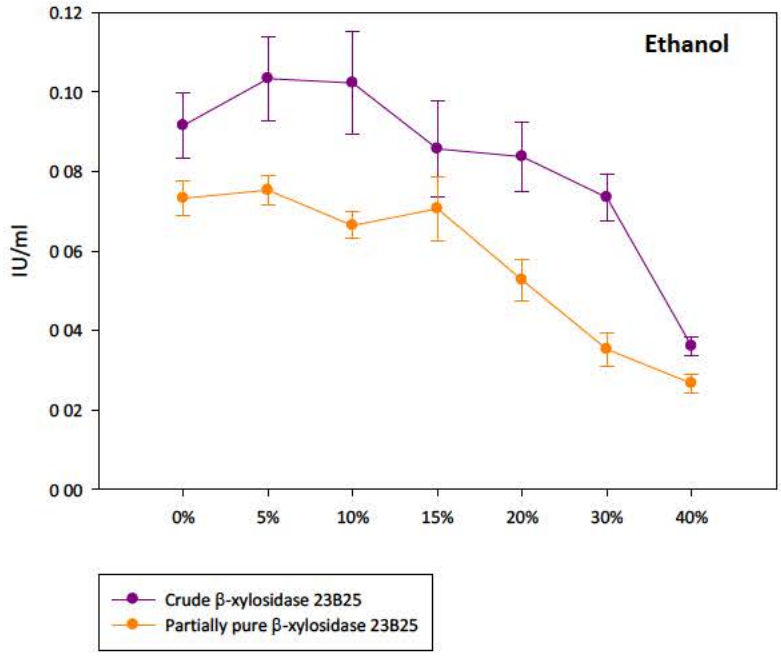

Fig. 4.12 The effect of ethanol on the crude and partially purified β-xylosidase 23B25 activity.

4.5 Discussion

A total of 2180 non-*Saccharomyces* yeast from the culture collection of the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa) were screened for β-xylosidase activity. The initial screening showed that 70 yeast isolates had xylanase activity. The 70 yeast isolates were further reduced by screening for β-xylosidase on the substrate *p*-nitrophenyl-β-D-xylopyranoside and 60 yeast strains showed to have β-xylosidase activity. Five yeasts were selected for showing equal or higher β-xylosidase activity than the positive control, *Aureobasidium pullulans* NRRL Y2311-11, for further analysis. Yeast isolates 129A12 and 130A19 was isolated from the indigenous Kei apple, isolate 23B25 and 23B24 were isolated from grapes from the Constantia vineyards and isolate 132B28 was from the indigenous stem fruit. Based on the rDNA D1/D2 domain, the yeasts were identified as *A. pullulans* (isolate 129A12, 130A19, 23B25 and 23B24) and *Hanseniaspora* sp. (isolate 132B28).

The yeast isolate displaying the highest volumetric β-xylosidase activity compared to *Aureobasidium pullulans* NRRL Y 2311-11 and the other yeast screened, was isolate 23B25. Yeast isolate 23B25 was further identified based on the ITS region and showed to have a 100% homology with *A. pullulans*. *Aureobasidium pullulans* was first described by de Bary (1866) as *Dematium pullulans*, but was later renamed when in 1910 Arnaud suggested that *D. pullulans* is conspecific with *Aureobasidium vitis* (Matsumoto *et al.*, 1987). *Aureobasidium pullulans* is a dimorphic ascomycete fungus (Karni *et al.*, 1993), which is also known as the

"black yeast" due to its melanin production (Hoog, 1993), and it was classified as a yeast (Kurtzman & Fell, 2000). *Aureobasidium pullulans* occur in three varieties which include *A. pullulans* var. *pullulans, A pullulans* var. *melanogenum* and *A. pullulans* var. *aubasidani* (Yurlova & de Hoog, 1997).

Aureobasidium pullulans appears in three morphological forms, which include the elongated branched septate filaments (hyphal formation), large chlamydospores or small elliptical yeast-like cells (Hoog, 1993). The colonies of *A. pullulans* initially appears as yellow, cream, light pink or light brown, after which it becomes black due to chlamydospore formation. It is broadly distributed in nature, where it has been isolated in soil, water, phylloplane, wood, various plants and rocks, monuments and limestone (Urzì *et al.*, 1999). The morphological form that *A. pullulans* displays are mostly dependent on the environmental conditions, particularly the carbon and nitrogen source of the culture medium (Karni *et al.*, 1993). When *A. pullulans* is in its hyphal form, it will mostly produce xylanase, glycoamylase, glucohydrolase and when in its yeast-like form it will produce pullulan, an exopolysaccharide branched polymer that consists of glucose residues (Federici & D'Elia, 1983; Leathers, 1986; Leathers, 2003). *A. pullulans* is known to be capable of producing cellulases and xylanases, lipase, amylase and mannanase, therefore its application for enzyme production is promising (Chi *et al.*, 2009).

To characterise the crude *A. pullulans* isolate 23B25 β-xylosidase, the enzyme production was optimised by selecting a carbon and nitrogen source, and the pH and temperature for culturing. The carbon source that induced the highest β-xylosidase production by the *A. pullulans* 23B25 was wheat bran and triticale bran, but triticale bran was selected due to its availability in the laboratory and as no statistical significant difference was observed between wheat bran and triticale bran. Wheat bran and triticale bran outperformed toilet paper, molasses, cheese whey and sawdust as carbon source, which could be because both has a higher cellulose and hemicelluloses content, and other components necessary for growth, than the other substrates chosen. The nitrogen source that induced the β-xylosidase activity was yeast nitrogen base, ammonium sulphate and yeast peptone, but ammonium sulphate was chosen as it is comparatively cheaper and the β-xylosidase production levels were not statistically significant between the three nitrogen sources. The pH and temperature conditions for optimal β-xylosidase production by *A. pullulans* 23B25 could not be determined and the arbitraty culturing condition of pH 4.5 and 30°C for 144 hours while shaking at 100 rpm was chosen.

It has been noted that the morphology of the *A. pullulans* has to be controlled because of its close relation to pullulan production (Catley, 1980; Heald & Kristiansen, 1985). The pH of the culture media has been reported to affect pullulan production by *Aureobasidium pullulans,* as it induces cell differentiation (Madi *et al.*, 1994). However, the relationship between the cell morphology and pH differs for each *A. pullulans* isolate, e.g. the isolate ATCC 48168 transformed from yeast-like to swollen cells at pH 2.4 and a swollen

cells developed into septate swollen cells at pH 2.1 (Park, 1984). Isolate CECT 2660 formed chlamydospores at pH 3 and had yeast-like cells when the pH was maintained at 6 (Bermejo *et al.*, 1981), and the isolate QM 3092 had yeast-like cells from pH 3 to pH 7 (Reeslev *et al.*, 1997).

To determine the effect of pH on the cell morphology and β-xylosidase production of *A. pllulans* 23B25, the yeast was cultured in buffered yeast dextrose (YD) and triticale medium. The *A. pullulans* 23B25 had hyphal growth at pH 1.5 and 2, and yeast-like cells at pH 3 to 7. β-xylosidase activity was observed at pH 2 to 7, therefore *A. pullulans* isolate 23B25 produces β-xylosidase predominantly in its yeast-like phase. This, however, is in contrast to previously reports of *A. pullulans* producing xylanases during its hyphal phase (Federici & D'Elia, 1983; Leathers, 1986; Leathers, 2003).

The crude β-xylosidase produced by the *A. pullulans* isolate 23B25 was characterised to evaluate its potential as hydrolysing enzyme for bioethanol production. Size exclusion using FPLC was also employed to partially purify the crude β-xylosidase 23B25 to evaluate whether the β-xylosidase 23B25 retains its characteristics when most of the components in the supernatant are removed. The fluorescent band/smear on the zymogram showed that the crude β-xylosidase 23B25 has a molecular mass between 150 kDa and 300 kDa. The partially purified β-xylosidase 23B25 produced a fluorescent band/smear on the zymogram with a molecular mass ranging from 180 kDa and 300 kDa. The difference in the molecular mass range of the crude and partially purified β-xylosidase 23B25 could be the result of the component in the medium influencing the migration of the protein in the zymogram and SDS-PAGE. Furthermore, the smear could be as a result of multiple β-xylosidases produced by *A. pullulans* isolate 23B25 or that the β-xylosidase is glycosylated to various degrees. The molecular mass of the *A. pullulans* isolate 23B25 β-xylosidase falls within the range of previously reported *A. pullulans* β-xylosidases, e.g. the cell-associated β-xylosidase from *A. pullulans* isolate ATCC 20524 had a molecular mass of 121 kDa (Ohta *et al.*, 2010) and the extracellular β-xylosidase from *A. pullulans* isolate CBS 58475 was 224 kDa (Dobberstein & Emeis, 1991).

The pH optima of the crude β-xylosidase 23B25 were between 2 and 3 and were stable for 6 hours at these pH levels. The enzyme was not optimal at pH 4 and pH 5, but was stable for 48 hours at pH 4 and 72 hours at pH 5. Reports has been made that the maximum β-xylosidase activity of the crude *Aureobasidium pullulans* NRRL Y 2311-1 β-xylosidase was at pH 4.5 and its pH optima ranged from 4 to 7 (Myburgh et al., 1991). The pH range of the *A. pullulans* 23B25 β-xylosidase was narrower than the *A. pullulans* NRRL Y 2311-1 β-xylosidase, but the β-xylosidase 23B25 was active at a lower pH of 2 and 3. *A. pullulans* NRRL Y 2311-1 β-xylosidase was stable for 24 hours at pH 4 and 5, but the *A. pullulans* 23B25 β-xylosidase surpassed this by being stable for 48 hours. Reports have been made that the cell-associated *A. pullulans*

isolate ATCC 20524 β-xylosidase had a pH optimum at pH 3.5 (Ohta *et al.*, 2010) and the extracellular *A. pullulans* isolate CBS 58475 β-xylosidase had a pH optimum at pH 4.5 (Dobberstein & Emeis, 1991).

The temperature optima of crude β-xylosidase 23B25 were at 80°C and 90°C, but the enzyme was only stable for 0.5 hours at 80°C and unstable at 90°C. The crude β-xylosidase 23B25, however, displayed exceptional thermal stability by being stable for 72 hours at 50, 60 and 70°C. The crude *A. pullulans* NRRL Y 2311-11 β-xylosidase had its temperature optima between 45 and 50°C (Myburgh et al., 1991). *A. pullulans* NRRL Y 2311-1 β-xylosidase was stable for only 3 hours at 30°C and lost 75% activity after 20 min incubation at 60°C. Therefore the *A. pullulans* 23B25 β-xylosidase performed superior by being stable at 72 hours at 50°C to 70°C. These high temperature optima has been reported for other *A. pullulans* β-xylosidase, such as the cell-associated *A. pullulans* isolate ATCC 20524 β-xylosidases which had a temperature optimum at 70°C (Ohta *et al.*, 2010) and the extracellular *A. pullulans* isolate CBS 58475 β-xylosidase that was optimal at 80°C (Dobberstein & Emeis, 1991).

