Cloning of the *XynA* gene from *Thermomyces lanuginosus* and expression in *Saccharomyces cerevisiae*

by

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B. Sc. Hons (UFS)

Submitted in fulfillment of the requirements

for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences

Department of Microbiology and Biochemistry

University of the Free State

Bloemfontein

South Africa

November 2001

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AKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following:

The National Research Foundation and Protein Research Trust for financial support during this study.

Prof. Suren Singh (ML Sultan Technikon, Durban) for the T. lanuginosus strain. Without it, this study wouldn't have been possible.

Dr Koos Albertyn, friend and mentor, whose passion for molecular biology was a true inspiration the past two years. In the words of William Arthur Ward: "The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires".

Fellow colleagues in the molecular biology laboratory, for endless stimulating conversations.

My friends, especially Olga, Jeanette and Janine, for emotional and intellectual support, and dearly appreciated distraction when the need aroused.

My sister, Jeanine, who was subjected to numerous bouts of temporary madness while writing this dissertation.

My parents, for their invaluable encouragement, prayers, moral and financial support throughout this study.

Above all, to God be the Glory, the Creator of man and microorganism.

This dissertation is dedicated to my father, Gerrit, whose hard work throughout his life was an inspiration and motivation for this study, and to my mother, Erna, whose love and support carried me through 18 years of school and study.

Sometimes a scream is better than a thesis
Ralph Waldo Emerson

Experience: that most brutal of teachers. But you learn, my God do you learn
C. S. Lewis

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CHAPTER ONE

Introduction

Hemicelluloses are non-cellulosic low-molecular-mass polysaccharides that are found together with cellulose in plant tissues. Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose (Timell, 1967). Heteropolysaccharides, based on a backbone structure of β-1,4-linked D-xylose residues, are collectively referred to as β-1,4-xylans and constitute the main polymeric compound of the hemicellulose fraction (Coughlan and Hazlewood, 1993). β-Xylanase (1,4-β-D-xylan-xylanohydrolase, EC 3.2.1.8) is capable of degrading xylans and has received considerable attention in the food, feed and paper industries (Graham and Inborr, 1992; Maat *et al*., 1992; Nissen *et al*., 1992; Wong and Saddler, 1993). A thermostable, cellulase-free xylanase from the filamentous fungus *Thermomyces lanuginosus* was isolated by Singh *et al*. (2000b). The xylanase from this fungus is not only remarkably thermostable, but is also active over a wide pH range (Singh *et al*., 2000a). The yeast *Saccharomyces cerevisiae* has several properties which have established it as a host for the expression of heterologous proteins of biotechnological interest, and several studies have been conducted on the secretion of heterologous xylanases by *S. cerevisiae* (Crous *et al*., 1995; Pérez-Gonzalez *et al*., 1996; La Grange *et al*., 1996).

1.1 Aim of the study

The aim of this study was to test the viability of an immobilized enzyme construct, with expression of this enzyme (the xylanase from *Thermomyces lanuginosus* SSBP) by *Saccharomyces cerevisiae*, and the subsequent partial characterization of the recombinant enzyme. This enzyme construct was designed on a molecular basis, which entailed the following:

1. cloning of the *XynA* gene from *T. lanuginosus*
2. cloning of the *Agα1* gene from *S. cerevisiae*
3. removal of the binding domain from this cloned *Agα1* gene
4. cloning of the *XynA* gene into this deleted binding domain region, which is adjacent to a region coding for a stalk-like protein (for the immobilization of the enzyme as it is expressed on the stalk)
5. cloning of this fused *Agα1::XynA* into two shuttle vectors (a single- and multicopy vector)
6. expression of the xylanase by *S. cerevisiae*
7. partial characterization of the expressed enzyme, and
8. comparison with the characteristics of the native enzyme as determined by Singh *et al*. (2000b)

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CHAPTER TWO

Literature Review

2.1 Occurrence of β-Xylanase in microorganisms

2.1.1 General

There are relatively few studies dealing with β-xylanase of bacterial origin, because eukaryotic microorganisms such as fungi are regarded to be better producers of β-xylanases. Studies on bacterial β-xylanase are limited to the genera *Bacillus*, *Streptomyces* (Dekker, 1985) and *Clostridium* (Lee *et al*., 1987).

As with other xylanolytic microorganisms, filamentous fungi produce multiple xylanases (Wong *et al*., 1988) whose genes have been cloned and sequenced from the following: *Aureobasidium pullulans* (Li and Ljungdahl, 1994), *Cochliobolus carbonum* (Apel *et al*., 1993), *Penicillium chrysogenum* (Haas *et al*., 1993), *Trichoderma reesei* (Saarelainen *et al*., 1993; Törrönen *et al*., 1992), *Aspergillus awamori* (Hessing *et al*., 1994), *Aspergillus kawachi* (Ito *et al*., 1992a, b) and *Aspergillus tubingenes* (De Graaff *et al*., 1994).

2.1.2 Thermomyces lanuginosus

Recently, a *Thermomyces lanuginosus* strain (SSBP) was isolated in Durban, South Africa (Singh *et al*., 2000b), which displayed high levels of thermostable,

cellulase-free xylanase activity (Singh *et al*., 2000b). The xylanase produced by this strain retained 100 % of its activity at 60 °C for over 14 days, with an optimum temperature and pH of 70 °C and 6.0-6.5 respectively (Singh *et al*., 2000a). Significantly higher levels of enzyme is produced by this strain compared to *T. lanuginosus* DSM 5826, which is currently used in mill scale trials in Europe (Purkarthofer *et al*., 1993).

2.1.2.1 Occurrence

T. lanuginosus is distributed all over the world as result of the common occurrence of self heating masses of organic debris (Emerson, 1968). This thermophilic fungus has been isolated in the British Isles, Denmark, Italy, USA, Canada, Nigeria, Ghana, South Africa, India, Indonesia, Brazil and Japan. *T. lanuginosus* was isolated from dry and waterlogged grassland, loamy garden soil and aquatic sediments. It has been associated with self heating grains of barley and wheat, the atmosphere around silos, pecans, tobacco products, various composting materials, dung and it has also been trapped from air in Indonesia and the British Isles where it was the second most abundant thermophilic species, and was surpassed only by *Aspergillus fumigatus* in abundance (Cooney and Emerson, 1964).

2.1.2.2 Morphology

Microscopic examination of young colonies of *T. lanuginosus* by Emerson (1968) revealed masses of developing aleuriospores borne single at the tips of aleuriophores. In young colonies, spores are colourless and smooth walled, but as maturation proceeds, they turn dark brown and the thick exospore becomes characteristically wrinkled. Mature spores are spherical, irregularly sculptured and range from 6-10 μm in diameter. Dipicolonic acid has been found in

aleurioconidia, which plays an important role in heat resistance. Aleuriospores are generally unbranched, but occasionally they branch once or twice near the base and appear to cluster. Septations commonly occur in the aleuriospores, but they are difficult to observe. The mycelium is partly superficial, and partly immersed while there are no stroma, setae and hyphopodia. Aleuriospores are micronematous, straight or flexous, colourless or brown and smooth (Cooney and Emerson, 1964).

2.1.2.3 Temperature relations

Various definitions exist for thermophily, with reference to different groups of organisms. However, Cooney and Emerson (1964) made a distinction between thermophilic and thermotolerant fungi. Thermotolerant fungi are those that have a growth temperature maximum of about 50 °C and a temperature minimum well below 20 °C. Thermophilic fungi were defined as having a growth temperature maximum of 50 °C or higher, and a termperature minimum of 20 °C or higher. The thermophilic fungi is thought to succeed not because of a high metabolic rate, but because of a capacity to thrive in an environment that their nearest competitors cannot tolerate (Emerson, 1968). There have been a few attempts to explore the basis of thermophily in fungi, however, no direct connection has yet been established between pigments, nucleotide ratios, or other features of metabolism and the thermophilic properties of fungi. Thus, the basis of their thermophilic qualities remain the most interesting and challenging feature of their physiology.

2.2 Mode of action of β-xylanases

2.2.1 Enzymes necessary for the complete hydrolysis of xylan

The complete enzymatic hydrolysis of xylan into its constituent monocarbohydrates requires the synergistic action of a consortium of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Coughlan and Hazlewood, 1993; Jeffries, 1996). The most important enzyme is endo-1,4-β-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylo-oligosaccharides. β-Xylosidase, debranching enzymes (α-L-arabinofuranosidase and α-glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase and ρ-coumaroyl esterase) allow the complete degradation of the xylo-oligosaccharides to their monomeric constituents (Wong *et al*., 1988).

Xylanases are classified into two groups: endo-β-1,4-xylanases and exo-β-1,4-xylanases (Biely, 1985: Christakopoulos, 1997). Exo-β-1,4-xylanases hydrolyze xylan from the non-reducing end of the polymer thus yielding only β-xylose as a hydrolysis product (Biely, 1985). Endo-β-1,4-xylanases hydrolyze xylan and xylo-oligomers with a depolymerization (DP) value greater than 2, the affinity increasing with increasing DP (Biely, 1985; Biely *et al*., 1997). Endoxylanases are distinguished from one another on the basis of the substrates on which they act, as well as their reaction products (Biely *et al*., 1997). Endoxylanases are secreted extracellularly, since xylan is a large polymer incapable of crossing the cell membrane (Biely, 1985). Polymeric xylans are usually cleaved at unsubstituted regions to yield mixtures of unsubstituted xylo-oligomers, as well as short and long chain substituted xylo-oligomers (Coughlan and Hazlewood, 1993). Removal of the substituent groups by ancillary enzymes create new substrates for endoxylanase action.

All glycosidases share a common mechanism, involving carboxyl groups with the classic paradigm of the carbohydrase action lysozyme which has been extensively investigated (Nath and Rao, 2001). Xylanases are classified into two families, 10 / F (high Mr / low pI values) and 11 / G (low Mr / high pI values) (Christakopoulos *et al*., 1997; Clarke *et al*., 1997; Jeffries, 1996), according to the similarity of amino acid sequences of their catalytic domain in hydrophobic cluster analysis (Henrissat, 1991).

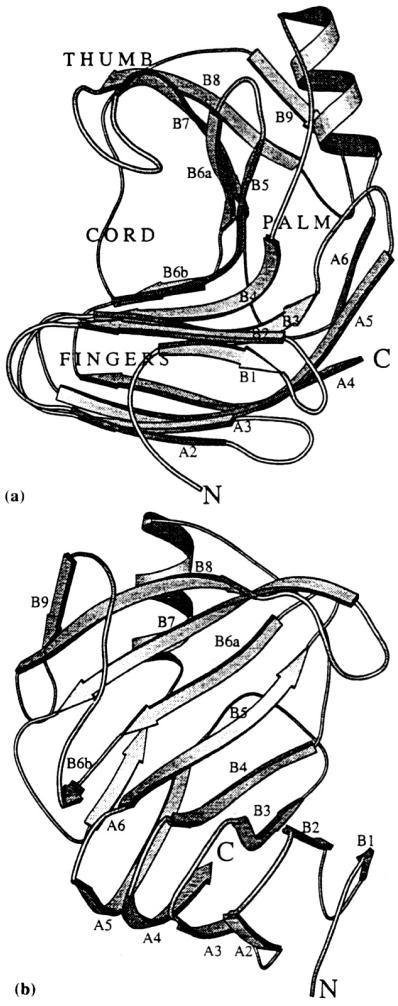

Fig. 2.1 (a) The schematic drawing of XYNII form *T. reesei* (figure generated using MOLSCRIPT, Kraulis, 1991); (b) as in (a) but rotated about 90°. (Törrönen and Rouvinen, 1997)

The overall structures of family 11 / G endoxylanases are similar and have been described as a partially closed hand. Xylanases fold into a single ellipsoidal domain comprising of two β-sheets (A and B) and a single three turn α-helix (Fig. 2.1). The sheets are mostly composed of anti-parallel strands and twisted at almost 90°. The hydrophobic faces of the two β-sheets pack together to form a sandwich which is described as fingers. The twisted part of the β-sheets form a cleft in one side of the molecule, which together with the helix are described as a palm. The active site is located at the concave side of the cleft. A long loop between the B8 and B7 strands is described as a thumb. An unusual feature is the chord which runs across the mouth of the celft, partly closing it at one side. It does not make any hydrogen bonds with other parts of the molecule (Törrönen *et al*., 1994). There has been some controversy in assigning the secondary structure elements in the xylanase fold. Campbell *et al*. (1993) have reported that the xylanase fold consists of three β-sheets instead of two. The twisted part of β-sheet B has been described as a separate sheet. However, the continuity in hydrogen bonding strongly support the idea of two sheets (Törrönen and Rouvinen, 1997). Plesniak *et al*. (1996) have also reported NMR studies and the secondary structure determination of *Bacillus circulans* xylanase. This matches perfectly with the crystal studies and indicates that the xylanase fold is similar in solution and in the crystalline state.

Reports from various family 11 / G xylanases show that these enzymes operate *via* a double displacement mechanism, in which the anomeric configuration is retained. The enzymes of family 11 / G are single domain proteins composed of three anti-parallel β-sheets and one α-helix, with the active site lying between the second and third sheets (Nath and Rao, 2001). Family 10 / F xylanases of thermophilic origin often have associated duplicated family IX cellulose-binding domains (CBD's) at the C terminus of the catalytic domain and thermostabilizing domains (TSD's, typically found in tandem domains), at the N-terminus

(Bergquist *et al*., 1999; Lee *et al*., 1993; Morris *et al*., 1999; Winterhalter *et al*., 1995; Zverlov *et al*., 1996).

Both families contain bacterial and fungal enzymes, suggesting that the acquisition of xylanase activity has involved, at some stage, lateral gene transfer between fungi and bacteria (Hazlewood and Gilbert, 1992).

2.3 Localization and structural composition of xylan in plants

Cellulose, hemicellulose and lignin constitute the major biopolymers found in wood. Four types of hemicelluloses are predominant in plants, namely xylan, mannan, galactan and arabinan (Whistler and Richards, 1970). Xylan is the second most abundant polysaccharide in nature and is surpassed only by cellulose in abundance (Whistler and Richards, 1970). Xylans are classified according to the nature of the linkages joining the xylose residues. β-1,3-Linked xylans are found only in marine algae, those containing a mixture of β-1,3- and β-1,4-linkages only in seaweeds and β-1,4-linked xylans occur in hardwoods, softwoods and grasses (Barry and Dillon, 1940; Dekker and Richards, 1976; Kato and Nevins, 1984; Timell, 1965). Hetero-β-1,4-D-xylans constitute the major portion of the hemicellulose-fraction in terrestrial plants (Timell, 1965; Whistler and Richards, 1970). Native xylans are complex polymers containing varying amounts of arabinose, 4-O-methylglucuronic acid and acetic acid groups attached to the main xylose chain, depending on the botanical origin of the xylan (Johannson and Samuelson, 1977; Puls and Schuseil, 1993).

Xylan accounts for 10-35 % of the dry weight of hardwoods (angiosperms) (Puls and Schuseil, 1993). The main hemicellulose in hardwood is O-acetyl-4-O-methylglucuronoxlan (Puls and Schuseil, 1993). On average, the degree of polymerization (DP) is 200 with 10 % of the backbone units substituted at C-2 with 4-O-methylglucuronic acid and 70 % of the xylopyranosyl units acetylated at

C-2 and/or C-3 (Lindberg *et al*., 1973; Puls and Schuseil, 1993). Small amounts of rhamnose and galacturonic acid may also form part of the main chain (Coughlan and Hazlewood, 1993).

Hetero-β-1,4-D-mannans (galactoglucomannan and glucomannan) comprise approximately two-thirds and arabino-4-O-methylglucuroxylan about one-third of the total hemicellulose found in softwoods (Johannson and Samuelson, 1977). Softwoods (gymnosperms) contain 10-15 % arabino-4-O-methylglucuronoxylan which is located mainly in the tertiary wall of pinewood (Puls and Schuseil, 1993). Softwood xylan is not acetylated and consists of a backbone containing β-1,4-linked xylose units, with α-1,2-linked 4-O-methylglucuronic acid and α-1,3-linked L-arabinofuranoside substituents (Joseleau *et al*., 1992; Puls and Schuseil, 1993). The ration of arabinose side-groups to xylose is 1:8 and on average, two out of ten xylose units are substituted with uronic acid (Joseleau *et al*., 1992; Puls and Schuseil, 1993). Softwood xylans contain less α-1,2-linked 4-O-methylglucuronic acid than hardwood xylans and the L-arabinofuranosyl side-chains are linked to the main chain *via* C-2 and/or C-3 (Joseleau *et al*., 1992; Puls and Schuseil, 1993). Some of the arabinosyl side-chains are substituted at C-5 with feruloyl or ρ-coumaroyl residues (Joseleau *et al*., 1992; Meuller-Harvey *et al*., 1986; Puls and Schuseil, 1993). Grass arabinoxylans differ from species to species and from tissue to tissue within the same species, regarding the proportion and composition of the xylan present (Meuller-Harvey *et al*., 1986; Puls and Schuseil, 1993).

2.4 Applications of β-xylanases

In general, microbial biotechnology is directed towards the improvement of resource utilization, optimization of current processes through the addition of microbially-derived enzymes, the production of flavour compounds, polysaccharides, pigments and anti-oxidants, as well as reducing the

environmental impact of large-scale, well-established industries (Knorr and Sinskey, 1985). During the past two decades, numerous applications have been found for endoxylanases. Table 2.1 represent just a few of the interesting areas for xylanase application. Apart from their important role in the hydrolysis of xylan-containing raw materials used in various industries, xylanases have also been found to play important roles in plant tissues. Xylanases are involved in fruit softening, seed germination and plant defense systems (Deising and Mendgen, 1991; Prade, 1995). Microbial xylanases have the ability to induce ethylene synthesis in plant cells, thus acting as elicitors of the plant defense systems (Apel *et al*., 1993; Deising and Mendgen, 1991). Evidence exists which supports the important role of β-xylanases in the pathogenicity of *Magnaporthe grisea.* It has been reported that treatment by commercial β-xylanases on cultured rice cells causes cell death and that a 21 kDa β-xylanase from *Trichoderma viride* induces defense responses in tobacco plants, including ethylene production, necrosis and the induction of pathogenesis-related proteins (Bailey *et al*., 1990). This recently new role of xylanases in plants might give rise to new applications for xylanases (Prade, 1995). It was also shown that antibodies for detecting various β-xylanases can be useful for the characterization of β-xylanase production, plant cell walls, and plant pathognesis (Wu *et al*., 1995).

