Cloning and expression of a β-glucosidase gene from *Acremonium cellulolyticus* in *Saccharomyces cerevisiae*

by
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DECLARATION

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Date: March 2013

ABSTRACT

Humanity is currently dependant on fossil fuels as an energy source. Increasing economic development and industrialization is, however, raising the demand for this unsustainable energy source. This increased pressure on dwindling reserves and growing concern over detrimental environmental effects associated with the use of these fuels have sparked great interest in the development of alternative sources. Bioethanol has surfaced as a good alternative to fossil fuels, as it can be produced from cheap, abundant, renewable, non-food sources. Bioethanol is also carbon-neutral, i.e. utilisation thereof leaves the net level of carbon dioxide in the atmosphere unperturbed. Lignocellulose, more specifically its cellulose fraction, has been identified as a possible feedstock for the production of bioethanol. The use of lignocellulose as feedstock will allow for a more sustainable supply and much needed energy security.

Lignocellulosic feedstocks can be divided into two main categories, i.e. wastes from processes other than fuel production and crops grown specifically for fuel production. Cereal crops such as triticale have been identified as good industrial crops for the production of energy. Triticale's higher biomass yield, moderate water and nutrient requirements, steadily increasing area of cultivation and main use as an animal feed and not a human food source, makes it attractive as feedstock for the production of bioethanol.

The combined activity of endoglucanases, exoglucanases and β-glucosidases is needed to hydrolyse crystalline cellulose to fermentable sugars. The high cost of these enzymes is, however, the most significant barrier to the economical production of bioethanol from cellulosic biomass. A promising strategy for a reduction in costs is the production of these cellulolytic enzymes, hydrolysis of biomass and fermentation of the resulting sugars to bioethanol in a single process step via a cellulolytic microorganism. The development of such a consolidated bioprocessing (CBP) organism can be achieved by the introduction of cellulolytic activity into a noncellulolytic microorganism that is able to ferment glucose to ethanol.

Saccharomyces cerevisiae is a good host candidate for CBP as this yeast's high tolerance towards ethanol and its use in industrial applications has been established. The enzymatic activities of endoglucanases and exoglucanases are, however,

inhibited by the build-up of cellobiose during the hydrolysis of cellulose. This effect may be alleviated with the introduction of a better functioning β-glucosidase into the system. β-Glucosidases hydrolyse cellobiose to glucose, alleviating the inhibition on the enzymatic activities of endoglucanases and exoglucanases.

Despite advances in enzyme production systems and engineering enzymes currently in use for higher stability and activity, there is still a demand to expand the current collection of enzymes. Bioprospecting for novel cellulolytic enzymes focuses on specific environment, with high turnover rates of cellulosic material or extreme conditions, such as the composting process. These enzymes are becoming more attractive compared to their mesophillic counterparts due to their potential industrial applications and the fact that they represent the lower natural limits of protein stability.

OPSOMMING

Die mensdom is hoofsaaklik van fossielbrandstowwe as 'n energiebron afhanklik. Toenemende ekonomiese ontwikkeling en industrialisasie verhoog egter die aanvraag na hierdie onvolhoubare energiebron. Druk op kwynende reserwes en groeiende kommer oor die nadelige gevolge vir die omgewing wat met die gebruik van hierdie brandstowwe gepaard gaan, het tot groot belangstelling in die ontwikkeling van alternatiewe bronne gelei. Bio-etanol is 'n goeie alternatief vir fossielbrandstowwe, want dit kan van goedkoop, vollop, hernubare nie-voedselbronne geproduseer word. Bio-etanol is ook koolstof-neutraal; die gebruik daarvan laat die netto vlak van koolstofdioksied in die atmosfeer onverstoord. Lignosellulose, en meer spesifiek die sellulose fraksie, is as moontlike grondstof vir die vervaardiging van bio-etanol geïdentifiseer. Die gebruik van lignosellulose as grondstof sal meer volhoubare voorsiening en broodnodige energie-sekuriteit verseker.

Sellulose grondstowwe kan in twee hoof kategorieë verdeel word, nl. Newe produkteafval van prosesse anders as brandstofproduksie en gewasse wat spesifiek vir brandstofproduksie gekweek word. Graangewasse soos korog is geïdentifiseer as 'n goeie industriële gewas vir die produksie van energie. Korog se hoër biomassa opbrengs, matige water en voedingstofvereistes, groeiende bewerkingsgebied en die gebruik as 'n veevoergewas eerder as 'n menslike voedselbron, maak dit aantreklik as 'n grondstof vir die vervaardiging van bio-etanol.

Die gesamentlike aktiwiteit van endoglukanases, eksoglukanases en β-glukosidases is nodig om kristallyne sellulose tot fermenteerbare suikers te hidroliseer. Die hoë koste van hierdie ensieme is egter die grootste hindernis vir die ekonomiese produksie van bio-etanol vanaf sellulosiese biomassa. 'n Belowende koste verminderingstrategie is die produksie van hierdie sellulolitiese ensieme, die hidrolise van biomassa, en die fermentasie van die suikers na bio-etanol in 'n enkelstap-proses via 'n sellulolitiese mikro-organisme. Die ontwikkeling van so 'n gekonsolideerde bioprosesserings (CBP) organisme kan deur die uitdrukking van sellulolitiese aktiwiteite in 'n nie-sellulolitiese mikro-organisme wat wel in staat is om glukose na etanol om te fermenteer, gerealiseer word.

Saccharomyces cerevisiae is 'n goeie kandidaat gasheer vir CBP, omdat hierdie gis ‘n hoë verdraagsaamheid teenoor etanol toon en sy gebruik in industriële toepassings gevestig is. Die ensiematiese aktiwiteite van endoglukanases en eksoglukanases word egter deur die ophoop van sellobiose gedurende die hidrolise van sellulose geïnhibeer. Hierdie effek kan met die byvoeging van meer effektiewe β-glukosidases verlig word. β-Glukosidases hidroliseer sellobiose na glukose en verlig dus die inhibisie op die endoglukanase en eksoglukanase ensiematiese aktiwiteite.

Ten spyte van vooruitgang in ensiemproduksie stelsels en ensiemmodifiserings strategieë wat tans vir hoër stabiliteit en aktiwiteit in gebruik is, bestaan daar steeds 'n behoefte om die bestaande versameling van ensieme uit te brei. Bioprospektering vir nuwe sellulolitiese ensieme fokus op spesifieke omgewings, met hoë omsetkoerse van sellulose materiaal of omgewings met uiterste toestande, soos die komposterings-proses. Hierdie ensieme is besig om meer aantreklik in vergelyking met hul mesofieliese eweknieë te raak as gevolg van hul potensiele industriële toepassings en die feit dat hulle die laer natuurlike grense van proteïen-stabiliteit verteenwoordig.

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CHAPTER 1:

INTRODUCTION AND AIMS OF STUDY

1.1. INTRODUCTION

Fossil fuels are a crucial energy source for humanity, so much so that wars have been fought in order to ensure energy security. This dependence, the looming depletion of fossil fuel reserves and growing public concern over detrimental environmental effects associated with burning of these fuels, have sparked great interest in the development of alternative energy sources (Li *et al*. 2009). Bioethanol production from lignocellulosic biomass is emerging as one of the most important alternative technologies for sustainable production of renewable transportation fuels.

Over the last three decades, extensive research has been conducted on the different approaches for the conversion of cellulose to bioethanol (Sun and Cheng 2002). These approaches differ mainly in the method of hydrolysis of cellulose to fermentable sugars and the subsequent fermentation of the liberated sugars (Lynd *et al.* 1991). Hydrolysis can be accomplished by the use of acids, alkalis or cellulolytic enzymes. The use of enzymatic hydrolysis is environmentally-friendly and is also devoid of the corrosion problem that results from the use of acid or alkaline hydrolysis (Duff and Murray 1996, Fang *et al.* 2009). Enzymatic hydrolysis also lowers utility costs and thus overall production costs, as it may be conducted at mild conditions (pH 4.8 and temperature 45 – 50℃).

Cellulases are highly specific enzymes that are grouped into three major classes according to their mode of catalytic activity (Béguin and Aubert 1994). The three classes are: exoglucanases, endoglucanases and β-glucosidases (Den Haan *et al.* 2007, Duan and Feng 2010, Kumar *et al.* 2008, Lynd *et al.* 2002, Mansfield and Meder 2003, Rabinovich *et al.* 2002, Zhang and Lynd 2004). The collective activity of these three classes is required to completely degrade cellulose to reducing sugars.

Cellulases are subjected to end-product inhibition, caused by cellobiose and to a lesser extent by glucose (Sun and Cheng 2002). This leads to a lower yield of reducing sugars available for fermentation by bacteria or fungi to ethanol. The supplementation of β-glucosidases to cellulases systems during hydrolysis alleviates this inhibition by hydrolysing cellobiose, resulting in better overall saccharification of crystalline cellulose (Exocoffier *et al*. 1991, Xin *et al.* 1993, Yan *et al.* 1998).

However, the high cost of cellulases remains the most significant barrier to the economical production of bioethanol from cellulosic biomass (Fang *et al.* 2009). This is due to the

requirement for high enzyme loading due to the relative low catalytic efficiency of the hydrolytic enzymes currently available (Margeot *et al.* 2009, Wen *et al.* 2009). The availability of cellulolytic enzymes produced at higher titers as well as higher specific activities is deemed critical to reduce the cost of bioethanol production (Singhania *et al.* 2010). Despite advances in enzyme production systems and engineering enzymes currently in use for higher stability and activity, there is still a demand to expand the current collection of enzymes (Heinzelman *et al.* 2009).

Bioprospecting is the research field defined as the purposeful evaluation of wild biological material in search of valuable novel products (Artuso 2002). Some of the valuable products that have been sourced from nature in the past include pharmaceuticals, agrochemicals, cosmetics, flavourings, fragrances and industrial important enzymes. Microorganisms may serve as a potential source of novel enzymes, as they have adapted to a wide range of different environmental conditions by the development of highly optimised enzymes, which are suited to perform optimally under the physicochemical conditions of their habitats (Pace 1997, Whitman *et al.* 1998).

The classical approach for the isolation of new microbial enzymes includes enrichment of microorganisms from environmental samples, their isolation in pure culture and screening for the desired enzymatic activity (Lämmle *et al.* 2007). This only allows for the isolation of enzymes from culturable microbes and not the unculturable majority. It has been estimated with the use of molecular phylogenetics that only a mere 1% of all microbes in the biosphere are culturable under normal laboratory conditions (Pace 1997, Rappe *et al.* 2003). In order to prevent this loss of diversity, culture-independent approaches have been developed to exploit the collective genomes of microbial communities (Rondon *et al.* 2000, Schloss and Handelsman 2003). Metagenomics and metatranscriptomics comprise the direct extraction of total genomic DNA or mRNA, respectively, from environmental samples, its cloning into suitable expression vectors and subsequent screening of the resultant libraries.

Bioprospecting for novel cellulolytic enzymes focuses on specific environments with high turnover rates of recalcitrant cellulosic material or extreme conditions, such as temperature extremes, pH extremes and the presence of oxidants. Enzymes sourced from the microbes inhabiting these environments are adapted to be active at temperature extremes and stable over a wide pH range (Kasana and Gulati 2011). These enzymes are becoming more attractive compared to their mesophillic counterparts because of their

potential industrial applications and the fact that they represent the lower natural limits of protein stability.

Cellulase producing microbes have been isolated from various extreme environments, e.g., a cold-adapted manure biogas digester (Akila and Chandra 2003), cattle's rumen (Iyo and Forsberg 1999), the Yellow Sea (Hou *et al.* 2007), cold environments in the Western Himalaya (Shipkowski and Brenchley 2005) and deep-sea sediment (Zeng *et al.* 2006). Another example of such an extreme environment that may harbour industrial important enzymes, is the composting process. Composting microbial communities are adapted to withstand large changes in temperature, redox conditions and water activity (Klamer and Bååth 1998, Goyal *et al.* 2005). This suggests the potential for discovering robust cellulolytic enzymes that can withstand the harsh pre-treatment approaches of bioethanol production such as dilute acid, ionic liquid and ammonia fibre expansion (Allgaier *et al.* 2010).

1.2 AIMS OF STUDY

The main objective of this study was the isolation of a novel eukaryotic β-glucosidase through bioprospecting a compost heap environment, and the heterologous expression and characterisation of the recombinant enzymatic activity in *Saccharomyces cerevisiae*.

The specific aims of the study included:

- The construction of an organic triticale compost heap and monitoring changes in physical conditions.
- Metatranscriptomic library construction and screening for β-glucosidase enzymes expressed in *S. cerevisiae*.
- The isolation and identification of culturable filamentous fungi producing β-glucosidase in the composting process.
- Cloning, functional expression and characterisation of a specific β-glucosidase gene in *Saccharomyces cerevisiae*.

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CHAPTER 2:

FEEDSTOCKS AND ENZYMES FOR THE PRODUCTION OF BIOETHANOL

2.1. The need for alternatives to fossil fuels

Fossil fuels play a crucial role in world economics, dominating the 1.5 trillion dollar energy market (Goldemberg 2006, Shafiee and Topal 2009). This will remain the case for at least the next few decades, as it is estimated that energy generated from fossil fuels is still expected to meet about 84% of global energy demand in 2030. In 2009, Shafiee and Topal proposed a new method to calculate when fossil fuels will be depleted, estimated to be around 35, 107 and 37 years for oil, coal and gas, respectively (Shafiee and Topal 2009). Increasing economic development and industrialization of countries along with an estimated doubling of energy usage in the next two decades, will further raise the demand for this unsustainable energy source (Fortman *et al.* 2008, Sun and Cheng 2002).

There is worldwide research into alternative reliable energy resources to replace the diminishing fossil fuels. This is mainly being driven due to the uncertainty surrounding the future supply of fossil fuels. Furthermore, burning fossil fuels leads to a net increase in atmospheric carbon dioxide, which is very likely contributing to the global warming process that is growing concern (Rojo 2008). Bioethanol has been identified as an alternative to fossil fuels as it can be produced from cheap, abundant, renewable non-food agricultural (waste) products and is carbon-neutral (Moreira and Goldemberg 1999, Fortman 2008, Rojo 2008). Burning these biomass-derived compounds will release carbon dioxide into the atmosphere that had been previously fixed by photosynthesis, leaving the net level of carbon dioxide in the atmosphere unperturbed. Estimated reductions in greenhouse gas emissions through bioethanol production from lignocellulose feedstocks are calculated to be in the order of 86% (Wang *et al.* 2007).

Currently, the bulk of bioethanol fuel is produced in Brazil, the European Union and the U.S.A. from crops like sugarcane, maize and other starch-rich grains (Hammerschlag 2006, Lynd *et al.* 1991). Starch- and sugar-based biofuels is, however, a growing concern as it raises the demand and thus the cost of already limiting food sources (Scharlemann and Laurance 2008, Sun and Cheng 2002). In answer to this, an extensive shift in research focus has taken place to develop lignocellulosic materials as feedstock for the production of second generation bioethanol. The production of ethanol from these renewable sources will allow for a more sustainable supply and much needed energy security (Fortman *et al.* 2008, Lynd *et al.* 1991).

2.2. Lignocellulose as feedstock for bioethanol production

The earth's surface receives approximately 2,3638 x 10^{24} joules in solar energy (Kumar *et al.* 2008). This equates to more than 12 000 times the current human energy requirement and an estimated 4 000 times the amount of energy humankind will need in 2050. Plants utilise this energy during the process of photosynthesis and are estimated to collectively store ten times the world's energy requirement. This makes the production of bioethanol from plant biomass an attractive alternative to the use of fossil fuels, as lignocellulosic biomass is an abundant, renewable and underutilized global carbon source (Sun and Cheng 2002).

Lignocellulose is a compact and in part crystalline complex material (Kumar *et al.* 2008, Leonowicz *et al.* 1999, Zhang 2008) consisting of cellulose (insoluble fibres of β-1,4-glucan) and hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans) intertwined with lignin (a complex polyphenolic structure) (Howard *et al.* 2003) (Fig. 2.1).

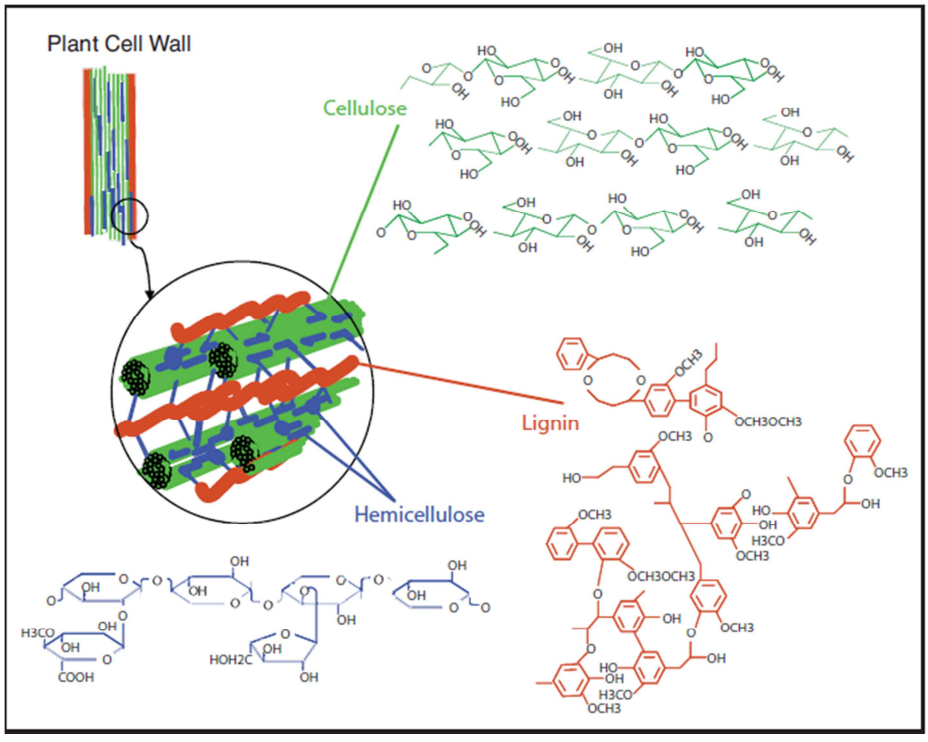

Fig. 2.1: A schematic representation of the structure of lignocellulose, showing how lignin, hemicellulose and cellulose are interlaced to form a complex biopolymer (adapted from biofuel.webgarden.com).

Possible sources of lignocellulosic feedstocks for the production of bioethanol can be divided into two main categories, i.e. wastes from processes other than fuel production and crops grown specifically for fuel production (Lynd *et al.* 1991, Sun and Cheng 2002). The waste feedstock options include agricultural residues, forestry residues and municipal solid waste, while woody or herbaceous high-productivity energy crops (HPECs), specifically grown to be used as biofuel feedstock or trees produced by conventional forestry, constitute the non-waste category of feedstocks (Kumar 2008, Sun and Cheng 2002).

There has been a growing interest in the use of cereal crops, such as triticale, as an industrial crop for the production of energy and chemicals in a biorefinery system. Triticale, a hybrid of wheat (*Triticum*) and rye (*Secale*), yields more grain, produces more biomass (straw) and requires moderate water and nutrient inputs compared to other cereal crops (Goyal *et al.* 2011, Martinek *et al.* 2008; Pejin *et al.* 2009, Salmon *et al.* 2004). As such, worldwide production and cultivated area of triticale have been steadily increasing (Salmon *et al.* 2004). The first hybrids were reported by Wilson in 1875 (Wilson 1875) and introduced as an agricultural crop in the 1960's, largely as an animal feed crop (Ammar *et al.* 2004, Mergoum *et al.* 2004, Salmon *et al.* 2004). Triticale consists out of 36-37% (dry weight basis) cellulose, 21-22% hemicellulose (17% xylose, 3% arabinose, 1% galactose, 1% mannose) and 16-21% lignin (Neureiter et al. 2004, Pronyk and Mazza 2012). Triticale's higher biomass yield, moderate water and nutrient requirements, steadily increasing area of cultivation and main use as an animal feed crop and not a human food source, makes it attractive as an feedstock for the production of bioethanol.