Crude β-xylosidase 23B25 was not inhibited by glucose, but was improved by approximately 22% in the presence of 200 mM glucose and by 21% in 450 mM glucose. At the time of the study, no literature was available reporting improvement of β-xylosidase activity by glucose. Xylose inhibited the crude β-xylosidase 23B25 where approximately 26% activity was lost at 10 mM xylose. Xylose inhibition of β-xylosidases varies and can range from as low as 2 mM xylose (*Trichoderma reesei*; Herrmann *et al.* (1997)) to as high as 200 mM xylose (*Scytalidium thermophilum*; Zanoelo *et al.* (2004)). Ethanol had an effect on the β-xylosidase activity, where 20% less activity at 30% (v/v) ethanol was observed for the crude β-xylosidase 23B25. The performance of the crude β-xylosidase 23B25 in the presence of 10% (v/v) ethanol was superior to that of the *Sporotrichum thermophile* β-xylosidase which had 30% less activity at this ethanol concentration. Contrarily, an improvement of β-xylosidase activity by ethanol was observed for *Candida utilis* β-xylosidase, which had an improved activity of 57% in the presence of 10% (v/v) ethanol (Yanai & Sato, 2001) and *Pichia anomala* β-xylosidase, which had an improved activity of 55% in 15% (v/v) ethanol (Manzanares *et al.*, 1999).

The partially purified β-xylosidase had a pH optima range from pH 2 to 4 and was also only stable for 6 hours. The temperature optimum for the partially purified β-xylosidase was at 80°C, but the enzyme was not stable at this temperature. However, at 30°C to 60°C the partially purified β-xylosidase retained stability at 72 hours. Glucose had no effect on the partially purified β-xylosidase 23B25 and only at the high 50 mM xylose concentration was the enzyme inhibited, having 57% less activity. Ethanol also inhibited the partially purified β-xylosidase 23B25 by 28% at the high ethanol concentration of 20% (v/v) ethanol, but as 10% (v/v) ethanol only 9% less activity was observed. The differences observed between the crude and partially purified β-xylosidase 23B25 suggest that the components in the medium or produced by the yeast

do have and impact on the characteristics of the enzyme, which might differ when the yeast is cultured in a different medium.

4.6 Conclusion

The crude *Aureobasidium pullulans* stain 23B25 β-xylosidase showed potential as a hydrolytic enzyme for lignocellulosic bioethanol production by having an acidic pH optima of 2 and 3, and high temperature optima of 80°C and 90°C. The β-xylosidase 23B25 was stable for 72 hours at pH 5 and 6, but was stable for only 6 hours at pH 2 and 3. It showed superior stability by being stable at 72 hours at 70°C and 60°C, but was unstable at 90°C and stable for only 30 min (0.5 hours) at 80°C. The crude β-xylosidase 23B25 was uninhibited by glucose and displayed an exceptional characteristic by being improved by 200 mM and 450 mM glucose. The crude β-xylosidase 23B25 was inhibited by xylose, but only 26% less activity was observed at 10 mM xylose. Ethanol inhibited the crude β-xylosidase 23B25, but only 20% less activity was observed at a high concentration of 30 % (v/v) ethanol. An attempt will be made to clone and heterologously express the β-xylosidase 23B25 gene in *Saccharomyce cerevisiae* for improved enzyme production and enzyme characterisation (refer to Addendum C).

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion and Conclusions

5.1 General Conclusions

The realization that bioenergy, especially bioethanol, can satisfy social and energy mandates has prompted the South African government to invest more attention in establishing a biofuel production process in South Africa to minimise the current usage of fossil fuels. Initially the focus will be to establish a first-generation production process, which involve using sugarcane and sugar beet as biomass for bioethanol production (Energy., c2007 [cited 2010 Mar 12]; Funke *et al.*, 2009). Subsequently, second-generation bioethanol production, which involves the utilisation of lignocelluloses as biomass, will have to be developed. The current drawback of this process is that using lignocellulose as biomass is not commercially competitive with sugarcane or maize (Balat *et al.*, 2008).

Technologies to convert lignocellulose to its respective monomers, such as glucose and xylose, are currently inefficient; therefore various researchers have focused on finding novel enzymes and the microorganisms that produce them. Characterised crude enzymes produced by microorganisms can be used as an addition/replacement for currently commercially used enzymes, such as Celluclast (Sigma-Aldrich, Steinheim, Germany) and Novozyme 188 (Sigma-Aldrich). The genes encoding for these enzymes can be expressed heterologously to improve enzyme production, or used for the creation of a consolidated bioprocess (CBP) microorganism.

In this study, the aim was to identify non-*Saccharomyces* yeast from a culture collection of the Wine and Fermentation Technology Division (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa) that produce a β-glucosidase or β-xylosidase, to characterise the enzyme, to determine whether it has potential as a hydrolytic enzyme for bioethanol production and to possibly obtain the enzyme gene for cloning and expression in *Saccharomyces cerevisiae*.

5.1.1 Screening for a yeast producing superior β-glucosidase and β-xylosidase activity and the identification thereof

Initially, 2180 non-*Saccharomyces* yeasts from the culture collection of the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa) were screened for β-glucosidase and β-xylosidase activity. The yeasts were isolated from various fruits from different regions in South Africa. The screening for β-glucosidase activity on cellobiose as carbon source resulted in 1272 yeast with a positive β-glucosidase phenotype (i.e. growth on cellobiose), and a further screening to confirm the growth on cellobiose resulted in 261 yeasts with strong growth ability on cellobiose as the only carbon source. The

261 yeasts were further evaluated for growth on cellobiose using growth curve analysis resulting in four yeast isolates displaying superior growth kinetics on cellobiose. The four yeasts (isolates 126A6, 131B2, 45A27W and 45A27) were screened for β-glucosidase activity on the *p*-nitrophenyl-β-D-glucopyranoside substrate and one yeast was selected for displaying superior β-glucosidase activity, compared to the positive control (*Saccharomyces cerevisiae* BGL) and the other non-*Saccharomyces* yeast screened.

Yeast isolate 126A6 was originally isolated from exotic fruit, Jaboticaba (*Myrciaria* spp.), and was preliminarily identified based on the rDNA D1/D2 domain as a *Candida* sp. Yeast isolate 131B2 was isolated from an indigenous fruit, stem fruit (*Englerophytum magalismontanu*) and was identified as a *Rhodotorula* sp. Yeast isolates 45A27W and 45A27R were isolated from grapes originating from the Rustenburg vineyards and were identified as a *Candida* sp. and a *Rhodotorula* sp., respectively. The yeast isolate selected for displaying superior β-glucosidase activity was yeast isolate 131B2 and was further identified based on the ITS region and phylogenetic analysis. Using the ITS region a 99% homology was obtained with *Rhodotorula slooffiae,* but a bootstrap value of only 73.6% was obtained for the clade formed with *Rhodotorula slooffiae*. As the bootstrap value is less than 95%, the clade can not be considered as definite and the yeast isolate 131B2 was labelled as a *Rhodotorula slooffiae*-like isolate.

In search of β-xylosidase producing yeasts, the same collection of 2180 non-*Saccharomyces* yeasts was screened using the substrate Remazol Brilliant Blue-xylan, which will indicate xylanase production, e.g. endoxylanases and β-xylosidase. Seventy yeasts showed xylanase activity and were further screened for β-xylosidase activity on the substrate *p*-nitrophenyl-β-D-xylopyranoside of which 60 isolates showed β-xylosidase activity. Five yeasts (isolates 129A12, 130A19, 23B25, 23B24 and 132B28) were selected for displaying higher or equal β-xylosidase activity than the positive control, *Aureobasidium pullulans* NRRL Y 2311-1.

Yeast isolates 129A12 and 130A19 originated from indigenous Kei apple (*Dovyalis caffra)*, isolate 23B25 and 23B24 from grapes from Constantia vineyards and isolate 132B28 from indigenous stem fruit (*Englerophytum magalismontanum*). The five selected yeasts were preliminarily identified based on the rDNA D1/D2 domain as *Aureobasidium pullulans* (isolates 129A12, 130A19, 23B25 and 23B24) and *Hanseniaspora* sp. (isolate 132B28). Yeast isolate 23B25 was eventually selected for its superior β-xylosidase activity compared to *A. pullulans* NRRL Y2311-1 and the other non-*Saccharomyces* yeasts screened. Yeast isolate 23B25 was further identified based on the ITS region and a homology of 100% was obtained in the D1/D2 domain and ITS region with *Aureobasidium pullulans*.

5.1.2 Characterisation of the β-glucosidase and β-xylosidase

The β-glucosidase produced by the *Rhodotorula slooffiae*-like isolate 131B2 and the β-xylosidase produced by the *Aureobasidium pullulans* isolate 23B25 were characterised to determine whether these enzymes has

potential for hydrolysis of lignocelluloses during bioethanol production. The β-glucosidase produced by the *Rhodotorula slooffiae*-like isolate 131B2 showed novel properties compared to the commercially available Novozyme 188 β-glucosidase. The crude β-glucosidase 131B2 displayed a pH optima from 3 to 5 and temperature optima at 80°C to 90°C. The crude β-glucosidase 131B2 was stable for 72 hours at the low pH of 3 to 5 and stable for 72 hours at the high temperature of 60°C and 70°C. The commercial *Aspergillus niger* β-glucosidase preparation (Novozyme 188) was in this study only stable 1 hour at these temperatures. Furthermore, the crude β-glucosidase 131B2 also had a broader optimum pH range of 3 to 5, compared to Novozyme 188 (optimal at pH 4 to 5). The crude β-glucosidase 131B2 outperformed the Novozyme 188 by being less inhibited by glucose and ethanol. The crude β-glucosidase 131B2 was also compared to Novozyme 188 as β-glucosidase supplement to the commercial *Trichoderma reesei* cellulase preparation (Celluclast) during hydrolysis of Avicel (microcrystalline cellulose) at pH 5 and 50°C. The results showed that the two enzymes performed similarly.

The crude β-xylosidase 23B25 showed potential at a lignocellulose hydrolytic enzyme by displaying an optimum pH range between 2 and 3, and an optimum temperature range at 80°C and 90°C. The enzyme was stable at 50°C to 70°C and a pH 5 and 6 for 72 hours. The crude β-xylosidase 23B25 was inhibited by xylose but not glucose and in fact showed an improved activity at 200 mM and 450 mM glucose. Furthermore, ethanol did inhibit the crude β-xylosidase 23B25, but only at a high concentration of 30% (v/v) ethanol and higher.

Conclusively, the results obtained in this study suggest that South Africa's natural biodiversity can serve as a source for bioprospecting enzymes with biotechnological application, as 261 yeast isolates of the non-*Saccharomyces* yeast culture collection showed β-glucosidase activity and 60 yeast isolates showed β-xylosidase activity. These isolates were narrowed down to two yeast isolates, a *Rhodotorula slooffiae*-like and an *Aureobasidium pullulans,* showing respectively superior β-glucosidase and β-xylosidase production during the screening. During the characterisation of the β-glucosidase and β-xylosidase these enzymes showed amendable qualities which validate its possible use during enzymatic hydrolysis of lignocelluloses during bioethanol production and warrant further research efforts to obtain the encoding gene sequences.