Table 2.1 Industrial applications of xylanolytic enzymes (Coughlan and Hazlewood, 1993)

Application
Elucidation of the structure of complex xylans
Extraction of juices, flavours, spices, oils and pigments
Clarification of juices and wines
Production of modified xylans as bulking agents used in food processing
Production of sweetners (xylitol) or flavours from xylan
Modification of cereal flours to enhance the volume, texture and staling properties of bread
Improvement of the nutritional value of silage, wheat- and rye-based animal feedstuffs
Retting of flux, hemp, jute, sisal and bast
Saccharification of agricultural and forestry residues for fermentation to fuels and chemical feedstocks
Pre-bleaching of pulp during paper manufacturing
Refining of dissolving pulp for the production of viscose rayon, cellulose esters and ethers

Although many interesting areas for xylanase application exist, only the three major areas where xylanases have made an impact on the industry will be discussed in this section.

2.4.1 Pulp and paper industry

Consumers today demand products of highest quality produced through processes that have as little as possible harmful effects on the environment. This is as result of a global trend in realizing that our planet's resources are limited and should be utilized responsibly. Apart from the public perception, governments worldwide are also increasing the restrictions placed on effluents,

toxic gasses and by-products resulting from industrial activities that are deleterious to the environment. Like any other large-scale industry, the pulp and paper industry is thus also faced with having to reduce their environmental impact (Viikari *et al*., 1994b).

The essence of pulp bleaching is to remove as much lignin as possible from the pulp, since residual lignin results in the pulp having a brownish colour (Buchert, *et al*., 1994). Currently, most bleaching sequences are based on elemental chlorine (Cl_2), chlorine dioxide, and alkaline extraction of the pulp. In response to new government regulations for emissions and environmental concerns about organochlorine concentration in bleachery effluents, the pulp and paper industry is investigating a variety of reduced chlorine-free bleaching sequences. Researchers are seeking methods for producing pulps using non-polluting chemicals, as well as trying to develop more efficient pulping methods to reduce the amount of residual lignin passing to the bleaching stage and to find alternative bleaching methods (Tolan and Foody, 1995; Viikari *et al*., 1994b). One promising option is the use of hemicellulases in a pre-bleaching treatment that renders the unbleached pulp lignin more easily removable (Davis *et al*., 1992).

Enzymatic bleaching, in particular xylanase-aided bleaching, has been found to be the most promising of the new bleaching technologies. Xylanases enable the specific removal of xylan, which avoids losses in pulp yield and quantity. Two hypothesis exist for the mechanism of xylanase-aided bleaching. Firstly, xylanase could enhance bleaching by rendering the fibre structure more porous and permeable, thus aiding the extraction of lignin (Viikari *et al*., 1994a,b), and secondly, xylanases could increase the extractability of lignin by reducing the amount of lignin-carbohydrate complexes (LCC) present in the pulp fibres (Viikari *et al*., 1994a,b; Yang and Erikkson, 1992). The beneficial effects of β-xylanase pre-treatment of kraft pulp bleachability were first reported by Viikari *et al*. (1986), at the VTT Biotechnical Laboratory in Finland. Evaluation of the effectiveness of

xylanase-aided bleaching consists of three aspects. Firstly, an increase in the amount of solubilized sugars is observed after incubation of xylanase with pulp (Prade, 1995). Secondly, pulp exhibit increased bleachability using conventional bleaching chemicals after xylanase treatment (Viikari *et al*., 1994a) and thirdly, xylanases facilitated the removal of lignin-carbohydrate complexes (LCC's) from pulp (Prade, 1995; Yang and Erikkson, 1992). Today, xylanase-aided bleaching is used in several kraft pulp mills all over the world (Vicuňa *et al*., 1995) and several commercial xylanase preparations are available (Table 2.2).

Table 2.2 Commercial xylanase preparations available for enzymatic bleaching of pulps (Vicuňa *et al*., 1995; Viikari *et al*., 1994b)

Product Name	Application pH	Application temperature (°C)	Supplier
Irgazyme 10	5-7	35-55	Gennencor International
Iragazyme 40	6-8	35-70	Gennencor International
Cartazyme HS	3-5	30-50	Sandoz Chemicals
Cartazyme HT	5-8	60-70	Sandoz Chemicals
Ecopulp	5-6	50-55	Alco, Ltd
Xylanase	5-6	55	Iogen Corp.
Xylanase	7-8	55	Iogen Corp.
VAI-xylanase	6-7.5	65-75	Voest-Alpine
Pulpzyme HB	6-8	50-55	Novo-Nordisk

The most important factor of these xylanase preparations is the presence of no or very low cellulase activity. Contaiminating cellulase activity has been found to be deleterious to pulp fibre strength (Baily *et al*., 1993; Tolan, 1995). Fully bleached kraft (sulphate) pulps were not obtained with the use of xylanases with chlorine-fee chemicals (Viikari *et al*., 1994b). However, the xylanases did reduce the amount of chlorine (Cl_2 and / or ClO_2) needed to produce the desired pulp brightness after bleaching, or higher pulp brightness could be achieved when the same amount of chlorine was used in addition to the xylanases (Buchert *et al*.,

1994; Viikari *et al*., 1994a). A reduction in chlorine consumption of up to 30 % has been reported (Buchert *et al*., 1992), as well as reductions in the organochlorine (AOX) of up to 50 % in the resulting effluents, after pre-treatment with xylanases (Bernier *et al*., 1994). Results from the Pulp and Paper Research Institute of Canada (Paprican) suggest that the Cl_2-sparing effect of an enzyme preparation depends primarily on its β-xylanase activity, not cellulase or mannanase activity (Paice *et al*., 1992).

The major drawbacks of enzyme-aided bleaching are the availability, cost and quality standard of enzyme preparations (Gamerith *et al*., 1992). These factors are particularly important in chemical pulp production, where an additional target of pulp bleaching is the selective removal of hemicellulose (Gamerith *et al*., 1992). The production process of kraft pulp is responsible for the extensive modifications of hemicelluloses (Buchert *et al*., 1995). Xylan is partly solubilized in the pulping liquor during the heating period of kraft cooking at high alkaline pH (Buchert *et al*., 1995; Viikari *et al*., 1994a,b). As the cooking process proceeds, the alkalinity decreases and degraded xylan of low depolymerization precipitates onto the surface of the cellulose microfibrils (Buchert *et al*., 1995). The precipitated xylan is thought to physically restrict the removal of high molecular weight lignin from the pulp fibres during the subsequent bleaching stages (Viikari *et al*., 1994a,b).

The use of thermostable β-xylanase for enzymatic hydrolysis or pre-treatment of pulp at high temperatures may contribute to making the process technically possible and economically viable. As the β-xylanase of mesophilic fungi, e.g. strains of *Aspergillus, Gliocladium, Schizophyllum* and *Trichoderma* are not thermostable at 50 °C or above, the hydrolytic efficiencies of these enzymes are low (Dekker, 1983; Poutanen *et al*., 1987; Steiner *et al*., 1987). The search for promising microorganisms capable of producing thermostable enzymes led to the isolation of *T. lanuginosus* (DSM 5826), which has been shown to produce a high level of cellulase free β-xylanase using beechwood xylan (Gomes *et al*., 1993).

T. lanuginosus grows well in various lignocellulosic substrates of commercial potential, but corn cobs was found to be the most effective substrate for β-xylanase production. Corn cobs is also of economical and technical importance for larger scale production of β-xylanase. This cellulase-free β-xylanase from *T. lanuginosus* showed excellent properties, including activity over a broad pH range, and very good stability (storage, pH and temperature) (Gomes *et al*., 1993).

2.4.2 Food and animal feed industry

2.4.2.1 Food industry

Along with that of cellulases and pectinases, the use of β-xylanases has also been suggested in applications including the clarification of juices (Biely, 1985), preparation of dextrins as food thickeners and the production of fluids and juices from plant materials (Woodward, 1984). There is also a constant need for methods and additives that will either improve dough processing or the quality of the baked products. A baking process is evaluated on the basis of criteria such as dough handling (machinability) and process yield. The quality of a baked product is evaluated on appearance (volume, colour, texture) and eating properties (crump elasticity, flavour, aroma). Xylanases from *Aspergillus niger* var. *awamori* have been found to substantially improve bread volume, reduce the stickyness of dough and improve the crump structure of bread (Maat *et al*., 1992). Other biotechnological applications of xylanases in the food industry include liquefaction of coffee mucilage, alteration of the rheological and organoleptic properties of musts and wines and the extraction of pigments and flavour compounds (Coughlan, 1992).

2.4.2.2 Animal feed industry

Feed ingredients of plant origin contain a number of anti-nutritive components (non-starch polysaccharides) that cannot be digested by monogastric species because of the lack of or insufficiency of endogenous enzyme secretions. In addition to being unavailable to the animal, these non-starch polysaccharides (NSP's) also lower the utilization of other dietary nutrients, which leads to a decrease in performance. Examples of such NSP's include pentosans in wheat, β-glucans in barley, and phytic acid, which is found in all plant feed ingredients (Ravindran *et al*., 1999). Low digestibility and litter problems are related to the NSP composition of rye, triticale, barley, oats and wheat when these grains are fed to poultry (Moran *et al*., 1969; White *et al*., 1983; Fengler and Marquardt, 1988b). Elimination of these anti-nutritive components increases the productivity of the diet and in doing so reduces manure output.

Much interest has been focused on the fact that animals do not have the enzymatic capabilities to digest cellulose, arabinoxylans, β-glucans or pectins, and on ways of removing these polymers which encapsulate the desired endosperm contents. Feed processing techniques such as pelleting and extruding result in significant damage to endosperm cell walls and gelatinisation of starch (Tovar *et al*., 1991). This often results in increased digestibility and improved weight gains and feed conversion efficiency.

Processing alone does not solve the problem of viscous NSP's, as feeding diets rich in rye and barley, especially to young poultry, have been known to present problems (Moran *et al*., 1969; White *et al*., 1983; Pettersson and Aman, 1988; Pettersson and Aman, 1989). As the concentration of these grains increases, growth rate and feed conversion efficiency are significantly depressed whilst the moisture content of the litter is elevated (Classen *et al*., 1995; Bedford and Classen, 1992).

Animal feed is supplemented with enzymes for two reasons. Firstly, enzyme supplementation degrades soluble fibre with anti-nutritional properties or improves the apparent metabolizable energy (AME) of cereals, and secondly, it supplements the animal's own digestive enzymes during maturation (Cowan, 1996).

For many years, feed compounders have found that switching from corn to high levels of inclusion of viscous cereals such as rye, barley, triticale, oats and wheat, will result in poorer performance and more noticeably, wet litter. Research suggested that the problem was related to the presence of a carbohydrate fraction present in the endosperm cell walls, such as an arabinoxylan in rye and wheat and a β-glucan in barley and oats (White *et al*., 1983; White *et al*., 1981; Antoniou and Marquardt., 1981; Antoniou *et al*., 1980). High intestinal viscosity has more precisely been attributed to a high molecular weight, soluble fraction of these compounds (White *et al*., 1983; Bedford and Classen, 1992) which is inert to the action of avian pancreatic enzymes. When these compounds dissolve in the intestine, they entangle and form a gel like structure which creates an obviously sticky or viscous digesta. Increased intestinal viscosity reduces the passage rate of digesta (Antoniou *et al*., 1981; Antoniou and Marquardt, 1983). The overall feed consumption (FC) would be less with a reduction in digesta passage rate, and could contribute to a decrease in live performance. As the viscosity increases, the rate of nutrient absorption decreases (Fengler and Marquardt, 1988a), which, in turn, could reduce the nutrient assimilation rate due to reduced enzyme-substrate reactions in the intestine (Ikeda and Kusano, 1983).

The choice of enzymes to be used depends on the type and relative percentage of the raw materials in the feed (Chesson, 1993). Cereals and vegetable protein raw materials used in animal feed can be subdivided into four main groups on the basis of their fibre composistion (Table 2.3).

Table 2.3 Classification of raw materials into four groups based on their fibre composition. (Adapted from Chesson, 1993; Cowan, 1996; Graham and Inborr, 1992)

Group	Raw materials	Main non-starch polysaccharide (NSP)	Main enzyme(s) used in supplementation
1	Barley, oats	β-glucan	β-glucanases
2	Wheat, rye, triticale	Arabinoxylan (pentosan)	β-xylanases
3	Sorghum (white), maize	-	α-amylases
4	Vegetable protein	Galactosaccharides and pectic materials	α-galactosidases, proteases, pectinases, phytases

Enzyme supplementation of broiler chicken feed improved feed conversion efficiency as a result of more efficient digestion and absorption of nutrients in the small intestine (Bedford and Classen, 1992). Broiler chickens fed rye as a cereal source fail to thrive due to the presence of arabinoxylan that increase digesta viscosity and consequently reduce digestibility of nutrients (Bedford and Classen, 1992; GrootWassink *et al*., 1989). However, addition of a xylanase-based enzyme preparation to a rye-based feed was shown to effectively improve growth performance of the chickens (GrootWassink *et al*., 1989). Xylanase-based products have also been shown to improve litter quality by reducing the viscosity and water-binding capacity of the excreta as a result of polysaccharide (arabinoxylan) degradation (Graham and Inborr, 1992). The addition of xylanases to a wheat-based feed has been shown to significantly increase the AME value of the feed, especially of feeds containing wheat with a low-AME value (Chesson, 1993).

Although the introduction of enzymes in animal feeds has been considered as one of the most significant advances in commercial animal nutrition in the past 30 years, there are still some questions relating to their use which have yet to be answered. One these is the issue of heat stability. By its nature, feed processing is strongly denaturing, whereas enzymes are protein catalysts and need to maintain their structural identity if they are to perform actively. Whilst one solution has been the application of liquid enzymes post-pelleting, the fact remains that the majority of feed enzymes are applied as dry powders and are thus exposed to the conditioning and pelleting process.

Pelleting can also have positive effects, such as better feed handling, increased feed utilization and better growth rate, which leads to enhanced bird performance (Leeson and Summers, 1991; Gibson, 1995). Pelleting can also be associated with negative effects, especially when the mixture is overheated. Overheating can result in resistance of protein (Araba and Dale, 1990) and starch (Brown, 1996) to digestion, inactivation of vitamins (Pickford, 1992), increased intestinal viscosity (Nissinen, 1994) and inactivation of endogenous enzymes (Inborr and Bedford, 1994).

Most of the inactivation takes place during conditioning, when the feed is heated with steam, rather than during extrusion of the pellets (Eeckhout *et al*., 1995). The problem of inactivation can be overcome by adding the enzyme as a liquid to finished pellets. The enzyme can be protected in substrate-bound and granulated enzyme preparations coated with hydrophobic compounds. Enzymes are added to the feed either directly or as a pre-mix along with vitamins, minerals and other feed additives such as choline chloride. However, the addition of liquid enzymes can result in a lack of homogeneity and requires special equipment. Despite these possibilities, many enzymes are mixed in the feed as a dry powder prior to pelleting because of simplicity (Silversides and Bedford, 1999).

2.4.3 Production of fuel ethanol from lignocellulosic waste

Probably the most compelling stimulus for research on hemicellulolytic enzymes was the oil crisis of the seventies, which sparked the search for alternative sources of liquid fuel and chemical feedstocks (Coughlan, 1992). Cellulose, starch and xylan are the three major carbohydrates found in available plant biomass from fuel crops, agricultural and forestry residues and wastes which are generated in vast tonnages annually. Xylan represents a large fraction of biomass that could be converted to xylose, ethanol and single cell protein, which will greatly contribute to solving both energy and food related problems (Wong *et al*., 1988).

The potential products obtainable with β-xylanases can be subdivided into two major categories: hydrolytic products and residual fibre. Selection among different β-xylanases may provide enzyme preparations with appropriate functional, physical and chemical properties. The judicious use of proper mixes of xylanolytic enzymes could result in cleaner reactions, higher yields and lower consumption of enzyme and energy, parameters vital to the economic feasibility of industrial processes (Puls and Schuseil, 1993).

Economic viability of biomass-based fuel alcohol production depends on the effective bioconversion of cellulose, but effective conversion of the xylan component will further enhance the economic gains of such a process (Hayn *et al*., 1993). A biological process for fuel-ethanol production from lignocellulose requires: (1) delignification to liberate cellulose and hemicellulose from lignin-carbohydrate complexes (LCC's), (2) depolymerization of cellulose and hemicellulose into their corresponding monomeric sugars and (3) fermentation of the mixed sugars, comprising hexose and pentose sugars, to ethanol (Lee, 1997). However, due to economic considerations the bioconversion of biomass has yet to be realized (Coughlan, 1992).

2.5 Heterologous protein production by *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* has been widely used as a host organism for the production of heterologous proteins such as enzymes, structural proteins, hormones, interferons and cytokines (Boreau *et al*., 1992; Hitzeman *et al*., 1981; Innis *et al*., 1985; Kniskern *et al*., 1991). Unlike bacteria, *S. cerevisiae* does not produce endotoxins, and products of yeast cells are considered safe for use in pharmaceutical and food products. Another advantage of using *S. cerevisiae* as host organism for heterologous protein production is that large-scale fermentation and downstream processing of the organism and its products are readily established, since this organism is one of the most commonly used species for industrial processes. Genetic manipulation of *S. cerevisiae* is done routinely, and this organism has several advantages over bacteria in that it carries out posttranslational modifications during the translocation of the proteins through the endoplasmic reticulum and the cell membrane. These modifications may include proper folding, glycosylation, disulfate bond formation and proteolysis (Li and Ljungdahl, 1996). Proteins secreted by yeast cells are protected from aggregation and protease degradation, and the secretion is facilitated by hydrophobic short signal peptides at the N-terminal regions of protein precursors. Several secreted yeast proteins and peptides, including invertase and mating α-factor pheromone (α-factor), have such signal peptides. These signal peptides are cleaved off by specific peptidases during the secretion process (Li and Ljungdahl, 1996). A number of heterologous proteins are often retained in periplasmic space or secreted into the culture medium at low yields when they are fused to these yeast signal peptides (Chaudhuri *et al*., 1992; Das and Shultz, 1987; Marten and Seo, 1991).

2.5.1 Targeting of a heterologous protein to the cell wall of Saccharomyces cerevisiae

The cell wall of *S. cerevisiae* consists of two major components, namely glucans and mannoprotein, and a minor component, chitin (Fleet and Phaff, 1981: Ballou, 1982; Cabib *et al*., 1982). Glucans is composed of both β-1,3- and β-1,6-linked glucose residues (Manners *et al*., 1973a,b). Mannoproteins can be divided into two classes: sodium dodecyl sulfate- (SDS) extractable and glucanase-extractable. SDS-extractable mannoproteins are in general of low molecular weight (Valentin *et al*., 1984), and glucanase-extractable mannoproteins of high molecular weight (Pastor *et al*., 1984; Frevert and Ballou, 1985).