2.3. Compost: Source of cellulases for the biofuels industry

Composting is defined as a dynamic biological process whereby heterogeneous organic matter is degraded by micro-organisms to carbon dioxide, water and a stable humus-like material, under moist, self-heating, aerobic conditions (Khalil *et al.* 2001, Ryckeboer *et al.* 2003, Steger *et al.* 2003). A complex consortium of aerobic mesophillic and thermophillic bacteria and fungi is predominantly responsible for this process. Decomposition occurs throughout both a thermophillic and mesophillic phase, and the microbes responsible are able to adapt to changes in temperature, redox conditions and water activity (Goyal *et al.* 2005, Klamer and Bååth 1998). Over a period of three months, temperatures steadily climb to reach a maximum of 60℃, after which it drops to approximately 25℃. These

temperature fluctuations and changes in activity are used to divide the process into four distinct phases (Ishii *et al.* 2000).

The initial phase of composting, the mesophillic phase, is characterized by the growth of mesophillic organisms (Steger *et al.* 2003). Degradation of plant and animal residues by these organisms leads to a rapid increase in temperature (Khalil *et al.* 2001). This heralds the next stage in the process, the thermophillic phase, during which temperatures can reach highs of up to 60℃. In this phase, thermoph illic organisms are responsible for the degradation of organic matter as the growth of mesophillic organisms is inhibited at these temperatures (Ishii *et al.* 2000, Goyal *et al.* 2005, Khalil *et al.* 2001, Steger *et al.* 2003). After reaching these highs, temperatures gradually drop and mark the beginning of the final two phases of composting, namely cooling and maturation, during which an entirely new mesophillic community develops (Steger et al. 2003). Fungal numbers are at their lowest during the thermophillic phase, where bacterial growth dominates the process (Klamer 2008, Ryckeboer 2003). However, as decomposting progresses, fungal numbers increase with the highest numbers observed during the maturation phase (Goyal *et al.* 2005).

Cellulose is a major component of composting, as plant material is used as bulking agents, amendments and organic substrates (Tuomela *et al.* 2000). This makes compost an excellent source of cellulases, as the capacity of microorganisms to degrade and assimilate this biopolymer relies on their ability to produce these enzymes. This and the ability of compost microbial communities to quickly adapt to major environmental perturbations, suggest that the composting is the ideal source of novel robust cellulolytic enzymes that can withstand the harsh industrial conditions linked to bioethanol production (Allgaier 2010). In this study, triticale was used as the main component in the stacking of a compost heap, allowing for the selection of microbial communities specially adapted to degrade the cellulose fraction of this potential feedstock for the industrial production of bioethanol.

2.4. Cellulose: Distribution and structure

The term cellulose was first used in 1839 to describe the resistant fibrous solid that remained after the treatment of different plant tissues with acids and ammonia, followed by extraction with water, alcohol and ether (Payen 1838). This was based on the work of a

French chemist, Anselme Payen, who also determined the molecular formula to be $C_6H_{10}O_5$, and the structure to be isomerically similar to that of starch. Cellulose is the most abundant form of renewable bioorganic macromolecule on earth, with an estimated annual carbon production of 60 $x10^{12}$ kg and 53 $x10^{12}$ kg, in terrestrial and marine ecosystems. This carbohydrate is produced by terrestrial plants, marine algae, bacteria and even some marine animals (Teeri 1997, Wilson 2008).

Plants synthesise cellulose by carbon dioxide fixation as main structural component of cell walls. On average it can comprise about 30-50% of plant cell dry weight (Brown 2004). As a structural component in plant cell walls, cellulose helps to resist turgor pressure, maintain the size, shape and the division/differentiation potential of the cell (Saxena and Brown 2005). Cellulose morphology involves a highly ordered structural design of fibrillar elements (Fig. 2.2). Individual chains consist of D-anhydroglucopyranose residues linked in a 180° angle to each other by β-1,4-glycosidic bonds, with the disaccharide anhydrocellobiose as the repetitive unit (Teeri 1997, Wilson 2008, Wood 1991). The degree of polymerisation of cellulose chains range from 100 to 20 000 D-anhydroglucopyranose residues (Krässig 1993, O' Sullivan 1997, Tomme *et al.* 1995, Zang *et al.* 2006, Zhang and Lynd 2004).

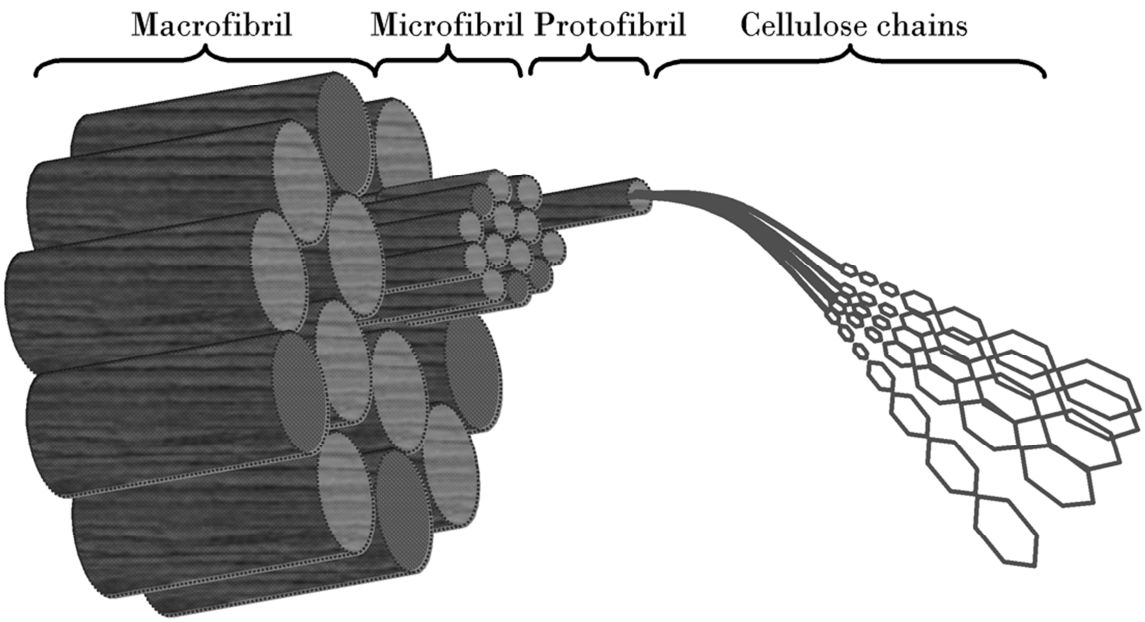

Fig. 2.2: A schematic representation of the macromolecular structure of cellulose. Individual cellulose chains are assembled into larger units known as protofibrils, which are packed into larger rod-like units called microfibrils. The microfibrils are associated through Van der Waals bonds to form a very rigid macromolecular structure called the macrofibril (Clarke 1997).

Approximately 36 individual cellulose chains are assembled into larger protofibrils (Brown *et al.* 1996). Protofibrils are tightly packed together into rod-like units called microfibrils that are associated though hydrogen and Van der Waals bonds, to form a very rigid macromolecular structure called a macrofibril. An individual microfibril consists out of approximately 20 protofibrils, while about 200 microfibrils are packed into a single macrofibril with a diameter in the micrometer range (Krässig 1993).

The order of the macrofibrils in a cellulose chain is not uniform throughout the entire structure. There are regions of very high crystalline order that are very stable (Wilson 2008). Crystalline cellulose has an estimated half-life of 5-8 million years (Wolfenden and Snider 2001). There are also regions of lower order, or so-called amorphous regions (Teeri 1997), where cellulose fibres contain abnormalities like kinks in the microfibrils or voids known as micropores, large pits and capillaries (Blouin *et al.* 1970, Cowling 1975, Fan *et al.* 1980). These heterogeneous regions allow the cellulose fibres to be partially penetrable by aqueous solutions and even larger entities like enzymes.

Both cellulases and hemicellulases, responsible for the degradation of crystalline cellulose and hemicellulose respectively, have been used successfully to produce fermentable sugars from lignocellulose (Fujii 2009). In order to make bioethanol produced with lignocellulolytic enzymes an economically feasible alternative to petroleum-based fuel, improvements need to be made to current production processes. The most significant reduction in production costs for bioethanol may be realised by either reducing the cost of the feedstock or the saccharification of the feedstock, i.e. improved enzymatic hydrolysis (Wooley *et al.* 1999, Fortman *et al.* 2008).

2.5. Microbial cellulose degradation

Cellulose may be degraded aerobically or anaerobically by either mesophillic or thermophillic microbes (Sun and Cheng 2002). Fungi are the major degraders of cellulose in soil and are well-known for their ability to do this aerobically (Lynd *et al.* 2002). This ability also extends to certain bacterial species, which include both filamentous (e.g. *Streptomyces, Micromonospora*) and non-filamentous (e.g. *Bacillus, Cellulomonas, Cytophaga*) genera (De Boer *et al.* 2005, Lynd *et al.* 2002). Anaerobic fungi from the phylum *Chytridiomycota*, which colonizes the intestinal tract of ruminant animals (Bornemann *et al.* 1989, Teunissen *et al.* 1991), and obligatory anaerobic bacteria

(*Acetovibrio, Clostridium, Ruminococcus*) are responsible for the degradation of cellulose in anaerobic environments (Leschine 1995, Lynd *et al.* 2002). These organisms use different cellulase systems to degrade cellulose to reducing sugars.

Anaerobic bacteria make use of effective complex cellulase systems, also known as cellulosomes, which allows for the synergistic action of the different types of cellulases (Doi and Kosugi 2004, Lynd *et al.* 2002, Shoham *et al.* 1999, Zhang and Lynd 2005). These complexes are formed on the cell wall of the organism and binds firmly to the substrate. This shortens the distance that hydrolysis products need to diffuse to reach the host cell, and positions the cell at the site of hydrolysis.

In contrast to this, aerobic fungi and bacteria produce non-complexed cellulase systems. These systems, compromising of endoglucanases, exoglucanases and β-glucosidases, are freely diffusible extracellular enzymes that degrade cellulose in a concerted mannner (Mansfield and Meder 2003, Lynd *et al*. 2002). This mechanism of cellulose degradation is employed in several aerobic environments, of which composting is one example.

2.6. Cellulases: The enzymes responsible for cellulose hydrolysis

Glycoside hydrolases with the ability to degrade the glycosidic bonds found in cellulose, are designated as cellulases (Himmel *et al.* 2007, Lynd *et al.* 2002). Cellulases are currently the third largest industrial commodity, by US dollar volume, due to their use in the textile industry for cotton softening and denim finishing; colour care in the detergent market; cleaning, anti-deposition and mashing in the food industry, in the paper industry for de-inking and fibre modification in the paper industry and as animal feed additive (Zhang *et al.* 2006, Wilson 2009). Cellulase enzymes currently in use in the industry are mostly derived from *Trichoderma* and *Aspergillus* species. These enzymes are chosen for their high catalytic efficiency on insoluble cellulose, stability at high temperatures and certain pH ranges, and also for higher tolerance to end-product inhibition (Zhang 2006).

Cellulases are grouped into three major classes according to their mode of catalytic activity, of which the collective activity facilitates the degradation of crystalline cellulose (Mansfield and Meder 2003, Zhang and Lynd 2004, Rabinovich *et al.* 2002, Den Haan *et al.* 2007, Lynd *et al.* 2002, Kumar *et al.* 2008, Duan and Feng 2010) (Fig. 2.3). The three groups are: (1) exoglucanases, which include cellodextrinases and cellobiohydrolases; (2) endoglucanases; and (3) β-glucosidases.

Exoglucanses remove cellobiose units in a processive manner from the reducing or non-reducing ends of cellulose chains. They are also active against microcrystalline cellulose, peeling cellulose chains from the microcrystalline structure. Endoglucanases randomly cut the cellulose chains at internal amorphous sites, generating oligosaccharides of various lengths and consequently providing the cellobiohydrolases with further chain ends to act upon (Teeri 1997). Finally, β-glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose, providing an easily metabolisable carbon source for the cellulolytic microorganism (Beguin 1990). In addition to the more typical cellulases, novel types of cellulases such as the *Trichoderma reesei* swollenin (SWOI) have been identified. SWOI has a high amino acid homology with plant expansins, which disrupt cellulose fibres, but lack hydrolytic activity. Therefore, swollenin is probably involved in making cellulose fibres more accessible for cellulases to act upon (Saloheimo et al. 2002).

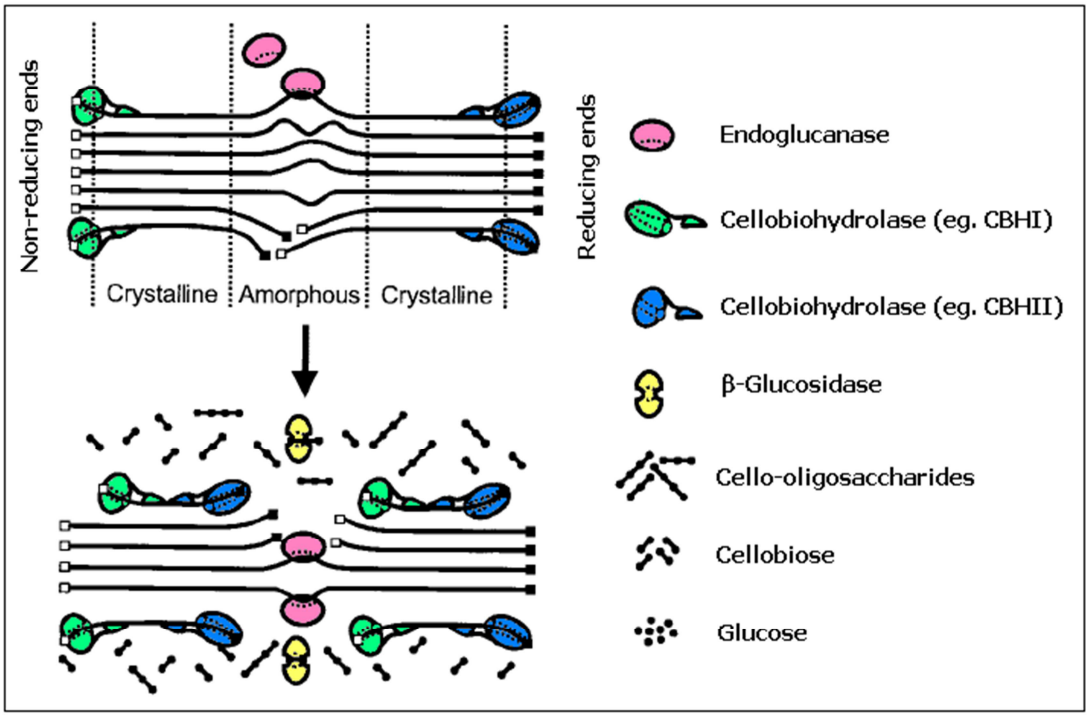

Fig. 2.3: A schematic representation of the mode of action of the cellulase enzymes in the hydrolysis of amorphous and crystalline cellulose (Lynd *et al.* 2002).

Cellulases are subjected to end-product inhibition caused by cellobiose and to a lesser extent by glucose (Sun and Cheng 2002). This leads to a lower yield of reducing sugars available for fermentation by bacteria or fungi to ethanol. The supplementation of β-glucosidases to cellulases systems during hydrolysis alleviates this inhibition by

hydrolysing cellobiose, resulting in overall better saccharification of crystalline cellulose (Exocoffier et al. 1991, Xin et al. 1993, Yan et al. 1998).

2.7. β-Glucosidases

β-Glucosidases are exo-acting glycosyl hydrolases that act upon compounds containing β-glucosidic linkages by splitting of the terminal, non-reducing β-D-glucose residues and releasing β-D-glucose (Sternberg *et al*. 1977). This group of enzymes occur universally in mammals, plants and microorganisms and are grouped into Families 1, 3 and 9 glycosyl hydrolases, according to the CAZy system of classification (Henrissat and Bairoch 1993, Woodward and Wiseman, 1982).

Commercial applications of β-glucosidases include its use in commercial feed additives, e.g. Barlican (a non-toxic cellulase enzyme mixture) for single-stomached animals such as chickens and pigs. This supplementation enhances cellulose degradation and thus improves nutrient utilization (Coenen *et al.* 1995, Zhang *et al.* 1996). Another important application of β-glucosidases is in the wine industry, were it is used to prevent sediment formation during the storage of bottles and to catalyse the release of flavour compounds (Gueguen *et al.* 1997). This same application is also used in the Japanese brewing industry, were treatment with β-glucosidases releases several free monoterpene alcohols that contribute to the characteristic flavour of sweet potato shochu (Iwashita *et al.* 1998).

From a bioethanol perspective, β-glucosidases are of great interest due to their ability to hydrolyse cellulosic material along with endo- and exoglucanases. The most popular cellulase preparations used for the saccharification of cellulose on industrial scale contain *T. reesei* cellulases supplemented with an *Aspergillus* β-glucosidase (Reczey *et al.* 1998, Sternberg *et al.* 1977). As the levels of β-glucosidase produced by *T. reesei* are only sufficient for sustaining growth on cellulose, it is not adequate for extensive saccharification of cellulose. Exogenous supplementation with β-glucosidase relieves the cellobiose-mediated inhibition of exoglucanases and endoglucanases, in turn leading to overall better saccharification of crystalline cellulose (Exocoffier *et al*. 1991, Xin *et al.* 1993, Yan *et al.* 1998).

2.8. Microbial β-glucosidases

Both cellulylolytic and non-cellulolytic microbes possess this well-charaterised group of enzymes. In cellulose-degrading bacteria and fungi, β-glucosidases predominantly hydrolyse cello-oligosaccharides and cellobiose to glucose. Microbial β-glucosidases are, however extremely diverse in genetic and enzymatic properties, with some microbes producing more than one β-glucosidase, e.g. intracellular, cell wall-bound and extracellular β-glucosidases produced by both *T. reesei* (Saloheimo *et al.* 2002, Chirico and Brown 1987, Umile and Kubicek 1986, Inglin *et al.* 1980) and *C. wickerhamii* (Skory *et al.* 1996, Himmel *et al.* 1986, Freer 1985). Another surprising phenomenon is the expression of three different enzymes from a single gene. This is seen in *A. kawachii,* where the cell wall-bound (CB-1) and two extracellular (EX-1 and EX-2) β-glucosidases are encoded for by the same *bglA* gene (Iwashita *et al.* 1999). Some β-glucosidases even exibit more than one activity, like that of *Talaromyces thermophilus* that exhibits both β-glucosidase and β-galactosidase activity (Nakkharat and Haltrich 2006).

Recombinant enzyme production has become a key technology in various industries, including the food, textile and pharmaceutical industries. A number of microbial β-glucosidases have been cloned and heterologously produced in prokaryotic (Rajoka *et al.* 1998, Hashimoto *et al.* 1998, Marri *et al.* 1995) and eukaryotic (Van Rooyen *et al.* 2005, Saloheimo *et al.* 2002, Dan *et al.* 2000, Iwashita *et al.* 1999, Rajoka *et al.* 1998, Machida *et al.* 1988, Murray *et al.* 2004, Donzelli *et al*. 2001, Barnett *et al.* 1991, Takashima *et al.* 1999) host expression systems. Compared to their prokaryotic counterparts, the heterologous production of fungal β-glucosidases is more challenging due to the additional complexity of glycosylation and the presence of introns in their structural genes.

Fungal β-glucosidases that have been heterologously produced in eukaryotic hosts resulted in enzymes that are mostly extracellular or associated with the periplasmic space (Van Rooyen *et al.* 2005). These recombinant enzymes have been charaterised to have pH and temperature optima of between 4-5 and 45-65°C respectively (Van Rooyen *et al.* 2005, Meeko *et al.* 2010, Dhake and Patil 2005, Pornphimon 2005, Zhou and Yin 2009), similiar to β-glucosidases purified from their native hosts (Table 2.1).

Table 2.1: Molecular properties (i.e. pH and temperature optima and substrate specificity) of various fungal β-glucosidases.