APPENDIX A

Graphs for the pH and temperature stability of β-glucosidase 131B2

A.1 pH stability of β-glucosidase 131B2

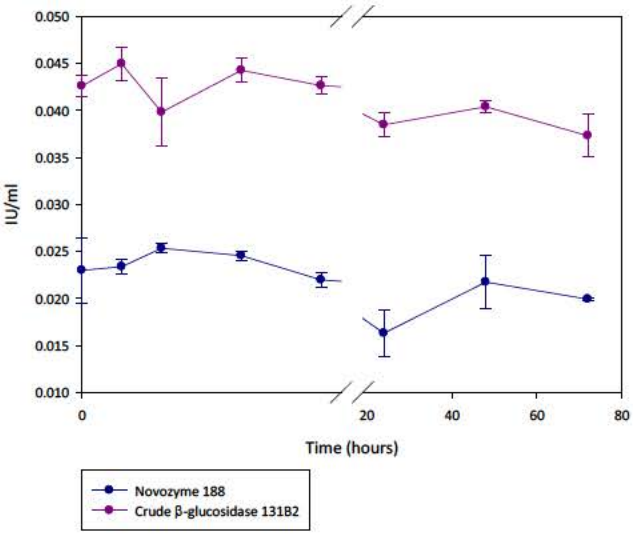

Fig. A.1.1 pH stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at pH 3 for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

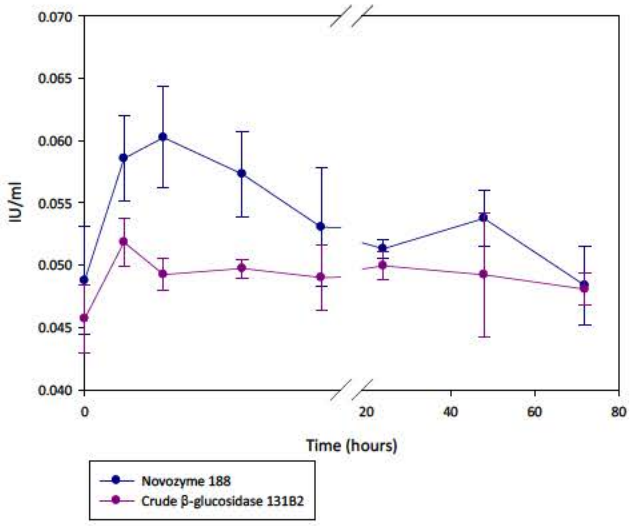

Fig. A.1.2 pH stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at pH 4 for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

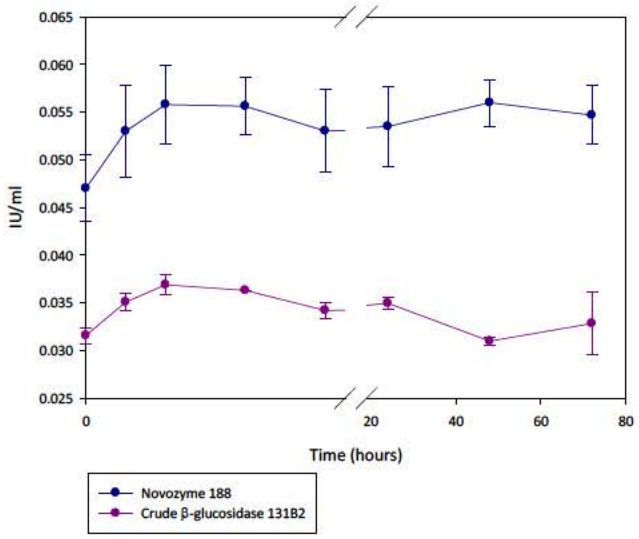

Fig. A.1.3 pH stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at pH 5 for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

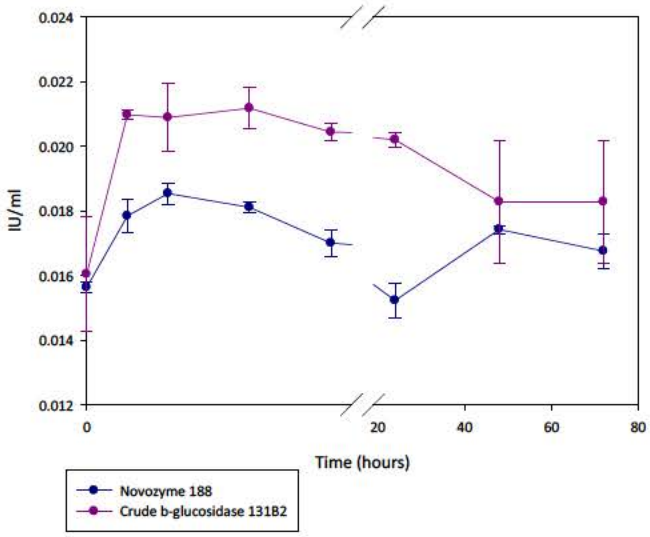

Fig. A.1.4 pH stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at pH 6 for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

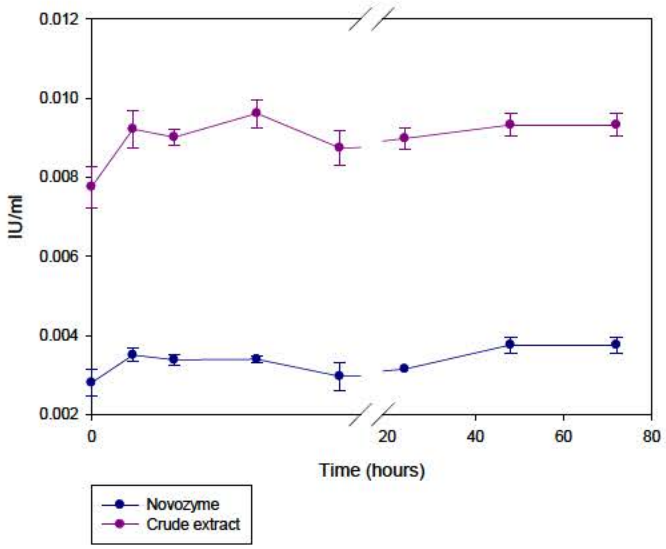

Fig. A.1.5 pH stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at pH 7 for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

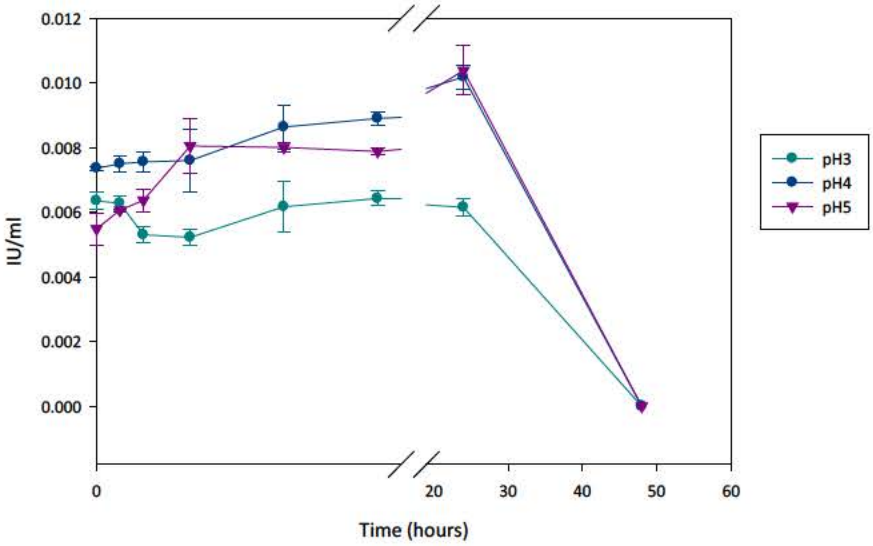

Fig. A.1.6 pH stability of the partially purified β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 at pH 3 to 5 at 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

A.2 Temperature stability of β-glucosidase 131B2

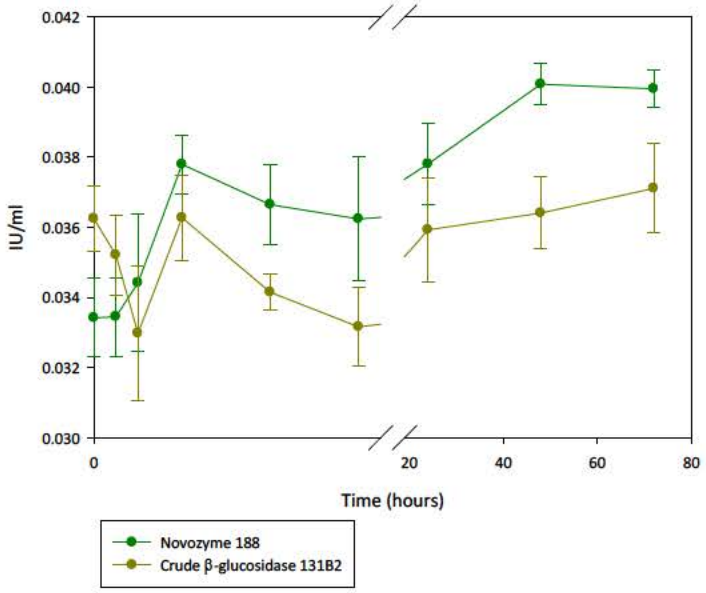

Fig. A.2.1 Temperature stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 30°C for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

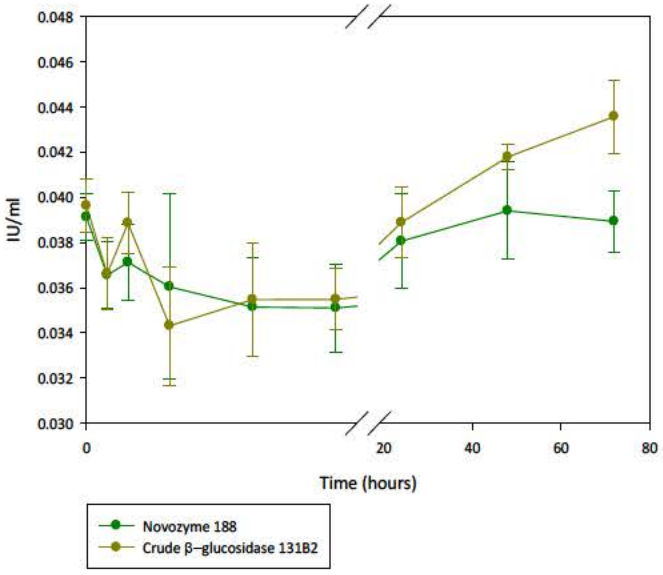

Fig. A.2.2 Temperature stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 40°C for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