Cell adhesion proteins mediate many cellular interactions, including mating reactions. The sexual agglutinins are cell adhesion proteins that mediate direct cell-cell contact during mating in budding yeasts (Lipke and Kurjan, 1992). α-Agglutinin, which is the sexual adhesion molecule of MATα cells, is a glucanase-extractable mannoprotein (Hauser and Tanner, 1989). The AGα1 gene encoding for the α-agglutinin, has been cloned and sequenced (Lipke *et al*., 1989). From the DNA sequence it was deduced that the N-terminal half of the molecule was likely to contain the α-agglutinin binding site. This was later confirmed by Cappellaro *et al*. (1991). The ability of the complementary agglutinins to interact with one another indicates that the binding domain of each agglutinin is accessible on the exterior surface of the cell wall (Lipke and Kurjan, 1992).

AGA1 and *AGα1* contain C-terminal hydrophobic sequences that lack the basic residues characteristic of transmembrane domains (Lipke *et al*., 1989; Roy *et al*., 1991). These hydrophobic domains are reminiscent of sequences present at the C-termini of precursors to eukaryotic cell surface proteins that are transported and linked to the cell membrane by glycosyl phosphatidylinositol (GPI) anchors (Cross, 1990; Low and Saltiel, 1988). GPI anchors are associated with cell

surface proteins of diverse function in both unicellular and multicellular eukaryotes. Such proteins are synthesized as precursors with hydrophobic C-terminal sequences (Fig. 2.2).

In an early step after transport into the endoplasmic reticulum, this sequence is cleaved and the remaining C-terminal amino acid becomes amide-linked to an ethanolamine residue, which is in turn linked through a phosphodiester bond to the C-6 position of a mannose residue in a complex glycan. The reducing terminus of the glycan is in turn linked to the inositol moiety of a phospho-inositide, resulting in attachment to the cell membrane.

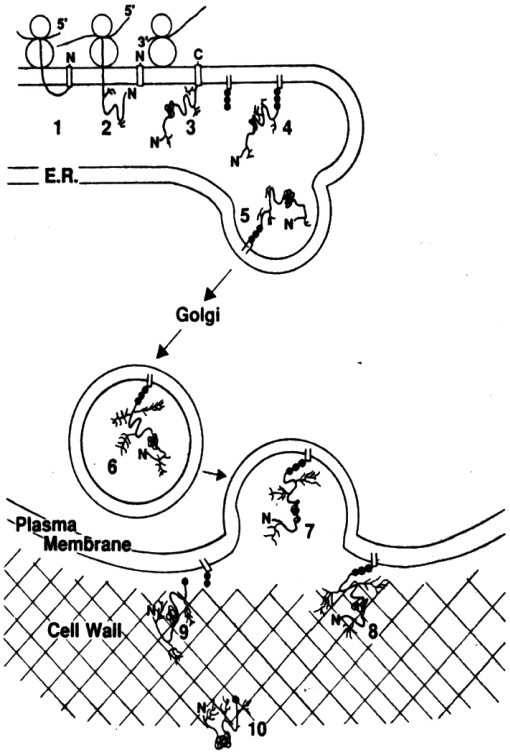

Fig 2.2 Model proposed by Lipke and Kurjan (1992) for agglutination localization. Boxes in the primary translation product indicate hydrophobic signal sequences. GPI anchors are associated with cell surface proteins of diverse function in both unicellular and multicellular eukaryotes. Such proteins are synthesized as precursors with hydrophobic C-terminal sequences

The C-terminal half of the α-agglutinin is not long enough to allow cell membrane anchorage of the mature form and simultaneous exposure of the binding domain at the surface of the cell wall, which is 1 000-2 000 Å (100-200 nm) thick (Ballou, 1982; Osumi *et al*., 1974). In addition, agglutinins are released from cells by treatment with β-glucanase, which would not occur if they remained attached to the membrane by a GPI anchor. α-Agglutinin is therefore likely to be bound to the cell wall. Lipke and Kurjan (1992) proposed that the α-agglutinin is transported to the cell surface by a GPI anchor and then released form the membrane and transferred into the matrix of the cell wall (Fig. 2.2) (Lipke *et al*., 1989). Transfer would involve release of a portion of the GPI moiety by *trans*-glycosylation or some other reaction. Unlike the sugars in N- and O-linked oligosaccharides, the reducing ends of the sugars in a GPI anchor are orientated away form the peptide, therefore a *trans*-glycosylation reaction is possible (Lipke and Kurjan, 1992).

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CHAPTER THREE

Cloning of the *XynA* gene of *Thermomyces lanuginosus* and Expression in *Saccharomyces cerevisiae*

3.1 Abstract

First strand cDNA was prepared from total RNA isolated from *Thermomyces lanuginosus* SSBP and the *XynA* gene amplified by using PCR. This gene was fused with a modified *Agα1* gene from *Saccharomyces cerevisiae* to form a cell surface immobilized enzyme construct. The modification of the *Agα1* entails the deletion of the binding domain region of this gene, and the cloning of the *XynA* into this region to construct a cell surface bound enzyme upon expression. This construct was inserted next to a *PDC1* promoter from *S. cerevisiae*, in two shuttle vectors, pRS416 (single copy) and pRS426 (multicopy). The recombinant *Agα1*::*XynA* was expressed in *S. cerevisiae*, and extracellular secretion of the xylanase was obtained, instead of the expected cell surface immobilization of the enzyme.

3.2 Introduction

The main polysaccharide-containing renewable resource in nature are plant cell walls, and they are composed of three major polymeric constituents: cellulose, hemicellulose and lignin (Biely, 1993). Xylan is a major component of hemicellulose and is comprised of a backbone of β-1,4-linked xylopyranose

residues which can be substituted with acetic acid, arabinose or 4-O-methylglucuronic acid side chains (Luttig *et al*., 1997).

Recently, much interest has been focused on the enzymatic hydrolysis of xylan, because of the potential application in the animal feed (Chesson, 1993), pulp and paper (Viikari *et al*., 1997) and baking industries (Maat *et al*., 1992). A variety of bacteria, yeasts and filamentous fungi have the ability to degrade xylan by producing a range of enzymes (Sunna and Antranikian, 1997). Several xylanolytic enzymes are required for the complete hydrolysis of this complex heteropolymer. The most important enzyme is endo-1,4-β-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylo-oligosaccharides, although other debranching enzymes are thought to play a synergistic role in the hydrolysis process (Biely, 1993).

Singh *et al.* (2000) isolated a high level of cellulase-free thermostable β-xylanase, produced by the deuteromycete *Thermomyces lanuginosus* strain SSBP. Little is known about the potential of *T. lanuginosus* strains to produce xylan-debranching enzymes such as α-glucuronidase, acetylxylan esterase and α-L-arabinofuranosidase (EC 3.2.1.55). The most extensively investigated strain, *T. lanuginosus* DSM 5826, was shown to produce very low amounts of debranching enzymes (Purkarthofer *et al*., 1993). There are no reports on the production in *T. lanuginosus* of other types of hemicellulose-hydrolyzing enzymes and there is only a single report on the production of pectinolytic enzymes (Gomes *et al*., 1992). Its selectivity for xylan, catalytic activity over a broad pH range, and especially its thermostability makes this enzyme extremely valuable in the paper and pulp industry. However, these qualities are also useful in the poultry feed industry, where feed enzymes are often exposed to high temperatures during feed processing (Eeckhout *et al*., 1995).

In this chapter the molecular cloning of a cDNA fragment coding for the *T. lanuginosus* (SSBP) β-xylanase is reported. Expression of the *XynA* gene was

investigated in *Saccharomyces cerevisiae*, with the aid of both single- and multicopy shuttle vectors. *S. cerevisiae* has several properties which have established it as a host for the expression of heterologous proteins of biotechnological interest. The yeast expression system offers a broader range of potential applications than bacterial expression systems. As an unicellular microorganism, yeast retains the advantages of bacterial systems in ease of manipulation and growth, while its eukaryotic sub-cellular organization is capable of post-translational processing and the modification of many heterologous proteins (Romanos *et al*., 1992*)*. Furthermore, *S. cerevisiae* has GRAS (Generally Regarded As Safe) status which allows for its use in the food industry. *S. cerevisiae* cannot utilize complex polysaccharides such as xylan (Crous *et al*., 1995).

The immobilization of the expressed enzyme was also investigated, with the fusion of an α-agglutinin, which is the sexual adhesion molecule of MATα cells of *S. cerevisiae*, and the expressed xylanase. This immobilized enzyme construct, expressed in *S. cerevisiae*, might be important for furthering biotechnology in areas of improved and large scale economic degradation of polymeric carbohydrates.

3.3 Materials and Methods

3.3.1 Strains

Thermomyces lanuginosus (SSBP) was kindly supplied by Prof. S. Singh (ML Sultan Technikon, Durban) and *Saccharomyces cerevisiae* (Σ12786) by Dr J. Albertyn (University of the Free State, Bloemfontein).

3.3.2 Plasmid cloning vectors

Expression of the *XynA* gene was investigated with the aid of two shuttle vectors, pRS416 (single copy vector) and pRS426 (multicopy vector). Both these vectors contain a *URA3* marker for selection and maintenance in yeast, as well as the β-lactamase gene of *E. coli* to confer ampicillin resistance. These vectors were propagated in *E. coli* Top 10 competent cells, with the *lacZ*-fusion protein as selector for positive transformants.

The pGEM®-T Easy vector (Promega, USA) was used for the sub-cloning of DNA fragments. This vector also contains a β-lactamase coding region, as well as a *lacZ* start codon. The T7 RNA polymerase promoter primer (5'-GTA ATA CGA CTC ACT ATA-3') from this vector was used to sequence ssDNA.

3.3.3 Primers and restriction enzymes

All primers were obtained from IDT (Integrated DNA Technologies, Inc.).

All restriction enzymes were purchased from Roche Molecular Biochemicals and used according to the conditions recommended by the supplier, unless otherwise stated.

3.3.4 Amplification of the promoter region

Primers *PDC1*-1F (5'-TGG GAT CCG AAA GAA GAT CAA GCG AGT CCA-3') and *PDC1*-1R (5'-GGA ATT CGA TTT GAC TGT GTT ATT TTG CG-3') (restriction sites for *Bam*HI and *Eco*RI respectively underlined) were used to amplify a 1191 bp fragment starting upstream from the initiation codon of a *PDC1* promoter from *Saccharomyces cerevisiae* strain W303-1A. DNA was amplified in

50 µl reaction mixtures containing 1 µl genomic DNA (*S. cerevisiae* W303-1A), 2 pmol of each primer, 0.2 mM of each dATP, dTTP, dCTP, dGTP, 5 µl of PCR reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 41 µl nuclease-free water and 5 units (U) Taq polymerase (Roche Molecular Biochemicals). The programme comprised of denaturation for 5 min at 94 °C and further denaturation, annealing and elongation for 30 sec at 94 °C, 30 sec at 55 °C and 2 min at 72 °C respectively for 25 cycles. The reaction was then held for a further 5 min at 72 °C to complete elongation. A 5 µl aliquot of the reaction was subjected to agarose gel electrophoresis on a 1% agarose (Promega, USA) TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) gel, separated at 85 mV and visualized by ethidium bromide staining under UV light (Herolab UVT-28 M).

3.3.5 Cloning of the PDC1 promoter into the two shuttle vectors

The 1191 bp fragment of the *PDC*1 promoter and both the shuttle vectors pRS416 and pRS426 were digested with *Eco*RI and *Bam*HI in a 25 µl reaction volume, consisting of 1 µl plasmid DNA, 10 U *Eco*RI, 10 U *Bam*HI, 2.5 µl SuRE/Cut Buffer H [50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol (DTT), pH 7.5] and 19.5 µl ddH_2O (double-distilled water) for 1 ½ h at 37 °C. Restriction endonuclease-digested DNA was eluted from the 1% agarose TAE gel with a Nucleospin® Extract kit (Machery-Nagel). The purified 1191 bp fragment of the *PDC1* promoter was ligated into both shuttle vectors using 0.5 U of T4 DNA ligase (Roche) with 2.3 µl ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM DTT, 10 mM ATP, pH 7.5) in a 23 µl reaction at 14 °C overnight.

3.3.6 DNA transformation

DNA was transformed according to the method described by Sambrook *et al*., 1989. *E. coli* Top 10 competent cells were thawed on ice. 10 µl of the ligation reaction was added to 80 µl of competent cells and incubated on ice for 30 min. This mixture was heat shocked for 40 sec at 42 °C. 800µl of LB media (0.5 % yeast extract, 1 % tryptone, 1 % NaCl) was added, and incubated for 1 h at 37 °C on a rotary shaker. This was then centrifuged for 2 min at 4 000 x g in a microcentrifuge (Biofuge 13, Heraeus Instruments) and the supernatant removed. The pellet was dissolved in 100 µl of LB media, and plated out on LB plates substituted with ampicillin (60 mg/l media), X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 mg/l media)] and IPTG [isopropylthio-β-D-galactoside (10 mg/l media)]. The plates were incubated at 37 °C overnight. Positive transformants (single white colonies) were inoculated to 5 ml LB-media substituted with 10 µl/ml (10 mg/ml stock) ampicillin and grown overnight at 37 °C on a shaker. DNA minipreparations were used to screen for the recombinant plasmid.

3.3.7 *DNA minipreparations*

Cells were harvested from LB media at 13 000 x g for 1 min. The supernatant was discarded, and the pellet re-suspended in 200 µl sterile STET-buffer [8 % sucrose, 5% Triton X-100 (v/v), 0.05 M Tris-HCl (pH 8,0), 5 mM EDTA pH 8,0]. 4 µl lysozyme (50 mg/ml) (Roche) was added and incubated for 1 min in a boiling waterbath. The tubes were placed on ice for 10 min and centrifuged for 15 min at 15 000 rpm. The pellet was discarded using a sterile toothpick, and 200 µl of isopropanol was added to the supernatant. The tubes were incubated at –20 °C for 10 min and centrifuged at 15 000 x g for 10 min. The supernatant was removed by aspiration and dried under vacuum in a SpeedVac Concentrator

SVC 100H (Savant). The pellet was resuspended in 50 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with RNase (50 μg/ml). The isolated plasmids were screened by restriction enzyme analysis with *Eco*RI and *Bam*HI in a 25μl reaction volume (as been described in section 3.3.5) at 37°C for 1 ½ h.

3.3.8 RNA isolation

RNA was isolated as has been described by Govender (1998). *T. lanuginosus* (SSBP) was cultivated in basal media (1.5 % yeast extract, 0.5 % KH_2PO_4) with 1.5 % oatspelts xylyn (Sigma, USA) as carbon source to induce β-xylanase production. The fungus was cultured in 250 ml Erlenmeyer flasks containing 100 ml media at 50 °C for 72 h on a rotary shaker. The fungal mycelium was harvested through cheese cloth and ground in liquid nitrogen. The frozen mycelium was ground to a fine powder with a mortar and pestle under liquid nitrogen, suspended in a mixture of 10 ml phenol (pre-heated at 65°C), 10 ml STE-buffer [100 mM NaCl, 250 mM Tris-HCl (pH 7.2), 10 mM EDTA] and 200 μl 10 % SDS (sodium dodecyl sulphate). This mixture was shaken for 4 min at 65 °C, and left on ice for 5 min. After centrifugation at 5 000 x g for 10 min, the upper layer was collected and extracted twice with equal volumes of phenol-chloroform-isoamylalcohol [25:24:1 (v/v/v)]. This was followed by precipitation with 1/50 volumes 5 M NaCl and 2 volumes 100% ethanol for 2 h at -20°C. The precipitate was pelleted, dried under vacuum for 5 min and re-hydrated in 100 μl diethyl pyrocarbonate (DEPC) treated water (2 % DEPC). The isolated RNA was subjected to electrophoresis on a formaldehyde RNA gel.

3.3.9 Amplification of the XynA gene

A two-step RT-PCR method (M-MLV Reverse Transcriptase RNase H minus, Promega) was used to synthesize complementary DNA (cDNA) from the isolated total RNA of *T. lanuginosus* to amplify the X*ynA* gene. The first step is responsible for first-strand cDNA synthesis, and occured in 22 µl reactions which consisted of 5 µl M-MulV reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 0.2 mM of each dATP, dCTP, dGTP, dTTP, 200 U M-MulV reverse transcriptase, 2 pmol of each XynA-1F (5'-AAG GAT CCA TGG TCG GCT TTA CCC CCG TTG-3') and XynA-1R (5'-AGA GTC GAC TTA GCC CAC GTC AGC AAC GGT C-3') primers (with restriction enzyme sites for *Bam*HI and *Sal*I underlined respectively), 5 µl of total RNA as target sequence and 8 µl nuclease-free water. The programme comprised of one cycle at 37 °C for 90 min for reverse transcriptation, and one cycle at 98 °C for 5 min to synthesize the first-strand cDNA. The reaction was cooled down to 4 °C for 15 min. The second step of the method is responsible for amplification of the double-strand DNA. This occurred in a 50 µl reaction, which consisted of 5 µl PCR buffer (containing 15 mM $MgCl_2$), 0.2 mM of each dCTP, dATP, dGTP, dTTP, 2 pmol of each XynA-1F and XynA-1R primers, 5 µl of cDNA (obtained from step one), 5 U Taq polymerase (Roche) and 37 µl nuclease free water. These reactions were subjected to denaturation, annealing and elongation for 30 sec at 94 °C, 1 min at 60 °C and 2 min at 68 °C respectively for 40 cycles. The reaction was then held for another 7 min at 68 °C to complete elongation. The amplified fragment was subjected to agarose gel electrophoresis.

3.3.10 DNA sequencing

The PCR-product was purified with the Nucleospin® Extract kit (Machery-Nagel), and ligated into a pGEM®-T Easy vector (Promega) according to the

manufacturer's instructions. 10 µl of the ligation reaction was transformed to *E. coli* Top 10 competent cells, and plasmids were extracted with the Nucleospin® Plus kit (Promega). Restriction enzyme analysis was performed to verify the orientation of the fragment in the vector, with the use of the restriction enzyme *Pvu*II according to conditions suggested by the supplier. The *Xyn*A gene was sequenced using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). The samples were prepared for PCR using half-reactions consisting of 4 µl pre-mix (as described by the manufacturers), 1.6 pmol T7 primer (5'-GTA ATA CGA CTC ACT ATA-3'), 2 µl PCR buffer (containing 15 mM $MgCl_2$), approximately 200-300 ng DNA and 10.5 µl nuclease-free water. The PCR programme comprised of 25 cycles consisting of denaturation, annealing and elongation for 10 sec at 96 °C, 5 sec at 50 °C and 4 min at 60 °C respectively. The obtained sequences were compared with existing sequences from NCBI, Entrez. The mRNA sequence of the *XynA* from *T. lanuginosus* (SSBP) was deposited into the NCBI library (Entrez).