Native host	pH optima	Temperature optima	Specific activity (U/mg)[1] on specific substrates[2]	Reference
Aspergillus niger	5.0	50ºC	198.5 (pNPβGlu) 5.27 (cellobiose) 4.59 (cellotriose) 3.24 (cellotetraose) 2.30 (cellopentaose)	Yan and Lin 1997
Aspergillus oryzae	5.0	60℃	-	Langston *et al.* 2006
Candida peltata	5.0	50ºC	108 (pNPβGlu)	Galas and Romanowska 1996
Daldinia eschscholzii	5.0	50ºC	85 (pNPβGlu) 3.2 (pNPβCel) 47 (cellobiose)	Karnchanatat *et al.* 2007
Debaryomyces vanrijiae	5.0	40ºC	-	Belancic *et al.* 2003
Melamocarpus sp.	6.0	60ºC	0.27 (pNPβCel)	Kaur *et al.* 2007
Metschnikowia pulcherrima	4.5	50ºC	1.3 (pNPβGlu) 0.28 (cellobiose)	González-Pombo *et al.* 2008
Monascus purpureus	5.5	50ºC	84.4 (pNPβGlu) 18.5 (pNPβCel) 84 (cellobiose)	Daroit *et al.* 2008
Neosartorya fischeri NRRL181	6.0	40ºC	710 (pNPβGlu) 2 (pNPβCel) 0.04 (cellobiose)	Kalyani *et al.* 2012
Penicillium funiculosum	4.5	60ºC	-	Karboune *et al.* 2008
Penicillium occitanis	4.5	60ºC	-	Bhiri *et al. 2008*
Trichoderma koningii AS3.2774	5.0	50ºC	994.6 (pNPβGlu)	Lin *et al.* 2010
Xylaria regalis	5.0	50ºC	65.3 (pNPβGlu) 65 (cellobiose)	Wei *et al.* 2006

[1]One unit (U) of enzymatic activity is defined as the amount of enzyme producing 1 µmol of reaction products in 1 min under assay conditions.

[2]*p*NPαGlu: *p*-nitrophenyl-α-D-glucoside; *p*NPβCel: *p*-nitrophenyl-β-D-cellobioside.

Currently, *S. cerevisiae* is the preferred organism for ethanol production and therefore an important recombinant host for the production of cellulose-hydrolyzing enzymes (Lynd *et al.* 2002). In addition, it has complete GRAS (Generally Regarded As Safe) status that is of particular interest when producing β-glucosidases for application in the food and wine industries.

2.9. Structure and mode of action

Substrate specificity and the mode of action of β-glucosidases are regulated by their detailed three-dimensional structures. Cleavage of the glycosidic bond in cellobiose occurs through an acid hydrolysis mechanism and results in either an overall retention or an inversion of anomeric configuration. This mechanism involves two critical residues: a proton donor and a nucleophile/base, usually represented by glutamic and aspartic acid (Sinnott 1990). The binding of the enzyme to the substrate positions the proton donor and nucleophile in exact proximity to each other. This also decreases the energy barrier as a result of substrate deformation, and ensures subsequent stabilisation of the chemical intermediate (McCarter and Withers 1994).

Inverting β-glucosidases utilise the charged state of the catalytic site to activate a water molecule for nucleophilic attack, while an acidic amino acid donates the necessary proton. The charged moiety becomes protonated as a result of the nucleophilic attack of the activated water, while the proton donor becomes the charged moiety that activates the water molecule for the next reaction (Davies and Henrissat 1995, Mosier *et al.* 1999). Since the protonation of the catalytic residues inverts at the completion of hydrolysis, the mechanism is designated as inverting. This single nucleophilic substitution yields a product with opposite stereochemistry (e.g. α-D-glucopyranoside) to the substrate (Sinnott 1990).

Retaining β-glucosidases utilize an acid residue in the active site for nucleophilic attack on the glycosyl bond of the substrate, while an opposing residue donates the necessary proton. This results in the formation of a covalently bound intermediate. The second nucleophilic attack by an activated water molecule releases the hydrolytic product, which consecutively recharges the catalytic residue that acts as the proton donor. This second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry as the substrate (Davies and Henrissat 1995, Mosier *et al.* 1999).

An additional feature of retaining β-glucosidases is that they are also able to synthesize glycosidic bonds. This may occur by two different approaches, i.e. reverse hydrolysis and transglycosylation The biosynthetic properties of these enzymes have received a lot of interest due to the prospect of using transglycosidic reactions to produce commercially important glycosides. The focus of this study is on the hydrolytic activity of β-glucosidases and not its biosynthetic activity, and as such only the former will be discussed.

Structurally β-glucosidases function as mono-, di-, tri- and tetramers and have a so-called pocket topology, which is optimal for the recognition of the non-reducing ends of a sugar (Jenkins *et al.* 1995). β-Glucosidases are tailored to accommodate substrates having a large number of these easy-accessible, non-reducing chain ends at the surface of the substrate. For this reason, this enzyme cannot act on its own on fibrous substrates like native cellulose, which has almost no free chain ends.

2.10. Synergy amongst cellulases

Synergy is the phenomenon were a mixture of cellulases exhibits higher specific activity, compared to the sum total of the individual enzymes (Lynd *et al.* 2002, Wood and McRae 1979). This effect is found amongst most cellulases and is most pronounced on crystalline cellulose (Irwin *et al.* 1993). To date, four types of synergy have been described: (i) endo-exo synergy, observed between endoglucanases and exoglucanases; (ii) exo-exo synergy, between two different exoglucanases that attack from different ends of the cellulose chain; (iii) synergy between exoglucanases and β-glucosidases, where these enzymes hydrolyse the end products of exoglucanases and in the process alleviates feedback inhibition; and (iv) intramolecular synergy between the catalytic domains and the carbohydrate-binding modules (CBMs) of different enzymes (Din *et al.* 1994, Lynd *et al.* 2002, Teeri 1997).

Three main factors affects synergism: (i) the ratio and concentrations of the cellulases in the reaction mixture; (ii) the ease of access to binding sites for the cellulases in the mixture; and (iii) the physical and chemical heterogeneity of the cellulose substrate (Jeoh *et al.* 2006). It is also thought that synergy may only occur if the different cellulases attack different regions of the cellulose microfibril, creating new sites for attachment for the other enzymes in the mixture. This would explain why most endoglucanases do not show synergy with each other, but only with exoglucanases, as no new sites for attachment are created by its working (Jeoh *et al.* 2006).

2.11. The discovery of β-glucosidases for use in bioethanol production

The demand for cellulases is expected to increase as cellulases are to be used to hydrolyse cellulose into fermentable sugars that may be used to produce ethanol and other bioproducts (Duan and Feng 2010, Wilson 2009). However, fuel ethanol produced from lignocellulose may prove to be too costly due to the low efficiency and high costs of many cellulases used in the hydrolysation of cellulose to glucose. A significant reduction in cost is needed to render enzymes economically feasible (Zhang *et al.* 2006). One way of facilitating this is the isolation of new cellulases from the environment with better properties suited for industrial applications. These properties include higher catalytic efficiencies on specific insoluble cellulose substrates, increased stability at higher temperatures and certain pH values, and higher tolerance to end-product inhibition (Duan and Feng 2010).

Given the complexities involved in the hydrolysis of lignocellulose as highlighted above, this study set out to discover and heterologously express novel β-glucosidase genes in an attempt to solve one of the major bottlenecks in cellulose hydrolysis. Our approach originally followed a broad screening with the construction of a triticale compost heap to select for microbial populations actively degrading this specific feedstock. We attempted to construct a metatranscriptomic library from mRNA isolated from the active stages of an active triticale compost heap, with the main aim to screen these cDNA libraries for novel β-glucosidase enzymes (see Addendum A). Parallel to the metatranscriptome approach, culturable fungal isolates from the active triticale compost heap were also screened for cellulase activities (Chapter 3). Due to major obstacles to construct metatranscriptome libraries and our inability to novel β-glucosidase genes using this approach (Addendum A), we redirected our focus to a genome mining approach to clone and heterologously express the cDNA version of a β-glucosidase gene from one of the culturable isolates obtained in this study, *Acremonium cellulyticus*, in *S. cerevisiae* (Chapter 3).

This psychrophilic microorganism was originally isolated from soil in north-eastern Japan in 1982 and has since been exploited in Japan as cellulolytic enzyme-producing industrial microorganism (Fang *et al.* 2009, Kasana and Gulati 2011). Yamanobe and co-workers isolated cellulases from this filamentous fungus strain that demonstrated significantly higher β-glucosidase activity than the cellulase derived from *T. reesei* (Yamanobe et al. 1987).

Psychrophiles are microorganisms that have adapted to survive and proliferate at low temperatures (D'Amico *et al.* 2006). These microbes have evolved to surmount the challenges associated with reduced enzymatic activity, decreased membrane fluidity, altered transport of nutrients and waste products, decreased rates of transcription, translation and cell division, protein cold-denaturation, inappropriate protein folding and intracellular ice formation (D'Amico *et al.* 2006). Enzymes sourced from psychrophiles are becoming more attractive compared to their mesophillic counterparts, due to their potential industrial applications and the fact that they represent the lower natural limits of protein stability (Kasana and Gulati 2011).

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CHAPTER 3:

THE HETEROLOGOUS EXPRESSION OF AN *ACREMONIUM CELLULOLYTICUS* β-GLUCOSIDASE GENE IN *SACCHAROMYCES CEREVISIAE*

3.1. ABSTRACT

A compost heap with triticale as main constituent was constructed and the composting process was monitored over a period of 152 days. A filamentous fungus showing high cellulase activity was isolated and preliminarily identified as an *Acremonium cellulolyticus* species. The 2577 bp β-glucosidases gene from this psychrophile was cloned and heterologously expressed in *Saccharomyces cerevisiae*. The β-glucosidase production profile of this recombinant organism was assessed and the expressed enzyme characterised. A maximum enzymatic activity of 0.56 nkat/mg total protein was measured using p-nitrophenyl-β-D-glucopyranoside as substrate. The recombinant protein displayed a pH optimum of 4.0 and temperature optimum of 70℃. Temperature stability was determined, with 70% of maximal enzymatic activity retained after 1 hour at 60℃. Enzymatic activity of the recombinant β-glucosidase was adversely affected by the presence of glucose and ethanol at higher concentrations, but xylose was found to have a stimulatory effect on enzymatic activity. Due to hyperglycosilation, an accurate estimation of the molecular weight of the recombinant β-glucosidase could not be made. Bioinformatic analysis placed the theoretical size of the 858 amino acid protein at 91.735 kDa.

3.2. INTRODUCTION

A procession of microbial species is present in a composting heap and the microbial variety and density change with the temperature and nutrition profile of the compost heap (Epstein 1997). Decomposition of organic matter occurs throughout both a thermophillic and mesophillic phase and the microbes responsible are able to adapt to changes in temperature, redox conditions and water activity (Goyal *et al.* 2005, Klamer and Bååth 1998). The main substrate acted upon by a complex consortium of aerobic mesophillic and thermophillic bacteria and fungi, is lignocellulose (Khalil et al. 2001, Ryckeboer *et al.* 2003, Steger *et al.* 2003). On average, cellulose can comprise 30-50% of plant cell dry weight (Brown 2004), which makes the process of composting an ideal source of microbial cellulases.

Cellulases have been exploited in various industrial processes for their ability to hydrolyse the complex structure of cellulose to its monomer glucose, which in turn can be converted to other value-added products (Wang *et al.* 2005). Some of the industrial processes

include cotton softening and denim finishing in the textile industry, colour care in the detergent market, cleaning, anti-deposition and mashing in the food industry, de-inking and fibre modification in the paper industry and as animal feed additive (Wilson 2009, Zhang *et al.* 2006). The hydrolysis of cellulosic waste materials to reducing sugars for bioethanol fermentation is another industrial application of cellulases.

Cellulolytic enzymes are composed of three main activities: endoglucanases, exoglucanases and β-glucosidases (Den Haan *et al.* 2007, Duan and Feng 2010, Kumar *et al.* 2008, Lynd *et al.* 2002, Mansfield and Meder 2003, Rabinovich *et al.* 2002, Zhang and Lynd 2004). The combined synergistic effect of these activities degrades the complex structure of cellulose to its glucose monomer. Filamentous fungal strains inherent to organic matter recycling processes, are known to produce and secrete large quantities of these enzymes (Goyal *et al.* 1991, Sehnem *et al.* 2006, Wen *et al.* 2009) and are thus one of the key role players during lignocellulosic composting.

Cellulases produced by the fungus *Trichoderma reesei* represent the best characterized and most used group of enzymes in the degradation of cellulose (Esterbauer *et al.* 1991). This organism, however, exhibits relatively low β-glucosidase activity (Sternberg and Mandels 1979), which often limits the amount of this enzyme in commercial cellulase preparations (Chauve 2010, Del Pozo 2012). Low levels of β-glucosidase results in the accumulation of cellobiose, which in turn is a strong inhibitor of cellobiohydrolase and endoglucanase activities during cellulose hydrolysis (Sehnem *et al.* 2006). This limitation can be alleviated either by overexpressing β-glucosidase in *T. reesei* or by adding extra β-glucosidase from other sources (Kumar 2008, Xin 1993). This supplementation with β-glucosidase has been shown to improve the overall efficiency of the hydrolysis process (Han 2009, Pallapolu 2011).

Yamanobe and co-workers isolated enzymes from the filamentous fungus *Acremonium cellulolyticus* Y-94 that exhibited a significantly higher β-glucosidase activity than that of *T. reesei* (Yamanobe *et al.* 1987). In 1985, Yamanobe *et al*. filed a patent for this organism and its β-glucosidase, which stated that this particular β-glucosidase excels in thermal stability and allows for saccharification of cellulose at 5 to 10℃ above that of cellulases usually employed (Yamanobe *et al*. 1985). This psychrophilic microorganism was originally isolated from soil in north-eastern Japan in 1982 and has since been exploited in Japan as cellulolytic enzyme-producing industrial microorganism (Fang *et al.* 2008, Fang *et al.* 2009, Kasana and Gulati 2011). Psychrophiles are microorganisms that

have adapted to survive and proliferate at low temperatures (D'Amico et al. 2006). Enzymes sourced from psychrophiles are becoming more attractive compared to their mesophillic counterparts, due to their potential industrial applications and the fact that they represent the lower natural limits of protein stability (Kasana and Gulati 2011).

The high cost of cellulases is the most significant barrier to the economical production of bioethanol from cellulosic biomass (Fang *et al.* 2008). This is due to the requirement for high enzyme loading given the relative low catalytic efficiency of the hydrolytic enzymes currently available (Margeot *et al.* 2009, Wen *et al.* 2009). The availability of cellulolytic enzymes produced at higher titers, as well as higher specific activities, is desirable as it is deemed critical to reduce the cost of bioethanol production (Singhania *et al.* 2010). Another promising strategy to reduce costs involves the production of cellulolytic enzymes, hydrolysis of biomass and fermentation of the resulting sugars to bioethanol in a single process step via a cellulolytic microorganism or consortium (Lynd *et al.* 2002).

This single step process, termed consolidated bioprocessing (CBP), offers large cost reductions if microorganisms that possess the required combination of substrate utilization and product formation properties can be developed successfully (Lynd et al. 1996). The development of microorganisms for cellulose conversion via CBP can be pursued according to two strategies (Lynd *et al.* 2002). The native cellulolytic strategy involves the improvement of product-related properties, such as yield and tolerance. The recombinant cellulolytic strategy involves engineering non-cellulolytic microorganisms that exhibit high product yields and tolerance so that they are able to utilize cellulose as a result of a heterologous cellulase system.

Heterologous expression of cellulases to enable growth has been investigated primarily in *S. cerevisiae*, enteric bacteria and *Z. mobilis* (Lynd 2002). The yeast *S. cerevisiae* has received the most attention with respect to heterologous cellulase expression, as well as the production of ethanol and other commodity products (Romanos 1992). In this study, a β-glucosidase gene sourced from the filamentous fungus, *A. cellulolyticus* isolated from a triticale compost heap, was cloned and heterologously expressed in *S. cerevisiae* and the recombinant enzymatic activity characterised.

3.3. MATERIALS AND METHODS

3.3.1. Triticale compost heap construction and sampling

A compost heap with triticale straw (sourced from Welgevallen experimental farm, University of Stellenbosch) as main constituent (Fig. 3.1), was constructed on an organic farm (Wechmars Hof, situated in the Helshoogte Valley in the Stellenbosch area). The heap, with final dimensions of 3 m x 2 m x 1.5 m, was stacked with three main layers each made up by several constituents, including organic constituents (mostly triticale straw, supplemented with chicken manure, bone meal, kelp, vermiculate, luzern, kikuyu grass and assorted herb clippings) and inorganics constituents (powdered clay, lime and rock dust). Bunches of reeds, resembling chimneys, were placed in the centre of the heap to allow for effective gas exchange and to enhance an aerobic composting process. The entire heap was layered with wheat straw to serve as insulation layer to maintain optimal composting temperatures. The compost heap was constructed in April 2009 and the final sample was taken in September 2009. The heap was maintained in the open and thus exposed to the prevailing autumn and winter weather conditions. Twenty permeable sample bags (made from a polyester textile, 20 cm x 25 cm) filled with the same triticale straw material, were placed in the central region of the heap. To enable easy sampling with minimal disturbance to the overall composting process, sample bags were closed with nylon rope that extended from the compost heap. Initially, sampling by careful extraction of one of the sample bags, was conducted on a weekly basis and later fortnightly, as measured enzymatic activity diminished over the twenty-two week period.

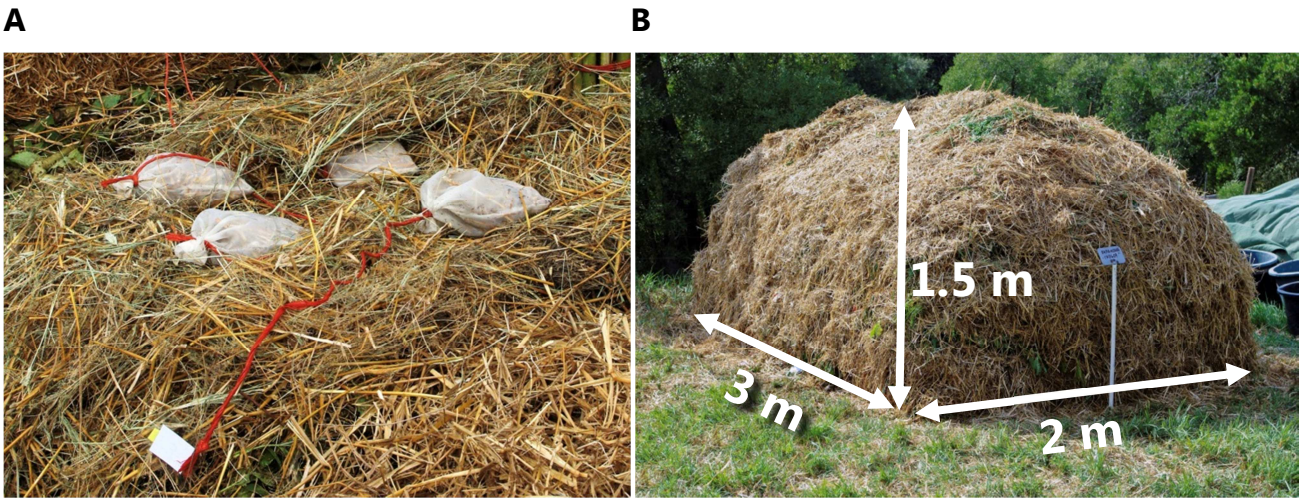

Fig. 3.1. Photographs of (A) compost heap construction showing placement of sampling bags and (B) the completed compost heap, after insulation with wheat straw.

3.3.2. Analysis of compost samples

3.3.2.1. Temperature and pH analysis. Coinciding with each sampling event, the internal compost heap temperature and pH were determined. The internal temperature of the compost heap was measured in the centre of the heap with the use of a TCL 305 digital thermometer, equipped with a 1 meter metal probe. The internal pH of the compost heap was determined in triplicate by measuring the pH of compost slurries (5 g compost in 25 mL 1 M potassium chloride shaken at 200 rpm for 15 min). Three measurements were taken from each of the slurries.

3.3.2.2. β-glucosidase activity assay determination. The collective β-glucosidase activity of the microbial composting community was determined by means of enzymatic activity assays. Extraction of total extracellular enzymes was done in triplicate by mixing 1 g compost in 0.05 M citrate buffer (pH 5) at 200 rpm for 15 min., followed by centrifugation at 13,000 rpm for 5 min. in a bench-top centrifuge. The resultant supernatant was used as catalyst in the enzymatic activity assay. In order to quantify the β-glucosidase activity of the composting community, adaptations were made to the methods used by McCleary and Harrington (1988). For this pupose, 2.5 µL 0.5 M p-nitrophenyl-β-D-glucopyranoside (dissolved in dimethylformamide) was added to 222.5 µL citrate buffer (0.05 M, pH 5) and incubated with 25 µL compost supernatant at 50℃ for 5 min. After incubation, 250 µL 1 M sodium carbonate was added to the reaction to terminate it and to aid in colour development. Absorbance was monitored at 405 nm and compared to a pNP standard curve (0 – 288 µM). The enzymatic activity was deduced from this and expressed as nkat/mg total protein secreted. Protein concentrations were determined using the Biorad DC protein assay kit, as prescribed by the manufacturer (Bio-Rad laboratories, Germany). Each reaction was conducted in triplicate.