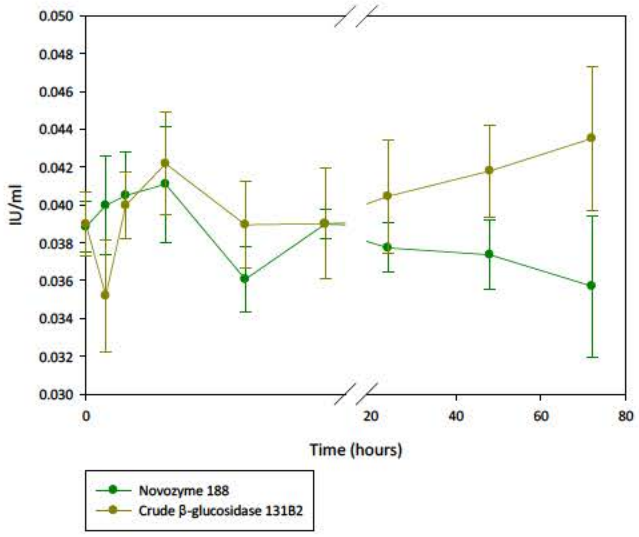

Fig. A.2.3 Temperature stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 50°C for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

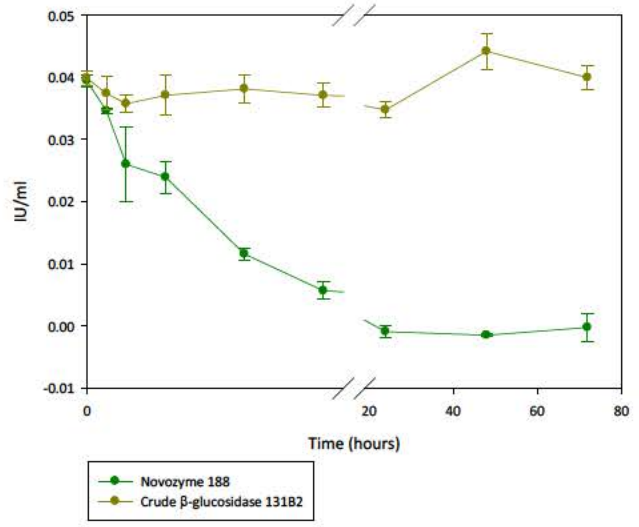

Fig. A.2.4 Temperature stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 60°C for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

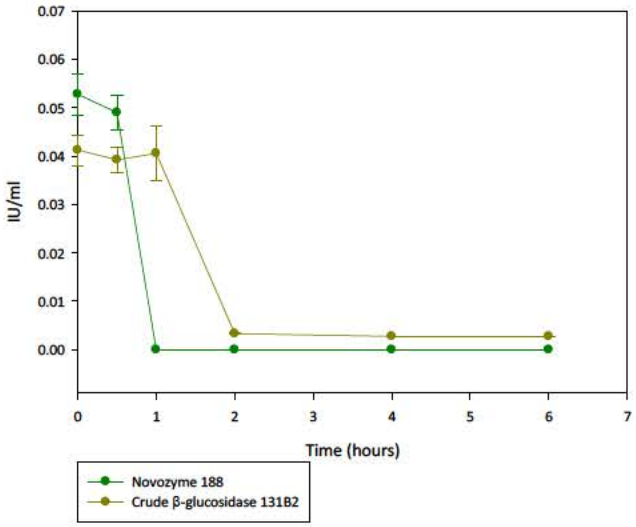

Fig. A.2.5 Temperature stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 70°C for 6 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

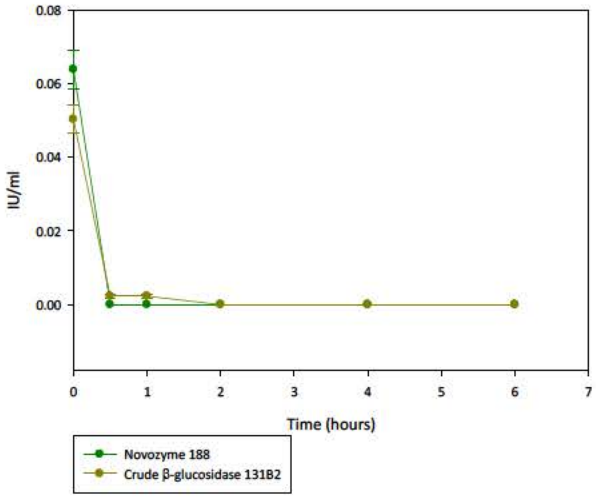

Fig. A.2.6 Temperature stability of the crude β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 and Novozyme 188, at 80°C for 6 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

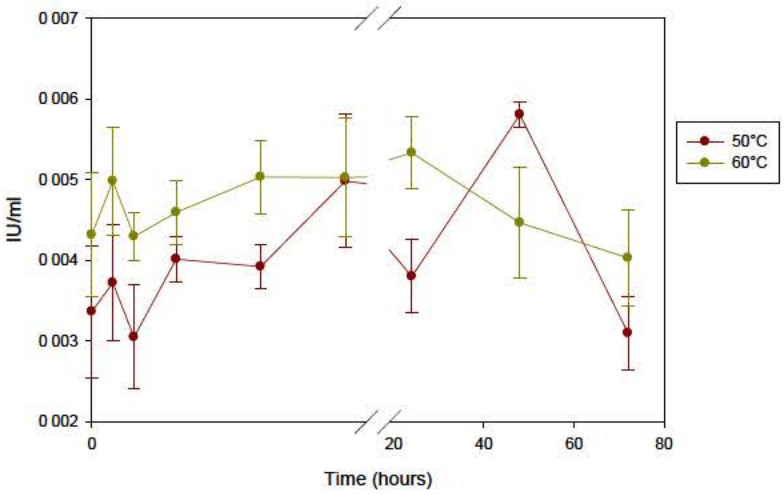

Fig. A.2.7 Temperature stability of the partially purified β-glucosidase produced by *Rhodotorula slooffiae*-like yeast isolate 131B2 at 50°C and 60°C for 72 hours. β-glucosidase activity was expressed as volumetric activity (IU/ml).

APPENDIX B

Graphs for the pH and temperature stability of β-xylosidase 23B25

B.1 pH stability of crude β-xylosidase 23B25

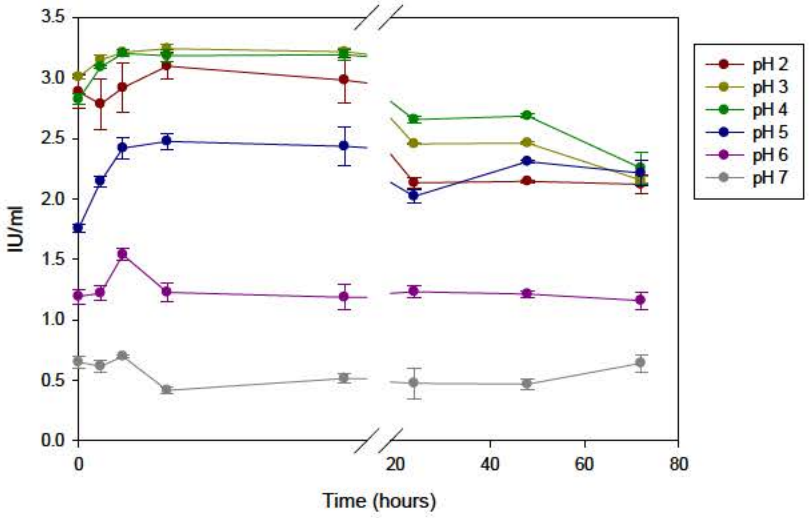

Fig. B.1.1 pH stability of the crude β-xylosidase produced by *Aureobasidium pullulans* isolate 23B25 at pH 2 to 7 for 72 hours. β-xylosidase activity was expressed as volumetric activity (IU/ml).

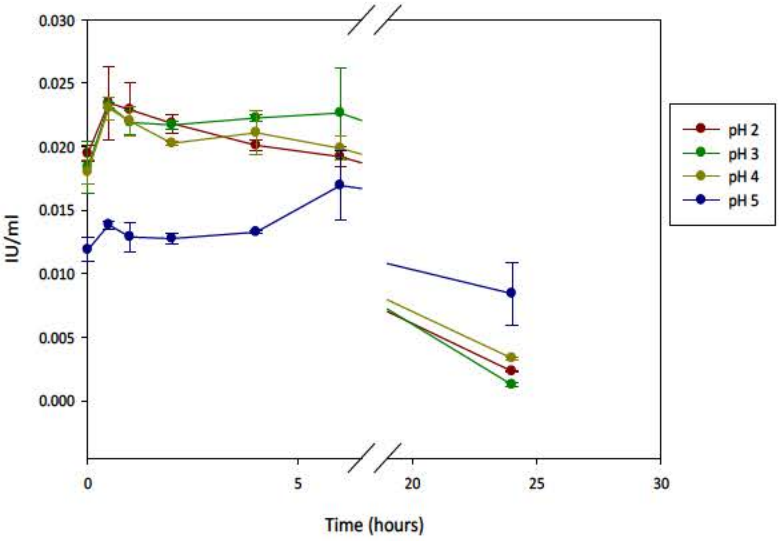

Fig. B.1.1 pH stability of the partially purified β-xylosidase produced by *Aureobasidium pullulans* isolate 23B25 at pH 2 to 5 for 24 hours. β-xylosidase activity was expressed as volumetric activity (IU/ml).

B.2 Temperature stability of β-xylosidase 23B25

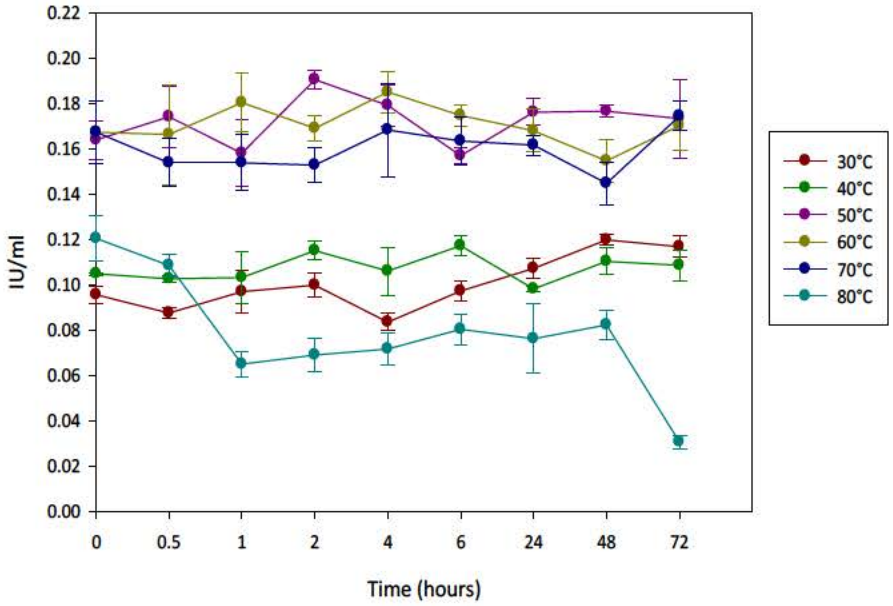

Fig. B.2.1 Temperature stability of the crude β-xylosidase produced by *Aureobasidium pullulans* isolate 23B25 at 30°C to 80°C for 72 hours. β-xylosidase activity was expressed as volumetric activity (IU/ml).

