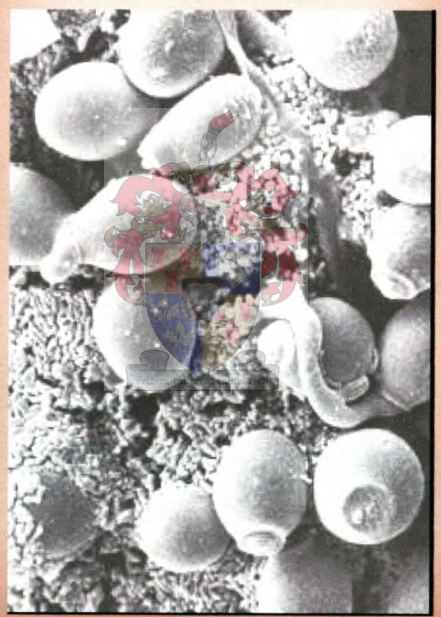
Engineering of *Pichia stipitis* for enhanced xylan utilization

Riaan den Haan

Dissertation presented for the Degree Doctor of Philosophy at the University of Stellenbosch



Supervisor: Prof. W. H. van Zyl

April 2003

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Date:

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SUMMARY

Plant biomass, the most abundant renewable resource in nature, consists of matrices of mainly lignin, cellulose, hemicellulose as well as inorganic components. Xylan, the major hemicellulose component in plant cell walls, is the most abundant polysaccharide after cellulose. This makes the main constituent sugar of xylan, D-xylose, the second most abundant renewable monosaccharide in nature. Very few hemicelluloses are either homopolymeric or entirely linear. Therefore, the variety of enzymes involved in their hydrolysis is more complex than the enzyme group responsible for the hydrolysis of cellulose. Although the ability to degrade xylan is common among bacteria and filamentous fungi, this trait is relatively rare among yeasts. However, some strains of the yeast *Pichia stipitis* are, amongst others, able to degrade xylan. As *P. stipitis* is also one of the best D-xylose fermenting yeasts thus far described, this yeast has the potential of fermenting polymeric xylan directly to ethanol. However, it was shown that the natural xylanolytic ability of this yeast is very weak.

In this study, xylanolytic genes were expressed in *P. stipitis* to test the ability of the yeast to produce heterologous proteins, and to determine the enhancement of xylan utilisation by the recombinant strain. The native xylose reductase gene (*XYL1*) and transketolase gene (*TKL*) and the heterologous *Saccharomyces cerevisiae* phosphoglycerate kinase (*PGK1*) gene promoter were cloned into *P. stipitis* transformation vectors and used to express the *Trichoderma reesei* β-xylanase encoding gene (*xyn2*) as reporter gene. It was shown that the *XYL1* promoter was induced in the presence of D-xylose and that the *TKL* promoter was constitutively expressed. The *PGK1* promoter of *S. cerevisiae* did not function in *P. stipitis*.

When the *T. reesei xyn2* gene and the *Aspergillus kawachii* β-xylanase encoding gene (*xynC*) were expressed under control of the *XYL1* promoter, extracellular β-xylanase activity of up to 136 nkat/ml and 171 nkat/ml was observed, respectively. This activity declined over time due to the presence of extracellular proteases, secreted by *P. stipitis*. Growing the cultures in a fermentor and controlling the pH level to pH 6 did not alleviate

the reduction of heterologous β-xylanase activity. When the *Aspergillus niger* β-xylosidase encoding gene (*xlnD*) was expressed as a fusion gene (designated *XLO2*) with the *S. cerevisiae* mating factor secretion signal (*MFα1*) under control of the *P. stipitis TKL* promoter, extracellular β-xylosidase activity of 0.132 nkat/ml was observed. Co-expression of the *xyn2* and *XLO2* genes led to β-xylanase and β-xylosidase activities of 128 nkat/ml and 0.113 nkat/ml, respectively. Co-expression of the *xynC* and *XLO2* genes led to β-xylanase and β-xylosidase activities of 165 nkat/ml and 0.124 nkat/ml, respectively.