3.3.3. Isolation, identification and enzyme characterisation of *Acremonium cellulolyticus* isolate

For the isolation of fungi with cellulolytic activities, carboxymethylcellulose (CMC) was used a sole carbon source. One gram compost was suspended in 10 mL physiological salt solution (0.85% NaCl), mixed by vortexing for 1 min. Serial dilutions (10^{-1} to 10^{-5}) of this mixture were then plated onto synthetic complete (SC) media containing the following components (w/v): 1% CMC, 0.176% yeast nitrogen base (YNB), 0.5% ammonium

sulphate, and 1.5% bacteriological agar. All plates were incubated at 30℃ to allow for growth. Pure cultures were prepared and maintained on malt extract agar (MEA) or stored on MEA slants at 4℃. All chemicals and growth med ium components were supplied by Sigma-Aldrich (USA) or Merck (Germany).

3.3.3.1. Screening isolates for cellulase activity. SC media containing 1% carboxymethylcellulose and 0.2% glucose as carbon sources was used to assess the cellulolytic activity of the different fungal isolates. For plate assays, isolates were cultivated at 30^{o}C for 2 days followed by staining with 0.1% Congo red and destaining with 1 M NaCl. Clearing zones were used as an indicator of cellulase activity. Although this assay is not quantitative, zone diameters were compared to that of the industrial important *Trichoderma reesei* RutC30 strain (Peterson and Nevalainen 2012), which served as a positive control during the screening. The fungal isolate (Trit15F8) was selected based on the zone diameter, which was comparable to that of the positive control.

The reducing sugar assay (Miller *et al.* 1960) was used for quantitative determination of the cellulolytic enzymatic activity of Trit15F8. The fungal isolate was cultivated in 50 mL SC medium with 1% triticale as sole carbon source, to ensure sufficient enzyme induction. Cultures were incubated in 250 mL erlenmyer flasks at 30℃ under constant agitation (200 rpm) for a period of 7 days. Culture supernatant samples from days 3, 5 and 7 were evaluated for endoglucanases activity.

3.3.3.2. Preliminary identification of fungal isolate Trit15F8. A putative identification was performed by sequencing the internal transcribed spacer regions (ITS1 and ITS2) of the nuclear ribosomal RNA gene. For this purpose, the fungal isolate Trit15F8 was cultivated in liquid SC medium with 2% glucose at 30℃ under constant agitation (100 rpm) for 5 days. Biomass was collected by filtration and genomic DNA isolated using the ZR Fungal/Bacterial DNA MiniPrep^{TM} kit (Zymo Research, USA) according to the manufacturer's guidelines. PCR was used to amplify the internal transcribed spacer regions (ITS1 and ITS2) of the nuclear ribosomal RNA gene using ITS1 and ITS4 primers (Table 3.2) and the isolated genomic DNA as template (White *et al*. 1990). The 25 µL PCR reactions contained, 100 ng genomic DNA, 0.2 µM of each primer, 10 µM deoxynucleotides, 1 x buffer and 0.25 µL Ex Taq (5 U) polymerase (WhiteSci,

South Africa). A GeneAmp PCR System 2400 (Perkin Elmer) was used with the following cycling conditions: 30 cycles of denaturing at 94℃ for 60 seconds; annealing at 58℃ for 60 seconds and extension at 72℃ for 60 seconds; and final extension at 72℃ for 7 min. The reaction mixture was separated on a 0.8% agarose (Sigma) gel at 80 V followed by ethidium bromide staining and visualised using UV light. The ±600 bp amplicon was exised and gel purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) and cloned using the InsTAclone™ PCR cloning Kit (Fermentas, Lithuania). Sequence determination was carried out at the Central Analytic Facility, Stellenbosch, South Africa. Analysis of the sequences was performed using DNAMAN software and subjected to similarity search by BLASTn (Basic Local Alignment Tool, http://blast.ncbi.nlm.nih.gov).

3.3.4. Cloning the full-length cDNA sequence of *A. cellulolyticus* β-glucosidase gene

3.3.4.1. Microbial strains and culture conditions. The genotypes of the microbial strains and plasmids used in this study are summarized in Table 3.1. The *A. cellulolyticus* Trit15F8 strain was cultivated in Czapek-Dox Broth (0.3% sodium nitrate, 0.1% dipotassium phosphate, 0.05% magnesium sulphate, 0.05% potassium chloride and 0.001% ferrous sulphate, 0.16% Yeast Nitrogen Base, 0.2% glucose) supplemented with 2% cellobiose (Sigma-Aldrich, USA). Two hundred millilitre media was inoculated with 10^6 spores/mL and incubated in a 1 L Erlenmeyer flask with continues shaking for 72 hours (200 rpm, 30℃). Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α (Invitrogen, USA) and cultivated at 37℃ in Luria-Bertani broth or on Luria-Bertani (LB) agar (Merck, Germany). Transformation was achieved by adhering to the manufacture's recommendations. To allow for selection of resistant bacteria, Ampicillin (Sigma-Aldrich, USA) was added to a final concentration of 100 µg/mL. The *S. cerevisiae* NI-C-D4 strain was cultivated in either yeast extract peptone dextrose (YPD) broth (Merck, Germany) or selective synthetic (SC) medium [2% carbon source, 0.67% yeast nitrogen base (Difco, USA)] containing amino acid supplements.

Table 3.1. Microbial strains and plasmids used in this study

Strain or plasmid	Relevant Genotype	Source or reference
Bacterial strain		
E. coli DH5α	*F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (r_k-, m_k+) phoA supE44 λ- thi-1 gyrA96 relA1*	Invitrogen (USA)
Yeast strain		
S. cerevisiae *NI-C-D4*	*MATα, trp1, ura3, pep4*	Wang *et al*. 2001
Fungal strain		
A. cellulolyticus Trit15F8	*Wild type*	This study
Plasmids		
pDWN001	*bla URA3 $ENO1_{PT}$*	This study
pDWN002	*bla URA3 $ENO1_{PT}$*	This study
ySFI	*bla URA3 $PGK1_P$-BglI- $PGK1_T$*	Van Rooyen *et al.* 2005
pDLG125	*bla URA3 $ENO1_{PT}$*	W.H. Van Zyl, Department of Microbiology, Stellenbosch University

3.3.4.2. DNA manipulations and vector construction. Standard procedures were used for DNA manipulation (Sambrook *et al.* 1989). Enzymes for DNA cleavage and ligation were purchased from either Roche (Germany) or Fermentas (Lithuania) and used as recommended by the supplier. Restriction endonuclease digested DNA and PCR products were visualized on 0.8% agarose gels by staining with Gelgreen^{TM} (Biotium, USA) and using a Dark Reader transillulinator (Clare Chemical Research, USA). DNA bands of interest were excised and purified with the Zymoclean gel DNA recovery kit (Zymo Research, U.S.A.), according to the manufacturer's instructions.

3.3.4.3. RNA isolation from the A. cellulolyticus isolate and first-strand cDNA synthesis. A manual method was used for the isolation of total RNA from the fungal isolate. This method involved the separation of biomass from the media by centrifugation (10,000 rpm, 15 min). To the biomass pellet, 400 µL STE Buffer (0.25 M Tris-HCl pH 7.2, 0.1 M NaCl, and 10mM EDTA) and 400 µL Phenol : Chloroform : Iso-Amyl Alcohol (PCI) (25:24:1) was added, followed by the addition of acid washed glass beads (500 µL) for cell lysis. This mixture was then vortex twice for 30 seconds at maximum speed, with 30

seconds cooling on ice between steps. The cell lysis mixture was centrifuged for 5 min. (13000 rpm at 4℃) and the supernatant transferred to a fresh eppendorf tube. To the supernatant, 1 volume of PCI was added and the extraction repeated until the supernatant was clear. After the final PCI step, 1 volume of Chloroform:Iso-Amyl Alcohol (CI) (24:1) was added to the clear supernatant, vortexed and centrifuged for 5 min. (13000 rpm at 4℃) and the supernatant transferred to a fresh epp endorf tube.

Total nucleic acids was precipitated by adding 1/50 volumes 5 M sodium chloride and 2 volumes 100% ethanol and incubated for 30 min. at -80℃. The sample was then centrifuged 10 min. (13000 rpm and 4℃), washed wit h 70% ethanol, dried and resuspended in 50 µL DEPC-treated nuclease-free water. Total RNA was selectively precipitated from the total nucleic acid mixture with the use of a 3 M lithium chloride solution (Manning 1991). This was followed by DNase treatment with the Turbo DNA-free kit (Ambion, U.S.A.) according to the manufacturer's instructions, to remove any residual genomic DNA before cDNA synthesis. The RevertAid Reverse Transcriptase kit (Fermentas, Lithuania) was used to synthesize first-strand cDNA. This was done according to the manufacturer's instructions.

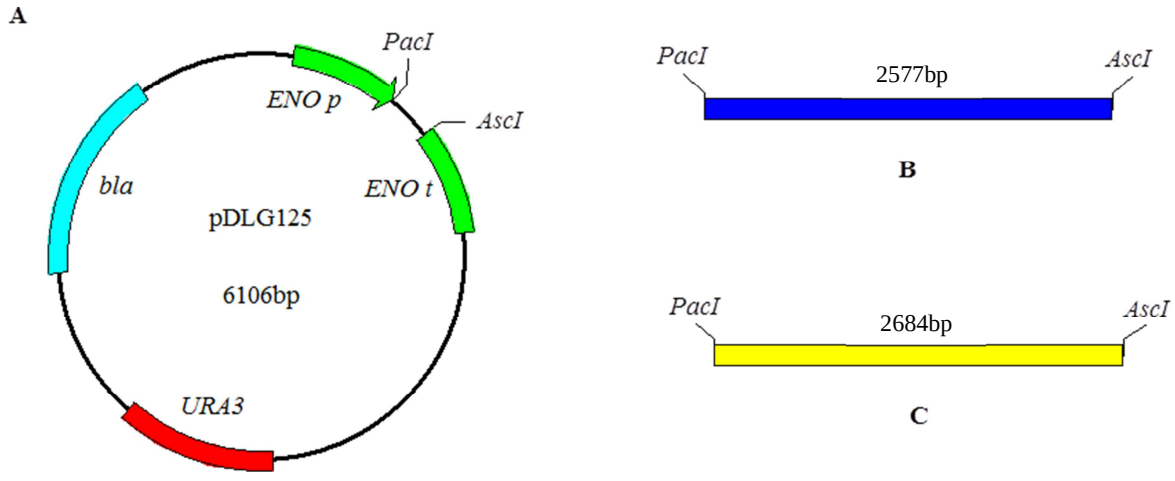

Fig. 3.2: Schematic representation of (A) the multicopy episomal shuttle vector pDLG125 and the genes used to construct the expression cassettes, (B) pDWN001 and (C) pSFI, used for the production and secretion of the recombinant β-glucosidases.

3.3.4.4. PCR amplification and cloning of the β-glucosidase genes. The sequence-specific primers used to isolate the genes encoding the β-glucosidase enzymes of interest, are presented in Table 3.2. Phusion High-Fidelity PCR Master Mix (Finzymes, Finland) was used and reaction parameters recommended by the manufacturer were followed. The *A. cellulolyticus* isolate first–strand cDNA and a plasmid, ySFI (Table 1), containing the complete *S. fibuligera* BGL1 gene was used as templates. Primer set Acbeta-F and Acbeta-R was used for the amplification of the *A. cellulolyticus* β-glucosidase gene and primer set SfiBgl-F and SfiBgl-R for the *S. fibuligera* β-glucosidase gene. PCR products obtained from each reaction were cloned into the multicopy episomal shuttle vector pDLG125 (Table 1). The 2 577 bp fragment amplified from the *A. cellulolyticus* first–strand cDNA was digested with *Pac*I and *Asc*I and ligated into pDLG125 (Fig. 3.2). The 2 684 bp fragment amplified from ySFI was digested with the same enzymes and also ligated into pDLG125, under the control of the constitutive ENO1 promoter. The resulting recombinant plasmids were designated as pDWN001 and pSFI, respectively. The native secretion signals of both genes were kept intact. Sequence determination of the individual constructs was carried out with the use of the ENO-F and ENO-R primers (Table 2). Sequencing was carried out at Inqaba Biotec, South Africa and all bioinformatics analysis of the cloned sequences was executed with CLC Genomics Workbench v5.5.1 software.

Table 3.2. β-Glucosidase genes, their origin and the PCR primers used for their amplification

Primer name	Sequence	Restriction enzyme	Source
Acbeta-F	5'-GACT**TTAATTAA**ATGATCACAATGCGGAACAGTTTATTG-3'	*PacI* (NEB, USA)	This study, GenBank
Acbeta-R	5'-GACT**GGCGCGCC**TTAGTGTCCAATGTTGAGAGCC-3'	*AscI* (NEB, USA)	Accession no. BD168028
OVRLprimer1	5'-CATTGCCCTCCCTCCCAGCCAACACCGGTCGTCAAG-3'	-	This study
OVRLprimer2	5'-CCTGACCGGTGTTGGCTGGGAGGGAGGGCAATG-3'	-	
SfiBgl-F	5'-GACT**TTAATTAA**ATGGTCTCCTTCACCTCCCTCCTCG-3'	*PacI* (NEB, USA)	Van Rooyen *et al.* 2005
SfiBgl-R	5'-GACT**GGCGCGCC**TCAAATAGTAAACAGGACAGATGTC-3'	*AscI* (NEB, USA)	
ENO-F	5'-GTAATCCCTTATTCCTTCTAGCTAT-3'	-	This study
ENO-R	5'-CTATTCTAAGAAAATGAAATAAATGACAA-3'	-	
ITS1	5´TCCGTAGGTGAACCTTGCGG-3´	-	Altschul *et al.* 1997
ITS4	5´-TCCTCCGCTTATTGATATGC-3´	-	

The restriction sites included in the primers are indicated in bold.

3.3.4.5. Intron splicing by OE-PCR. Overlap Extension PCR was used to remove an intron between basepair 188 and 242 of the β-glucosidase gene in pDWN001. This entailed a two staged PCR amplification of the pDWN001 insert. During the first stage, the gene specific primers are used in conjunction with specifically designed primers, OVRLprimer1 and OVRLprimer2 (Table 3.2) to create 17 bp nucleotide overhangs complementary to the regions flanking the intron. The PCR reaction mixtures (50 µL) were as follows: 120 ng pDWN001, 250 pmol Acbeta-F or Acbeta-R, 250 pmol OVRLprimer-1 or OVRLprimer2 and Phusion High-Fidelity PCR Master Mix (Finzymes), with cycling parameters as recommended by the manufacturer. Following cycling, the two PCR reactions were pooled and purified using the High Pure PCR Cleanup kit (Roche, Germany). During the second stage of the process, the combined products of stage one was used as template in PCR reaction using only the gene specific primers in order to yield a single full-length intronless product. The component quantities and cycling parameters are the same as in stage one. The product of this reaction were cloned as previously described into the modified pDLG125 plasmid, giving rise to the recombinant plasmid pDWN002. Sequencing and bioinformatics analysis, as previously described, was used to confirm the successful removal of the intron.

3.3.4.6. Yeast transformation. *S. cerevisiae* NI-C-D4 was transformed with the individual recombinant plasmids, pSFI, pDLG125 and pDWN002 by the LiAc/SS carrier DNA/PEG method described by Gietz and Schiestl (2007). Disruption of the uracil phosphoribosyltransferase (FUR1) gene in the plasmid-containing *S. cerevisiae* transformants was performed to ensure autoselection of the *URA3*-bearing expression plasmids in non-selective medium (Kern *et al.* 1990). The fur1::LEU2 autoselective transformants were screened for on selective plates deficient in uracil and leucine.

3.3.5. β-glucosidase production profile by the recombinant

A growth curve analysis was conducted to determine the recombinant β-glucosidase production profile by the *S. cerevisiae* NI-C-D4 transformants, as well as the effect the recombinant *A. cellulolyticus* β-glucosidase gene might have on yeast cell growth. Cells of *S. cerevisiae* NI-C-D4[pDWN002], *S. cerevisiae* NI-C-D4[pSFI] (positive control) and *S. cerevisiae* NI-C-D4[pDLG125] (negative control) was inoculated in triplicate to A_{600nm} = 0.1

from overnight precultures, into 250 mL Elermyer flasks containing 50 mL SC^{-ura} broth, with glucose as sole carbon source. The selective media was supplemented with the antibiotics chloramphenicol (300 µg/mL) and streptomycin (500 µg/mL) to inhibit bacterial growth. After inoculation, flasks were incubated on an orbital shaker (200 rpm) at 30℃. After 8 hours of initial growth, samples (400 µL) were removed in triplicate from each flask and both, A_{600nm} and β-glucosidase activity (as previously described) were measured. Sampling was repeated every 3 hours until 44 hours of growth time was reached. Thereafter, sampling was conducted at 12 hours intervals, up to 104 hours of growth.

3.3.6. Characterisation of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002]

3.3.6.1. Effect of pH and temperature on enzymatic activity. The optimal pH of the β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was determined using culture supernant as enzyme preparation and pNPG as substrate (50℃, pH 3.0 − 8.0). Different buffers were used to prepare reactions (as previously described), leading to individual reactions at different pH values, i.e. 50 mM citrate buffer (pH 3.0 − 4.0), 50 mM phosphate-citrate buffer (pH 5.0−6.0) and 50 mM phosphate buffer (pH 7.0 − 8.0). The optimum temperature of the recombinant β-glucosidase was determined in the same manner by measuring enzymatic activity (pH 5.0) in the temperature range of 20 − 90℃. Standard enzymatic reactions were prepared and incubated at the different temperatures. Temperature stability was measured by analysing residual activity after incubation of enzyme aliquots for 0 - 60 min. at 60℃, 70℃ and 8 0℃, respectively. Enzymatic activity was expressed relative to maximal activity observed for that specific parameter.

3.3.6.2. Effect of selected monosaccharides and ethanol on enzymatic activity. The effect of selected monosaccharides (glucose and xylose) and ethanol on the enzymatic activity of the β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was investigated using pNPG as substrate at pH 5.0 and 50℃. Reactions were prepared (as previously described) with the use of culture supernatant as enzymatic preparation and the addition of glucose, xylose or ethanol to various final concentrations. Individual reactions were prepared to contain final concentrations of glucose or xylose (ranging from 0 – 250

mM) or ethanol (ranging from 0 - 50%). Enzymatic activity was expressed relative to the activity observed from reactions containing none of the supplementary compounds.

3.3.7. Isolation of extracellular and intracellular protein fractions from *S. cerevisiae* NI-C-D4

Extracellular protein fractions were isolated by harvesting culture supernatant. Overnight cultures of *S. cerevisiae* NI-C-D4[pDWN002], *S. cerevisiae* NI-C-D4[pSFI] and *S. cerevisiae* NI-C-D4[pDLG125] were grown with constant shaking (37℃, 200rpm) in 50 mL SC^{-ura} media. From this, 15 mL was centrifuged for 10 min. (3 000 x g, 4℃). The supernatant was transferred to a Vivaspin 15 (10 000 MWCO Polyethersulfone) centrifugal filter device (Millipore, USA) and centrifuged in a swinging bucket rotor (4˚C, 3000 x g, 2 hours). The 100 µL concentrated supernatant was transferred to a 1.5 mL eppendorf tube and stored at 4˚C. For the isolation of the intracellular protein fractions, pelleted yeast cells from the same overnight cultures were subjected to the YeastBuster protein extraction reagent (MERCK, Germany) and intracellular protein isolated according to the manufacturer's specifications. The isolated proteins were stored at -20˚C.

3.3.8. SDS-PAGE analysis of recombinant *A. cellulolyticus* β-glucosidases expressed in *S. cerevisiae* NI-C-D4

The concentrated extracellular and intracellular protein fractions of *S. cerevisiae* NI-C-D4[pDWN002], *S. cerevisiae* NI-C-D4[pSFI] and *S. cerevisiae* NI-C-D4[pDLG125] was subjected to SDS-PAGE analysis. To 15 µl of each sample, 5 µl of reducing sample buffer [12% SDS (w/v); 6% β-Mercaptoetanol (v/v); 30% Glycerol (w/v); 0.05% Coomassie blue G-250 (w/v) and 150mM Tris-HCl (pH7.0)] was added and the samples boiled (5 min., 95℃). For size determination, the PageRuler Prest ained Protein Ladder (10-170 kDa; Fermentas, Lithuania) was used. The protein fractions were separated by an 8 % (v/v) SDS-PAGE according to the method of Laemmli (1970). Electrophoresis was conducted at 120 volts for approximately 1.5 hours. After gel electrophoresis, the rapid silver staining method was used according to Bloom et al. (1987).