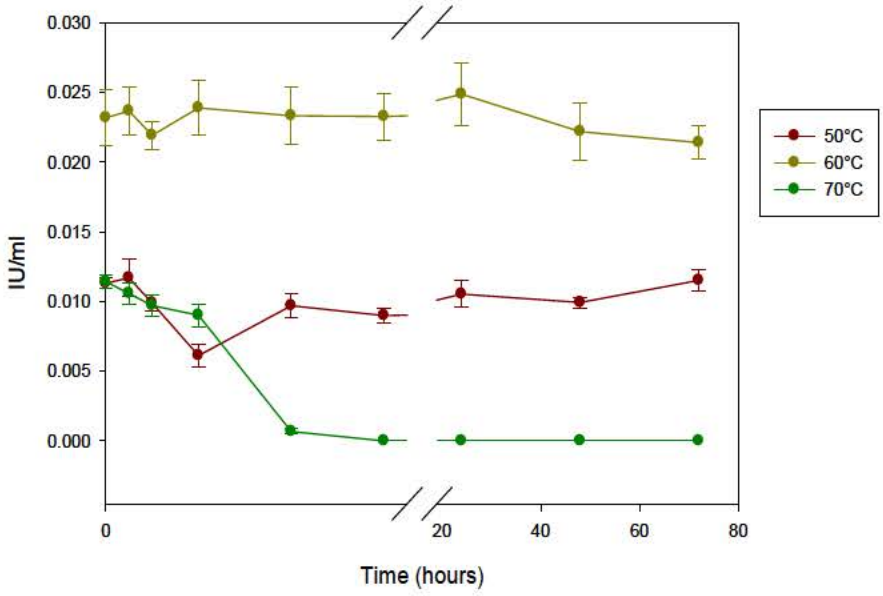

Fig. B.2.2 Temperature stability of the partially purified β-xylosidase produced by *Aureobasidium pullulans* isolate 23B25 at 50°C to 70°C for 72 hours. β-xylosidase activity was expressed as volumetric activity (IU/ml).

APPENDIX C

Cloning strategies for heterologous expression of a β-glucosidase and β-xylosidase in *Saccharomyces cerevisiae* using a PCR and cDNA library approach

C.1 Abstract

In a previous study, two non-*Saccharomyces* yeast were selected, one for β-glucosidase activity and the other for β-xylosidase activity. The yeast with β-glucosidase activity was identified as a *Rhodotorula slooffiae*-like yeast (isolate 131B2) and the yeast with β-xylosidase activity as *Aureobasidium pullulans* (isolate 23B25). Gene-specific primers were used and cDNA libraries were constructed to obtain the genes that code for these enzymes. Amplification of the genes using the designed primers were unsuccessful and during the cDNA library construction various impediments was experienced, such as a low recombination efficiency and a high small to large insert ratio, rendering this approach also unsuccessful to obtain the desired genes. However, the study was successful in reducing the number of small inserts cloned and increased the recombination efficiency.

C.2 Introduction

Bioethanol production from lignocellulosic biomass is too expensive to compete within the commercial market (Banerjee *et al.*, 2010), which employs sucrose-containing (sugarcane and sugar beet) or starch-containing (mostly corn) as feedstock. One of the proposed methods of reducing the cost of bioethanol production is to use enzymatic hydrolysis as a method to hydrolyse the polysaccharides of lignocellulose to their respective monomeric sugars. Although various enzymatic hydrolytic methods exits, consolidated bioprocessing (CBP) shows potential to be the most cost effective approach for the commercialisation of bioethanol production from lignocellulosic biomass (Cardona & Sánchez, 2007; Lynd *et al.*, 2005). It implies that a single microorganism or a consortium of microorganisms convert lignocellulose to ethanol in a single reactor. However, no microorganism naturally has all the required attributes to be a CBP microorganism. They either produce enzymes with hydrolytic activity, but does not ferment the sugar to ethanol or produce high ethanol yields, but not the necessary enzymes for cellulose hydrolysis (Lynd *et al.*, 2002; Lynd *et al.*, 2005). A solution is to genetically modify a

microorganism to have some of the desired traits. *Saccharomyces cerevisiae* is such a microorganism that can be modified as a CBP microorganism because of its robustness in industrial processes, its use in large-scale ethanol fermentation from sugarcane and starch feedstock material and its established molecular tools for genetic engineering (Hahn-Hägerdal *et al.*, 2007). *S. cerevisiae* is, however, not able to hydrolyse cellulose and hemicellulose to their respective monomers and therefore various attempts have been made to heterologously express genes encoding cellulases and hemicellulases in the yeast (Hahn-Hägerdal *et al.*, 2001).

Cellulases and hemicellulases are involved in hydrolysing the cellulose and hemicellulose fraction of lignocellulosic biomass. Cellulases hydrolyse amorphous and crystalline cellulose and include endoglucanase, cellobiohydrolase and β-glucosidase (Zhang & Lynd, 2004). Hemicellulases include endoxylanase, β-xylosidase and several accessory enzymes (debranching enzymes and esterases), such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase for hemicelluloses hydrolysis (Saha, 2003). Various cellulases and hemicellulases have been expressed in *S. cerevisiae*, for example from the soft-rot fungus, *Trichoderma reesei,* the endoglucanases (Arsdell *et al.*, 1987; Fujita *et al.*, 2002; Saloheimo *et al.*, 1997), cellobiohydrolase (Den Haan *et al.*, 2007; Penttilä *et al.*, 1988; Reinikainen *et al.*, 1992), β-glucosidase (Cummings & Fowler, 1996), xylanase (La Grange *et al.*, 1996; La Grange *et al.*, 2001) and β-xylosidase (Margolles-Clark *et al.*, 1996).

Considering the large biodiversity available, various researchers have been searching or screening for novel microorganisms displaying potentially superior lignocellulolytic activity. In the first and second part of this study wild yeasts isolated from vineyards and various fruit orchards by the Wine and Fermentation Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa), were screened for novel β-glucosidases and β-xylosidases. A wild yeast identified as a *Rhodotorula slooffiae*-like isolate was selected for displaying superior β-glucosidase activity under the selected experimental conditions (refer to chapter 3), and the wild yeast identified as *Aureobasidium pullulans* for displaying superior β-xylosidase activity (refer to chapter 4). The focus of this study was to clone and heterologously express the β-glucosidase and β-xylosidase gene in *Saccharomyces cerevisiae*.

A transcriptomic approach was taken to obtain the desired gene and involved isolating the total RNA after inducing for β-glucosidase or β-xylosidase expression, to amplify the β-glucosidase and

β-xylosidase gene using gene specific primers and to express the genes heterologously in *S. cerevisiae*. An alternative approach was the constructing of copy (c)DNA libraries, which were screened in *S. cerevisiae*. A cDNA library can be very useful when the enzyme gene's sequence is unknown to design gene-specific primers. It is also favoured over genomic libraries because the introns are removed before introducing the gene in host cells, which might be unable to process the non-coding regions accurately.

C.3 Materials and Methods

C.3.1 Yeast isolates and culture conditions

The yeast selected was *Rhodotorula slooffiae*-like yeast (isolate 131B2), displaying superior β-glucosidase activity, and *Aureobasidium pullulans* (isolate 23B25), displaying superior β-xylosidase activity. The yeast cultures were cultured on yeast peptone dextrose (YPD; Biolab, Merck, Gauteng, SA) solid medium and routinely re-cultured. Yeast cultures were grown in 5 ml YPD (Biolab) liquid medium overnight at 30°C for subsequent inoculations.

To induce the β-glucosidase and β-xylosidase enzyme activities, the respective yeast cultures were cultured for one to seven days at 30°C in 20 ml triticale media, containing 1% (m/v) triticale bran and 0.68% (m/v) yeast nitrogen base (YNB; Sigma-Aldrich, Stienheim, Germany). The triticale bran used contained 42.9% glucan, 15.2% xylan, 6.8% arabinose and 31.3% starch (analysis conducted by María del Prado Garcia Aparicio, Process Engineering, University of Stellenbosch).

C.3.2 Primer design and amplification of β-glucosidase and β-xylosidase gene

The primers (primer RhodoBGluc_F and RhodoBGluc_R) for the amplification of the *Rhodotorula slooffiae*-like yeast (isolate 131B2) β-glucosidase gene were based on the β-glucosidase sequence from the *Rhodotorula glutinis* isolate ATCC 204091 (GenBank: GL989656) available on NCBI (http://www.ncbi.nlm.nih.gov/). Control isolates included *Rhodotorula slooffiae* and *Rhodotorula glutinis* (Stellenbosch University). Sequences of the respective primers are indicated in Table C.1.

Table C.1 Primers used in polymerase chain reaction (PCR)

Enzyme	Primer	Direction	Sequence
β-glucosidase	RhodoBGluc_F	Forward	5'-GATC**TTAATTAA**ATGGCCCCCCTCACGCTCGAAGAGTTCGCA-3'
	RhodoBGluc_R	Reverse	5'-GATC**GGCGCGCC**CTACAAACCCCGCCAATACACCGTCGAGT-3'
β-xylosidase	XYL1F	Forward	5'-CTAC**AAGCTT**ATGGCTGCTCTACTTTCTCTTTC-3'
	XYL1R	Reverse	5'-GCTA**CTCGAG**CTAAGATCCAGGGGCAAGG-3'
	AbPXYL_F	Forward	5´-TGGGGCAGAGGCCAGGAGACCCCCGGTGAA-3´
	AbPXYL_R1	Reverse	5´-GGTACCGCAGTCAAGATCAGTACCAGC-3´
	AbPXYL_R2	Reverse	5´-CTGGGTGGTAGCGTTGGCCCAGCTTCCGATCAGGGC-3´

The primers for the amplification of the β-xylosidase gene were based on the cell-associated β-xylosidase from the *Aureobasidium pullulans* var. *melanigenum* isolate ATCC 20524 (GenBank: AB531130.1) (Table C.1). Protein sequence alignments were performed in CLC Genomics Workbench 4.7.1 (CLC Bio, Aarhus, Denmark) of eukaryotic β-xylosidases available on the NCBI database and regions of homology were selected to design an additional set of gene specific primers (AbPXYL_F, AbPXYL_R1 and AbPXYL_R2) (Fig. C.1 and Table C.1). *Aureobasidium pullulans* NRRL Y2311-1 served as the positive control isolate.

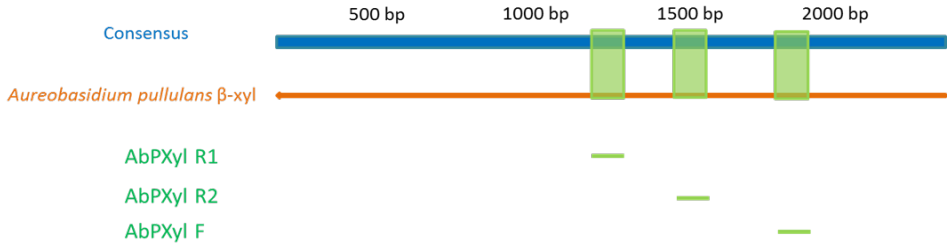

Fig. C.1 Diagram illustrating the design of the primers for the *Aureobasidium pullulans* isolate 23B25 based on the protein sequences similarities of eukaryotic β-xylosidases on the NCBI (June 2010).