3.3.11 Cloning of XynA into vectors

The amplified *XynA* gene from *T. lanuginosus* was cloned into the two shuttle vectors adjacent to the *PDC1* promoter. The *XynA* gene was removed from the pGEM®-T Easy vector with restriction enzymes *Eco*RI and *Sal*I, and the pRS416 and pRS426 vectors were digested with the same enzymes in 25 µl reactions consisting of 5 µl DNA, 10 U *Eco*RI, 10 U *Sal*I and 2.5 µl SuRE/Cut Buffer H. Digestion occurred at 37 °C for 90 min. The *XynA* gene was ligated into the pRS vectors using 0.5 U of T4 DNA ligase (Roche) with 2.3 µl ligation buffer in a 23 µl reaction at 14°C overnight. DNA transformation and plasmid extraction were performed as been described in sections 3.3.6 and 3.3.7. Restriction enzyme analysis confirmed that the correct insert was obtained.

3.3.12 Construction of expression system

3.3.12.1 Deletion of binding domain of Agα1 of Saccharomyces. cerevisiae

The binding domain region of the *Agα*1 gene of *S. cerevisiae* was deleted using the Expand Long Template PCR System kit (Roche). Primers AGA1-2F (5' ATA GTC GAC GGT ACA GCT AGC GCC AAA 3') and AGA1-2R (5' CGC AGA TCT ATC GTT GAT ATT TAT AGC 3'), with restriction enzyme digestion sites for *Sal*I and *Bgl*II underlined respectively, was used to amplifiy a 4336 bp fragment consisting of the *Agα1* (without binding domain region) in a pUC 18 expression vector. A 50 µl PCR reaction was prepared consisting of 1 µl template DNA (*Agα1* cloned into pUC18), 2 pmol of each primer, 0.2 mM of each dCTP, dATP, dGTP, dTTP, 5 µl of PCR buffer 1 (containing 17.5 mM $MgCl_2$), 40 µl of nuclease-free water and 3.5 U Taq-polymerase. An initial denaturation cycle of 94 °C for 3 min was followed by further denaturation, annealing and elongation for 30 sec at 94 °C, 30 sec at 55 °C and 3 min 30 sec at 68 °C respectively. Final elongation at 72 °C for 7 min completed the PCR reaction. The amplified product was subjected to agarose gel electrophoresis

3.3.12.2 Cloning of XynA into the modified Agα*1*

The *XynA* was digested from the pGEM®-T Easy vector with the use of restriction enzymes *Bam*HI and *Sal*I in 25 µl reactions. The first reaction consisted of 5 µl plasmid DNA, 10 U *Bam*HI, 2.5 µl SuRE/Cut Buffer B (10 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, pH 8.0) and 16.5 µl water. This reaction-mixture was incubated at 37 °C for 90 min. It was necessary to

precipitate the digested DNA for further digestion with *Sal*I, because of non-complementary digestion buffers of the two restriction enzymes. The DNA was precipitated by the addition of 1/10 of the sample volume 3 M sodium acetate and twice the total volume cold 100 % ethanol. This was incubated at –20 °C for 15 min, and centrifuged at 15 000 x g for 10 min at 4 °C. The supernatant was removed by aspiration and 1 ml 70 % ethanol was added. This was centrifuged for 5 min at 15 000 x g, the supernatant removed and the pellet dried under vacuum. The pellet was re-suspended in 16.5 µl ddH_2O, and was then subjected to digestion with *Sal*I. This reaction consisted of 16.5 µl of DNA suspended in ddH_2O, 10 U *Sal*I and 2.5 µl SuRE/Cut Buffer H (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.5). Incubation at 37 °C for 90 min completed the digestion process, which left the fragment with sticky ends on both ends.

The amplified fragment consisting of the modified Agα1 in the pUC18 vector was subjected to restriction enzyme digestion with *Bgl*II and *Sal*I in a 25 µl reaction which consisted of 2 µl DNA, 10 U *Bgl*II, 10 U *Sal*I, 2.5 µl SuRE/Cut Buffer H (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, pH 7.5) and 18.5 µl water. This reaction was incubated for 3-4 h at 37 °C, and resulted in compatible sticky ends with the digested *XynA*. Restriction endonuclease-digested DNA was eluted from a 1% agarose TAE gel with a Nucleospin® Extract kit (Machery-Nagel), and the two fragments were ligated with 0.5 U T4 DNA ligase (Roche), in 2.3 µl ligation buffer (10x) at 14-16 °C overnight.

3.3.12.3 Preparation of competent cells and DNA transformation

E. coli Sure™ 2 cells were prepared competent according to the method by Inoue *et al.* (1990). 10 µl of the ligation reaction was transformed to the *E. coli* Sure™ 2 competent cells as been described in section 3.3.6, and plasmid extraction was performed as been described in section 3.3.7. Restriction

enzyme analysis was performed with the use of *Acc*65I (Promega) according to instructions from the manufacturers, to confirm that the ligation was successful.

3.3.12.4 Amplification of modified Agα1

PCR was used to amplify the modified *Agα*1 (which contains the *XynA* gene in the place of the binding domain), with the use of primers AGA1-1F (5' CGC AAG CTT ATG TTC ACT TTT CTC AAA 3'), and AGA1-4R (5' CGA CTC GAG TTA GAA TAG CAG GAT CGA 3'), with the restriction enzyme digestion sites for *Hind*III and *Xho*I underlined respectively. 50 µl PCR reactions consisted of 1 µl template DNA, 2 pmol of each primer, 0.2 mM of each dATP, dGTP, dCTP, dTTP, 5 µl PCR buffer (containing 15 mM $MgCl_2$), 41 µl nuclease-free water and 5 U Taq-polymerase (Roche). After an initial denaturing cycle of 5 min at 94 °C, the reactions were subjected to 25 cycles of denaturation, annealing and elongation which comprised of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C respectively. The reactions were incubated for a further 5 min at 72 °C to complete elongation. The PCR products were visualized on a 1% agarose TAE gel stained with ethidium bromide.

3.3.12.5 *Cloning of modified Agα1 into pRS-shuttle vectors*

Both the amplified modified *Agα1* and the pRS416 and pRS426 shuttle vectors (containing the *PDC*1 promoter) were digested with *Hind*III and *Xho*I. Digestion reactions consisted of 6 µl of DNA, 10 U *Hind*III, 10 U *Xho*I, 2.5 µl SuRE/Cut Buffer B and 14.5 µl water for the digestion of the modified *Agα1*. For the digestion of the shuttle vectors, which contain *Hind*III and *Xho*I restriction sites in their multiple cloning sites, 2 µl of DNA was digested with *Hind*III and *Xho*I as described above. Both digestion reactions were incubated at 37 °C for 2 h, upon

which the DNA was purified with the use of a GFX™ PCR and Gel Band Purification kit (Amersham Pharmacia Biotech Inc.). DNA transformations and plasmid extraction were performed as been described in sections 3.3.6 and 3.3.7 respectively. Restriction enzyme analysis were performed to confirm that the correct insert was obtained.

3.3.13 Yeast transformation

S. cerevisiae (Σ12786) was inoculated from a day old culture into 5 ml YPD (1 % yeast extract, 2 % peptone and 2 % glucose) and incubated at 30 °C under shaking conditions for 14-16 h. 1 ml of the yeast culture was centrifuged for 4 sec at 13 000 x g in Eppendorf tubes, and the supernatant was removed by aspiration. 25 µl (of a 2 mg/ml stock) salmon sperm DNA (after boiling for 10 min and placed on ice) and 5 µl of plasmid DNA (modified *Agα1* in the pRS-shuttle vectors as well as the *XynA* gene in the pRS vectors) were added to the pellet. 100 µl one-step buffer (0.5 ml 2 M lithium acetate, 4 ml 50 % polyethelene glycol, 0.5 ml 1 M DTT) was added per tube, and incubated for 30 min at 42 °C. This was centrifuged for 8 sec at 13 000 x g, and the supernatant was completely removed by aspiration. The pellet was resuspended in 250 µl of YPD, and the cells were plated out on selective SC^{-ura} medium [20 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids (US Biological), 0.6 g/l complete supplement mixture minus histidine, leucine, tryptophan and uracil (Bio 101, Inc.), 30 mg/l of each tryptophan, leucine and histidine and 15 g/l agar]. The plates were incubated at 30 °C for 2-3 days.

3.3.14 Screening for β-xylanase activity

Transformants were screened for their xylan degrading ability by plating transformed colonies on oatspelts xylan agar plates (per litre of distilled water):

10 g oatspelts xylan (Sigma), 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 5 g $(NH_4)_2SO_4$, 1 g $CaCl_2$, 0.1 g yeast extract, 0.1 g NaCl, 0.3 g chloramphenicol, 15 g agar and 1 ml trace element solution [1000 fold concentrate: 950 ml distilled water, 50 ml HCl (concentrated), 35 g $FeSO_4.7H_2O$, 7 g $MnSO_4.H_2O$, 11 g $ZnSO_4.7H_2O$, 1 g $CuSO_4.5H_2O$, 2 g $CoCl_2.6H_2O$, 1.3 g $Na_2MoO_4.2H_2O$, 2 g H_3BO_3, 0.35 g KI, 0.5 g $Al_2(SO_4)_3$] substituted with 2 % glucose. The initial pH of the medium was adjusted to 5.0. Positive transformants were identified by zones of hydrolysis around the colonies.

3.4 Results

3.4.1 Amplification of the PDC1 promoter

The *PDC1* promoter of *S. cerevisiae* (W303-1A) was successfully amplified by PCR.

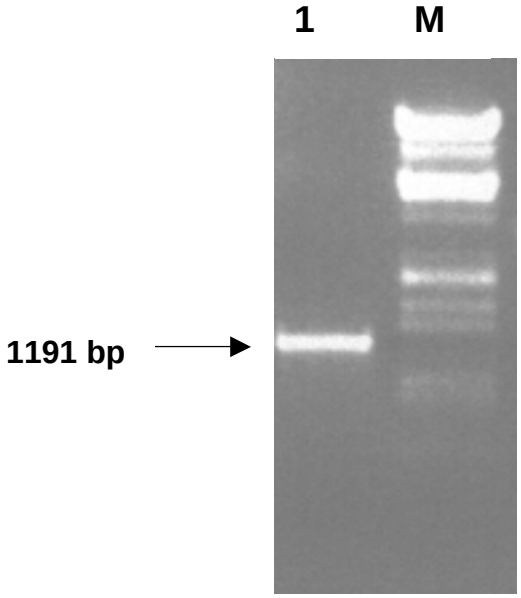

Fig. 3.1 Agarose gel electrophoresis of the amplified fragment containing the *PDC1* promoter. Lane M : λ phage DNA digested with *Eco*RI and *Hind*III. Lane 1: 1191 bp PCR product.

3.4.2 Cloning of the PDC1 promoter into pRS416 and pRS426 shuttle vectors

The 1191 bp fragment of the *PDC1* promoter was amplified by PCR and ligated into the two shuttle vectors pRS416 and pRS426. These ligations were transformed to *E. coli* Top 10 competent cells, and subsequent restriction enzyme analysis performed on extracted plasmids confirmed positive clones. Figure 3.2 represent the fragments obtained upon restriction enzyme analysis with *Eco*RI and *Bam*HI.

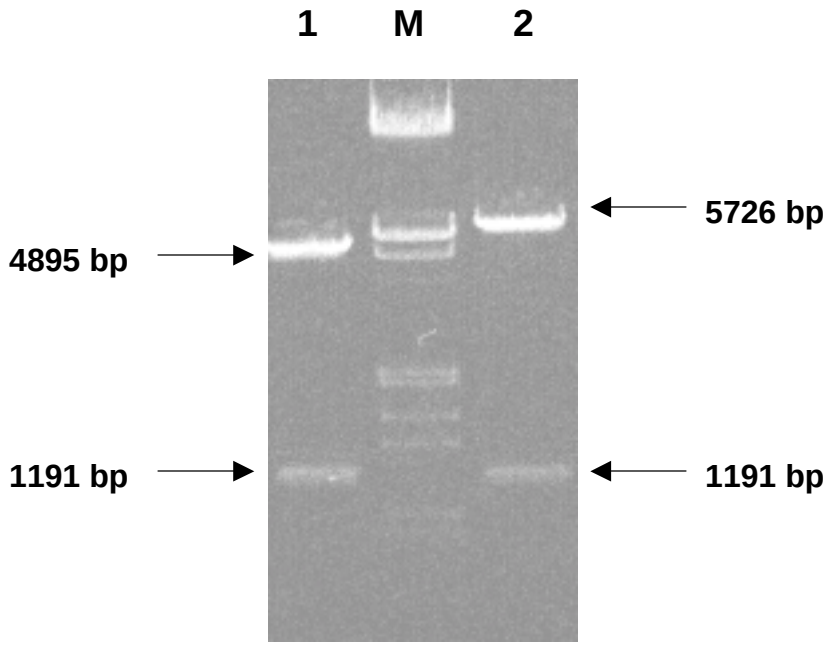

Fig. 3.2 Agarose gel electrophoresis of plasmid DNA from transformed *E. coli* Top 10 competent cells. Lane M: λ phage DNA digested with *Eco*RI and *Hind*III. Lane 1: pRS416 (4 895 bp) containing the 1191 bp insert. Lane 2: pRS426 plasmid (5726 bp) with the 1191 bp insert.

3.4.3 RNA isolation

T. lanuginosus was cultured on a basal medium containing 1.5 % oatspelts xylan, which is responsible for the induction of xylanase activity displayed by this fungus. The RNA isolation method used proved to be very successful, as three bands were obtained (Fig. 3.3). The bands obtained represent the 28S RNA, 18S RNA, 5.8S RNA, 5S RNA and tRNA.

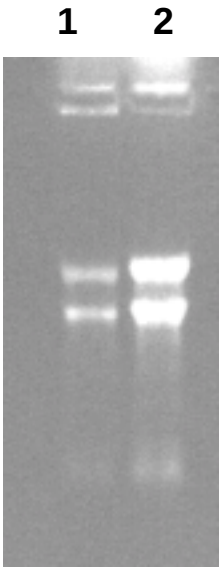

Fig. 3.3 Agarose gel electrophoresis of total RNA (lanes 1 and 2) from *T. lanuginosus* (SSBP).

3.4.4 Amplification of the XynA gene

The total RNA isolated was subjected to a two-step RT-PCR method, with the use of sequence specific primers for the amplification of the *XynA* gene. The

reverse transcriptase is responsible for first-strand cDNA synthesis, followed by the amplification of the double-strand DNA.

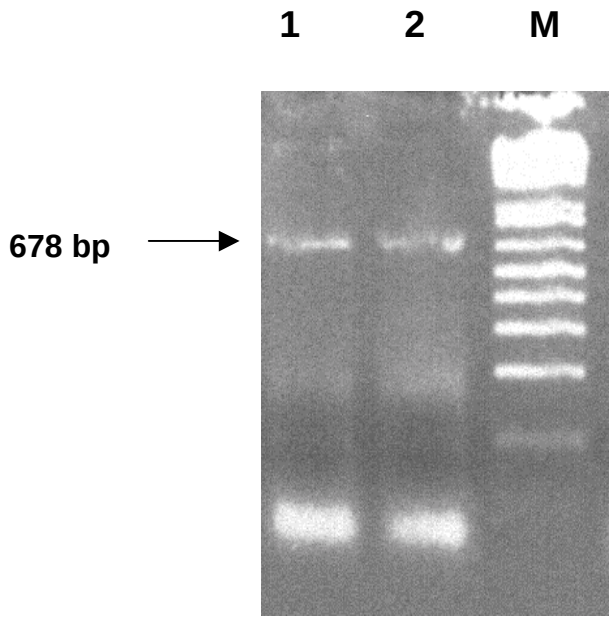

Fig. 3.4 Agarose gel electrophoresis of the amplified fragment (678 bp) containing the *XynA* gene of *T. lanuginosus* (SSBP). Lane M: λ phage DNA digested with *Eco*RI and *Hind*III. Lanes 1 and 2: fragments containing the 678 bp RT-PCR product.

*3.4.5 DNA sequencing of the X*ynA *gene*

The expected 678 bp fragments, containing the β-xylanase coding gene of *T. lanuginosus*, were purified with the use of the Nucleospin® Extract kit. These fragments were cloned into the pGEM®-T Easy vector (Promega) and subjected to restriction enzyme analysis with restriction enzyme *Pvu*II. Three bands were obtained upon agarose gel electrophoresis. The 2567 bp fragment correspond to the plasmid-backbone, whilst the pair of 630 bp and 499 bp fragments represent the insert in one orientation in the plasmid, and the pair of 757 bp and 372 bp fragments represent the insert in the opposite orientation in the plasmid.

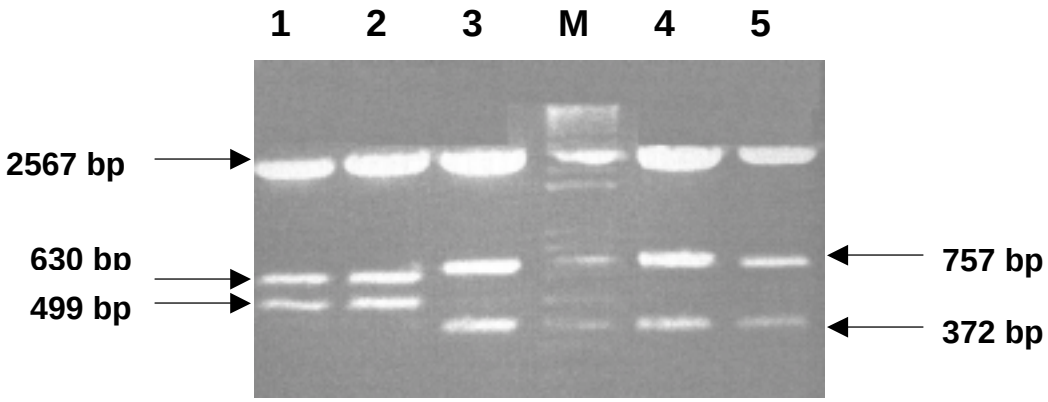

Fig. 3.5 Agarose gel electrophoresis of the cloned *xynA* gene of *T. lanuginosus* (SSBP) after restriction enzyme analysis was performed with *Pvu*II. Lane M: 1 kb marker. Lanes 1 and 2: represent the insert in one orientation in the plasmid (with fragments corresponding to 630 bp and 499 bp in size). Lanes 3-5: represent the insert in the opposite orientation in the plasmid (with fragments corresponding to 757 bp and 372 bp in size). The 2567 bp fragments in lanes 1-5 represent the plasmid-backbone.

The *xynA* gene sequence was obtained using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). Fig. 3.6 represent the nucleotide sequence of the *XynA* gene of *T. lanuginosus* aligned with the known genomic sequence of *T. lanuginosus XynA*.

The DNA sequence of the *XynA* gene from *T. lanuginosus* comprise of 678bp. The 106 bp intron present in the genomic DNA was eliminated through the first strand cDNA step of the RT-PCR performed on the total RNA.