3.3.9. Zymogram analysis of recombinant *A. cellulolyticus* β-glucosidases expressed in *S. cerevisiae* NI-C-D4

SDS-PAGE was performed as described above except for the following differences: 0.2% 4-methylumbelliferyl β-D-glucopyranoside (MUG; Sigma-Aldrich, USA) was added to the resolving gel to visualise the β-glucosidase activity. To 15 µl of each sample, 5 µl of reducing sample buffer without β-Mercaptoethanol was added and the samples incubated for 5 min at 60℃. After gel electrophoresis the SDS was removed from the gel by incubating the gel in 2.5% Triton X-100 (30 min., room temperature). The Triton X-100 was decanted and the gel incubated at 50℃ for 30 min in a hybridization oven. Protein bands were visualised by fluorescence under UV illumination.

3.4. RESULTS AND DISCUSSION

3.4.1. Triticale composting and β-glucosidase enzymatic activity profile

Over a period of 152 days, the temperature of the compost heap fluctuated between a high of 57.2℃ and a low of 16.1℃. The measured temperatures correlates with what is expected of a self-heating, aerobic composting heap (Goyal *et al.* 2005, Ishii *et al.* 2000, Khalil *et al.* 2001, Steger *et al.* 2003). Temperature evolution is considered to be a reflection of the metabolic activity of the microbial populations involved in the composting process (Fernandes *et al.* 1994) and is used to divide the process into four distinctive phases (Ishii *et al.* 2000). These phases are the mesophillic, thermophillic, cooling and maturation phases. The mesophillic phase is characterized by the growth and development of a mesophillic composting microbial community (Steger *et al.* 2003). The degradation of organic matter by this community leads to a rapid increase in temperature (Khalil *et al.* 2001) and to the next phase in the composting process. During the thermophillic phase, a thermophillic microbial composting community develops and is responsible for the degradation of organic matter (Ishii *et al.* 2000, Goyal *et al.* 2005, Khalil *et al.* 2001, Steger *et al.* 2003). At the end of this phase, temperatures drop gradually and leads to the final two phases of composting, cooling and maturation,when an entirely new mesophillic community develops (Steger et al. 2003).

The four distinctive phases in composting can be clearly identified from the resultant data (Fig. 3.3). After a short initial mesophillic phase (20 – 50℃), a thermophillic phase

(>50℃) followed where the temperature reached a high of 57.2℃, which then gradually drops after day 5 down to mesophillic ranges. This heralds the initiation of the cooling and maturation phases. Over the full sampling period of 152 days, the pH (data not shown) of the compost heap remained fairly stable, only fluctuating between a high of 8.24, at the end of the thermophillic phase and a low of 7.33 late into the maturation phase.

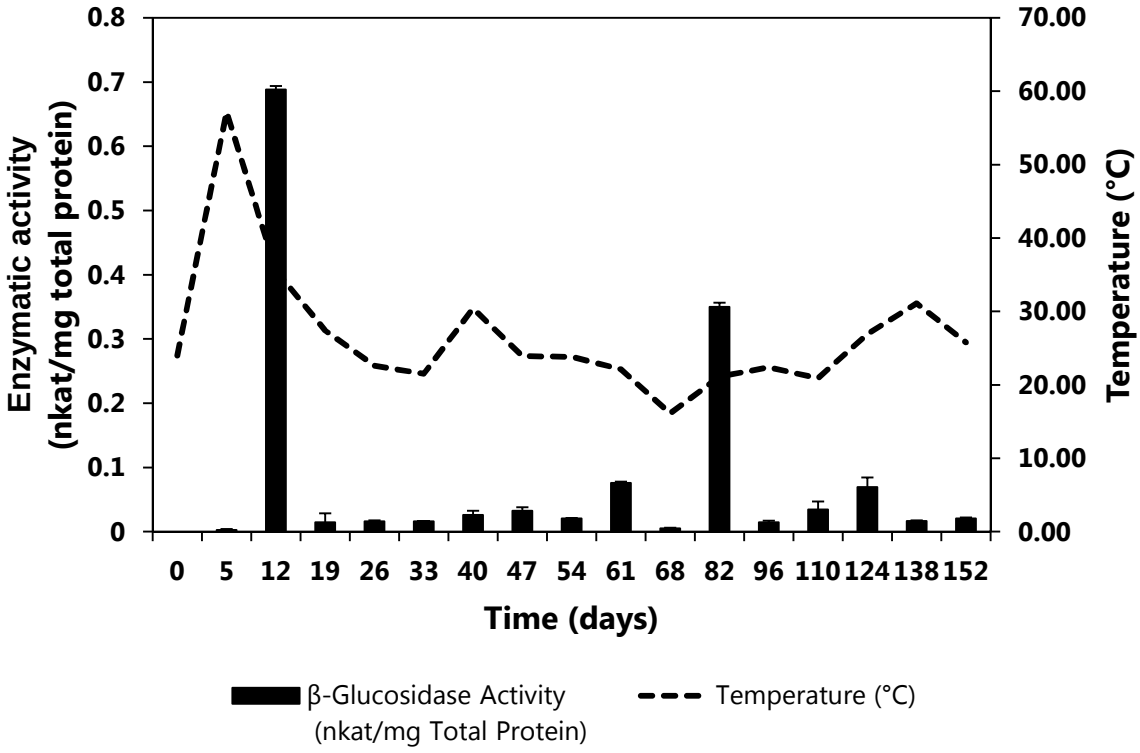

Fig. 3.3: Graph showing both the temperature and collective β-glucosidase activity profile of the triticale compost heap over the sampling period of 152 days.

The β-glucosidase activities within the compost heap peaked at 0.69 nkat/mg total protein secreted twelve days into the process. This correlates with the transition from the mesophillic phase to the thermophillic phase and the stage of highest expected degradation of organic material. During the cooling and maturing phases, a completely new mesophillic population of microbes will have developed, which according to literature mainly constitutes filamentous fungal species (Klamer 2008, Ryckeboer 2003, Steger et al. 2003, Goyal *et al.* 2005). Eighty-two days into the composting process, the highest β-glucosidase activity of this new mesophillic population was measured at 0.35 nkat/mg total protein.

3.4.2. Isolation and identification of an *Acremonium cellulolyticus* isolate from the triticale compost heap

The fungal isolate Trit15F8 was originally isolated during the mesophillic stage of the triticale composting process having the ability to grow on CMC as sole carbon source. Closer inspection of isolate Trit15F8 showed a high level of cellulase activity after seven days when grown on triticale as sole carbon source (Fig. 3.4). The cellulase activity was greater compared to the *T. reesei* RutC30 strain. The hypersecreting catabolite-derepressed mutant *T. reesei* RutC30 strain was originally produced in the late 1970's (Montenecourt and Eveleigh 1979) and is regarded as a benchmark for cellulase production in fungi (Peterson and Nevalainen 2012). Preliminary identification of isolate Trit15F8 based on the ±600 bp of the amplified ITS region showed 99% homology using NCBI's BLAST to the available internal transcribed spacer region sequence of *A. cellulolyticus* (Altschul *et al*. 1997).

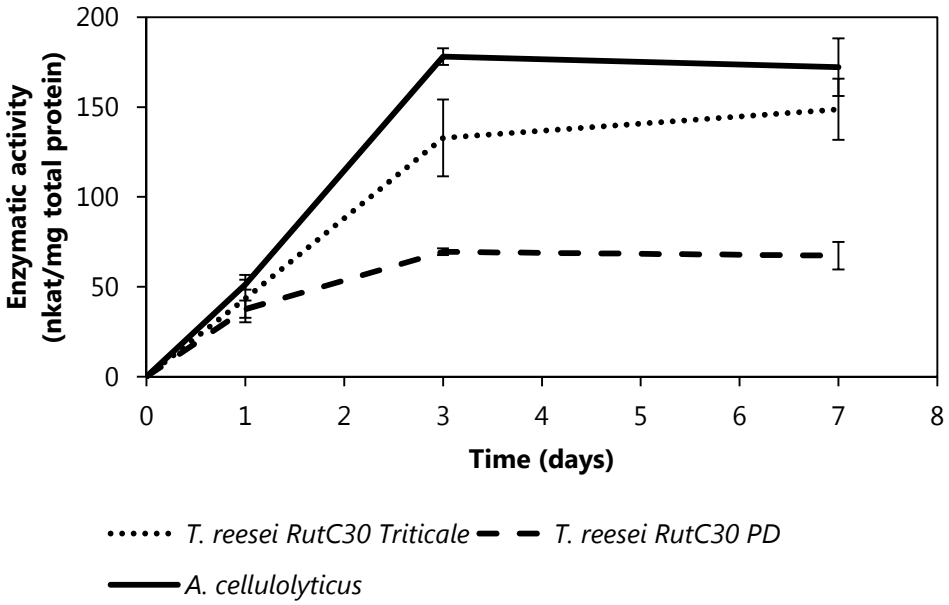

Fig. 3.4: Endo-glucanase activity of the fungal isolate Trit15F8 when cultivated on 1% triticale bran for 7 days. The measured activity was compared to that of *Trichoderma reesei* RutC30 cultivated on both 1% triticale bran and potato dextrose (PD).

3.4.3. Cloning and expression of the *A. cellulolyticus* β-glucosidase gene in *S. cerevisiae*

RNA was isolated from *A. cellulolyticus* grown on cellobiose as sole carbon source for 72 hours (30℃, 200 rpm). RNA devoid of any DNA conta mination (after LiCl precipitation and DNase treatment) was then used to obtain cDNA copies of the β-glucosidase gene (2577 bp), with the aid of gene-specific primers (Table 3.2) and a reverse transcriptase reaction. This gene was then cloned using restriction enzymes *PacI* and *AscI* into the shuttle vector pDLG125 (linearized with the same restriction enzymes) and the resulting expression plasmid (pDWN001) was used to transform *S. cerevisiae* NI-C-D4. The resulting yeast transformants containing the expression vector pDWN001 however showed retarded and poor overall growth on cellobiose (as sole carbon source) and no significant β-glucosidase activity could be detected in subsequent enzyme assays (data not shown).

After in-depth sequence analysis, the presence of an intron at 188 bp downstream from the 5' end of the β-glucosidase, was confirmed (Fig. 3.5). The intron was successfully removed by overlap-extension PCR and confirmed by sequencing of the entire open reading frame (Fig. 3.5). Comparison of the final cDNA β-glucosidase gene revealed a total of nine basepair changes in the β-glucosidase obtained in this study compared to the NCBI gene sequence (Accession number: BD168028), which translated into four amino acid (aa) changes (V418A, T484S, L796I, I821V) in the final β-glucosidase protein sequence when compared to the protein sequence on the NCBI database (Fig. 3.6). In summary, all four of the aa changes constituted changes between aa with the same properties in terms of polarity and charge. For example, at aa position 418, the nonpolar, neutral aa valine changed into nonpolar, neutral aa alanine.

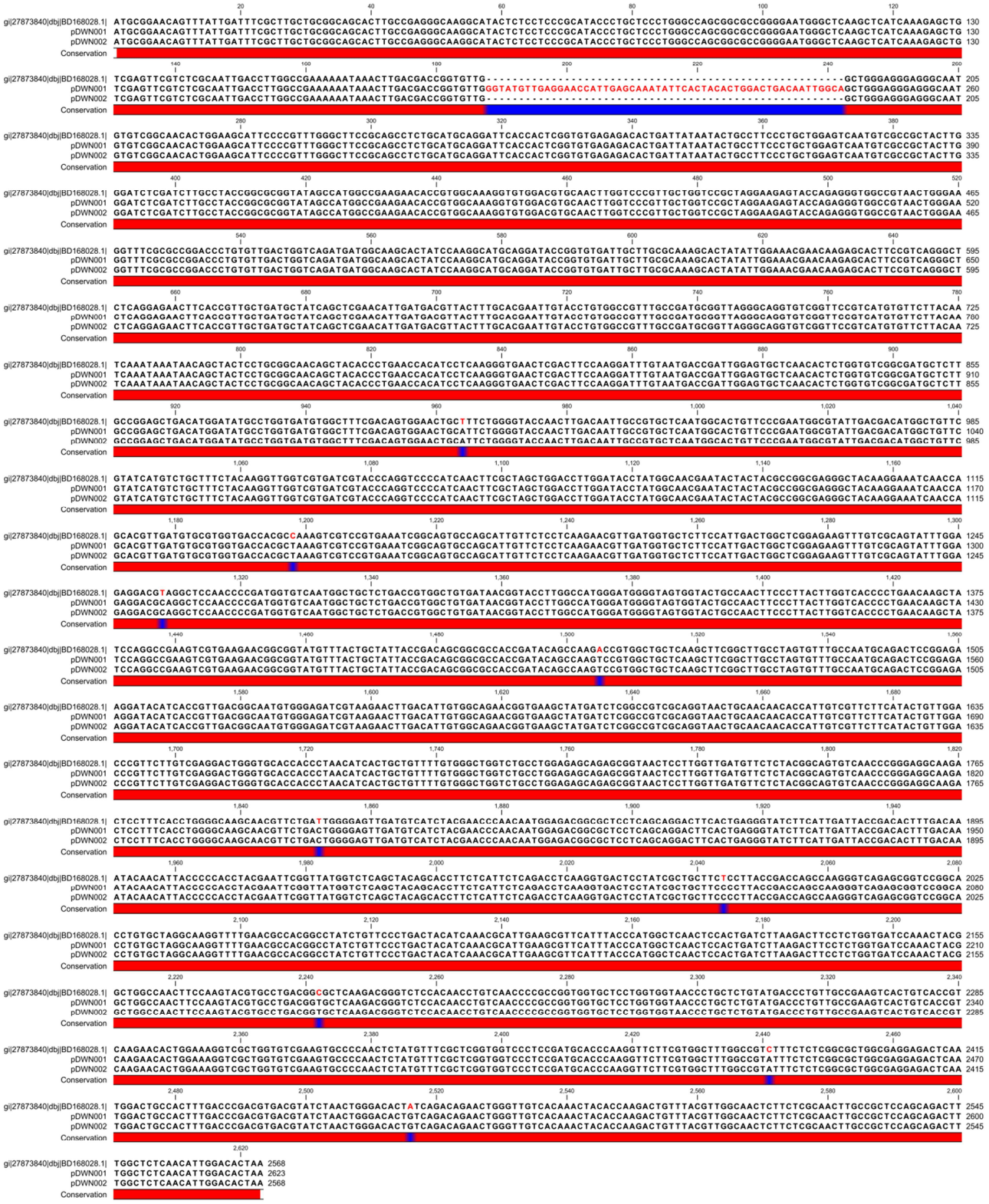

Fig. 3.5: Sequence alignment of the β-glucosidase genes from *A. cellulolyticus* obtained from NCBI (Accession number: BD168028), compared to the first cDNA clone obtained (pDWN001) in this study and the final cDNA clone (pDWN002) after a further round of overlap-extension PCR to remove the remaining intron between basepair 188 and 242. Conserved basepairs are indicated by the red bar and nucleotide changes by the blue bar.

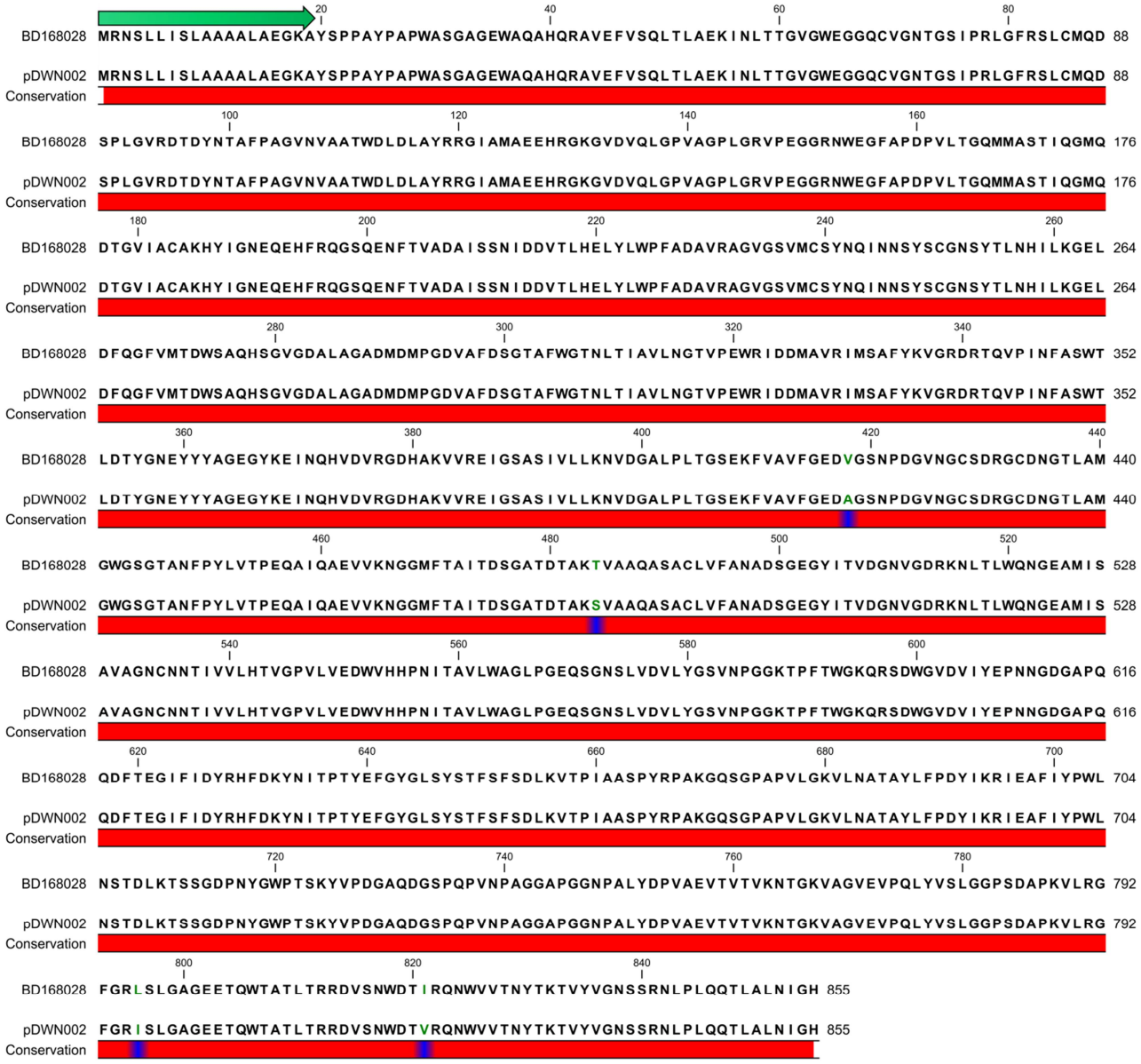

Fig. 3.6: Amino acid sequence alignment of the β-glucosidase protein from *A. cellulolyticus* obtained from NCBI (Accession number: BD168028), compared to final cDNA clone (pDWN002) obtained in this study. Conserved aa are indicated by the red bar and aa changes by the blue bar. The native secretion signal as predicted with CLC Genomics Workbench v5.5.1 software is indicated by the green arrow.

3.4.4. Growth analysis and β-glucosidase production profile by the recombinant *S. cerevisiae* NI-C-D4[pDWN002]

The Growth analysis and β-glucosidase production profile by the recombinant organisms were monitored for 104 hours. After a short lag phase, the *S. cerevisiae* NI-C-D4[pDWN002], and both the positive *S.cerevisiae* NI-C-D4[pSFI] and negative control *S. cerevisiae* NI-C-D4[pDLG125] entered the exponential growth phase (Fig. 3.7). The *S.*

cerevisiae NI-C-D4[pDLG125] strain entered the exponential growth phase slightly faster than the other two recombinant strains. This indicates that the production of the recombinant β-glucosidases had a minor metabolic burden on the host cells, with the recombinant β-glucosidase (*BGL1*) originating from *Saccharomycopsis fibuligera*, having the most significant effect, with the slowest exit from the lag phase (*S. cerevisiae* NI-C-D4[pSFI]). It is, however, important to note that all three strains reached an eventual maximum growth of A_{600} of 3.4 during the stationary phase of cell growth after 32 hours.