The expression of the fungal xylanolytic genes in *P. stipitis* also led to an increased biomass yield when the recombinant strains were cultured on birchwood xylan as sole carbon source. The strain co-expressing the *A. kawachii* β-xylanase and *A. niger* β-xylosidase encoding genes was the most successful, yielding a 3.2-fold higher biomass level than the control strain. Biomass levels of the recombinant strains were further improved on average by 85% by growing them in a fermentor under conditions of high oxygenation. The strains were also tested for direct conversion of xylan to ethanol and the strain co-expressing the *A. kawachii* β-xylanase and *A. niger* β-xylosidase encoding genes produced 1.35 g/L ethanol, which represents a 3.6-fold increase in ethanol yield over the reference strain. These strains represent a step towards the efficient degradation and utilisation of hemicellulosic materials by ethanol-producing yeasts.

OPSOMMING

Plant biomassa, die volopste hernubare koolstofbron in die natuur, bestaan uit matrikse van lignien, sellulose en hemisellulose. Xilaan, die hoof hemisellulose komponent in plantselwande, is na sellulose die volopste polisakkaried. Gevolglik is die hoof suikerkomponent van xilaan, naamlik D-xilose, die tweede volopste hernubare monosakkaried in die natuur. Baie min hemisellulose molekules is homopolimere of heeltemal linieêr. Daarom is die ensieme betrokke by die afbraak van hemiselluloses meer kompleks as die ensieme betrokke by die afbraak van sellulose. Bakterieë en filamentagtige fungi wat oor die vermoë om xilaan af te breek beskik, kom wydversprei voor maar relatief min giste kan xilaan benut. Sommige rasse van die gisspesie *Pichia stipitis* het egter beperkte vermoë om xilaan af te breek. *P. stipitis* is ook een van die beste D-xilose fermenterende giste wat tot dusver beskryf is en het dus die potensiaal om etanol vanaf polimeriese xilaan te produseer.

In hierdie studie is gene wat kodeer vir xilaanafbrekende ensieme in *P. stipitis* uitgedruk om die vermoë van die gis as heteroloë uitdrukking sisteem te evalueer. Verder is die effek van die heteroloë xilaanafbrekende ensieme tydens groei op xilaan as enigste koolstofbron getoets. Die promoters van die xilosereduktasegeen (*XYL1*), die transketolasegeen (*TKL*) van *P. stipitis* en die fosfogliseraatkinasegeen (*PGK1*) van *Saccharomyces cerevisiae* is in *P. stipitis* transformasie vektore gekloneer en gebruik om die *Trichoderma reesei* β-xilanasegeen (*xyn2*) as verklikkergeen uit te druk. Dit het bewys dat die *XYL1* promotor induseerbaar is in die teenwoordigheid van D-xilose terwyl die *TKL* geen konstant uitgedruk was. Die *PGK1* promotor van *S. cerevisiae* was nie funksioneel in *P. stipitis* nie.

Ekstrasellulêre β-xilanase aktiwiteit van onderskeidelik 136 nkat/ml en 171 nkat/ml kon waargeneem word wanneer die *T. reesei xyn2* geen of die *Aspergillus kawachii* β-xilanasegeen (*xynC*) onder beheer van die *XYL1* promotor uitgedruk is. Hierdie aktiwiteit het afgeneem na gelang van tyd a.g.v. die teenwoordigheid van ekstrasellulêre proteases wat deur *P. stipitis* uitgeskei word. Die afname van ekstrasellulêre β-xilanase

aktiwiteit kon nie voorkom word deur die kulture in 'n fermentor te groei en die pH vlak tot pH 6 te beheer nie. Tydens uitdrukking van die *Aspergillus niger* β-xilosidase geen (*xlnD*) as 'n fusiegeen (genoem *XLO2*) met die paringsfaktor sekresiesein (*MFα1*) van *S. cerevisiae* onder transkripsionele beheer van die *P. stipitis TKL* promotor, kon ekstrasellulêre β-xilosidase aktiwiteit van 0.132 nkat/ml waargeneem word. Gesamentlike uitdrukking van die *xyn2* en *XLO2* gene het gelei tot β-xilanase en β-xilosidase aktiwiteite van 128 nkat/ml and 0.113 nkat/ml, onderskeidelik. Gesamentlike uitdrukking van die *xynC* en *XLO2* gene het gelei tot β-xilanase en β-xilosidase aktiwiteite van 165 nkat/ml and 0.124 nkat/ml, onderskeidelik.