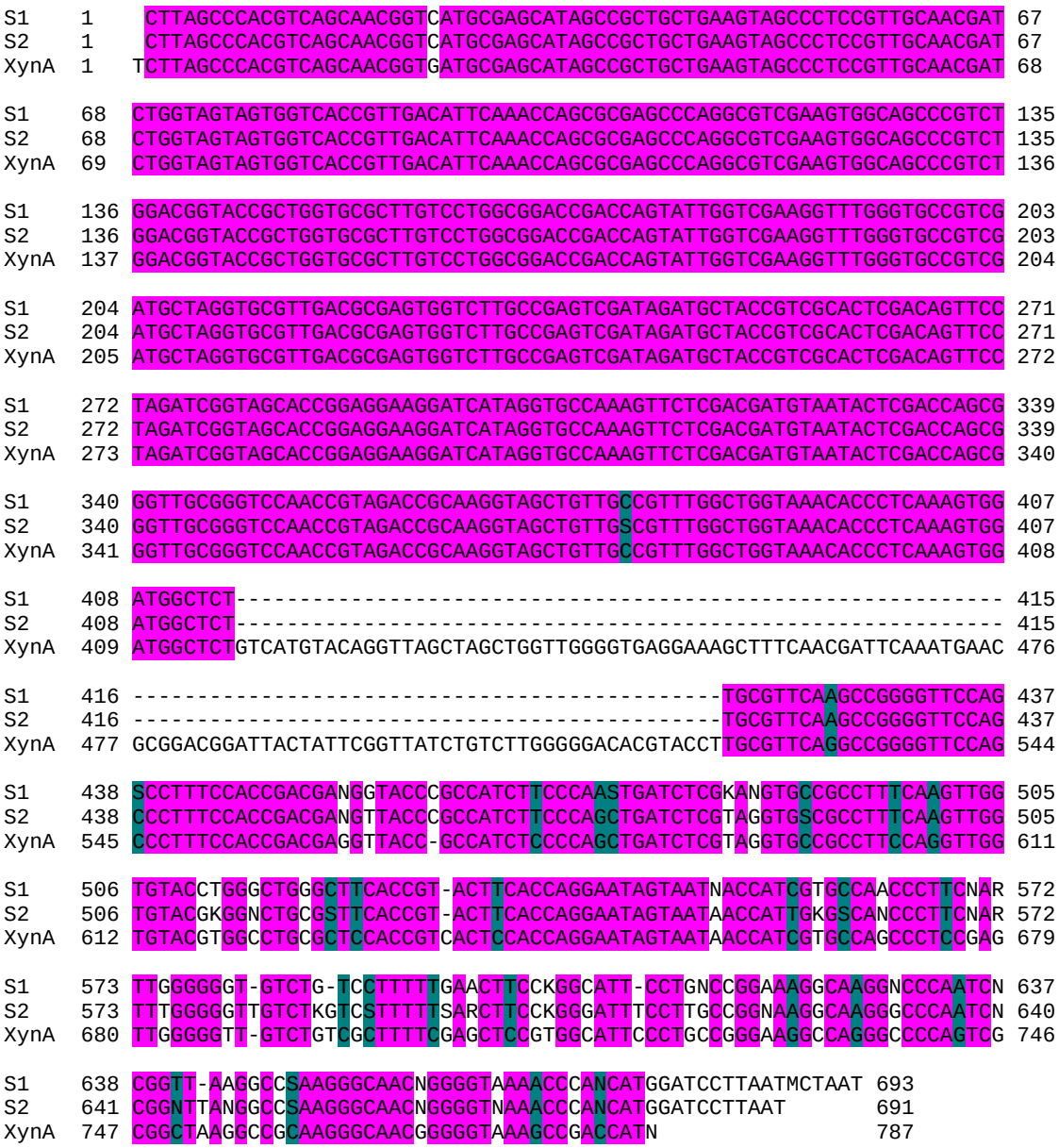

Fig. 3.6(a) Clones S1 and S2 were aligned with the reverse orientation of the known genomic sequence of *T. lanuginosus XynA*.

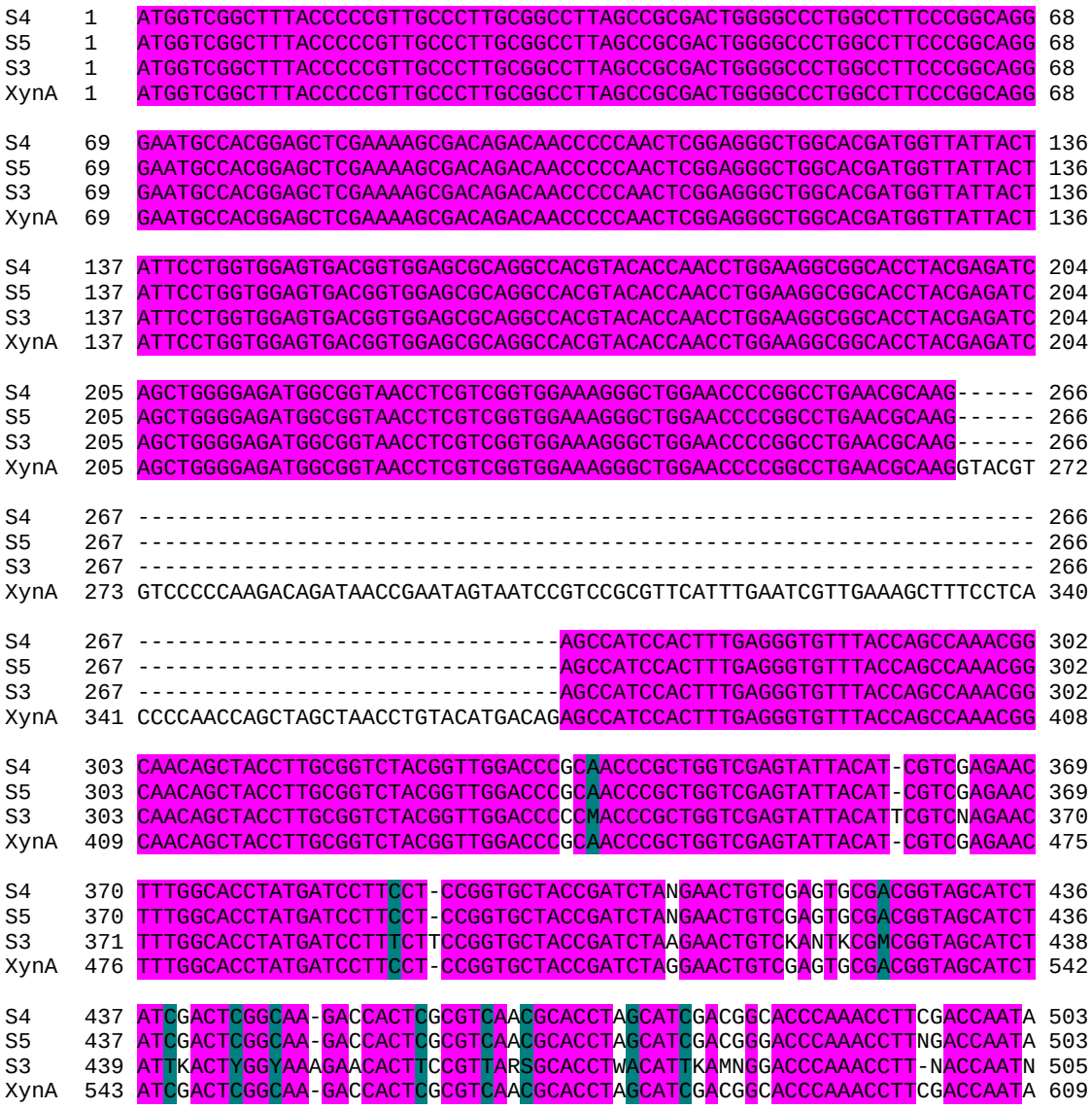

Fig. 3.6(b) Clones S3, S4, and S5 were aligned with the forward orientation of the known genomic sequence of *T. lanuginosus XynA*.

3.4.6 Construction of an immobilized-enzyme expression system in S. cerevisiae

3.4.6.1 Deletion of binding domain of Agα1 gene of S. cerevisiae

The binding domain of the *Agα1* of *S. cerevisiae*, cloned into a pUC18 expression vector, was deleted using the Expand Long Template PCR System kit (Roche) with sequence specific primers, containing restriction enzyme digestion sites for *Sal*I and *Bgl*II (Fig. 3.7).

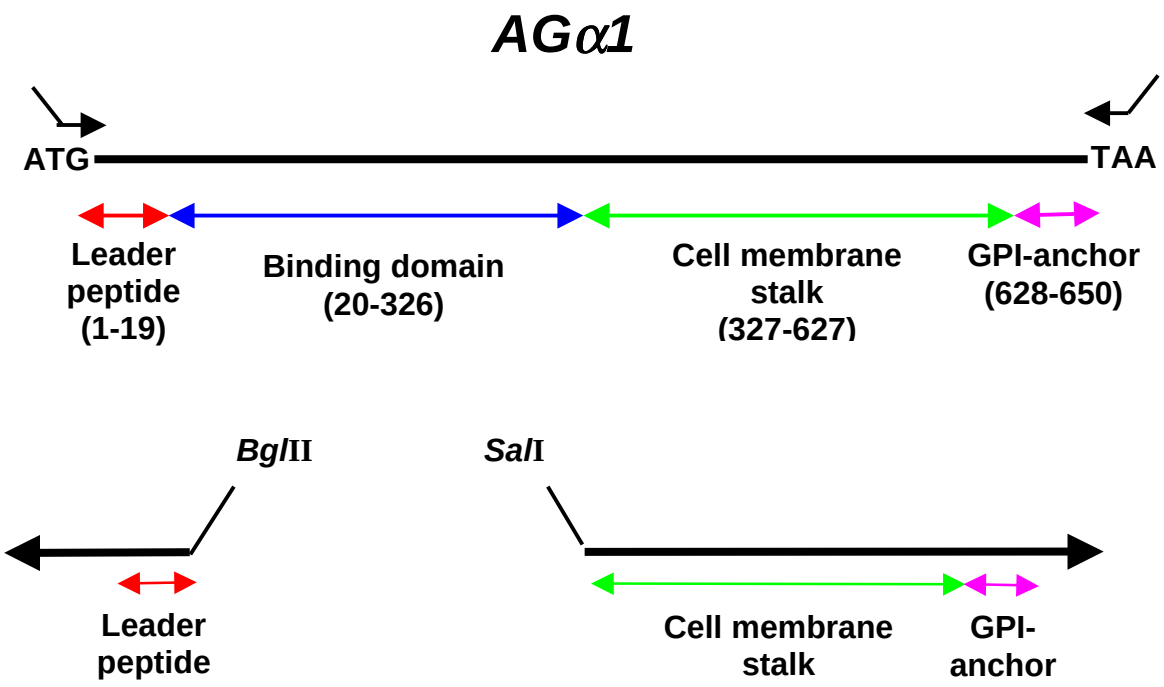

Fig. 3.7 Schematic representation of the construction of the expression cassette, with the deletion of the binding domain coding region.

The expected 4336 bp fragment, representing the *Agα1* without the binding domain region cloned into pUC18, was obtained upon agarose gel electrophoresis of the PCR products (Fig 3.8).

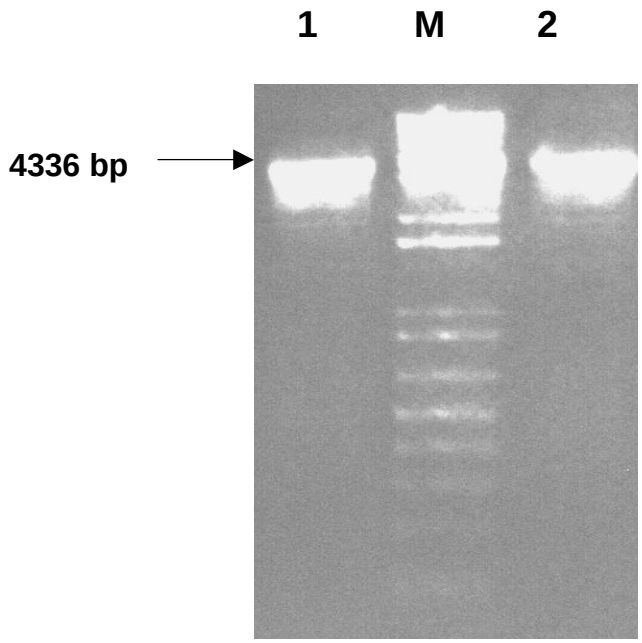

Fig. 3.8 Agarose gel electrophoresis of the PCR products representing the *Agα1* gene, without the binding domain region, cloned into a pUC18 expression vector. Lane M: 1 kb marker. Lanes 1 and 2: fragments containing the 4336 bp PCR product.

3.4.6.2 Cloning of XynA into modified Agα1

The cloned *XynA* gene was digested from the pGEM®-T Easy vectors with the use of restriction enzymes *Bam*HI and *Sal*I, and ligated into the space from the deleted binding domain of the *Agα1* gene (Fig. 3.9) (with *Bam*HI and *Bgl*II sharing complementary sticky ends).

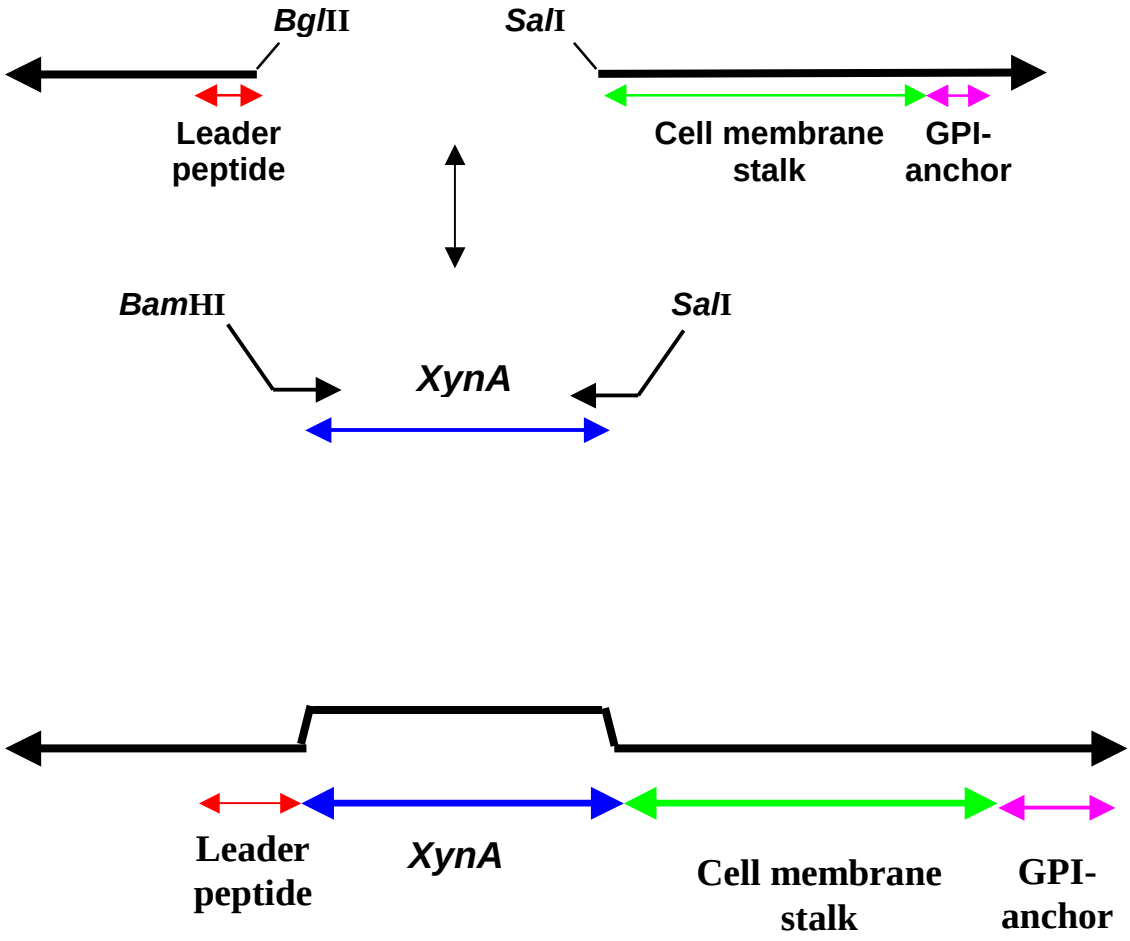

Fig. 3.9 Schematic representation of the cloning of the *XynA* gene into the modified *Agα1*.

3.4.6.3 Amplification on the modified Agα1

The modified *Agα1*, which contained the *XynA* gene in the place of the deleted binding domain, was amplified by PCR with sequence-specific primers. Restriction enzyme analysis confirmed that the correct insert was obtained (Fig. 3.10).

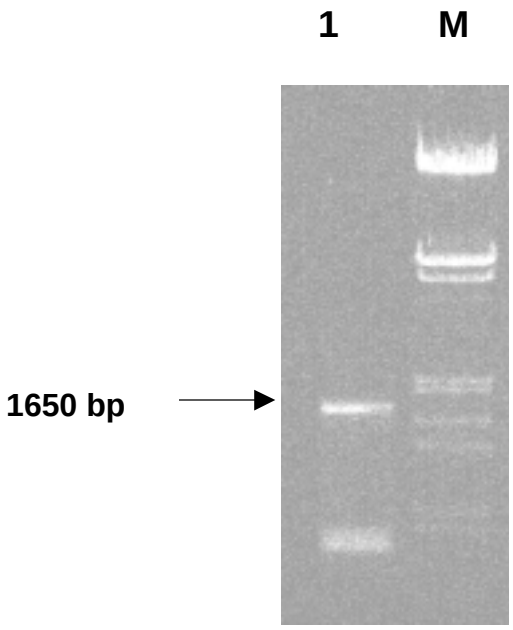

Fig. 3.10 Agarose gel electrophoresis of the PCR product representing the amplification of the modified *Agα1.* Lane M: λ phage DNA digested with *Eco*RI and *Hind*III. Lane 1: fragment containing the 1650 bp PCR product.

3.4.6.4 Cloning of the modified Agα1 into the pRS-shuttle vectors

The *Agα1::XynA* was cloned into the pRS416 and pRS426 shuttle vectors, adjacent to the *PDC1* promoter (Fig. 3.11).

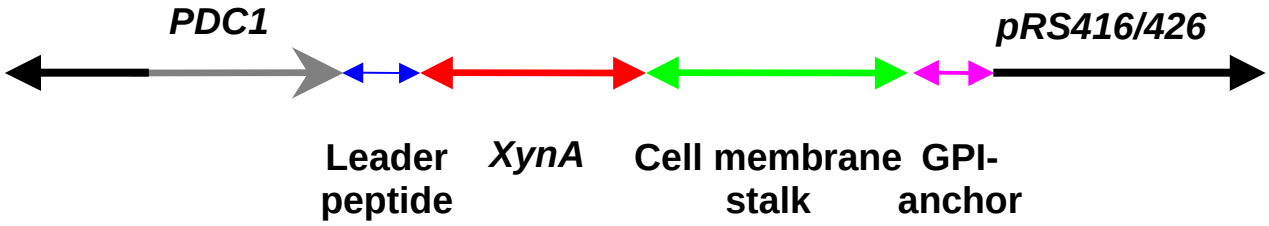

Fig. 3.11 Schematic representation of the completed expression cassette, cloned into the two shuttle vectors pRS416 and pRS426, adjacent to the *PDC1* promoter.