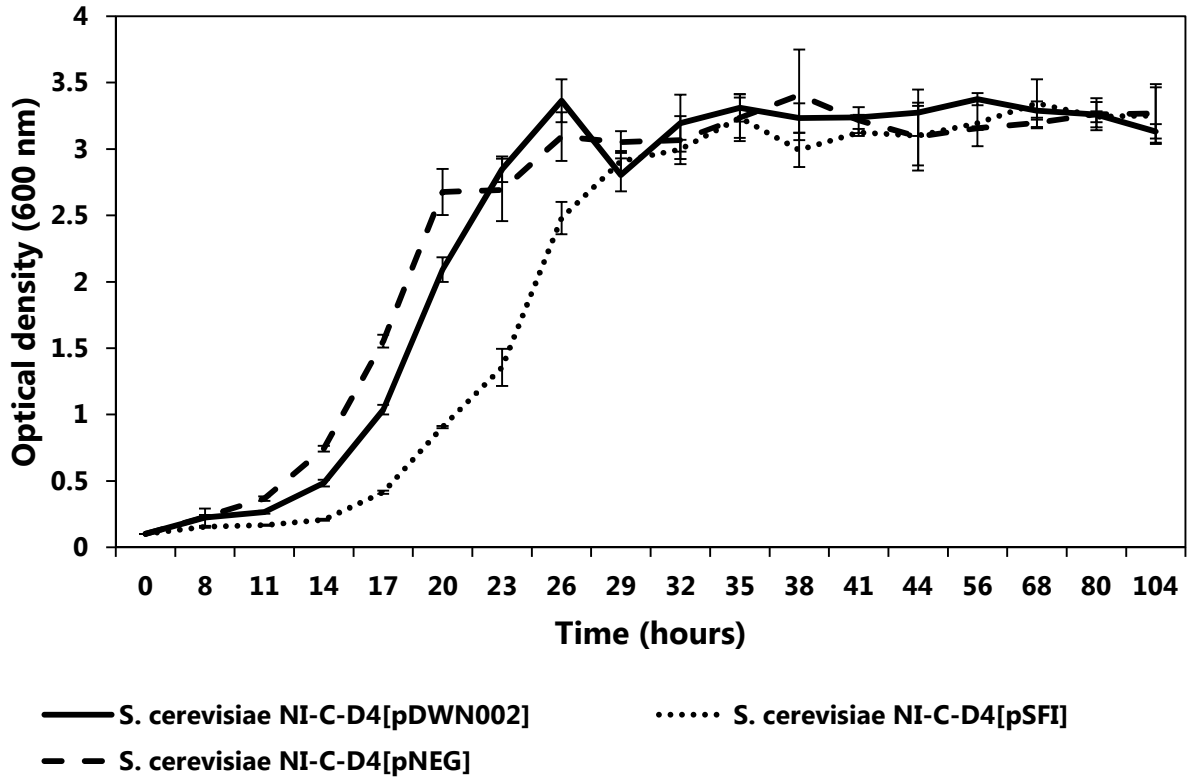

Fig. 3.7: Graphical comparison of the growth of the *Saccharomyces cerevisiae* NI-C-D4[pDWN002], containing the recombinant *A. cellulolyticus* β-glucosidase gene, to that of both a positive and a negative control. Cultures were cultivated in SC^{-ura} broth selective media supplemented with antibiotics, with constant shaking (200 rpm, 37℃).

β-glucosidase enzymatic activity assays on culture supernatant, also conducted in this time frame, showed that both recombinant β-glucosidases *S. cerevisiae* NI-C-D4[pDWN002] and *S. cerevisiae* NI-C-D4[pSFI]) were expressed during the exponential growth phase (Fig. 3.8). This also indicates that the native secretion signal of both recombinant genes are intact and working (see section 3.4.7). The recombinant *A. cellulolyticus* β-glucosidase (*S. cerevisiae* NI-C-D4[pDWN002]) was expressed as early on

as 20 hours after inoculation, with the *S. fibuligera* β-glucosidase (*S. cerevisiae* NI-C-D4[pSFI]) following closely at 23 hours. Enzymatic activity was expressed as nkat/mg total protein secreted, with the *S. fibuligera* β-glucosidase produced by *S. cerevisiae* NI-C-D4[pSFI] displaying a 2-fold β-glucosidase enzymatic activity compared to that of *S. cerevisiae* NI-C-D4[pDWN002]. This and the fact that the two recombinant β-glucosidases were cloned and expressed using the same shuttle vector and *S. cerevisiae* strain, lead to the assumption that the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pSFI] has twice the specific activity than that of *S. cerevisiae* NI-C-D4[pDWN002].

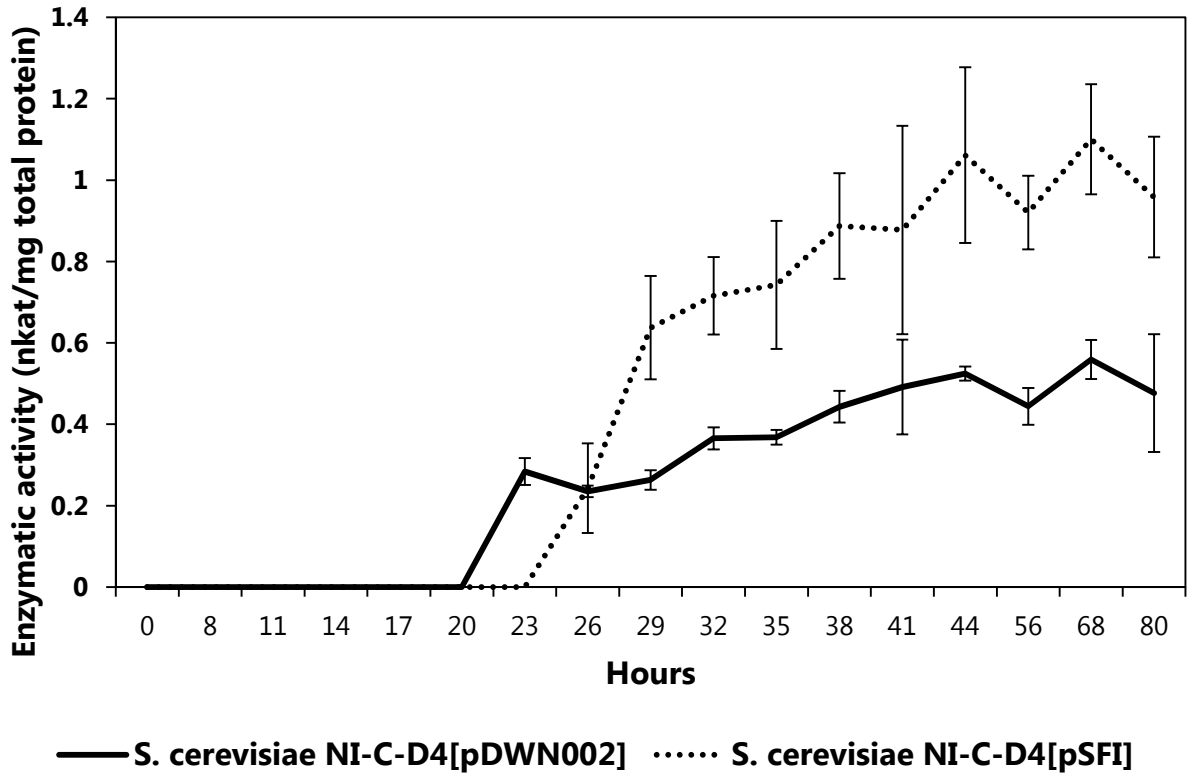

Fig. 3.8: Graph showing the enzymatic activity of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002], compared to that of *S. cerevisiae* NI-C-D4[pSFI]. Measured activity of the recombinant β-glucosidases was expressed as nkat/mg total protein secreted

3.4.5. Effect of pH and temperature on enzymatic activity

The recombinant *A. cellulolyticus* β-glucosidase protein produced by *S. cerevisiae* NI-C-D4[pDWN002] showed maximal relative enzymatic activity at 70℃ (Fig. 3.9). This correlates to optimal temperatures measured by both Yamanobe *et al.* (1985) and Fukasawa *et al.* (2007) for β-glucosidase isolated from the same organism. In the patent

filed by Yamanobe and co-workers, it is claimed that the enzyme excels in thermostability and allows for saccharification at 5 to 10℃ higher than other commonly used enzymes. It was noted from reviewed literature that the optimal temperatures of β-glucosidases isolated from various fungal species falls in the range of 40 – 60℃ (Belancic *et al.* 2003, Bhiri *et al.* 2008, Daroit *et al.* 2008, Galas and Romanowska 1996, González-Pombo *et al.* 2008, Lin *et al.* 2010, Kalyani *et al.* 2012, Karboune *et al.* 2008, Karnchanatat *et al.* 2007, Kaur *et al.* 2007, Langston *et al.* 2006, Wei *et al.* 2006, Yan and Lin 1997). Also supporting the claim made by Yamanobe and co-workers, that the β-glucosidase isolated from *A. cellulolyticus* allows for substrate saccharification at elevated temperatures.

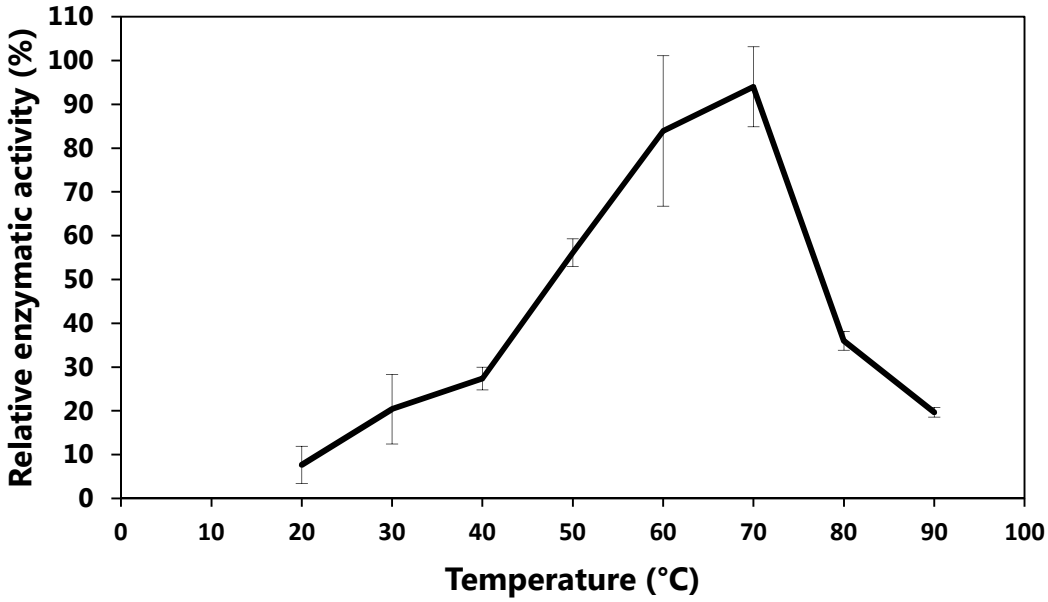

Fig.3.9: Graph depicting the relative activity of the recombinant *A. cellulolyticus* β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002], as measured in triplicate in the temperature range of 20 − 90℃.

The recombinant *A. cellulolyticus* β-glucosidase protein produced by *S. cerevisiae* NI-C-D4[pDWN002] showed good stability (Fig. 3.10) at 60℃, retaining more than 70% of its maximum relative activity for up to 60 min. Stability of its activity at 70℃ gradually dropped from a 100% to less than 30% over a period of 60 min. Stability of the recombinant *A. cellulolyticus* β-glucosidase expressed by *S. cerevisiae* NI-C-D4[pDWN002] was poor at 80℃; enzymatic activity dr opped from 100% to less than 30% after only 5 min incubation. The data for stability of enzymatic activity correlates to the findings of other investigators for the β-glucosidase isolated from the same organism (Yamanobe *et al.* 1985). Interest in identifying novel β-glucosidases with high optimal

temperature and thermostability have been increasing in the last decade as most applications of β-glucosidases require higher temperature (50℃ or a bove) (Li *et al.* 2012). Data obtained in this study shows that the recombinant *A. cellulolyticus* β-glucosidase expressed by *S. cerevisiae* NI-C-D4[pDWN002] excels in both thermostability and activity at temperatures higher than 50℃ .

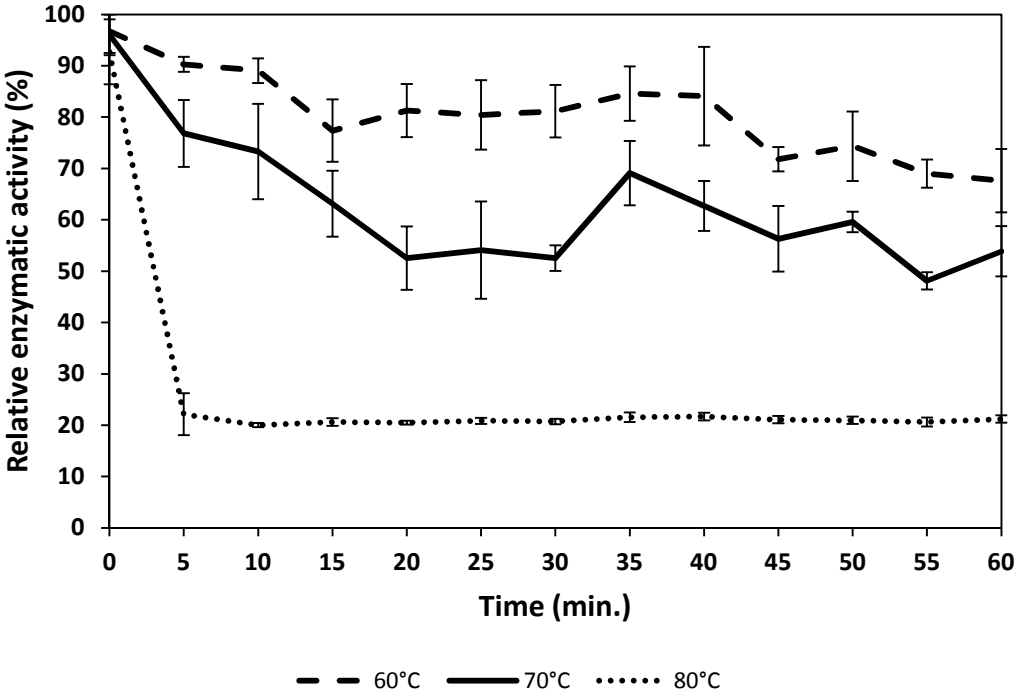

Fig. 3.10: Graph depicting the temperature stability of the recombinant *A. cellulolyticus* β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002], as measured in triplicate at 60°C, 70℃ and 80℃, respectively.

The enzymatic activity of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was measured at various pH values (3.0 − 8.0) using pNPG as substrate (Fig. 3.11). The enzyme displayed moderate to high activity (47% - 100%) at pH values between 3.0 and 6.0 with a pH optimum of 4.0. The measured pH optima correlates to the finding of Yamanobe *et al.* (1985) and Fukasawa *et al.* (2007) for a β-glucosidase isolated from the same organism. From reviewed literature for β-glucosidases isolated from various fungal species (Belancic *et al.* 2003, Bhiri *et al.* 2008, Daroit *et al.* 2008, Galas and Romanowska 1996, González-Pombo *et al.* 2008, Lin *et al.* 2010, Kalyani *et al.* 2012, Karboune *et al.* 2008, Karnchanatat *et al.* 2007, Kaur *et al.* 2007, Langston *et al.* 2006, Wei *et al.* 2006, Yan and Lin 1997), it can be deduced that the

pH optima (4.0) of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] falls in the lower ranges.

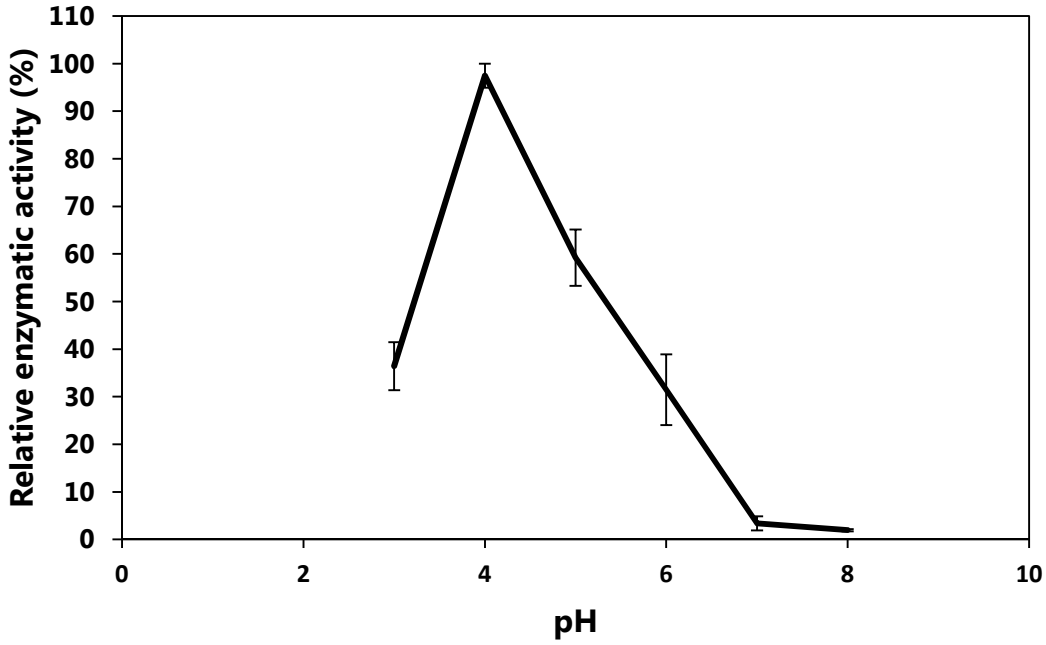

Fig. 3.11. Graphical depiction of the relative activity of the recombinant *A. cellulolyticus* β-glucosidase enzyme across a range of pH values. Buffers at set pH values, were used to setup enzymatic reactions in triplicate.

3.4.6. The effect of selected monosaccharides and ethanol on the enzymatic activity of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002]

Many β-glucosidases are strongly inhibited by monosaccharides such as glucose and xylose, constituting one of the main limiting factors in the efficient and complete hydrolysis of cellulose (Bhatia *et al.* 2002, Zanoelo *et al.* 2004). The effect of glucose and xylose on the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was investigated in a range of concentrations (0 – 250mM).

Results showed that the presence of glucose severely affected the enzymatic activity (Fig. 3.12), with a drop of 35% in maximal activity observed at a final glucose concentration of 10 mM. As expected, increasing levels of glucose amplified the effect, with only 50% and 30% of the enzyme's maximum activity remaining at 30 mM and 250 mM glucose, respectively. The data and current literature suggests that the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] has low tolerance to glucose

(Li *et al.* 2012). Most reported microbial β-glucosidases are also strongly inhibited by glucose, losing half of their maximal enzymatic activity at glucose concentrations of 0.35-100 mM when assayed with pNPG as substrate (Dotsenko *et al.* 2012; Karnchanatat *et al.* 2007; Krisch *et al.* 2012).