Die uitdrukking van xilaanafbrekende ensieme in *P. stipitis* het verhoogbe biomassaproduksie teweeg gebring wanneer die rekombinante gisrasse op birchwood xilaan as enigste koolstofbron gegroei het. Die rekombinante ras wat die *A. kawachii* β-xilanasegeen en die *A. niger* β-xilosidase geen gesamentlik uitdruk, was die mees suksesvolle ras en het 3.2-voudig hoër biomassa as die kontrole ras opgelewer. Die biomassa van die rekombinante rasse tydens groei op xilaan as enigste koolstofbron kon gemiddeld met 85% verhoog word deur die giste onder hoë suurstofkonsentrase in 'n fermentor te kweek. Die rekombinante rasse is verder ook getoets vir hul vermoë om xilaan direk tot etanol om te skakel. Die rekombinante ras wat die *A. kawachii* β-xilanasegeen en die *A. niger* β-xilosidase geen gesamentlik uitgedruk het, het 'n 3.6-voudige verhoging in etanolproduksie getoon en 1.35 g/L ethanol gelewer. Hierdie rekombinante gisrasse verteenwoordig 'n stap nader aan die doeltreffende afbraak en benutting van hemisellulose deur etanolproduserende giste.

This thesis is dedicated to Marlese and Welma den Haan

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence." - **Louis Pasteur**

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The Almighty, for this opportunity.

PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the journal to which the manuscript was submitted.

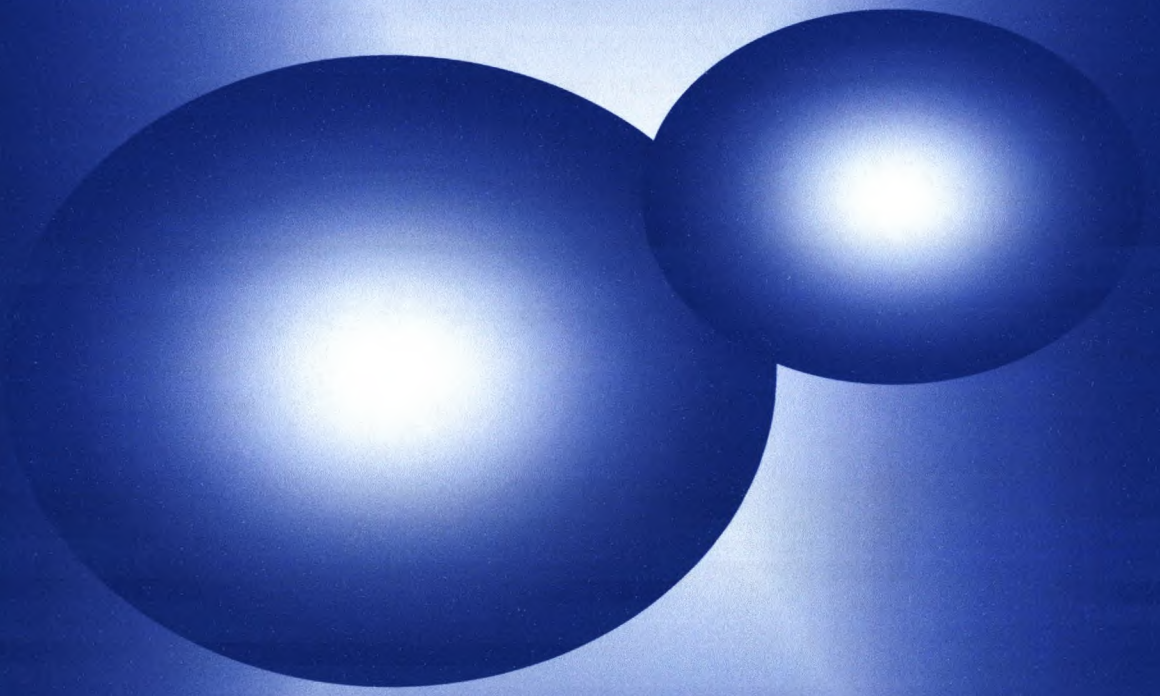
Chapter 3 "Differential expression of the *Trichoderma reesei* β-xylanase II (*xyn2*) gene in the xylose fermenting yeast *Pichia stipitis* " has been published in *Applied Microbiology and Biotechnology* 57:521-527.