Restriction enzyme analysis was performed with the enzymes *Bam*HI and *Xho*I, to confirm that the correct inserts have been obtained. Three bands were obtained upon restriction enzyme analysis, with sizes 5726 bp and 4895 bp corresponding to the pRS16 and pRS426 backbones respectively.

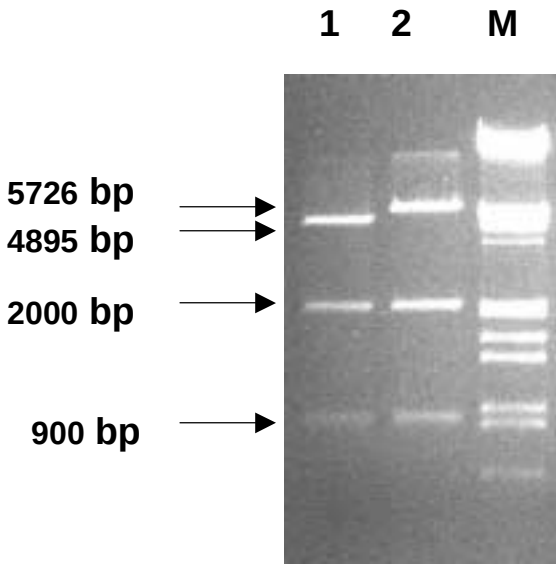

Fig. 3.12 Agarose gel electrophoresis of the restriction enzyme analysis representing the completed expression system. Lane M: λ phage DNA digested with *Eco*RI and *Hind*III. Lane 1: amplified Agα1 in the pRS416 vector. Lane 2: amplified Agα1 in the pRS426 vector.

3.4.7 Screening for β-xylanase activity

Recombinant *S. cerevisiae* Σ12786 strains with plasmids pRS416 and pRS426 containing only the *PDC1* promoter and the amplified *XynA* gene from *T. lanuginosus*, as well as the plasmids containing the fused *Agα1* gene with the *XynA*, were assayed qualitatively for β-xylanase production and secretion on oatspelts xylan agar plates over 5 days of incubation at 30 °C. Clear zones of hydrolysis around colonies indicate β-xylanase activity (Fig.3.13).

Fig. 3.13 Qualitative assay of β-xylanase activity. Clear zones around colonies represent hydrolyzed xylan. Colony 1 represent the transformed *S. cerevisiae* with the multicopy (pRS426) plasmid containing the cassette, and colony 2 represent the transformed *S.* cerevisiae with the single copy (pRS416) plasmid containing the cassette. Colonies 3-5 represent the control (wild type *S. cerevisiae*).

3.5 Discussion

Glycolytic promoters belong to the strongest promoters known in yeast. Many of them have been used to overexpress yeast genes normally transcribed only at low levels or genes from different species (Schmitt *et al*., 1983). A glycolytic

promoter of *S. cerevisiae*, *PDC1*, was chosen for the efficient transcription of the xylanase from *T. lanuginosus*. Genes coding for enzymes acting in the lower part of glycolysis are induced upon glucose addition to respiratory growing cells (Hauf *et al*., 2000). *PDC1* are induced up to 20-fold with growth on glucose as sole carbon source (Schmitt *et al*., 1983). The induction is triggered by the formation of glycolytic metabolites, leading to an autoactivation of glycolysis (Boles and Zimmerman, 1993a,b; Müller *et al*., 1995). This promoter was successfully cloned into two shuttle vectors, one a yeast centromeric plasmid (YCp), pRS416, and the other a yeast episomal plasmid (YEp), pRS426. Yeast centromeric plasmids include a CEN/ARS cassette that provides mitotic stability and the ability to be autonomously replicated. Yeast episomal plasmids (YEp) replicate autonomously at high copy number in the yeast nucleus by virtue of sequences derived from the natural 2μ circular yeast plasmid (Christianson *et al*., 1992). The marker used for selection of the transformants was *URA3* used in a corresponding mutant strain, which was auxotrophic for uracil. Continued selection required the use of minimal growth media lacking the relevant nutrient.

It was necessary to isolate total RNA from the xylanolytic fungus *T. lanuginosus*, as the presence of an intron in the *XynA* gene prevented the use of genomic DNA. cDNA was constructed and the *XynA* open reading frame amplified with the use of a two-step RT-PCR method using M-MulV reverse transcriptase. The amplified *XynA* was cloned into a pGEM®-T Easy vector (Promega) and was subjected to DNA sequencing to confirm that it was indeed the *XynA* gene from *T. lanuginosus* which was amplified (Fig. 3.6)

The α-agglutinin (*Agα1*) gene of *S. cerevisiae* was used for the construction of an immobilized enzyme construct. The sexual agglutinins are cell adhesion proteins that mediate direct cell-cell contact during mating in budding yeasts (Lipke and Kurjan, 1992). The ability of the complementary agglutinins to interact with one another indicates that the binding domain of each agglutinin is accessible on the exterior surface of the cell wall (Lipke and Kurjan, 1992). The

binding domain of the *Agα1* gene was deleted, and the amplified *XynA* was inserted in its place, as immobilization can increase the stability of enzymes (Monsan and Combes, 1988) and cell walls can be used as a source of immoblilized enzymes (Sakai *et al*., 1991). Schreuder *et al*. (1993) have shown that the C-terminal half of the α-agglutinin functions as a cell wall anchoring domain. This raises the question of which particular features of the C-terminal half of the α-agglutinin are involved in cell wall anchoring. The coding sequencing of the C-terminal half predicts (a) that it is exceptionally rich in serine and threonine, and (b) that it contains a hydrophobic tail that functions as an attachment signal for a glycosyl-phosphatidyl-inositol (GPI) anchor. When the C-terminal hydrophobic tail of the α-agglutinin and the Ggp/Gas protein (Nuoffer *et al*., 1991) are deleted, these proteins are secreted into the growth medium indicating that GPI anchors are essential for cell surface retention (Schreuder *et al*., 1993).

The modified *Agα1* (fused with the *XynA* gene of *T. lanuginosus*) was ligated into the two shuttle vectors adjacent to the *PDC1* promoter, and transformed to *S. cerevisiae* (Σ12786). *S. cerevisiae* has been widely used as host organism for the production of heterologous proteins. Unlike bacteria, *S. cerevisiae* does not produce endotoxins, and products of yeast cells are considered safe for uses in pharmaceutical and food products. Another advantage of using *S. cerevisiae* as a host organism for heterologous protein production is that large-scale fermentation and downstream processing of the organism and its products are readily established, since this organism is one of the most commonly used species for industrial processes. Genetic manipulation of *S. cerevisiae* is done routinely, and this organism has several advantages over bacteria in that it carries out posttranslational modifications during the translocation of proteins through the endoplasmic reticulum and the cell membrane. These modifications may include proper folding, glycosylation, disulfate bond formation, and proteolysis. Proteins secreted by yeast cells are protected from aggregation and protease degradation, and they are easily purified. The secretion of proteins is

facilitated by hydrophobic short signal peptides at the N-terminal regions of protein precursors. These signal peptides are cleaved off by specific peptidases during the secretion process (Li and Ljungdahl, 1996).

Qualitative enzyme assays performed on the transformed *S. cerevisiae* cells by means of activity plates, confirmed the extracellular secretion of the transformed xylanase from *T. lanuginosus*. However, it also confirmed the secretion of the enzyme from the *PDC1::Agα1::XynA* construct, where immobilization of the xylanase was expected.

The possibility of incorporating proteins in the cell wall of *S. cerevisiae* raises interesting biotechnological possibilities. The cell wall incorporated proteins stay cell associated and are present outside the plasma membrane. Cells producing such proteins can be easily harvested and can be used as a source of immobilized enzymes (Schreuder *et al*., 1993). With the growing interest in proteins being generated by bioengineering, it is becoming increasingly important to understand the roles different expression systems play in the synthesis process. The work reported here is an example of recombinant DNA technology to develop an immobilized cell surface expression system in *S. cerevisiae*. The initial steps in this approach have been accomplished with the isolation and characterization of the *XynA* gene of *T. lanuginosus*, and the construction of the immobilized enzyme system. Expression of this gene has been obtained in *S. cerevisiae*, although further investigation into the immobilization of the enzyme could enhance the potential of such a system.

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CHAPTER FOUR

Partial characterization of the cloned xylanase from *Thermomyces lanuginosus* expressed in *Saccharomyces cerevisiae*

4.1 Abstract

The xylanase from *Thermomyces lanuginosus* (*XynA*) was cloned into two shuttle vectors, pRS416 (single copy vector) and pRS426 (multi-copy vector) adjacent to a *PDC1* promoter (designated pRS416:*XynA* and pRS426:*XynA*). An expression cassette for this xylanase was constructed by cloning of the *XynA* gene into a modified *α*-agglutinin (*Aga1*) gene from *Saccharomyces cerevisiae*. This modification entailed the deletion of the binding domain coding region of the *Aga1*, and the cloning of the *XynA* gene into this deleted binding domain region, which is flanked by a stalk-like protein coding region. This fusion protein was cloned into two shuttle vectors (pRS416 and pRS426), flanking the *PDC1* promoter (designated pRS416:*Aga1*::*XynA* and pRS426:*Aga1*::*XynA*). The aim of the cassette was to immobilize the expressed enzyme on the cell surface of the yeast cell with the expression of the xylanase on the stalk of the *Aga1*, however, extracellular secretion of the enzyme was obtained upon expression. Enzyme assays performed on pRS416:*XynA* and pRS426:*XynA* yielded very low activity [0.1505 U/ml (2.5088 nKat/ml) and 0.0909 U/ml (1.5153 nKat/ml) respectively], whereas pRS416:*Aga*1::*XynA* and pRS426:*Aga1*::*XynA* yielded activities of 1.7035 U/ml (28.3973 nKat/ml) and 1.7319 U/ml (28.8707 nKat/ml) respectively. The partial characterization of this extracellular secreted recombinant xylanase (pRS416:*Aga*1::*XynA* and pRS426:*Aga1*::*XynA*) yielded

an optimum temperature of 70 °C and an optimum pH of 6.0-7.0. Thermal stability for the recombinant xylanase was determined for temperatures 50 °C, 60 °C and 70 °C, and the activation energy for pRS416:*Agα*1::*XynA* and pRS426:*Agα1*::*XynA* were calculated as 34.86 kJ/mol and 53.59 kJ/mol respectively.

4.2 Introduction

Xylan is a major component of the cell walls of monocots and hardwoods, representing up to 35 % of the dry weight of these plants (Puls and Schuseil, 1993). Xylan is second only to cellulose in natural abundance and represents a major reserve of fixed carbon in the environment. Unlike cellulose, xylan is a complex polymer consisting of a backbone of 1,4-glycosidically linked β-D-xylose with branches containing xylose, other pentoses, hexoses and uronic, ferrulic, cinnamic and acetic acids (Biely, 1985). The branches of sugars and sugar acids are also linked glycosidically to the backbone of xylan whereas acids are attached by ester linkages (Biely, 1985).

The complete breakdown of the xylan requires several different side-branch-splitting enzymes. The most important enzyme is endo-1,4-β-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylo-oligosaccharides, although other debranching enzymes are thought to play a synergistic role in the hydrolysis process (Biely, 1993).

In recent years, interest in thermostable enzymes has increased dramatically as resistance to thermal inactivation has become a desirable property of the enzymes used in many industrial applications (Singh *et al*., 2000c). Applications for enzymatic saccharification of xylan include the animal feed (Chesson, 1993), pulp and paper (Viikari *et al*., 1997) and baking industries (Maat *et al*., 1992).

Hydrolysis of lignocellulosic wastes by biological routes could also yield feedstocks for production of chemicals and fuels (Lynd *et al*., 1991).

Several microbial sources have been investigated for β-xylanase production, however, the thermophilic fungus *Thermomyces lanuginosus* is one of the best xylanase producers yet reported (Singh *et al*., 2000b). Strains of *T. lanuginosus* thrive at temperatures of up to 60 °C and are capable of producing high titres of cellulase-free xylanase. In addition to its thermostability, the xylanase from this fungus is also active over a wide pH range (Singh *et al*., 2000a).

The yeast *S. cerevisiae* has been widely used as a host organism for the production of heterologous proteins such as enzymes, structural proteins, hormones, interferons and cytokines (Boreau *et al*., 1992; Hitzeman *et al*., 1981; Innis *et al*., 1985; Kniskern *et al*., 1991). An *Aspergillus kawachii* endo-1,4-β-xylanase was cloned and expressed in *S. cerevisiae* by Crous *et al*. (1995). The xylanase gene (*xynC*) was inserted between the yeast phosphoglycerate kinase (*PGK1*) gene promoter ($PGK1_p$) and terminator ($PGK1_T$) sequences. This $PGK1_p$-*xynC*-$PGK1_T$ construct (designated XYN3) was cloned into a multi-copy episomal plasmid and the XYN3 gene was expressed in *S. cerevisiae*. The secreted β-xylanase (Xyn3) was stable between 30 °C and 50 °C, the optimum temperature was 60 °C and optimum pH lower than pH 3. An autoselective *fur1::LEU2 XYN3* recombinant strain was developed that allowed β-xylanase production at a level of 300 nkat/ml in a non-selective medium (Crous *et al*., 1995).

Two β-xylanase-encoding genes from *Aspergillus niger* were cloned and expressed in *S. cerevisiae* by Luttig *et al*. (1997). Each gene was inserted between the yeast alcohol dehydrogenase II promoter ($ADH2_p$) and terminator ($ADH2_T$) sequences in a yeast multi-copy vector. Both enzymes exhibited pH and temperature optima of pH 4 and 60 °C respectively. The maximum β-

xylanase activity obtained in complex medium was 91 nkat/ml (Xyn4) and 73 nkat/ml (Xyn5) for the two enzymes respectively (Luttig *et al*., 1997).

La Grange *et al*. (1996) described the cloning of the *Trichoderma reesei* β-xylanase gene (*XYN2*) and expression of this gene in *S. cerevisiae*. Expression was obtained with the aid of multi-copy plasmids, using two different *S. cerevisiae* promoter-terminator expression cassettes, derived from the inducible alcohol dehydrogenase II (*ADH2*) gene and the constitutive 3-phosphoglycerate kinase (*PGK1*) gene. The autoselective *S. cerevisiae* strains produced 1 200 nkat/ml and 160 nkat/ml of β-xylanase activity, under the control of the *ADH2 and PGK1* promoters respectively. The recombinant enzyme showed highest activity at pH 6 and 60 °C, and retained more that 90 % of its activity after 60 min at 50 °C (La Grange *et al*., 1996).

This chapter describes the assessment of the properties of the cloned xylanase from *T. lanuginosus* SSBP expressed fused with the α-agglutinin gene from *Saccharomyces cerevisiae*, and the comparison of this recombinant protein with the novel xylanase from *T. lanuginosus* SSBP as isolated and characterized by Singh *et al*. (2000c).

4.3 Materials and Methods

4.3.1 Materials

Xylan from oatspelts and birchwood were obtained from Sigma (USA). All other chemicals and reagents were of analytical grade, and no further purification was needed.

4.3.2 Media and growth conditions

Yeast transformants (transformed with the pRS416*:XynA*, pRS426*:XynA*, pRS416*:Aga1::XynA* and pRS426*:Aga1::XynA* constructs) were inoculated into 5 ml yeast nitrogen base (US Biological) substituted with all amino acids except uracil with the addition of 2 % glucose for the induction of the *PDC1* promoter. The pre-inoculum was grown at 30 °C overnight on a rotary shaker. From this pre-inoculum, 500 µl were inoculated into 50 ml YPD (1 % yeast extract, 2 % peptone and 2 % glucose) and incubated at 30 °C for 12-14 h.

4.3.3 Harvesting of enzyme

The culture medium was centrifuged for 10 min at 5 000 x g, and enzyme assays were performed on whole cells, as well as the dialyzed supernatant. The obtained supernatant was dialyzed against a 0.05 M cocktail buffer (consisting of 0.1 M Tris-HCl and 0.1 M tri-sodiumcitrate), pH 6.5, at 4 °C overnight.

4.3.4 Enzyme assays

Endo-1,4-β-D-xylanase activity was assayed according to Bailey *et al*. (1992), with the necessary modifications made for the analysis of a thermophilic xylanase, as has been suggested by the authors. Activity was determined by incubating 100 µl of enzyme solution at pH 6.5 and a temperature of 70 °C for 5 min in 900 µl of substrate solution [1 % (w/v) birchwood xylan in 0.05 M cocktail buffer, pH 6.5). Reducing sugars were assayed by the addition of 3 ml DNS (per litre: 16 g NaOH, 10 g dinitrosalycylic acid and 300 g potassium sodium tartrate tetrahydrate crystals), boiling for 5 min, cooling, and measuring the absorbance at 540 nm, against the reagent blank. Reagent blanks were constructed by the incubation of 900 µl of substrate for 5 min at 70 °C, addition of 3 ml DNS and 100

µl buffer (0.05 M cocktail buffer, pH 6.5), boiling and cooling, in that order. Enzyme blanks were constructed for the correction of absorbance of background colour in the enzyme blank, by incubating 900 µl of substrate solution at 70 ° C for 5 min, addition of 3 ml DNS and 100 µl enzyme solution, boiling and cooling, in that order. Absorbance values obtained at A_{540} for enzyme blanks were subtracted from absorbance values obtained from assayed samples.

Whole cell enzyme assays were performed on harvested cells, suspended in 0.05 M cocktail buffer. Enzyme assays were performed on the dialyzed supernatant obtained upon centrifugation. As control, the untransformed *S. cerevisiae* (Ó12786) was subjected to the same conditions as transformed clones. A standard curve was constructed with xylose as standard. One unit of enzyme activity is defined as the formation of 1 µmole product (xylose) per minute per milliliter of enzyme used. Data analysis were performed with GraphPad Prism version 3.0.

4.3.5 Partial characterization of the recombinant xylanase from T. lanuginosus

4.3.5.1 Optimum temperature

The optimum temperature for the expressed xylanase was determined over a temperature range of 30 –100 °C, incubated for 5 min, as has been described in section 4.3.4. Assays were performed in triplicate, and the standard deviations were calculated for each temperature.

4.3.5.2 Optimum pH

The optimum pH was determined over a range of pH 3-12. The enzyme extract was diluted 1:1 with 0.05 M cocktail buffer, pH ranging from pH 3-12. Standard deviations were calculated for each pH value.