Genomic (g)DNA and cDNA was used as template during the polymerase chain reaction (PCR). gDNA was prepared using the protocol described by Ausubel *et al.* (1994) and cDNA was prepared as prescribed in the RevertAid™ first-strand cDNA synthesis kit (Fermentas, Lithuania). Amplifications were performed using the Kapa2G™ Robust PCR kit (PeqLab, Erlangen, Germany) and the cycling conditions were 95°C for 10 minutes for pre-denaturation, 30 cycles of 95°C for 30 seconds denaturation, 50°C to 60°C for 30 seconds for annealing, 72°C for 2 minutes for extention and a final extension step of 72°C for 30 seconds.

C.3.3 Extraction of total RNA

The yeast were cultured in 20 ml triticale medium and harvested at 5000 rpm for 5 min followed by a wash with 10 ml ice cold diethylpyrocarbonate (DEPC)-treated MilliQ water. Five hundred microliters of extraction buffer (1 mM EDTA, 100 mM LiCl, 100 mM Tris-HCl), 500 µl acid-washed glass beads and 500 µl phenol: chloroform: isoamyl alcohol (PCI, pH 8.0, 25:24:1 by vol.; Sigma-Aldrich) were added to the cell pellet. The cells were disrupted by subjecting the mixture to a Fastprep dissicator (BIO 101) for 20 sec at 6m/sec, cooling the cells for one minute on ice. This procedure was performed twice. The broken cells were centrifuged for 9000 rpm for 10 min and the supernatant were transferred to a clean eppendorf tube containing 500 µl PCI. The excess protein was removed by repeatedly washing the supernatant with 500 µl PCI until a clean interface was visible. The nucleic acids were precipitated with potassium acetate (40% m/v; pH 5.5) and 2 volumes of 100% ethanol and the mixture were incubated for 30 min at -20°C prior to centrifugation. Following the washing of the pellet with 70% ethanol, the nucleic acids were re-dissolved in 50 µl DEPC-treated MilliQ water. The total RNA concentration was estimated by spectrophotometry using the NanoDrop ND-1000 Spectrometer (NanoDrop, Wilmington, DE, USA) and the total RNA quality was estimated by gel electrophoresis by running samples on 1% SeaKem® LE Agarose (Lonza, Rockland, USA) with ethidium bromide (EtdBr).

C.3.4 Preparation of shuttle plasmid/vector and cloning of cDNA library

The plasmids used were pYeSfiI-GAL1 (Bailly *et al.*, 2007), pYeSfi-PGK1 or pDLG125PacIAscI-Eno1 (obtained from Danie C. la Grange, Department of Microbiology, University of Stellenbosch) (Fig. C.2). The plasmid was extracted using the method prescribed by Del Sal *et al.* (1988) and purified using the DNA Clean and Concentrator™-5 kit (Zymo Research Inc., Orange, California, US). The plasmid was digested with the appropriate enzymes and purified using the High Pure PCR Purification kit (Roche, Mannheim, Germany). The digested plasmid was dephosporylated using the FastAP™ Thermosensitive Alkaline Phosphatase (MBI Fermentas) to prevent the re-ligation of plasmid and its concentration was estimated by spectrophotometry using the NanoDrop ND-1000 Spectrometer (NanoDrop).

The plasmid was evaluated for incomplete digestion or religation, by ligating 1 µg of the digested plasmid using the T4 DNA ligase (MBI Fermentas) and incubating the reaction at 16°C overnight in the thermal cycler. The reaction was transformed into electrocompetent One Shot MAX Efficiency *E. coli* DH10B'™T1R cells (Invitrogen) and plated out onto selective LB-ampicillin solid medium.

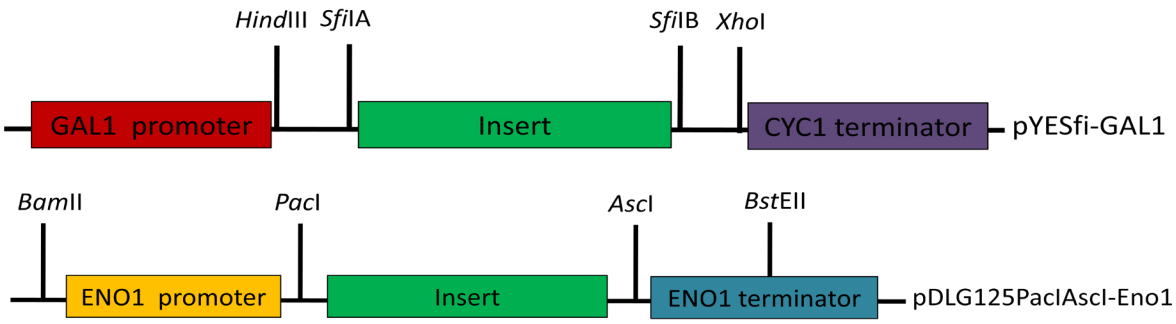

Fig. C.2 Diagram illustrating the design of the primers and the relevant restriction sites used during the cDNA library construction.

C.3.5 Construction of cDNA library

C.3.5.1 Synthesis of cDNA library

Prior to the construction of the cDNA, the residual DNA was removed from the total RNA by following the method prescribed by the Turbo DNA-*free*™ DNase Treatment and Removal kit (Ambion Inc., Austin, TX, USA). The cDNA library was constructed by using the DNase-treated total RNA and following the manufacturer's recommendations of the Creator™ SMART™ cDNA Library construction kit (Clontech, USA). The cDNA library synthesis was based on the method prescribed by Bailly *et al.* (2007).

The first-strand of the cDNA was synthesized by using a modified poly-dT oligonucleotide that anneals to the 3'-poly(A) tail of the mRNA. The Prime Script RTase (Takara Bio Inc., Ohtsu, Japan), a MMLV reverse transcriptase, was used as prescribed by the kit. The second-strand was synthesized by amplifying the different first-strand cDNA with polymerase chain reaction (PCR), between 18 and 36 cycles, using a primer (CDS III/3' PCR Primer) that binds to the modified poly-dT oligonucleotide. As the reverse transcriptase reaches the 5'-end of the mRNA, an additional oligo(C) was added to the 3'-end of the cDNA to which the SMART IV Oligo, with an olig(G) at the 3'-end, binds. The reverse transcriptase replicates the additional SMART IV Oligo to create a complete template. Single-stranded (ss) cDNA can only be amplified in the subsequent step with the universal primers (5'-end PCR primer) if it contains the SMART IV Oligo end. Assymetrical *Sfi*I restriction enzyme sites were also incorporated at the 5'- and 3'-ends for directional cloning of the cDNA into the corresponding sites of the plasmid (pYeSfi-GAL1). Additional primers were also designed to produce the restriction sites *Pac*I and *Asc*I at the 5'- and 3'-end of the cDNA for directional cloning into plasmid pDLG125PacIAscI-Eno1.

The second-strand cDNA was synthesised using the Advantage 2 PCR Polymerase (Clontech, USA). The Advantage 2 PCR reactions were carried out in a final volume of 100 µl, containing 2 µl of the first-strand cDNA, 2 µl of each oligonucleotide primer (12 µM), 10 µl 10x Advantage 2 PCR buffer, 2 µl of 50x dNTP mix and 2 µl 50x Advantage 2 polymerase mix. The PCR amplification conditions was 95°C for 1 min, followed by cycles of 95°C for 15 sec and 68°C for 4 min. The cycles were optimised by removing 80 µl of the reaction after 24 cycles and storing it at 4°C. The remaining 20 µl was returned to the thermal cycler and after every 2 cycles 5 µl was removed. Each 5µl-sample was run on 1% SeaKem® LE Agarose and visualised with GR Green Nucleic Acid Stain (Biolabo, Switzerland) alongside a DNA molecular size marker. The optimal number of cycles was determined by visually establishing the number of cycles necessary to produce sufficient cDNA without artefacts. The remaining 80 µl of PCR reaction was returned to the thermal cycler and subjected to the additional number of cycles. The produced cDNA was subsequently digested with restriction enzymes *Sfi*I (MBI Fermentas, Lithuania) or *Pac*I and *Asc*I (New England Biolab, Beverly, MA).

Table C.2 Primers used in polymerase chain reaction (PCR)

Restriction sites	**Application**	**Primer**	**Sequence**
*Sfi*I	First-strand cDNA synthesis	SMART IV Oligonucleotide	5’-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3’
		CD III/3’ PCR primer	5’-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}N_{-1}N$-3’ (N = A, G, C, or T; N_{-1} = A, G, or C)
	Second-strand cDNA synthesis	5’ PCR Primer	5’-AAGCAGTGGTATCAACGCAGAGT-3’
*Pac*I *and Asc*I	First-strand cDNA synthesis	SMART IV Oligonucleotide	5’-AAGCAGTGGTATCAACGCAGAGTTTAATTAAGGG -3’
		CD III/3’ PCR primer	5'-ATTCTAGAGGCGCGCCGACATG-d(T)30N_{-1}N-3' (N = A, G, C, or T; N_{-1} = A, G, or C)
	Second-strand cDNA synthesis	5’ PCR Primer	5’-AAGCAGTGGTATCAACGCAGAGT-3’

C.3.5.2 Size fractionation of cDNA library

Size fractionation of the cDNA was performed with three different methods: (1) CHROMA SPIN-400 Column provided by the Creator™ SMART™ cDNA Library construction kit (Clontech), (2) a sucrose gradient method from Current Protocols (Quertermous, 2001) with the exception of performed the fractionation step by piercing the bottom of the centrifuge tube (Beckman Instruments, Fullerton, CA) and dripping 250 µl into a 1.5 ml eppendorf tube at a time, and (3) the Agarose Gel PCR Purification kit (Roche Diagnostics, Mannheim, Germany) as prescribed by the manufacturer.

The cDNA fractions and plasmid were cleaned from residual salt using the DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA) and ligated using T4 DNA ligase (MBI Fermentas) at 16°C overnight in the thermal cycler.

C.3.5.3 Amplification of cDNA library

The cDNA library were introduced into electrocompetent One Shot MAX Efficiency *E. coli* DH10B'™T1R cells (Invitrogen) as prescribed by the manufacturer. Prior to the amplification of the cDNA library, the library titer in each transformation were determined as prescribed by the manufacturer of the Creator™ SMART™ cDNA Library construction kit (Clontech). The percentage recombinant clones were also determined by selecting colonies, and performing a miniprep (Del Sal *et al.*, 1988) and restriction enzyme digest of the plasmid with *Xho*I and *Hind*III for pYeSfi-GAL1, and *Bst*EII and *Bam*II for pDLG125PacIAscI-Eno1. The percentage of inserts > 500 bp were also determined as this would be an indicator whether the size fractionation was successful; less than 50% small inserts are desired.