Chapter 4 "Enhanced xylan degradation and utilization by *Pichia stipitis* overproducing fungal xylanolytic enzymes " has been submitted to Enzyme and Microbial Technology.

Chapter 5 "Xylan degradation and β-xylanase expression of recombinant *Pichia stipitis* strains in a fermentor" is in preparation.

The **South African Provisional Patent** (no. 2002/6062) that has been filed encompassing the work detailed in chapter 4 is provided as an **Appendix**.

Chapter 1



General introduction and project aims

1 INTRODUCTION

Plant biomass represents a useful and valuable resource to man (Lynd et al. 1999; Aristidou and Penttilä, 2000; Mielenz, 2001). Plant biomass consists of matrices of mainly lignin, cellulose, hemicellulose as well as various extractives and inorganic components (Jeffries and Jin, 2000). Therefore it is commonly referred to as lignocellulose. Lignocellulose is the most abundant and renewable polysaccharide in nature and is exploited for the generation of numerous products. Several research efforts are focussed on the exploitation of microbial capabilities of lignocellulose biodegradation to expand the use of this biomass resource (Jeffries and Jin, 2000; Hahn-Hägerdal et al. 2001; Lynd et al. 2002). The composition of lignocellulosic materials varies widely with plant species, age, time of harvest and condition or stage of growth of the plant. About 45% of the total dry weight of wood and up to 35% of agricultural residues is cellulose, the degradation of which yields D-glucose, making D-glucose the most abundant carbohydrate in terrestrial plants and the most abundant renewable carbon source in nature (Lynd et al. 2002).

The hemicelluloses of plant structural tissues, which include all the non-pectic, non-cellulosic polysaccharides, have traditionally been classified by the sugar residues present in the main chain or backbone of the molecule (Lappalainen, 1986). The major groups include xylans, galactans, glucans and glucomannans. Hemicelluloses are coupled to cellulose and lignin by covalent and non-covalent bonds. Very few hemicelluloses are either homopolymeric or entirely linear. Therefore, the variety of enzymes involved in their hydrolysis is more complex than the enzyme group responsible for the hydrolysis of cellulose. Xylan, the major hemicellulose, can represent a third of the total carbohydrate content of plant biomass (Lappalainen, 1986). This makes the main constituent sugar of xylan, D-xylose, the second most abundant renewable monosaccharide in nature (Jeffries and Jin, 2000). If biomass-based processes of producing commodities such as ethanol are to be cost effective, bioconversion of the xylan component is as important as that of cellulose (Lynd et al. 1999).

The structure of xylan is variable, involving not only linear β-1,4-linked chains of D-xylose, but also branched heteropolysaccharides, therefore complete degradation requires the synergistic action of a range of different enzymes (Kormelink et al. 1992; Thomson, 1993; Sunna and Antranikian, 1997). Degradation of the β-1,4-xylan backbone requires the action of endo-β-1,4-xylanases and β-xylosidases. The production of xylanolytic enzymes is wide-spread among filamentous fungi and bacteria (Hazelwood and Gilbert, 1993; Sunna and Antranikian, 1997). However, this trait is not as common among naturally occurring yeasts. Biely et al. (1978) tested the ability of 95 strains of yeast and yeast-like organisms, spanning 35 genera, for the ability to grow in media containing D-xylose or xylan as sole carbon source. Of the 54 strains that grew on D-xylose, only 13 strains, of the genera *Aureobasidium*, *Cryptococcus* and *Trichosporon*, could utilise xylan as sole carbon source. These strains were found to secrete xylanolytic enzymes that degraded the xylan in the medium to D-xylose and xylo-oligosaccharides. In addition, it was found that certain strains of the genera *Bullera*, *Candida*, *Geotrichum*, *Pheococcomyces*, *Sporothrix*, *Pichia, Pseudozyma* and *Rhodotorula* also hydrolyse xylan (Kremnický et al. 1996; Schäfer et al. 1996; Middelhoven, 1997).

Yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* are attractive eukaryotic host systems for the expression of heterologous proteins (Hadfield et al. 1993; Sudberry, 1996). Several researchers have successfully expressed heterologous xylanolytic genes in a variety of yeast hosts (Crous et al. 1995; Berrin et al. 2000; La Grange et al. 2001). However, these hosts are not capable of D-xylose fermentation. However, *Pichia stipitis* is able to utilise and ferment the main component sugar of xylan to ethanol, in fact it is among the best D-xylose-fermenting yeasts thus far described (Slininger et al. 1985; Jeffries and Kurtzman, 1994). Therefore, a xylan-degrading *P. stipitis* strain can be applied for theoretical direct conversion of xylan to ethanol. However, the maximum β-xylanase activity that was observed in a naturally occurring xylanolytic strain of *P. stipitis* was only 2.5-3% of the activity reported for *Cryptococcus albidus* (Basaran et al. 2001). An efficient transformation system for *P. stipitis* based on the *P. stipitis* oroditine-5'-phosphate decarboxylase (*URA3*) encoding gene and an autonomously replicating sequence (*ARS2*) has been developed (Yang et al. 1994). Using this

transformation system, heterologous protein production in *P. stipitis* could be tested and xylanolytic ability of the yeast could be improved by expression of foreign xylanolytic enzymes.

1.1 AIMS OF THE STUDY

In this study the potential of the D-xylose utilising yeast *P. stipitis* for heterologous protein production was investigated by the expression of fungal xylanolytic genes under control of native and heterologous promoters. The specific aims of the study were:

1. Cloning of the promoter and terminator sequences of the xylose reductase (*XYL1*) and transketolase (*TKL*) encoding genes into vectors for *P. stipitis* transformation.
2. Study of the regulation of the *XYL1* and *TKL* promoters.
3. The co-expression of fungal β-xylanases and a β-xylosidase in *P. stipitis*.
4. The characterisation of the recombinant enzyme activities produced by *P. stipitis*.
5. The characterisation of the growth and ethanol production of recombinant xylanolytic *P. stipitis* strains on xylan as sole carbon source.
6. The characterisation of extracellular protease production by strains of *P. stipitis*.

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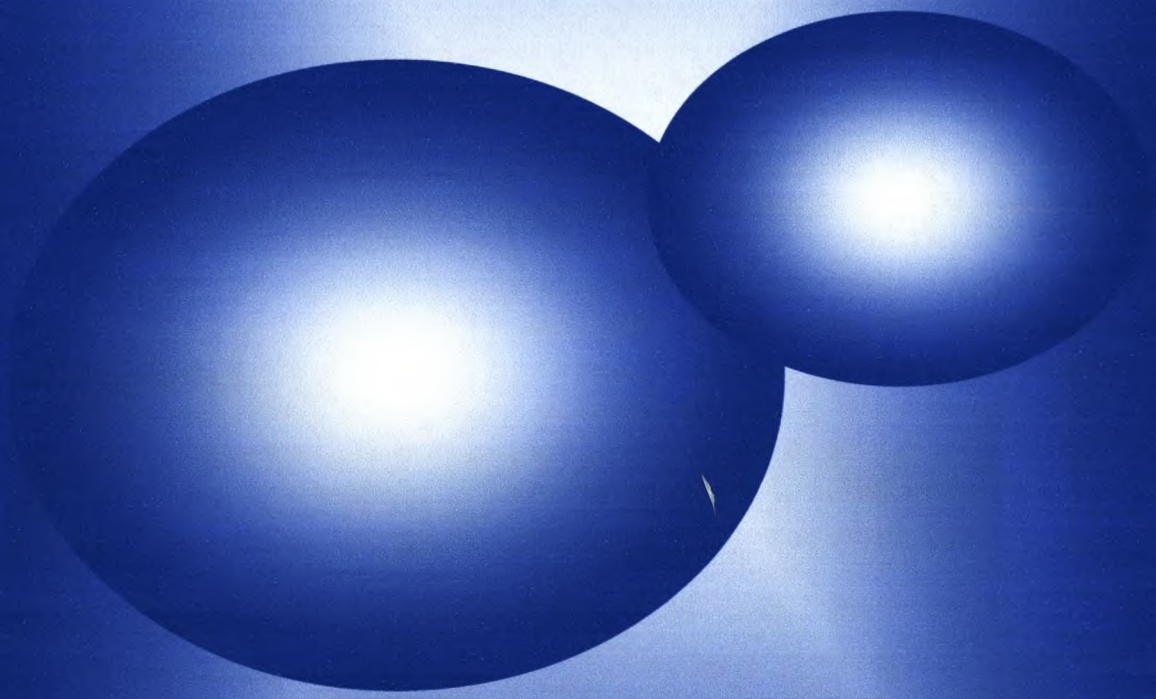
Chapter 2
Literature Review