4.3.5.3 Thermal stability

The temperature stability was determined at temperatures 50 °C, 60 °C and 70 °C. The enzyme was incubated at different temperatures for different time intervals and aliquots were periodically withdrawn and placed on ice, before residual xylanase activity was measured. The total incubation time was 48 h. Assays were performed in triplicate, and the standard deviations calculated.

4.3.5.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Saccharomyces cerevisiae transformants (containing the pRS416:*XynA*, pRS426:*XynA*, pRS416:*Agα1::XynA* and pRS426:*Agα1::XynA* constructs) were grown in 5 ml YPD (1 % yeast extract, 2 % peptone and 2 % glucose) at 30 °C overnight on a rotary shaker as pre-inoculum. From this pre-inoculum, 1 ml were inoculated in 100 ml YNB (yeast nitrogen base, with amino acids and with ammonium sulphate) (US Biological) supplemented with 30 mg/l uracil (for untransformed cells as control only) and 1% glucose. This inoculum was incubated at 30 °C, on a rotary shaker for 12-14 h followed by centrifugation for 10 min at 5000 x g. Extracellular proteins were precipitated by the addition of 100 ml 40 % TCA (tri-chloro-acetic acid) to 100 ml of the supernatant, incubated on ice for 1 h and centrifuged at 10 000 x g for 1 h at 4 °C. The pellets were re-suspended in 1 ml ice-cold acetone, transferred to an Eppendorf tube, and centrifuged for 5 min at 13 000 x g. The supernatant was discarded, and the pellets were re-suspended in 100 µl ice-cold acetone. This suspension was centrifuged for 5 min at 13 000 x g. The supernatant was discarded, and the pellet was resuspended in 1 % SDS, 1.5 M Tris (pH 8.8). Equal volumes of protein and 2 x sample disruption mixture (125 mM Tris-HCl, pH 6.8, 10 % 2-

mercaptoethanol, 10 % SDS, 10 % glycerol and little bromophenol blue) were boiled for 5 min and cooled on ice prior loading of gel.

SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli *et al*. (1970).

4.4 Results

4.4.1 Partial characterization of the recombinant T. lanuginosus xylanase

Cells harvested upon centrifugation were washed twice with 0.05 M cocktail buffer (pH 6.5) after which the cells were re-suspended in this buffer. Enzyme assays were performed on the whole-cell suspensions, as well as on the dialyzed supernatants from the pRS416:*XynA*, pRS426:*XynA*, pRS416:*Aga*1::*XynA* and pRS426:*Aga1*::*XynA* constructs. Assays were performed in triplicate. No activity was obtained from the whole-cell fractions, however, endo-1,4-β-D-xylanase activity was found in the dialyzed supernatant from pRS416:*Aga*1::*XynA* and pRS426:*Aga1*::*XynA*, and thus further experiments were performed on these constructs' supernatants as crude enzyme extract. The dialyzed supernatant from pRS416:*XynA* and pRS426:*XynA* yielded very little activity (see table 4.1). The control (wild type *S. cerevisiae* Ó12786) yielded no activity when assayed under experimental conditions.

Table 4.1 Activity of respective recombinant xylanase constructs.

Construct	Activity (Units/ml)	Activity (nKat/ml)
pRS416:*XynA*	0.1505	2.5088
pRS426:*XynA*	0.0909	1.5153
pRS416:*Aga1*::*XynA*	1.7035	28.3973
pRS426:*Aga1*::*XynA*	1.7319	28.8707

Activity in Units is defined as the formation of 1 µmol of xylose per minute per milliliter enzyme, whereas 1 Kat corresponds to the conversion of 1 mol xylan per second. Therefore, 1 Unit corresponds to 16.67 nKat.

A standard curve was constructed with xylose as standard for the conversion of absorbance values to enzyme activity in units, where one unit is defined as the formation of 1 µmol of xylose per minute per milliliter enzyme.

A 1.5 g/l stock solution of xylose was used for a dilution series to construct the standard curve, diluted with 0.05 M cocktail buffer (pH 6.5). The standard solutions were treated and assayed under conditions described for enzyme blanks.

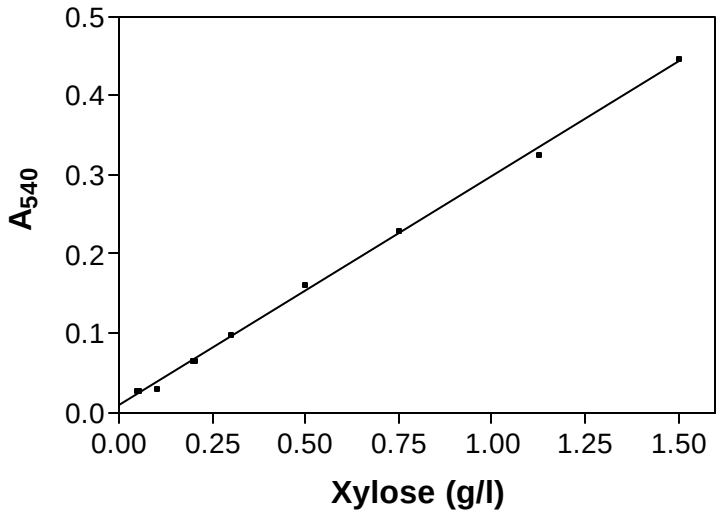

Fig. 4.4.1 Standard curve constructed with xylose as standard for the conversion of absorbance values to enzyme activity in units. Standard deviations for triplicate determinations are smaller than the symbols used for the data points.

4.4.1.1 Optimum temperature

The optimum temperature for the xylanase from *T. lanuginosus*, expressed in both single- and multicopy vectors as part of a fusion protein with the *Agα1* gene from *S. cerevisiae*, was determined over a temperature range of 30 °C – 100 °C.

Fig. 4.4.2(a) represents the optimum temperature for the xylanase expressed by the single copy vector (pRS416:*Agα1*::*XynA*), and fig. 4.4.2(b) depicts the optimum temperature for the xylanase expressed by the multicopy vector (pRS426:*Agα1*::*XynA*).

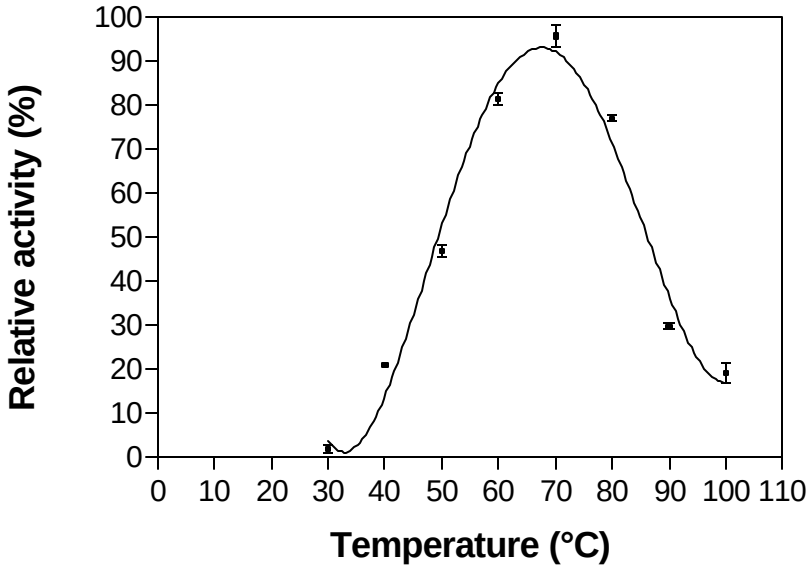

Fig. 4.4.2(a) Optimum temperature of the xylanase from *T. lanuginosus* expressed by a single copy vector, pRS416. Standard deviations for triplicate determinations are shown as error bars.

Although the enzyme is active over a wide range of temperatures, the optimum temperature for the recombinant xylanse from *T. lanuginosus* expressed by the pRS416:*Agα1*::*XynA* construct, was found to be 70 °C.

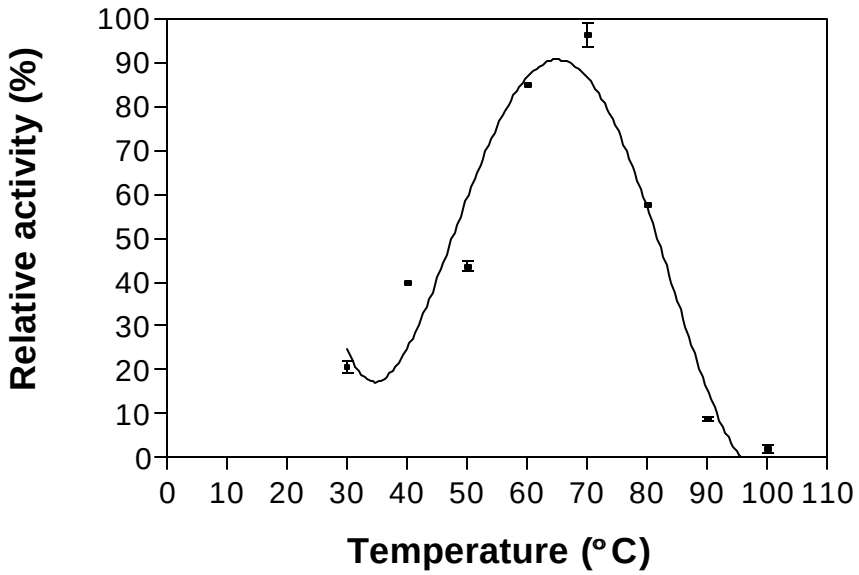

Fig. 4.4.2(b) Optimum temperature of the xylanase from *T. lanuginosus* expressed by a multicopy vector, pRS426. Standard deviations for triplicate determinations are shown as error bars.

The same trend is observed in Fig. 4.2.2 (b) as seen in Fig. 4.2.2 (a). The pRS426:*Agα1*::*XynA* construct also display highest amount of activity at 70 °C.

4.4.1.2 Optimum pH

The activity was determined over a range of pH 3-12. The pH range was constructed by adjustment of the pH of the substrate used. For each pH, the remaining activity was analyzed in triplicate and standard deviations determined, as depicted in Fig. 4.4.3 (a) and (b).

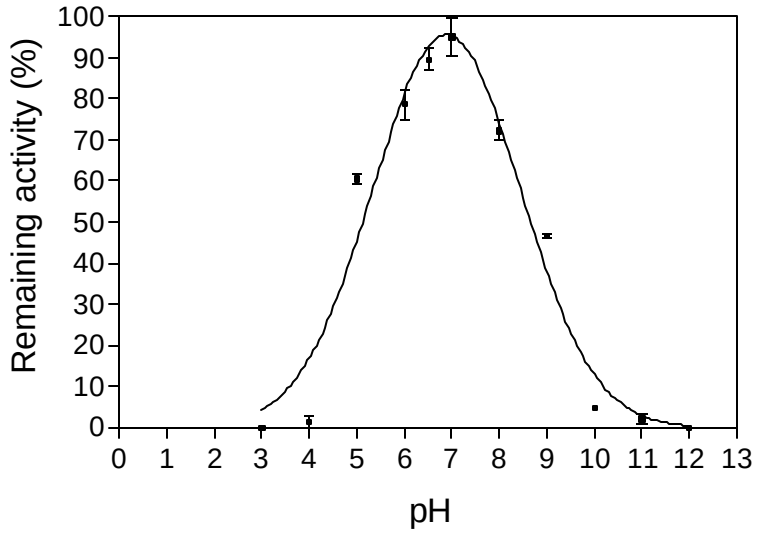

Fig. 4.4.3(a) Optimum pH of the xylanase from *T. lanuginosus* expressed by a single copy vector, pRS416. Standard deviations for triplicate determinations are shown as error bars.

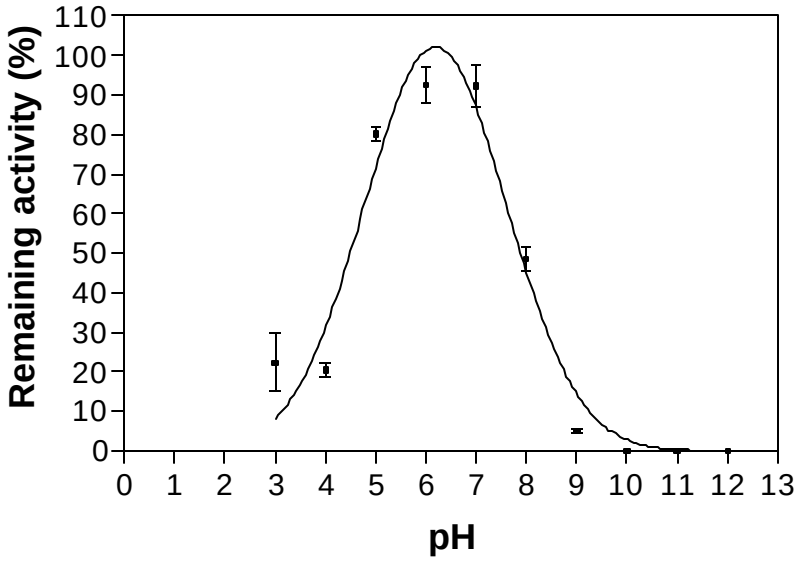

Fig. 4.4.3(b) Optimum pH of the xylanase from *T. lanuginosus* expressed by a multicopy vector, pRS426. Standard deviations for triplicate determinations are shown as error bars.

The optimum pH for the expressed xylanase was found to be pH 6.0-7.0 for the two constructs. A similar trend in activity for corresponding pH values for both constructs was obtained.

4.4.1.3 Thermal stability

The temperature stability was determined at temperatures 50 °C, 60 °C and 70 °C. Aliquots were periodically withdrawn over a 48 h period, and placed on ice. Residual activity was determined in triplicate, and standard deviations calculated. Fig. 4.4.4 (a) and (b) depict the inactivation curves for the xylanase at these temperatures over the 48 h period for both expression vectors.

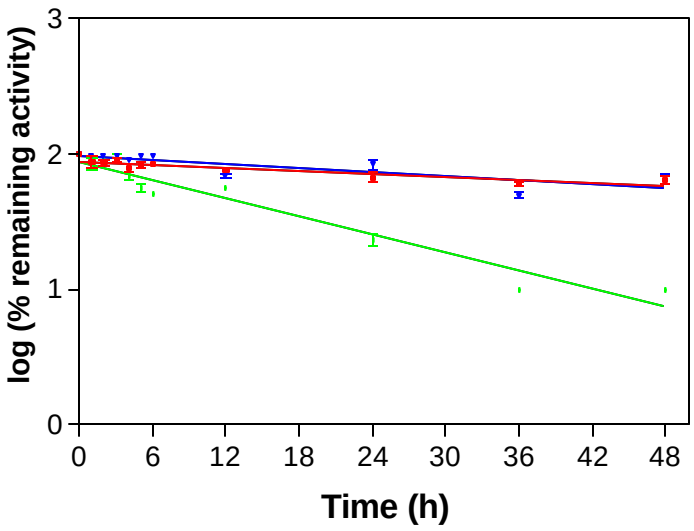

Fig. 4.4.4(a) Typical inactivation curves for the inactivation of the recombinant xylanase from *T. lanuginosus* expressed by a single copy vector, at different temperatures over 48 h. Standard deviations are shown for each determination [50 °C (■), 60 °C (▼), 70 °C (◆)].

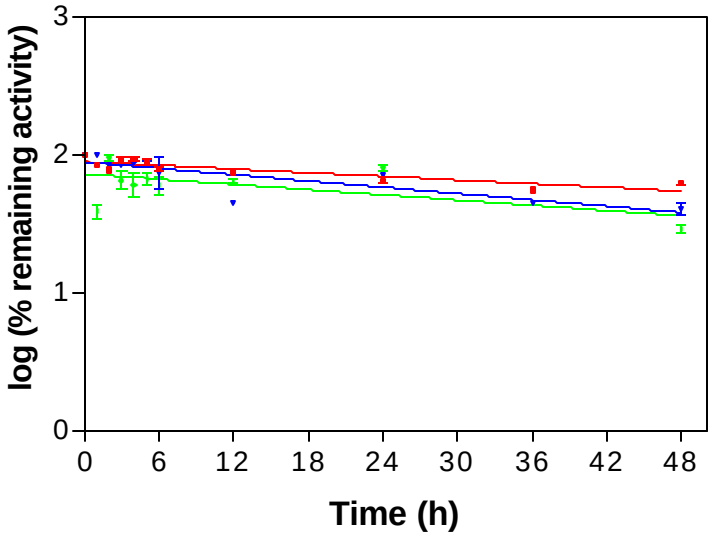

Fig. 4.4.4(b) Typical inactivation curves for the inactivation of the recombinant xylanase from *T. lanuginosus* expressed by a multicopy vector, at different temperatures over 48 h. Standard deviations are shown for each determination [50 °C (■), 60 °C (▼), 70 °C (◆)].

The inactivation energy is calculated from the gradient and the pre-exponential factor from the intercept of the Arrhenius plots [Fig. 4.4.5(a) and (b)]. The Arrhenius plots are based on the expression $k = Ae^{(-Ea/RT)}$ where k is the inactivation rate constant, A is the pre-exponential factor, R is the gas constant (8.3144 J/Kmol), T is the absolute temperature, and E_a is the activation energy for the reaction (Cornish-Bowden, 1995). Rate constants for the inactivation were calculated by linear regression analysis of the plot of log remaining activity (%) vs. time at different temperatures (50 ° C, 60 °C, 70 °C).

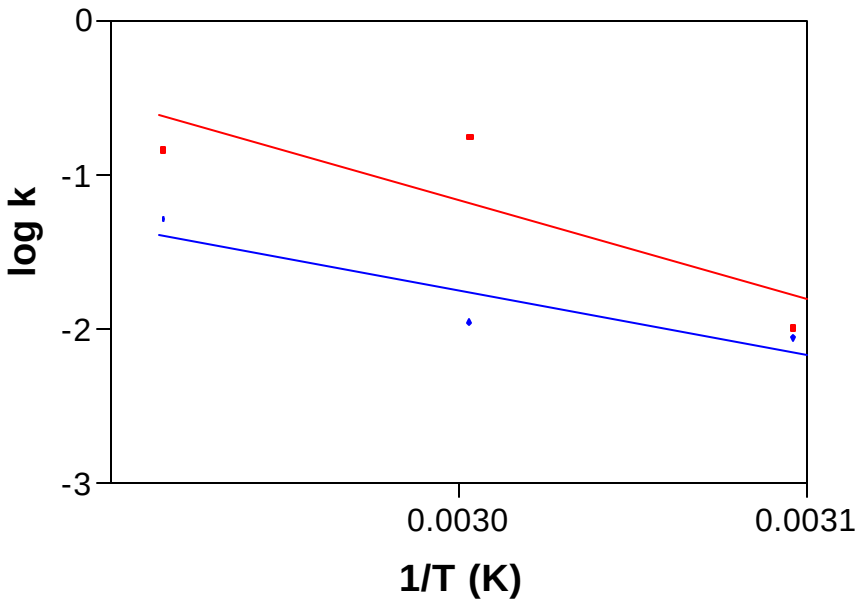

Fig. 4.4.5 Arrhenius plots for the inactivation of the xylanase from *T. lanuginosus*, expressed by a single copy vector (◆), and multicopy vector (■).