Amplification of the transformed *E. coli* were performed as prescribed by the Creator™ SMART™ cDNA Library construction kit (Clontech). An aliquate of the amplified cDNA library were cultured in selective LB-ampicillin medium and a plasmid extraction was performed with the Qiafilter plasmid maxi kit (Qiagen, Courtaboeuf, France). The plasmid concentration was estimated by spectrophotometry using the NanoDrop ND-1000 Spectrometer (NanoDrop)

C.3.6 Transformation of cDNA library into *Saccharomyces cerevisiae*

The haploid *S. cerevisiae* isolate Y294 (*MATα, leu2-3,112, ura3-52, his3, trp1-289*; ATCC 201160) was used to screen the cDNA library. The yeast cultivation and transformation by electroporation was done as described by Benatuil *et al.* (2010): 4 µg of plasmid-insert DNA was added to 400 µl electroporation reaction and transfering the DNA/cell mixture to a pre-chilled electroporation cuvette (0.2 cm electrode gap). The cells were electroporated at 2.5 kV, 200 Ω and 25 µF, transfered to 2.6 ml of 1:1 mix of 1 M sorbitol : Yeast Peptone Dextrose (YPD) broth and incubated for 1 h at 30°C. To determine the dilution necessary for optimal cell growth and screening of the cDNA library, a dilution series (10X, 20X, 50X, 100X and 200X) was performed.

C.3.7 Screening of cDNA library

The screening strategy for the β-glucosidase cDNA library cloned into the pYeSfi-GAL1 plasmid involved culturing the library on 0.68% Yeast Nitrogen Base (YNB) media, supplemented with auxotrophic requirements and 2% glucose for four days followed by replica-plating the library on 2% galactose for induction. Each plate was pooled together and grown again in a 96-deep-well plate with each well containing 2% galactose liquid medium. The cells were pelleted and the supernatant evaluated on *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma-Aldrich) for β-glucosidase activity (described in section 3.3.2). If activity was observed, the cells would be re-plated on 2% galactose solid medium and each colony selected for screening on *p*NPG (Sigma-Aldrich) as substrate. The β-glucosidase cDNA library cloned into the pDLG125PacIAscI-ENO1 plasmid were screened on solid medium containing 0.68% Yeast Nitrogen Base (YNB) media, 2.0% agar (Agar Noble, Difco), 0.2% glucose, supplemented with auxotrophic requirements and cellobiose (Sigma-Aldrich, Stienheim, Germany).

The β-xylosidase cDNA library cloned into the pYeSfi-GAL1 and pDLG125PacIAscI-ENO1 plasmid were screened for activity on solid medium containing 0.68% Yeast Nitrogen Base (YNB) media, 2.0% agar (Agar Noble, Difco), 0.2% glucose, supplemented with auxotrophic requirements and 0.005% 5-bromo-4-chloro-3-indolyl-β-D-xylopyranoside (X-Xyl; Sigma-Aldrich, Stienheim, Germany). When the substrate X-Xyl is hydrolysed, the 5-bromo-4-chloro-3-indolyl dye is released and a blue colony is observed.

C.4 Results and Discussion

C.4.1 Amplification of β-glucosidase and β-xylosidase genes with gene-specific primers

The primers used during the amplification of the *Rhodotorula slooffiae*-like 131B2 β-glucosidase gene was based on the sequence of the *Rhodotorula glutinis* ATCC 204091 β-glucosidase available on NCBI (ftp://ncbi.nlm.nih.gov). The open reading frame (ORF) of the β-glucosidase gene of *Rhodotorula glutinis* ATCC 204091 was approximately 3466 bp with 12 introns and a fragment size of approximately 2607 bp was expected for the cDNA. The amplification of the β-glucosidase 131B2 gene from the gDNA and cDNA using the gene-specific primers was, however, unsuccessful. This could be the result of gene variation between species.

The ORF of the cell-associated β-xylosidase gene for the *Aureobasidium pullulans* ATCC 20524 was approximately 2415 bp, interrupted by two introns of 54 and 52 bp, and the fragment size expected for the amplification of the *Aureobasidium pullulans* 23B25 cDNA was approximately 2309 bp using the XYL1 forward and reverse primers. Amplification of the β-xylosidase gene for the *Aureobasidium pullulans*

23B25 and *Aureobasidium pullulans* NRRL Y2311-1 (positive control) was unsuccessful using the XYL primer set (Fig. C.2), which could be the result of gene variation between isolates.

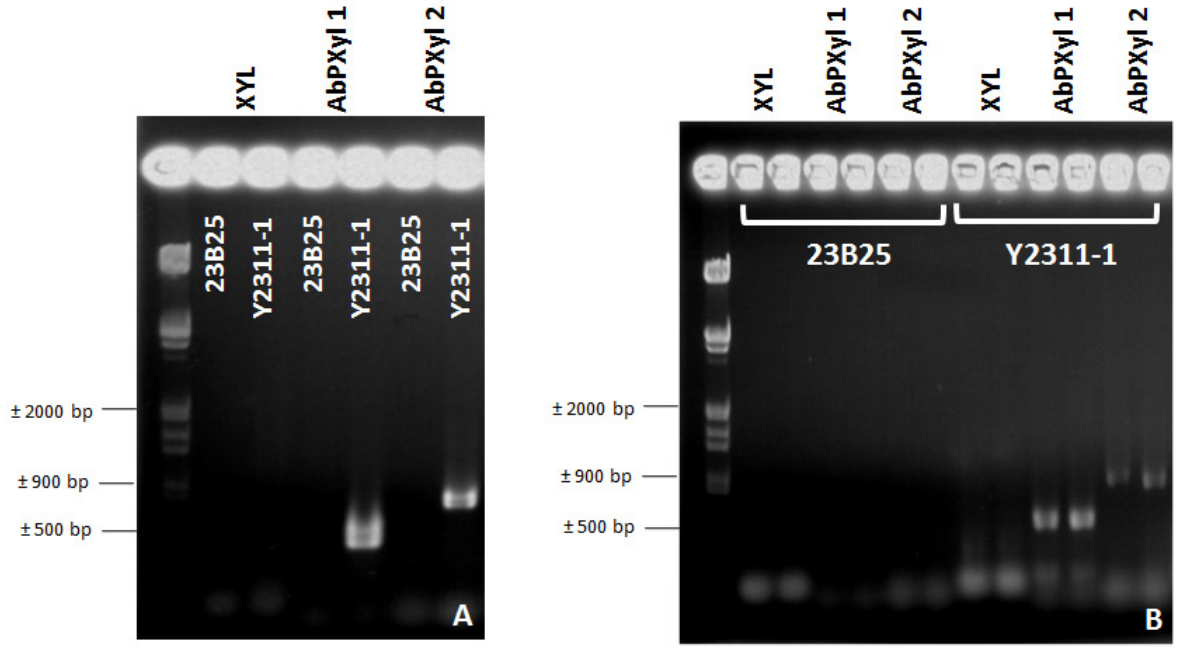

Fig C.3 (A) Amplification of gDNA isolated from *Aureobasidium pullulans* isolate 23B25 and *Aureobasidium pullulans* NRRL Y2311-1 (positive control) (B) Amplification of cDNA from *Aureobasidium pullulans* isolate 23B25 and *Aureobasidium pullulans* NRRL Y2311-1 (positive control). The primers sets used were XYL (XYL1F and XYLR), AbPXyl 1 (AbPXyl_F and AbPXyl_R1) and AbPXyl 2 (AbPXyl_F and AbPXyl_R1). Lane M: Lambda DNA Eco RI/ Hind III digest (MBI Fermentas).

The fragment size expected for the AbPXyl primer set 1 (AbPXyl_F and AbPXyl_R1) and AbPXyl primer set 2 (AbPXyl_F and AbPXyl_R2) was approximately 700 bp and 500 bp, respectively. An amplified product was obtained for the gDNA and cDNA of *Aureobasidium pullulans* NRRL Y2311-1 (positive controle), but not for the *Aureobasidium pullulans* isolate 23B25 (Fig. C.3 (B)). Amplification of the β-xylosidase of the *Aureobasidium pullulans* NRRL Y2311-1 and not of the *Aureobasidium pullulans* isolate 23B25 could also be due to gene variation between isolates.

C.4.2 Construction of cDNA libraries

The isolation of the total RNA was of sufficient quality as the 28S and 18S rRNA were intact (Life technologies, 2012) (Fig C.4 (A)). To prevent possible RNA degradation, extraction of the messenger (m) RNA prior to first-strand synthesis was omitted and SUPERase In™ RNase Inhibitor was added during the total RNA extraction, first-strand synthesis and double strand synthesis of the cDNA. The quality of total RNA generated was also evaluated by gel electrophoresis on 1% SeaKem® LE Agarose (Lonza) with ethidium bromide (EtBr) (Fig C.4 (A)).

The Creator™ SMART™ cDNA Library construction kit manual had an example of good quality ds cDNA, which was generated from human placenta poly(A) RNA. The manual considered ds cDNA smaller than 4000 bp as the cause of undercycling and ds cDNA less than 100 bp as non-cDNA contaminant (primer dimers, SMART oligonucleotides and unincorporated primers). Bearing this in mind, ds cDNA smaller than 4000 bp and larger than 100 bp were selected such as in Fig C.4 (B), Lane 4.

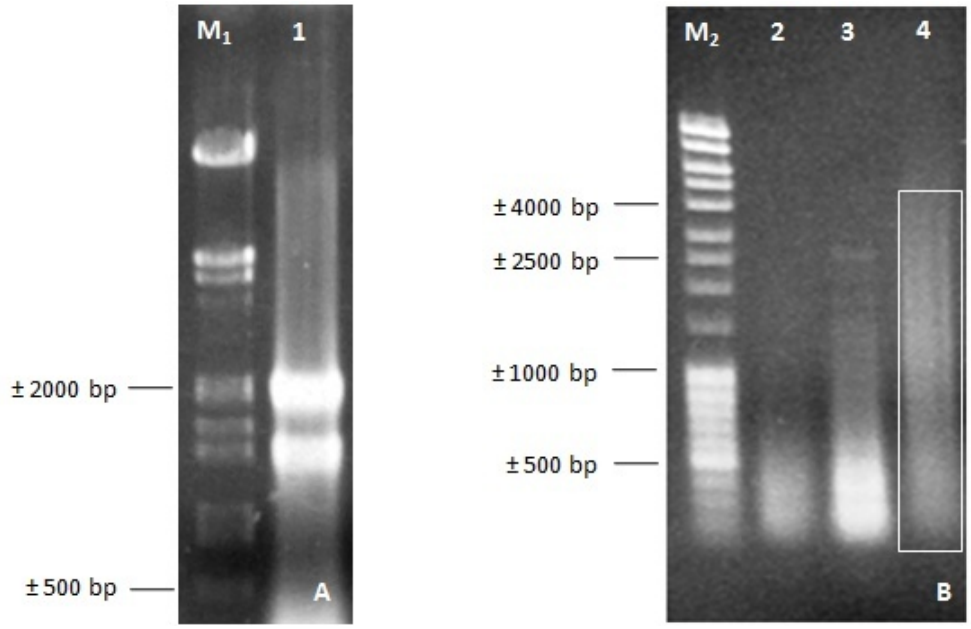

Fig C.4 (A, Lane 1) Total RNA on 1% SeaKem® LE Agarose (Lonza) stained with ethidium bromide (B) ds cDNA generated of (Lane 2) cycle 26, (Lane 3) 28 and (Lane 4) 30 stained with GR Green Nucleic Acid Stain (Biolabo). M_1: Lambda DNA *Eco* RI/ *Hind* III digest (MBI Fermentas); M_2: MassRuler™ DNA Ladder Mix (MBI Fermentas). The white block indicates the ds cDNA selected.