The role of naturally occurring and recombinant yeasts in the microbial bioconversion of hemicellulosic feedstocks to ethanol

THE ROLE OF NATURALLY OCCURRING AND RECOMBINANT YEASTS IN THE MICROBIAL BIOCONVERSION OF HEMICELLULOSIC FEEDSTOCKS TO ETHANOL

2.1 INTRODUCTION

Biomass represents a useful and valuable resource to man (Lynd et al. 1999; Aristidou and Penttilä, 2000; Mielenz, 2001). For millennia man has exploited the solar energy, stored in the chemical bonds of biomass, by burning it as fuel or eating plants as nutritional energy. More recently humans have exploited the fossilised biomass in the form of coal and oil, the additional chemical bonds in coal and oil represent a more concentrated source of energy as fuel. However, as it takes millions of years to convert biomass into coal and oil these fossil fuels are not renewable in the timeframe that they are used in. Plant biomass in contrast represents the only foreseeable, sustainable resource of organic fuels and significant efforts are underway to harness this potential energy source.

Plant biomass consists of matrices of mainly lignin, cellulose, hemicellulose as well as various extractives and inorganic components (Jeffries and Jin, 2000). Therefore it is commonly referred to as lignocellulose. Lignocellulose is the most abundant and renewable polysaccharide in nature and is therefore exploited for the generation of numerous products. Several research efforts are focussed on the exploitation of microbial capabilities of lignocellulose biodegradation to expand the use of this biomass resource (Jeffries and Jin, 2000; Hahn-Hägerdal et al. 2001; Lynd et al. 2002).

The composition of lignocellulosic materials varies widely with plant species, age, time of harvest and condition or stage of growth of the plant. Fig. 1 is a schematic representation of the dry weight composition of hardwood, softwood and bagasse. Lignin is the most abundant aromatic polymer in nature (Zaldivar et al. 2001). This phenolic macromolecule is the dehydration product of three monomeric alcohols (lignols), namely trans-*p*-coumaryl alcohol, trans-*p*-coniferyl alcohol and trans-*p*-sinapyl alcohol. Lignin

provides mechanical strength and serves as an effective barrier against microbial access and digestion of the plant carbohydrates. About 45% of the total dry weight of wood and up to 35% of agricultural residues is cellulose, the degradation of which yields D-glucose, making D-glucose the most abundant monosaccharide in terrestrial plants and the most abundant renewable carbon source in nature (Lynd et al. 2002).