The activation energy of the xylanase was 34,86 kJ/mol for the pRs416:*Agα1*::*XynA* construct, and 53,59 kJ/mol for the pRs426:*Agα1*::*XynA* construct.

Half-life's calculated from the semi-logarithmic plots of the percentage of remaining xylanase activity over 48 h at temperatures 50 °C, 60 °C and 70 °C are shown in Table 4.1 for the single copy (pRS416) and multi-copy (pRS426) vector expressed xylanase respectively.

Table 4.2 Half-lifes (min) of β-xylanase activity by the cloned *XynA* gene as part of a fusion protein with the *Aga1* gene of *S. cerevisiae*, expressed in both single- and multi-copy shuttle vectors by *S. cerevisiae*.

	pRS416 (single copy) $t_{1/2}$ **(min)**	**pRS426 (multi-copy)** $t_{1/2}$ **(min)**
50	78.48	67.92
60	62.22	39.54
70	13.5	0.079

4.4.1.4 SDS-PAGE profile

Singh *et al*. (2000a) reported a prominent band with a molecular mass of 24.7 kDa in eight *Thermomyces lanuginosus* strains when SDS-PAGE was performed. This molecular mass falls within the range of values reported for crude and purified xylanases from *T. lanuginosus* strains (Kitpreechavanich *et al*., 1993; Anand *et al*., 1990; Purkarthofer *et al*., 1993; Cesar and Mrša, 1996; Bennett *et al*., 1998), but is greater than the reported calculated value of 21.314

kDa for the sequenced xylanase from *T. lanuginosus* strain DSM 5826 (Schlacher *et al*., 1996).

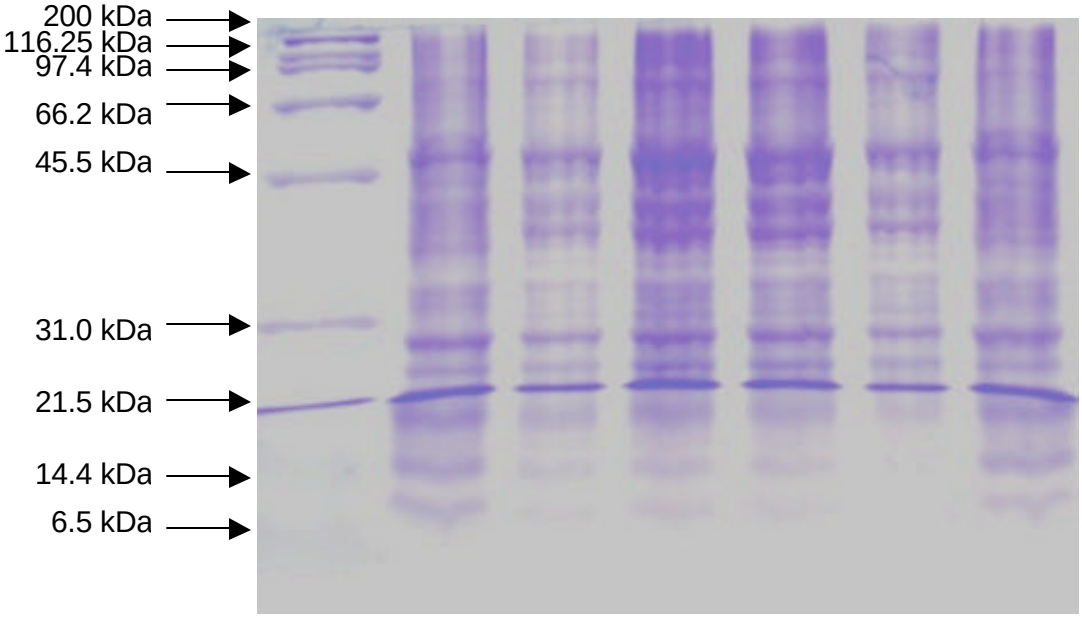

Fig. 4.4.6 SDS-PAGE analysis of the expression cassettes pRS416:*XynA* (lane 2), pRS426:*XynA* (lane 3), pRS416:*Agα1*::*XynA* (lane 4) and pRS426:*Agα1*::*XynA* (lane 5). Lanes 1 and 6 represent the wild type *S. cerevisiae* as control. Protein molecular mass standards (Bio-Rad Laboratories) used were myosin (200 kDa), β-glactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.5 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa).

The xylanase expressed by cassettes pRS416:*XynA* and pRS426:*XynA* was expected to have a molecular mass of between 21 kDa and 24 kDa, according to data published by Singh *et al*. (2000a), Kitpreechavanich *et al*. (1993), Anand *et*

al. (1990), Purkarthofer *et al*. (1993), Cesar and Mrša (1996), Bennett *et al*. (1998) and Schlacher *et al*. (1996). However, no significant difference could be detected upon comparison of these two cassettes with the untransformed *S. cerevisiae* control, in which the cassettes were expressed. Due to the native proteins excreted by *S. cerevisiae* (Fig. 4.4.6, lanes 1 and 6) it was not possible to distinguish between native secreted proteins and heterologous expressed proteins. The same problem occurred with the detection of the expressed xylanase by cassettes pRS416:*Aga1*::*XynA* and pRS426:*Aga1*::*XynA*, where a protein of molecular mass of approximately 60.5 kDa was expected. This size protein represents the leader peptide, cloned *XynA*, stalk and GPI-anchor, excluding the binding domain region. However, as a result of the amount of native *S. cerevisiae* secreted proteins, it is difficult to detect the expressed xylanase.

4.5 Discussion

The most thermostable xylanase reported up to date is that from the extremely thermophilic anaerobic bacterium *Thermotoga* strain, which has a $t_{1/2}$ of more than 20 min at 105 °C (Bragger *et al*., 1989). However, the commercial development of xylanase production by archaebacteria has been impeded, despite offering a potentially rich source of a variety of very stable enzymes, by the difficulties of cultivation of these organisms. Therefore, thermophilic fungi such as *Thermomyces lanuginosus*, are an attractive alternative source of thermostable xylanase (Singh *et al*., 2000c).

The cloned xylanase from *T. lanuginosus* SSBP fused with the modified *Aga1* from *S. cerevisiae*, expressed in both single- and multi-copy vectors by *S. cerevisiae* displayed an optimum temperature and pH of 70 °C and optimum pH of 6.0-7.0. This corresponds to data obtained by Singh *et al*. (2000a), who compared *T. lanuginosus* SSBP to seven other strains of *T. lanuginosus*. The

optimum temperature of 70 °C and optimum pH of 6.0 or 6.5 of the xylanase in crude extracts of all strains tested by Singh and co-workers (2000a), confirmed data published by Cesar and Mrša (1996), Bennett *et al*. (1998), and Puchart *et al*. (1999).

Singh *et al*. (2000c) reported that the xylanase from *T. lanuginosus* SSBP retain its full activity at temperatures of up to 65 °C and retained 45 % of its activity after 30 min at 100 °C. The enzyme also retained its total activity after 14 days at 60 °C. $T_{1/2}$ (half life time) determinations are more accurate and reliable for comparison of stability properties of enzymes. The $t_{1/2}$ values of 337 min and 257 min obtained for the xylanase of *T. lanuginosus* SSBP (Singh *et al*., 2000c) at 70 °C and 75 °C respectively, at pH 6.5, were significantly higher than those obtained for the recombinant xylanase constructed in this study (Table 4.1).

The activity of the recombinant xylanase expressed by pRS416:*Aga1*::*XynA* and pRS426:*Aga1*::*XynA* at 70 °C and pH 6.5 was considerably lower (28.3973 and 28.8707 respectively) than the reported 59 600 nkat/ml for the novel xylanase produced by *T. lanuginosus* SSBP by Singh *et al.* (2000a). However, the xylanase expressed by the pRS416:*XynA* and pRS426:*XynA* cassettes yielded insignificant amounts of activity compared to that of the fusion protein, which leads the author to suggest that some enhancement in the folding of the expressed protein is mediated by the fusion with the modified α-agglutinin gene, and thus leading to a protein stabilized to the extent of displaying (however low) β-xylanase activity.

Schreuder *et al*. (1993) reported on a similar study, of targeting a heterologous protein to the cell wall of *S. cerevisiae*. A fusion protein was constructed consisting of the signal sequence of yeast invertase, guar α-galactosidase and the C-terminal half of the α-agglutinin. Two cassettes were constructed, one containing only the reporter α-galactosidase gene, and the other containing the

α-GAL/AC1 fusion protein. Colonies of cells producing α-galactosidase were light blue with large faint halos, and colonies of cells producing αGAL/AC1 fusion protein were dark blue surrounded by a small deep blue halo. These results indicate that the α-GAL/AC1 has α-galactosidase activity and stays close to the colony, whereas α-galactosidase diffuses away from the colony. During batch culture, α-galactosidase activities were determined for cells and growth medium, and it was found that α-galactosidase was almost exclusively present in the growth medium, whereas the α-GAL/AC1 fusion protein was almost exclusively cell associated. Only small amounts of the reporter enzyme guar α-galactosidase were cell associated, and almost all was periplasmic. The small amount that was cell wall-bound was bound non-covalently. Most of the α-GAL/AC1 fusion protein was cell associated, while only a small amount was periplasmic. Of the cell wall-bound α-GAL/AC1 fusion protein, most was extractable with laminarinase, which show that the αGAL/AC1 is intimately associated with cell wall glucan like the α-agglutinin, whereas the α-galactosidase is not. This confirms that the C-terminal half of the α-agglutinin is responsible for incorporation of αGAL/AC1 fusion protein into the cell wall.

Immunofluorescent labeling with anti-α-galactosidase serum was performed on both intact α-GAL/AC1 cells and SDS-extracted cells. SDS-extracted cells showed similar labeling as intact cells, and buds of various sizes, even very small ones, were uniformly labeled. This show that αGAL/AC1 fusion protein is continuously incorporated into the cell wall throughout the cell cycle and that it instantly becomes tightly linked to the glucan from the cell wall (Schreuder *et al*., 1993). The fact that the α-galactosidase part of the α-GAL/AC1 fusion protein is accessible to antibodies indicates that the α-galactosidase is present in the mannoprotein layer on the outside of the cell wall. This indicates that the α-GAL/AC1 fusion protein is incorporated in the cell wall like the α-agglutinin since the N-terminal part of the α-agglutinin, which contains the a-agglutinin binding

domain, must be exposed on the outside to mediate agglutination (Schreuder *et al*., 1993).

An SDS-PAGE profile could have prove useful to determine whether the whole construct (consisting of the leader peptide, cloned *XynA*, stalk and GPI-anchor, excluding the binding domain region) was expressed extracellularly, where a difference in protein molecular mass between this expressed protein and that of the expressed xylanase from cassettes pRS416:*XynA* and pRS426:*XynA* should have been detected. However, because of the amount of secreted native proteins from *S. cerevisiae*, this was not possible. Thus is it difficult to determine whether the construct as a unit was expressed, or whether only the *XynA* was expressed.

Immunofluorescent labeling with anti-β-xylanase serum might prove useful in the determination of the presence and distribution of the *Aga1*::*XynA* fusion protein in or around the cell wall. This might explain the lack of activity found in whole-cells, and the low amounts of activity found in the extracellular environment.

Immobilization can increase the stability of enzymes (Monsan and Combes, 1988) and cell walls can be used as a source of immobilized enzymes (Sakai *et al*., 1991). However, Schreuder *et al*. (1993) found no differences in either thermo- or pH-stability when the stability of a cell wall-incorporated αGal/AC1 fusion protein was compared to the stability of free α-galactosidase. Simpson *et al*. (1991) found an improvement in xylanase stability in the presence of polyhydric alcohols such as glycerol and sorbitol, and this suggest that those and other components could be useful in improving enzyme stability. Singh *et al*. (2000c) found that components of different substrates enhance the thermostability of crude enzymes. The investigation into these and other factors might explain the lack of activity and stability of the expressed and recombinant xylanase reported in this study.

4.6 References

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CHAPTER FIVE

Concluding Remarks

Microbial biotechnology is directed towards the improvement of resource utilization, optimization of current processes through the addition of microbially-derived enzymes as well as the reduction of environmental impact of large-scale, well-established industries. During the past two decades, numerous applications have been found for endoxylanases, as has been described in chapter two.

Although the knowledge of microbial xylanolytic systems has increased the past few years, further research is necessary to fully understand the complete microbial degradation of xylan. Such an understanding is a prerequisite for increased economically feasible biotechnological applications of xylanolytic systems. Research could be directed towards the engineering of microorganisms capable of degrading xylan directly to single cell protein, fuels and chemicals. The manipulation of *S. cerevisiae* may be attractive in this regard. Due to the constant development of cloning techniques, it will be possible to transfer the genes encoding these enzymes from extremophiles to good protein producers, and will thus create improved β-xylanases produced in commercial quantities at a reasonable price.

According to the author's knowledge, this study is the first to report on the successful expression of the β-xylanase gene (*XynA*) from the thermophile *Thermomyces lanuginosus*. Expression of a heterologous protein does not guarantee appearance of the desired activity, as has been seen in this study. The protein must escape proteolysis, fold properly, accomplish any necessary assembly and prosthetic group acquisition, be suitably localized, have access to

all required substrates and not encounter an inhibitory environment. Unanticipated cell responses to a genetic modification may complicate rational practice of the metabolic engineering cycle. Introduction of a cloning vector alone may result in a cascade of metabolic changes, many of which are difficult to anticipate.

Further investigation into the use of the *Aga1* gene as immobilizing-agent, and the use thereof in the search for an immobilized cell surface expression system, could lead to vast new possibilities in biotechnological engineering, ready to be explored.

SUMMARY

The aim of the study was to construct an immobilized cell surface expression cassette for the xylanase (*XynA*) from *Thermomyces lanuginosus*. This cassette was constructed by deletion of the binding domain coding region of an α-agglutinin (*Aga1*) gene from *Saccharomyces cerevisiae*, and the cloning of the *XynA* gene from *T. lanuginosus* into the deleted region, which is flanked by a stalk-like protein coding region. The motivation for this was that the enzyme would be expressed on this stalk, on the surface of the yeast cell wall, and that would create an immobilized enzyme system.

Total RNA from *T. lanuginosus* was isolated, and the *XynA* gene amplified with a two-step RT-PCR method using sequence specific primers. This amplified *XynA* was successfully cloned and sequenced. A *PDC1* promoter from *S. cerevisiae* was cloned into two shuttle vectors, pRS416 (single copy) and pRS426 (multi-copy). This modified *Aga1* (with the *XynA* cloned into the deleted binding domain region) was cloned into the two shuttle vectors, adjacent to the *PDC1* promoter, and transformed to *S. cerevisiae*. Upon expression, it was clear that the enzyme was not immobilized, as no enzyme activity was detected when whole-cell enzyme assays were performed. However, activity was found in the extracellular fraction, which indicates that the enzyme was extracellularly secreted. Enzyme assays were thus performed on the extracellular fraction as crude enzyme extract, to complete the partial characterization of the secreted xylanase.

Upon characterization, it was found that the optimum temperature of 70 °C and optimum pH of 6.0-7.0 corresponds to values published in literature. However, the total activity displayed by the recombinant xylanase was significant lower than that of published data. The thermal stability of the recombinant xylanase was also not comparable to that reported. However, it did seem as if the *Aga1*-construct yielded a certain form of stability to the expressed xylanase, as no or very little activity and no stability were found when the xylanase expressed in *S. cerevisiae* without the construct was subjected to enzyme assays.

Further investigation into the reasons why low activity and stability were obtained and no immobilization of the enzyme upon expression by the cassettes could prove valuable in the search for an immobilized cell surface expression system in *S. cerevisiae*.

OPSOMMING

Die doel van die studie was die konstruksie van ‘n geï mobiliseerde sel oppervlak uitdrukkingskasset vir die xilanase (*XynA*) van *Thermomyces lanuginosus*. Hierdie kasset is gemaak deur die verwydering van die bindingsdomein koderings gebied van ‘n α-agglutinasie geen (*Aga1*) van *Saccharomyces cerevisiae*, en die klonering van die *XynA* geen in hierdie gebied, wat aangrensend is aan ‘n steelagtige proteien koderingsgebied. Die motivering hiervoor was dat die ensiem op hierdie steel uitgedruk kan word, op die oppervlak van die gis-selwand, and so ‘n geï mobiliseerde ensiemsisteem kan skep.

Totale RNS van *T. lanuginosus* is geï soleer, en die *XynA* geen geamplifiseer met ‘n twee-stap omgekeerde transkripsie polimerase ketting reaksie met spesifieke inleiers. Hierdie geamplifiseerde *XynA* is suksesvol gekloneer en basispaar opeenvolging orde bepaal. ‘n *PDC1* promotor van *S. cerevisiae* is kloneer in twee oordragingsvektore, pRS416 (enkel kopie) en pRS426 (multikopie). Hierdie gemodifiseerde *Aga1* (met die *XynA* gekloon in the verwyderde bindingsgebied), is gekloneer in die twee oordragingsvektore, langs die *PDC1* promoter, en getransformeer na *S. cerevisiae*. Na uitdrukking was dit duidelik dat die ensiem nie geï mobiliseer is nie, want geen aktiwiteit is verkry nadat ensiem aktiwiteitsbepalings op die heel selle uitgevoer is nie. Aktiwiteit is wel gekry in die ekstrasellulêre fraksie, wat wys dat die ensiem ekstrasellulêr uitgeskei is. Verdere ensiem aktiwiteitsbepalings is uitgevoer op die ekstrasellulêre fraksie as ru-ensiem ekstrak om die gedeeltelike karakterisering van die uitgeskeide ensiem te voltooi.

Die optimum temperatuur van die uitgedrukte ensiem is bepaal as 70 °C, en optimum pH was 6.0-7.0. Dit is gelykstaande aan waardes wat in literatuur gebubliseer is. Die totale aktiwiteit van die rekombinanate xilanase is egter laer as die van gepubliseerde data. Die temperatuur stabiliteit van die rekombinante xilanase is ook nie vergelykbaar met gepubliseerde data nie. Dit wil egter voorkom of the *Agα1*-konstruk ‘n mate van stabiliteit aan die uitgedrukte ensiem verskaf, want min of geen aktiwiteit is gevind met die xilanase uitgedruk in *S. cerevisiae* sonder die konstruk nie.

Verdere ondersoeke na die redes waarom min aktiwiteit en stabiliteit verkry is en geen immobilisering met uitdrukking van die ensiem in die uitdrukkingskasette nie, kan waardevol wees in die soeke na ‘n geï mobiliseerde seloppervlak uitdrukkingssisteem in *S. cerevisiae*.