During the construction of the cDNA library using the Creator™ SMART™ cDNA Library construction kit (Clontech, USA), various technical problems were encountered such as the screening of the β-glucosidase cDNA library using the pYeSfi-GAL1 plasmid and predominantly small inserts (< 500 bp) cloned into the plasmid. During the screening of the β-glucosidase cDNA library, substrates such as Esculin (Esculin → Esculetin and Glucose), 4-methylumbelliferyl-β-D-glucopyranoside (MUG) (MUG → 4-MU and Glucopyranoside) and cellobiose (Cellobiose → Glucose and Glucose) were used. These substrates produce glucose as an end-product when hydrolysed by β-glucosidase. The GAL1 promoter, however, is inhibited by glucose, which complicates the screening of β-glucosidase cDNA libraries. pYeSfi-GAL1 plasmid was replaced by the pDLG125PacIAscI-ENO1 plasmid, which has the constitutive ENO1 promoter that is not inhibited by glucose.

Small cDNA fragments, primers and primer dimers are usually preferentially ligated into the plasmid and should be removed before cDNA-plasmid ligation. When the cDNA library has a higher smaller:larger insert ratio, the cause could be either poor quality double stranded (ds) cDNA produced or insufficient size

fractionation. The CHROMA SPIN-400 Column drip procedure was used for size fractionation as prescribed by the Creator™ SMART™ cDNA Library construction kit (Clontech). However, after evaluating the fractions 1.1% agarose/EtBr gel, inefficient size fractionation was observed as each fraction predominately contained smaller cDNA. The CHROMA SPIN-400 Column was replaced by the sucrose gradient method from Current Protocols (Quertermous, 2001). Fractions between approximately 800 bp and 2000 bp were selected (Fig. C.5). The cDNA fractions were cloned into pYesSfiI-GAL1 vector and introduced into electrocompetent One Shot MAX Efficiency *E. coli* DH10B'™T1R cells. However, insert confirmation showed that the cDNA library contained predominantly small insert (< 50% inserts smaller than 500bp) (Fig. C.6). An agarose gel purification was subsequently chosen for size fractionation, which produced a β-xylosidase cDNA library with 89% inserts larger than 500 bp (Fig. C.7). However, only 8% of the inserts were larger than 2300 bp and the expected size of the β-xylosidase gene of *Aureobasidium pullulans* 23B25 is about 2300 bp which reduces the probability of obtaining the gene.

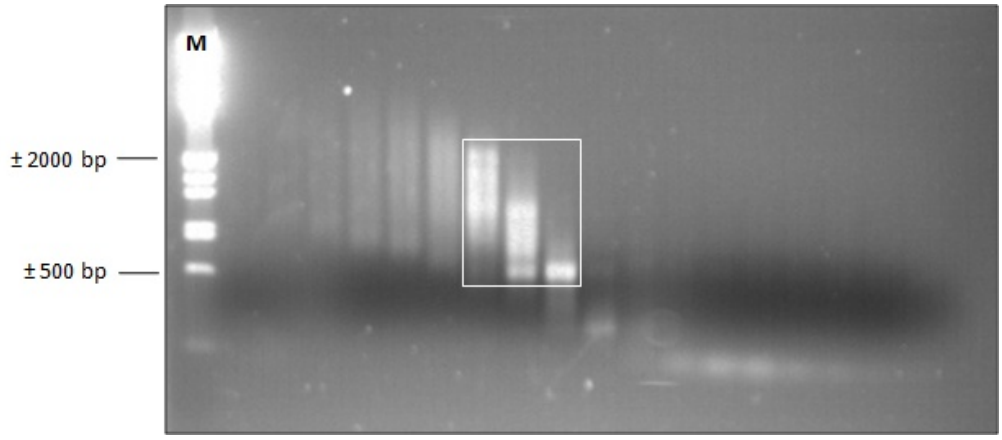

Fig. C.5 Size fractionation of β-glucosidase cDNA library of the *Rhodotorula slooffiae*-like yeast isolate 131B2 using the sucrose gradient method. M: Lambda DNA Eco RI/ Hind III digest (MBI Fermentas). The white block indicates the ds cDNA selected.

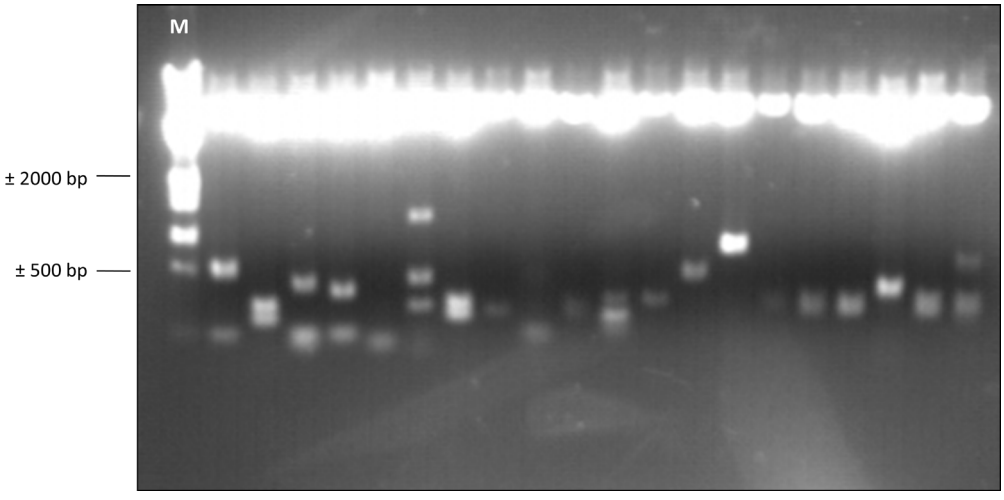

Fig C.6 Insert confirmation for β-glucosidase cDNA library of the *Rhodotorula slooffiae*-like yeast isolate 131B2 under the GAL1 promoter. M: Lambda DNA Eco RI/ Hind III digest (MBI Fermentas).

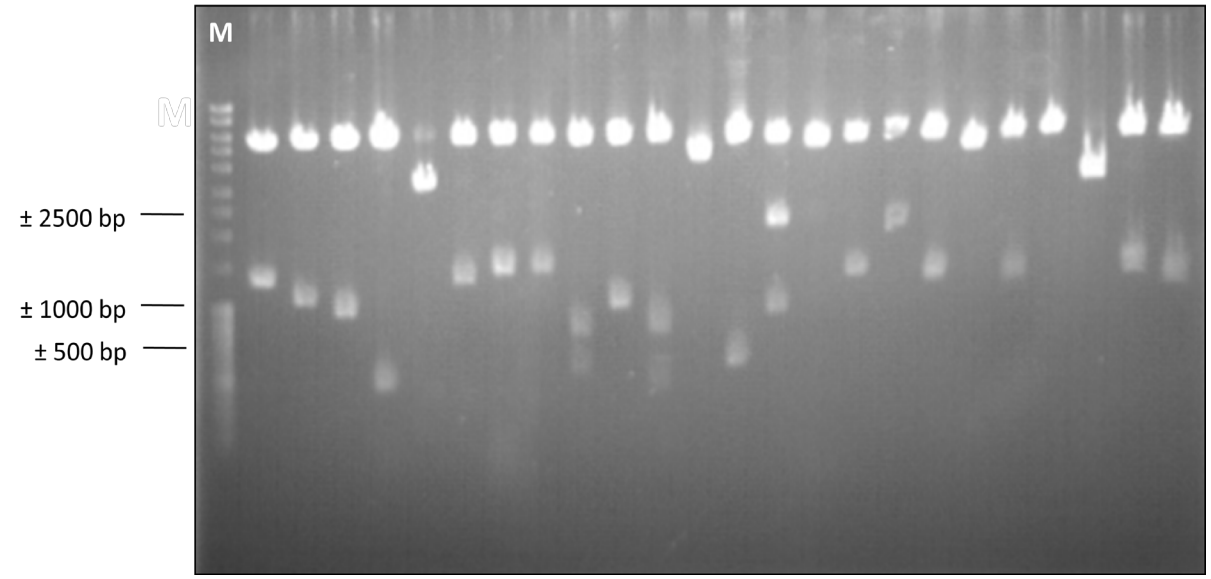

Fig C.7 The insert confirmation of β-xylosidase cDNA library under the ENO1 promoter of the *Aureobasidium pullulans* isolate 23B25. M: MassRuler™ DNA Ladder Mix (MBI Fermentas).

During the screening of the cDNA libraries in Fig C.6 and Fig C.7 no enzyme activity was observed. The reasons for no enzyme activity could be that a cDNA copy of the enzyme was not present or that problems arose for the *Saccharomyces cerevisiae* Y294 isolate during transcription of the foreign gene. The foreign protein might be unstable resulting in no enzyme activity detection during the screening the cDNA library. The normal functioning of the foreign protein can be influenced by hyper-glycosylation and the expression of the foreign gene at a high level might create a metabolic burden on the host cell which could affect its growth rate or its efficiency in expressing the gene (Romanos *et al.*, 1992). Factors such as the molecular mass of the foreign protein, glycosylation, and the charge of the protein might influence the transport of the foreign proteins across the cell wall. The host proteins which are involved in folding and disulphide bond formation might differ from the organism from which the foreign gene has its origin and may negatively affect the folding of the foreign protein. If malfolding of the foreign protein occurs it can be retained in the endoplasmic reticulum (ER) and degrade. Foreign proteins which are retained results in intracellular accumulation of proteins within the secretory pathway which might create a blockage or bottleneck within the secretory pathway and interfere with the secretion of the host proteins. For instance, within *Saccharomyces cerevisiae* folding of secreted proteins take place within the ER and requires accessory proteins, such as BiP and the disulphide isomerase protein (Freedman, 1989). BiP bind to proteins co-translocationally, but is released when the protein is folded; however, when malfolding takes place the protein binds permanently to BiP and is retained within the ER (Romanos *et al.*, 1992).

C.5 Conclusions

In this study, the β-glucosidase and β-xylosidase genes were not obtained using the gene-specific primers which might be due to gene variation between species. The constructing of cDNA libraries was also unsuccessful to obtain the β-glucosidase and β-xylosidase genes due to inefficient size fractionation of the cDNA. However, during this study the cDNA library strategy was improved by increasing the percentage large inserts cloned from 40% using the CHROMA SPIN-400 Column drip procedure prescribed by the Creator™ SMART™ cDNA Library construction kit (Clontech), to 89% using the Agarose Gel PCR Purification kit (Roche Diagnostics).

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