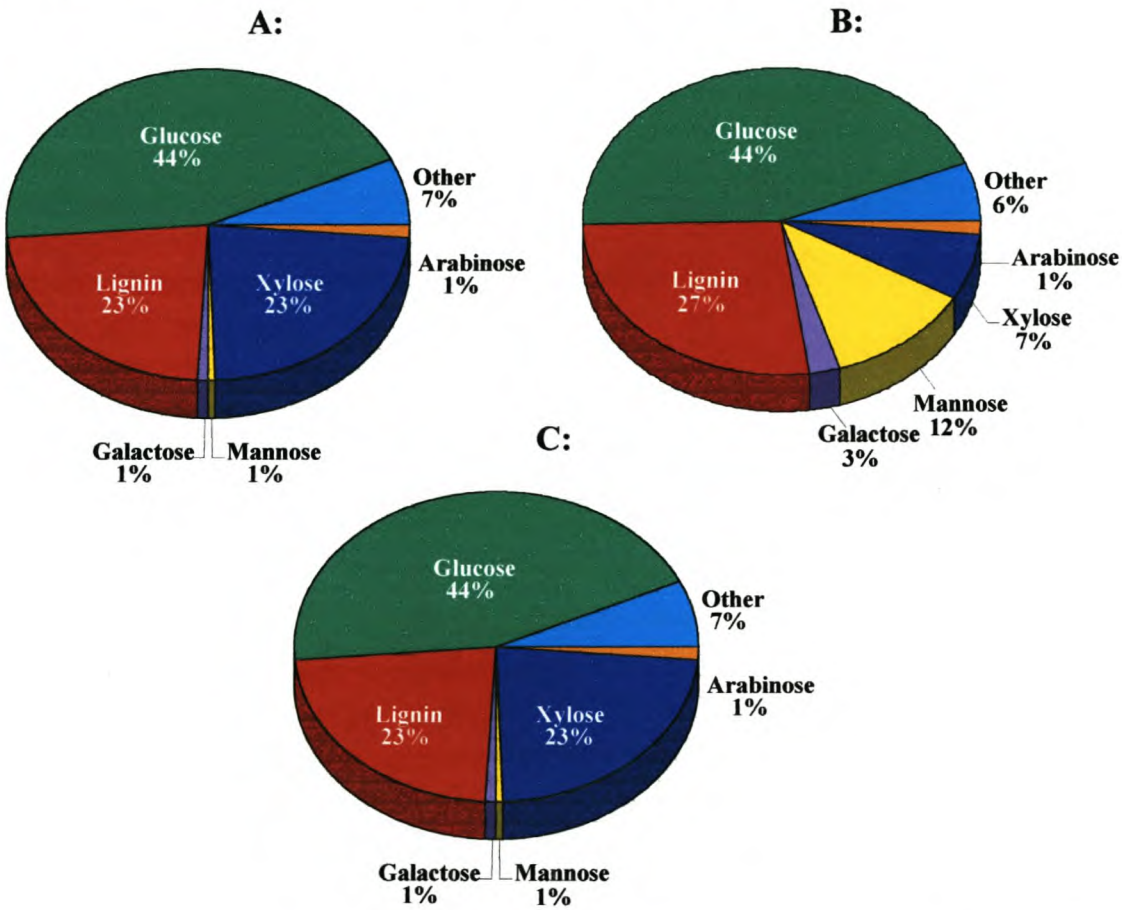


Fig. 1 A schematic representation of the dry weight composition of (**A**) hardwood (**B**) softwood and (**C**) bagasse. The carbohydrate composition of the wood may vary (Puls and Schuseil, 1993; Haltrich et al. 1996).

Cellulose is a fibrous, high-molecular weight, β-1,4 linked D-glucose polysaccharide with a degree of polymerization of 4000 to 8000 D-glucose residues or higher (Van Rensburg et al. 1998; Aristidou and Penttilä, 2000). Bundles of the linear polymer are held together by Van der Waals interactions and hydrogen bonding. The highly ordered nature and

insoluble structure of crystalline cellulose renders it recalcitrant to enzymatic degradation. Enzymatic degradation of cellulose therefore requires the synergistic action of several enzymes collectively referred to as cellulases. The precise structure of cellulose and the enzymes required for its complete degradation will not be discussed further. An extensive review on the structure, microbial degradation and biotechnological aspects of cellulose was recently published (Lynd et al. 2002).

The hemicelluloses of plant structural tissues, which include all the non-pectic, non-cellulosic polysaccharides, have traditionally been classified by the sugar residues present in the main chain or backbone of the molecule (Lappalainen, 1986). The major groups include xylans, galactans, glucans and glucomannans. Hemicelluloses are coupled to cellulose and lignin by covalent and non-covalent bonds. Very few hemicelluloses are either homopolymeric or entirely linear. Therefore, the variety of enzymes involved in their hydrolysis is more complex than the enzyme group responsible for the hydrolysis of cellulose. Xylan, the major hemicellulose, can represent a third of the total carbohydrate content of plant biomass (Lappalainen, 1986). If biomass-based processes of producing commodities such as ethanol are to be cost effective, bioconversion of the xylan component is as important as that of cellulose (Lynd et al. 1999). This review will be concerned with the structure and degradation of xylan and its main sugar component, D-xylose, focussing on yeasts that perform these functions.