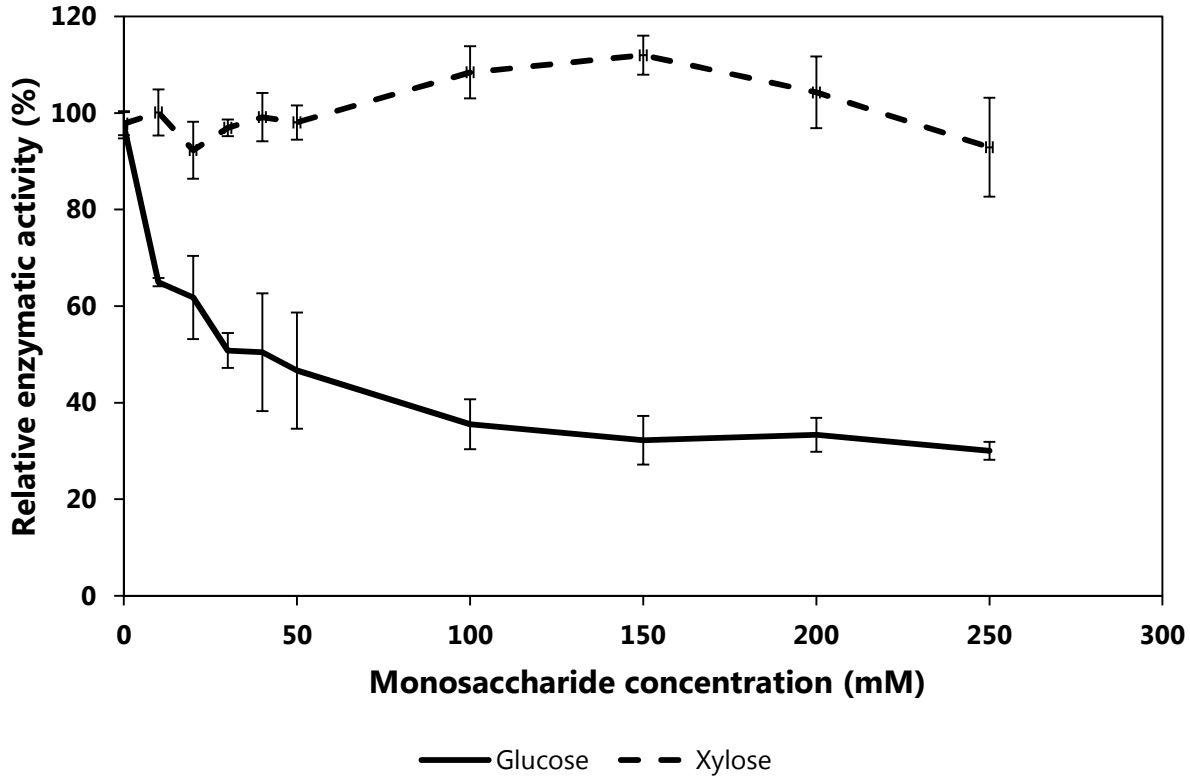

Fig. 3.12. Graph showing the relative effect of different concentrations of glucose and xylose respectively, of the recombinant *A. cellulolyticus* β-glucosidase enzyme produced by *S.cerevisae* NI-C-D4[pDWN002]

Surprisingly, the enzymatic activity of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was slightly enhanced in the presence of xylose (Fig. 3.12). The maximal stimulatory effect of about 10% was observed at a xylose concentration of 150 mM. This stimulatory effect of xylose on β-glucosidases has been reported by other researchers, with a maximal effect of up to a 2.4-fold increase in activity (Zhao *et al*. 2012, Zanoelo *et al.* 2004).

The effect of ethanol on the enzymatic activity of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was investigated in the range of 0 – 50% (Fig. 3.13). Ethanol at a certain range of concentrations has been proven to increase the activity of some β-glucosidases (Gopalan *et al.* 1989, Grogan 1991, Plant *et al.* 1988). In this study,

ethanol proved to have an inhibitory, rather than a stimulating effect on enzymatic activity. At a final ethanol concentration of 5%, the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] retained approximately 94% maximal enzymatic activity. From 5% to 15 % ethanol concentration, an approximate drop of 58% in enzymatic activity was observed. When ethanol concentrations were further increased enzymatic activity stabilised, with only a gradual decrease in activity. Enzymatic activity levels of approximately 33% – 25% was observed in the range 20% – 50% ethanol.

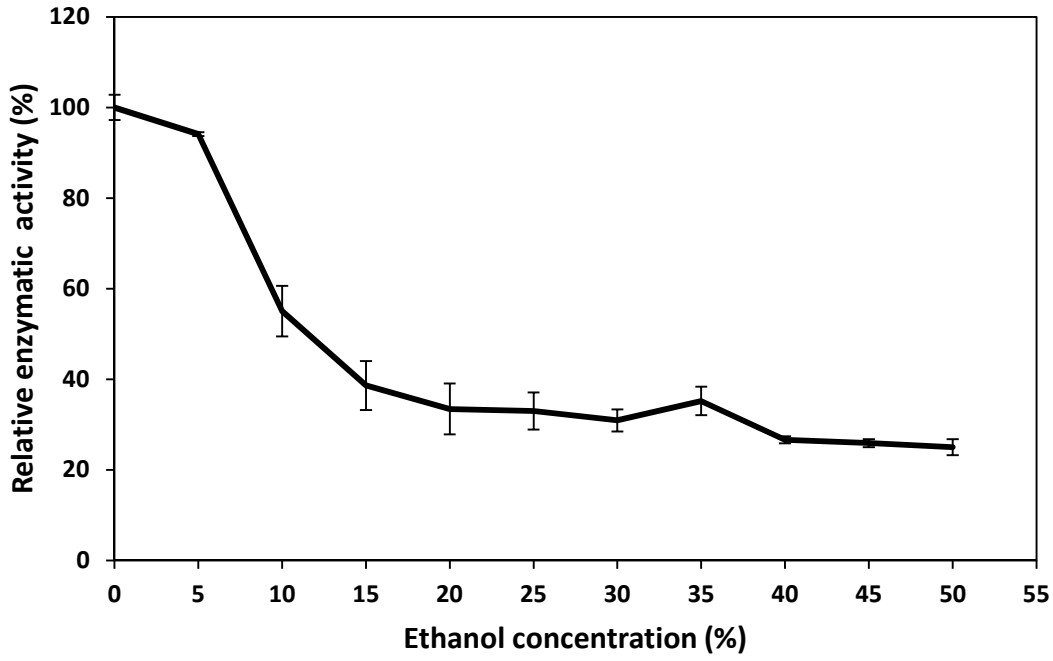

Fig. 3.13. Graph showing the tolerance towards ethanol, exhibited by the recombinant *Acremonium cellulolyticus* β-glucosidase enzyme produced by *S. cerevisiae* NI-C-D4[pDWN002]. Tollerance was determined in triplicate by incubating standard enzymatic activity reactions in the prescence of different concentrations of ethanol.

3.4.7. Protein size determination and Zymogram analysis

In order to determine the apparent molecular weight of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002], concentrated culture supernatant was subjected to SDS-PAGE analysis (Fig. 3.14). In order to test the effectivity of its native secretion signal, an intracellular protein fraction (lane 2) was subjected to the same treatment. In conjunction to SDS-PAGE, Zymogramphy was conducted on concentrated culture supernatant of *S. cerevisiae* NI-C-D4[pDWN002] (lane 8). Concentrated culture

supernatant and intracellular protein fractions of *S. cerevisiae* NI-C-D4[pSFI] (lanes 3, 6 and 9) and *S. cerevisiae* NI-C-D4[pDLG125] (lane 4, 7 and 10) was used as positive and negative control, respectively.

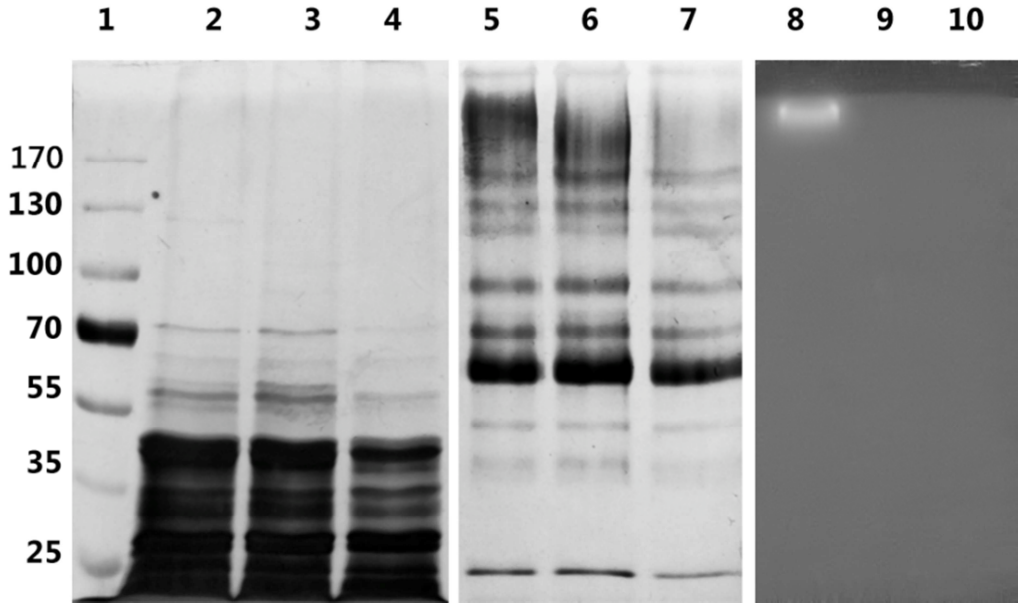

Fig. 3.14. SDS PAGE gel analysis and Zymogram detection of recombinant β-glucosidase. Lane 1: PageRuler Prestained Protein Ladder (10-170 kDa), Lane 2-4: Intracellular protein fractions of *S. cerevisiae* NI-C-D4[pDWN002] (lane 2), *S. cerevisiae* NI-C-D4[pSFI] (lane 3) and *S. cerevisiae* NI-C-D4[pDLG125] (lane 4), Lane 5-7: Concentrated supernatant of *S. cerevisiae* NI-C-D4[pDWN002] (lane 5), *S. cerevisiae* NI-C-D4[pSFI] (lane 6) and *S. cerevisiae* NI-C-D4[pDLG125] (lane 7), Lane 8-10: Zymogram analysis using fluorescent MUG substrate of *S. cerevisiae* NI-C-D4[pDWN002] (lane 8), *S. cerevisiae* NI-C-D4[pSFI] (lane 9) and *S. cerevisiae* NI-C-D4[pDLG125] (lane 10).

Hyperglycosilation of the recombinant β-glucosidases is apparent from the smearing pattern observed in the respective lanes (lanes 5 and 6). Due to this an accurate estimation of protein size could not be made. Strains of *S. cerevisiae* are known to hyperglycosilate proteins (Trahl-Bolsinger and Tanner 1990), but NI-C-D4 is an oversecreting, low glycosylation mutant of this yeast (Wang *et al.* 2001). The theoretical molecular weight of the 858 aa recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] was calculated to be 91.735 kDa (CLC Genomics Workbench v5.5.1). Zymography, however shows β-glucosidase activity of the protein at approximately 185 kDa (lane 8), approximately double the teoretical molecular weight of 91.735 kDa (CLC Genomics Workbench v5.5.1).

SDS-PAGE analysis was conducted under stringent denaturing conditions, negating the idea that the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN002] may be a dimeric protein in its native state. In addition to this, Fukasawa *et al.* (2007) subjected a β-glucosidase purified from *A. cellulolyticus* to SDS-PAGE analysis and observed a single band of 108 kDa. Even though NI-C-D4 has a low glycosylation phenotype, hyperglycosilation may still occur at some of thirteen theoretical glycosylation sites found within the expressed protein's sequence (Fig. 3.15) (as determined using CLC Genomics Workbench v5.5.1 in conjunction with the Pfam Protein Domain database), which leads to an increase in molecular weight of the protein (Kim 2003, Skory *et al.* 1996). No proteins bands were corresponding to the hyperglycosilated proteins produced by *S. cerevisiae* NI-C-D4, was observed in the lanes containing the intracellular fractions. This indicates that recombinant proteins under control of their respective native secretion signals, were successfully excreted.

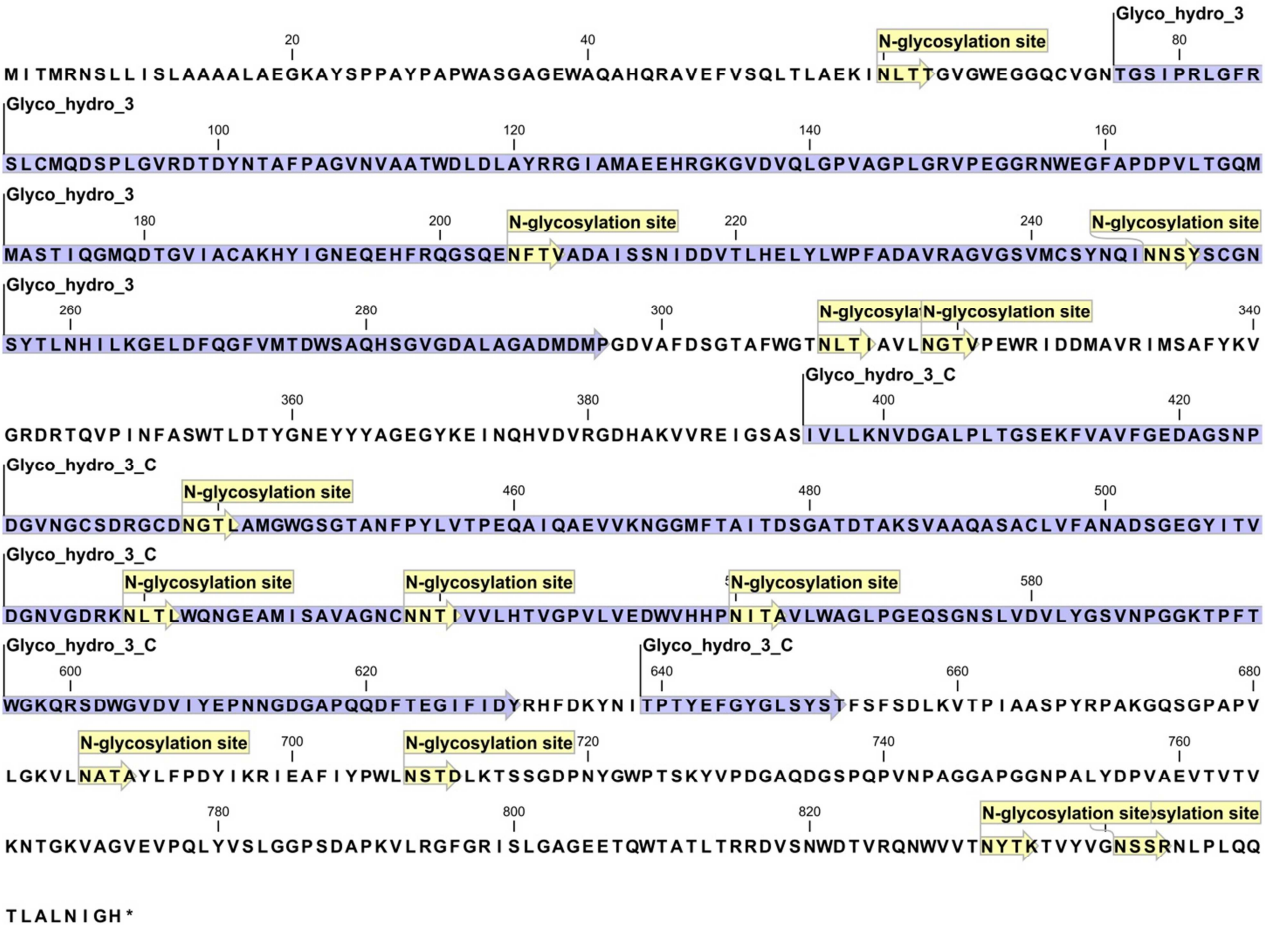

Fig. 3.15: Graphical representation of the thirteen theoretical glycosylation sites found within the sequence of the recombinant β-glucosidase produced by *S. cerevisiae* NI-C-D4[pDWN02], as determined using CLC Genomics Workbench v5.5.1 in conjunction with the Pfam Protein Domain database

3.5. CONCLUSION

During the process of composting, organic matter is degraded by a complex consortium of microorganisms. Filamentous fungal strains inherent to organic matter recycling processes, are known to produce and secrete large quantities of cellulases and are thus one of the key role players during composting. For this reason the process of composting was deemed as good source of these enzymes.

Cellulases have been exploited in various industrial processes for their ability to hydrolyse the complex structure of cellulose to its monomer glucose. During the hydrolysis of cellulose, low levels of β-glucosidase lead to the accumulation of cellobiose, which in turn is a strong inhibitor of cellobiohydrolases and endoglucanases. This effect can be alleviated with the addition of β-glucosidase from another source.

A fungal isolate from the species *Acremonium cellulolyticus* was isolated from a compost heap and its β-glucosidase gene was cloned and heterologously expressed in *S. cerevisiae*. The β-glucosidase production profile of this recombinant organism was assessed and the expressed enzyme characterised. The recombinant β-glucosidase showed relatively low enzymatic activity, compared to previously isolated enzymes. However, the recombinant β-glucosidase did proof to be stable and active at a wide range of temperatures and pH values. These attributes can be ascribed to the environment from which the enzyme was isolated, as microbial composting communities are able to adapt to changes in temperature, redox conditions and water activity.

Enzymatic activity of the recombinant β-glucosidase was adversely affected by the presence of glucose and ethanol, but only at higher concentrations. Interestingly, xylose was found to have a slight stimulatory effect on the enzymatic activity. This effect has been reported in the past, but the biochemical mechanisms for this are still unclear. The ability of the recombinant β-glucosidase to retain stability and activity in a wide range of temperatures and pH and the presence of inhibitory compounds, make the enzyme a promising candidate for the use in industrial applications.

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CHAPTER 4:

GENERAL DISCUSSION AND CONCLUSIONS

4.1. GENERAL DISCUSSION AND CONCLUSIONS

Dependence on depleting fossil fuel reserves and growing public concern over detrimental environmental effects associated with their use, have sparked great interest in alternative fuel sources (Li *et al*. 2009). The development of technologies for the conversion of renewable lignocellulosic biomass to bioethanol is emerging as one of the most popular alternatives to fossil fuels. Over the last three decades, extensive research has been conducted on the different approaches for the conversion of the cellulose fraction of lignocellulose to bioethanol (Sun and Cheng 2002). These approaches differ mainly in the method of hydrolysis of cellulose to fermentable sugars and the subsequent fermentation of the liberated sugars (Lynd *et al.* 1991).

The use of enzymatic hydrolysis of cellulose is environmentally-friendly compared the use of strong acids and alkali (Duff and Murray 1996, Fang *et al.* 2009). The high cost of cellulases is, however, the most significant barrier to the economical production of bioethanol from cellulosic biomass (Fang *et al.* 2008). Consolidated bioprocessing (CBP) is a promising strategy to reduce costs, through the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of the resulting sugars to bioethanol in a single process step via a cellulolytic microorganism or consortium (Lynd *et al*. 1996, Lynd *et al.* 2002). The development of CBP organisms may be achieved by engineering non-cellulolytic microorganisms that exhibit high product yields and tolerance so that they are able to utilize cellulose.

With this in mind, the main objective of this study was the isolation of an eukaryotic β-glucosidase through bioprospecting a compost heap environment and the heterologous expression and characterisation of the recombinant enzymatic activity in *Saccharomyces cerevisiae*.

Chapter 2 is a review of current literature and highlights the need for the development of technologies for the production of alternatives to fossil fuels. In this chapter, lignocellulose and more specifically its cellulose fraction, is evaluated as a possible feedstock for the production of bioethanol. The role of cellulases in bioethanol production as well as possible sources of novel and improved enzymes is also evaluated.

Chapter 3 (The Heterologous Expression of an *Acremonium cellulolyticus* β-Glucosidase Gene, in *Saccharomyces cerevisiae*) describes the construction and monitoring of a compost heap comprising triticale as main substrate. A fungal species exhibiting high

cellulolytic activity was isolated from the process and preliminarily identified as *Acremonium cellulolyticus*.

This psychrophilic microorganism was originally isolated from soil in north-eastern Japan and its β-glucosidase has been reported to exhibit higher activity than that of *T. reesei* (Yamanobe *et al.* 1987). This β-glucosidase also excels in thermal stability and allows for saccharification of cellulose at 5 to 10℃, above that of cellulases usually employed (Yamanobe *et al*. 1985). In order to gage the potential of this enzyme for the use in the construction of a CBP organism, it was heterologously expressed and the resultant recombinant organisms' growth and β-glucosidase production profile was investigated. In conjunction to this, the enzymatic activity of the recombinant β-glucosidase was measured and the enzyme characterised.

The recombinant β-glucosidase was found to have an insignificant metabolic burden on the host strains, compared to that of a previously reported recombinant β-glucosidase originating from *S. fibuligera*. Normalised enzymatic activity of the recombinant *A. cellulolyticus* β-glucosidase was, however, shown to be approximately half of that of the *S. fibuligera* enzyme. The recombinant *A. cellulolyticus* β-glucosidase however showed enzymatic activity and stability across a range of temperature and pH values. These findings correlated to that of Yamanobe *et al*. (1985) and proof the enzyme's suitability for the use in industrial applications.

The effect of selected monosaccharides and ethanol on enzymatic activity was also determined as the influence of inhibitors on the process design of hydrolysis of lignocelluloses is a crucial factor that needs to be taken into consideration. The recombinant *A. cellulolyticus* β-glucosidase is subject to end-product inhibition by glucose, not unlike most reported microbial β-glucosidases (Dotsenko *et al.* 2012, Karnchanatat *et al.* 2007, Krisch *et al.* 2012). Surprisingly, the enzymatic activity of the recombinant *A. cellulolyticus* β-glucosidase was slightly enhanced in the presence of xylose. This stimulatory effect of xylose on β-glucosidases has been reported by other researchers (Zhao *et al*. 2012, Zanoelo *et al.* 2004) and is a beneficial trait in the hydrolysis of lignocellulose, as xylose is one of the products of hemicellulose degradation. Ethanol at certain levels has been proven to increase the activity of some β-glucosidases (Gopalan *et al.* 1989, Grogan 1991, Plant *et al.* 1988). In this study, ethanol proved to have an inhibitory effect on enzymatic activity. The inhibitory effects of both glucose and ethanol on enzymatic activity of the recombinant β-glucosidases do not negate the potential use of

this enzyme in the production of bioethanol from renewable biomass, as these effects are only observed at concentrations not usually encountered during a CBP process.

Traditional culturing methods for the isolation of new microbial enzymes allow for the isolation of enzymes from culturable microbes and not the unculturable majority. It has been estimated that only a mere 1% of all microbes in the biosphere are culturable under normal laboratory conditions (Pace 1997, Rappe *et al.* 2003). In order to prevent this loss of diversity, culture independent approaches have been developed to exploit the collective genomes of microbial communities (Rondon *et al.* 2000, Schloss and Handelsman 2003).

The approach originally followed in this study (see Addendum I) with the construction of the triticale compost heap, was to select for microbial populations actively degrading this specific feedstock, the subsequent construction of a metatranscriptomic library from mRNA from the active stages. The main aim of this was to screen the cDNA library for novel β-glucosidase enzymes. Due to major obstacles in the construction of a metatranscriptomic library and the inability to isolate novel β-glucosidase genes using this approach, focus was redirected to the approach documented in chapter 3.

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ADDENDUM A:

METATRANSCRIPTOMIC STRATEGY FOR CLONING B-GLUCOSIDASE GENES

5.1. INTRODUCTION

A compost heap with triticale straw as main constituent, was constructed in April 2009 as described in Chapter 3. During the biological process of composting, a diverse consortium of microorganisms is responsible for the degradation of organic matter under aerobic conditions (Khalil *et al.* 2001, Ryckeboer *et al.* 2003, Steger *et al.* 2003). The composting process can be considered an ideal "natural" environment where a high turnover rate of cellulose-rich feedstocks can be obtained through microbial action and an environment enriched for cellulases and hemicellulases. Culturable fungal isolates from the compost heap was isolated and selected using traditional microbiological techniques and a cDNA copy of a β-glucosidase gene from one of these fungal isolates, *Acremonium cellulolyticus*, was cloned using PCR primers based on a gene sequence in the NCBI database and heterologously expressed in *S. cerevisiae*.

However, it is generally accepted that traditional microbial culturing methods only allow microbiologists to study a small fraction of the microbial population within a specific niche, as the majority of microbes (95.0 - 99.9%) are not culturable using standard microbiological media (Li *et al.* 2011). The vast majority of microbial diversity remains undiscovered and unexploited by these techniques, and requires culture-independent approaches, such as metagenomic analysis, to tap into the rich genetic resource present within natural microbial populations.

Metagenomic studies involves the direct extraction of genomic DNA from environmental samples followed by either functional (activity-based) screening of a metagenome library using suitable cloning vectors and hosts or next generation sequencing (sequence-based screening) of the entire metagenome to discover novel genes using a bioinformatics approach. Several studies have previously been reported where both the functional screening and sequencing approaches have led to the discovery of novel genes (Duan and Feng 2010). However, there is a challenge associated with the functional metagenome approach. Functional screening of metagenome libraries are often limited to the use of bacterial hosts such as *E. coli*, with insufficient or biased expression of foreign genes. This strategy has a strong bias towards prokaryotic genes, as eukaryotic gene promoters do not function in the bacterial host and bacteria have no mechanism for mRNA splicing for the removal of introns often found in eukaryotic genes.

In an attempt to overcome this drawback, metatranscriptomic approaches are deemed less biased towards eukaryotic genes. Several research papers on this approach have

appeared in the last six years (Grant *et al.* 2006, Bailly *et al.* 2007, Frias-Lopez *et al.* 2008, Gilbert *et al.* 2008, Kellner *et al.* 2011). Similarly to metagenomic studies, during the metatranscriptomic approach RNA is directly extracted from environmental samples, converted to cDNA using mRNA as template, followed by either functional (activity-based) screening of a cDNA library using suitable cloning vectors and hosts, or next generation sequencing (sequence-based screening) of the entire metatranscriptome to discover novel genes using a bioinformatics approach. The advantage of this approach is that only coding DNA sequences without non-coding regions such as introns are obtained, and expression of the genes are driven by regulatory sequences supplied in the final cloning vector. There is, however, some risks associated with working with RNA, which relates mostly to the instability or ribonuclease-sensitivity of mRNA as well as the short half-life of some mRNA species compared to genomic DNA.

In this study, we attempted to isolate total RNA from the triticale composting process and construct a metatranscriptomic (cDNA) library of all the genes expressed during the composting process. The aim was to screen the cDNA library expressed in *S. cerevisiae* for the presence of novel β-glucosidase genes.

5.2. MATERIALS AND METHODS

5.2.1. Compost sampling, RNA extraction and cDNA synthesis

Samples were taken from the triticale compost heap at weekly/fortnightly intervals, followed by liquid nitrogen-freezing and grinding, RNA stabilization with RNAlater (Ambion, USA) and storage at -80℃. Frozen compost/fresh soil samples were allowed to thaw and washed three times with diethylpyrocarbonate (DEPC) treated water to remove residual RNAlater, followed by RNA extraction. Total RNA was extracted from about 30-90 g of RNAlater-treated compost samples using the RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to the supplier's instructions. Prior to cDNA synthesis, residual DNA was removed from the total RNA by following the method prescribed by the Turbo DNA-freeTM DNase Treatment and Removal kit (Ambion Inc., Austin, TX, USA). Poly(A)-tailed mRNA was extracted from about 300-600 µg of total RNA using the poly(A) Purist MAG kit (Ambion). First strand cDNA synthesis was performed using Smart cDNA library construction kit (BD Biosciences, Clontech). 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 first-strand cDNA, 2 µL of each oligonucleotide primer (12 µM), 10 µL 10x Advantage 2 PCR buffer, 2 µL 50x dNTP mix and 2 µL 50x Advantage 2 polymerase mix. The PCR amplification conditions were 95℃ for 1 min, followed by cycles of 95℃ for 15 sec and 68℃ for 4 min. The amount of cycles was optimised by removing 80 µl of the reaction after 24 cycles and storing it at 4℃. The remaining 20 µl was returned to the thermal cycler and 5 µL was removed after every 2 cycles. Each 5 µL 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 (Fermentas, Lithuania) or PacI and AscI (New England Biolabs, Beverly, MA).

5.2.2. Preparation of shuttle plasmid/vector and cloning of cDNA library

The plasmid, pYESfi-URA3 shuttle plasmid (Bailly *et al.* 2007) 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 *Escherichia coli*–*Saccharomyces cerevisiae* pYESfi-URA3 shuttle plasmid also contains the *Sfi*1A/B restriction sites and was used for the directional cloning of the cDNA after restriction nuclease digestion and purification using the High Pure PCR Purification kit (Roche, Mannheim, Germany). The digested plasmid was dephosphorylated using the FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas, Lithuania) 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 (Fermentas, Lithuania) and incubating the reaction at 16℃ overnight in a thermal cycler. The reaction was transformed into electro-competent One Shot MAX Efficiency *E. coli* DH10B'™T1R cells (Invitrogen) and plated out onto selective LB-ampicillin solid medium.

5.2.3. Size fractionation of cDNA library

Size fractionation of the cDNA was performed with two different methods: (1) CHROMA SPIN-400 Column provided by the Creator™ SMART™ cDNA Library construction kit (Clontech), and (2) a sucrose gradient method from Current Protocols (Quertermous 2001). The sucrose gradients were prepared from 20% to 50% using 5% intervals in STE Buffer, and the cDNA was carefully layered on top of the sucrose gradient. The samples were centrifuged at 20℃, 32 000 rpm for 24 hours. A small hole was pierced at the bottom of the tube and twenty 250 µl fractions were collected. Subsequently, 25 µl of each fraction was visualized on a 1% agarose gel.

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 T_4 DNA ligase (Fermentas, Lithuania) at 16℃ overnight in a therm al cycler.

5.2.4. Amplification of cDNA library

The cDNA library was introduced into electro-competent 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 was determined as prescribed by the manufacturer of the Creator™ SMART™ cDNA Library construction kit (Clontech). The percentage recombinant clones was also determined by selecting colonies and performing a plasmid isolation (Del Sal *et al.* 1988) and restriction enzyme digest of the plasmid with *Sfi*I. The percentage of inserts > 500 bp were also determined as this would be an indicator whether the size fractionation was successful; less than 30% small inserts were desired.

Amplification of the transformed *E. coli* was performed as prescribed by the Creator™ SMART™ cDNA Library construction kit (Clontech). An aliquot of the amplified cDNA library was 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)

5.2.5. Transformation of cDNA library into *Saccharomyces cerevisiae*

The haploid *S. cerevisiae* strain 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). The electroporation protocol involved growing a yeast culture to an A_{600nm} of 0.7-1.5 and pelleting the cells by centrifuging at 5000 rpm for 5 min. at 4℃. The yeast pellet was resuspended in 25 ml LiAc/DTT/TE (0.1 M lithium acetate, 10 mM dithiothrietol, 10 mM Tris-HCL (pH7.5), 1 mM EDTA). After incubating the cells for 1 hour at room temperature, the cells were pelleted again at 5 000 rpm for 5 min at 4℃ and resuspended in 25 mL ice-cold deionized water. After pelleting, the cells were resuspended in 10 mL of ice-cold 1 M sorbitol. Finally, the cells were pelleted and finally resuspended in 100 µl of 1 M sorbitol. A total of 4 µg of plasmid-insert DNA was added to 400 µL electroporation reaction, followed by transferring 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, transferred to 2.6 ml of 1:1 mix of 1 M sorbitol:Yeast Peptone Dextrose (YPD) broth and incubated for 1 h at 30℃. 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.

5.2.6. Screening of cDNA library

The screening strategy for the β-glucosidase cDNA library cloned into the pYESfi-URA3 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 0.68% Yeast Nitrogen Base (YNB) media, supplemented with auxotrophic requirements and 2% galactose for induction and 1mM 4-methyllumbelliferyl-β-D-glucopyranoside (MUG) to detect β-glucosidase activity. The β-glucosidase enzyme will hydrolyse the substrate releasing the fluorescent substrate, 4-methyllumbelliferyl, MU), which can be observed when exposed to UV light on a transilluminator.

5.3. RESULTS AND DISCUSSION

5.3.1. RNA extraction and cDNA synthesis

The isolation of intact total RNA from the RNAlater (Ambion, USA) treated triticale compost samples with the use of the RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Inc., USA), proofed to be successful. Samples of the isolated total RNA was analysed on a 1% SeaKem® LE Agarose gel and visualised with GR Green Nucleic Acid Stain (Biolabo, Switzerland). Clear ribosomal RNA bands (Fig. 1) were observed, indicating intact RNA. The presence of mRNA and contaminating residual DNA could also be observed.

Contaminating residual DNA was removed with the use of the Turbo DNA-freeTM DNase Treatment and Removal kit (Ambion Inc., Austin, TX, USA) and poly(A)-tailed mRNA was extracted from total RNA using the poly(A) Purist MAG kit (Ambion). First strand cDNA was synthesized from the purified mRNA using the Smart cDNA library construction kit (BD Biosciences, Clontech) and second-strand cDNA was synthesised using Advantage 2 PCR Polymerase (Clontech, USA). Following electrophoretic analysis of the samples taken during optimization of second-strand synthesis, it was decided that 34 cycles yielded sufficient cDNA without artefacts.

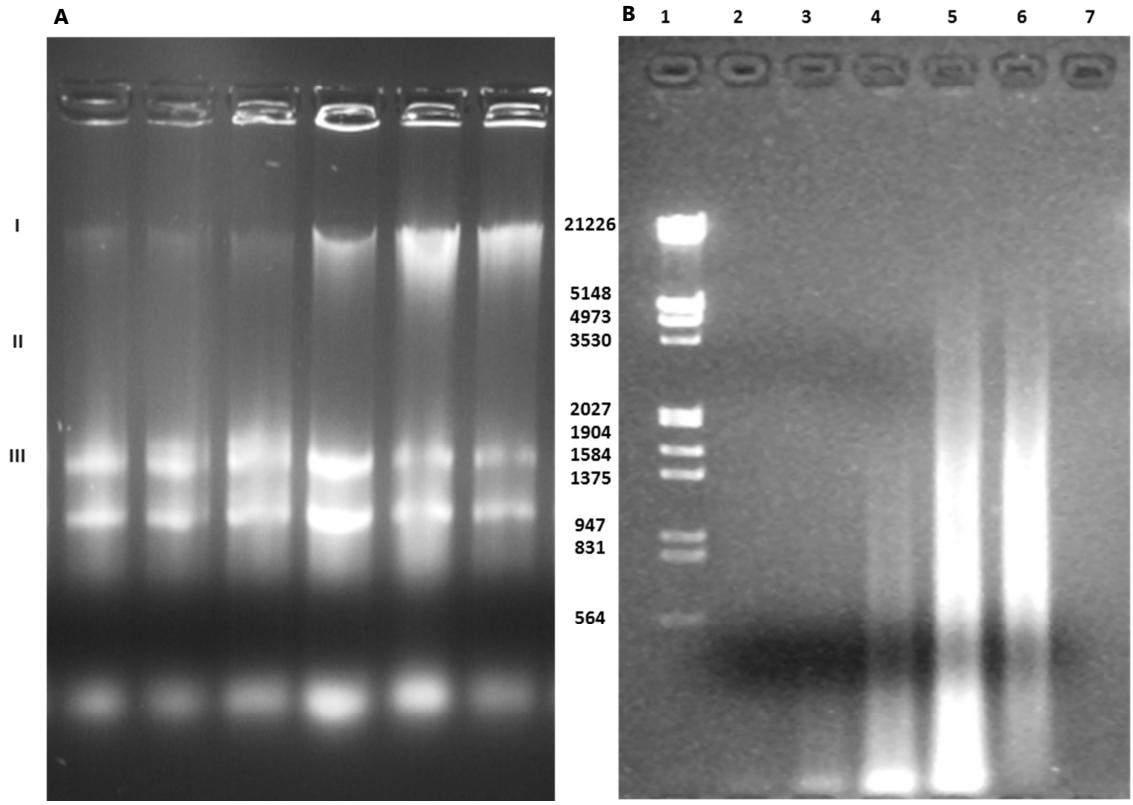

Fig. 1: Agarose gel photos of (A) intact total RNA isolated from RNAlater (Ambion, USA) treated triticale compost samples with (I) contaminating residual DNA, (II) mRNA smears and (III) ribosomal RNA visible and (B) samples taken during second stand cDNA synthesis optimization at 26, 28, 30, 32 and 34 cycles, respectively.

5.3.2. Size fractionation of cDNA library

Small cDNA fragments, primers and primer dimers are usually preferentially ligated into the plasmid and should be removed before cDNA-plasmid ligation. When a 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 on a 1.1% agarose/EtBr gel, inefficient size fractionation was observed as each fraction predominately contained smaller cDNA (data not shown). The CHROMA SPIN-400 Column was replaced by the sucrose gradient method from Current Protocols (Quertermous, 2001), which yielded good overall separation of the cDNA, as can be seen from the samples analysed by electrophoresis (Fig. 2). Sucrose gradient fractions 6 and 7 (corresponding to lanes 7 and 8) were chosen to proceed with in the construction of a

metatranscriptomic library. These fractions were chosen as they fall within the desired size range of 500 – 5000 bp.

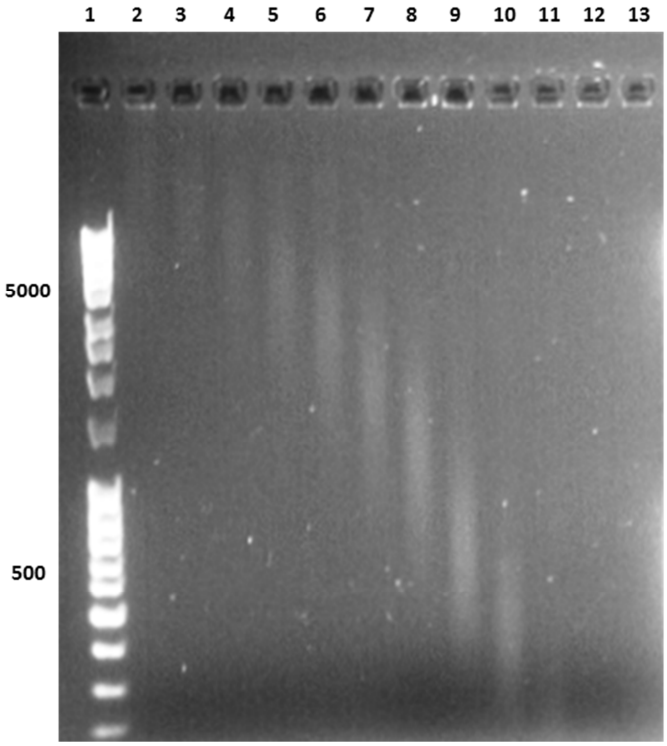

Fig. 2: Agarose gel photo showing the size fractionation of cDNA with the use of a sucrose gradient in conjunction with ultra-centrifugation.

5.3.3. Amplification of cDNA library

Prior to the amplification of the cDNA library, the titer of the unamplified library was determined to be 1.2 x 10^4. Low-titer libraries result from the use of the incorrect amount of cDNA in library construction. Too much starting cDNA is known to yield poor cloning efficiencies and low-titer libraries. The percentage recombinant clones were also determined prior to amplification. Insert confirmation showed that the cDNA library contained predominantly small insert (< 50% inserts smaller than 500bp) (data not shown). The presence of inserts with small sizes can be ascribed to the unsuccessful size fractionation of the cDNA preparation. It was decided to amplify the library even if a low titer and predominantly small sized inserts were observed.

5.3.4. Transformation of cDNA library into *Saccharomyces cerevisiae* and screening

The amplified library was cultured in selective LB-ampicillin medium and a plasmid extraction was performed. The resultant plasmid DNA was used to transform *S. cerevisiae* Y294. During the functional screening of the cDNA libraries in *S. cerevisiae,* no β-glucosidase enzymatic activity was observed. Possible reasons for this could include: (1) the lack of a cDNA copy of the enzyme in the library; (2) the inability of the *S. cerevisiae* Y294 strain to transcribe the foreign gene; (3) low stability of the foreign protein; (4) hyperglycosilation of the foreign protein or (5) the incorrect folding or disulphide bond formation due to physilogical differences between *S. cerevisiae* and the organism from which the foreign gene was obtained.

5.4. CONCLUSIONS

The metatranscriptomic approach developed in this study did not deliver any cDNA libraries with functional hits in *S. cerevisiae*. After significant optimization steps, a cDNA library with a 40-50% insert rate of relative large inserts was generated. Screening of this library did not identify any new genes. The isolation of intact total RNA from compost and removal of contaminating DNA proofed to be successful with the methods described. In order to improve upon library titers, the method employed for cDNA synthesis must be re-evaluated. The use of correct amounts of intact cDNA is crucial in the construction of metatranscriptomic libraries with high titres. The choice of expression host should also be re-evaluated as incompatibilities between the foreign proteins and *S. cerevisiae* Y294 physiology may be the cause for the lack of the desired enzymatic activity. Along with evaluation of expression host, the choice of shuttle vector should be also be re-evaluated.

During the screening of the β-glucosidase cDNA library, 4-methylumbelliferyl-β-D-glucopyranoside (MUG) (MUG → 4-MU and glucopyranoside) glucose is released as an end-product when hydrolysed by β-glucosidase. The GAL1 promoter of pYESfi-URA3 is under glucose represion, which could complicate the screening of β-glucosidase cDNA libraries. If all the above-mentioned problems can be addressed along with those highlighted in the text, the method developed in this study should yield high quality cDNA libraries for the functional screening of novel enzymes from the environment.

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