2.2 XYLAN: STRUCTURE AND DEGRADATION.

2.2.1 Xylan structure

Xylan, the major hemicellulose component in plant cell walls, is the most abundant polysaccharide after cellulose (Lappalainen, 1986). This makes the main constituent sugar of xylan, D-xylose, the second most abundant renewable monosaccharide in nature (Jeffries and Jin, 2000). The heterogenous xylan polymer consists of a main chain of β-1,4 linked D-xylose residues. The C-2 and C-3 positions of these D-xylose moieties can be substituted with L-arabinofuranose or 4-*O*-methyl glucuronic acid residues, or they can be esterified with acetic acid. Furthermore, the L-arabinofuranosyl residues in the side chains can be esterified with ferulic and *p*-coumaric acid (Tenkanen et al. 1996; Van

Peij et al. 1997). It is usually these ferulic acid substituents that engage in covalent crosslinking of xylan molecules with lignin or with other xylan molecules (Coughlan and Hazlewood, 1993). The solubility of xylan is influenced by the substituents in that solubility is directly proportional to the number of substituents. The frequency and composition of substituents in xylan differ depending on the plant origin, for example, the xylan chains in plant cell walls from hardwood and softwood are differently substituted (Puls and Schuseil, 1993; Li et al. 2000; Beg et al. 2001). Fig. 2 shows a schematic representation of the differences in the xylan structure of hardwood and softwood.

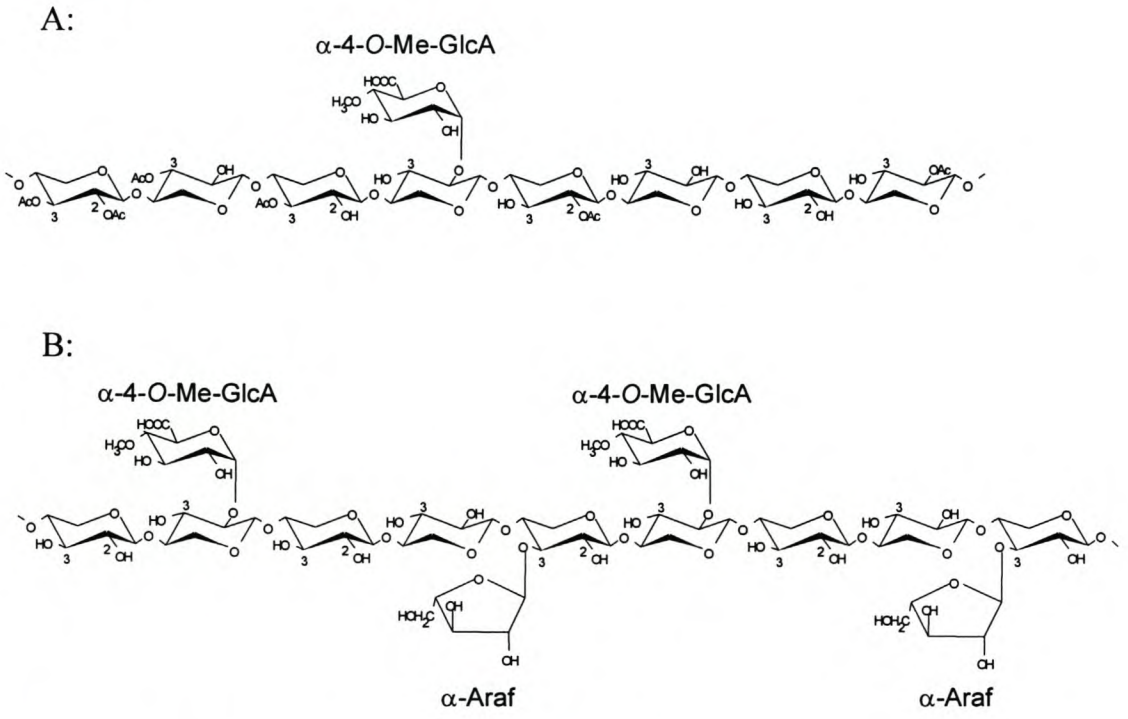


Fig. 2 The differences in the xylan structure of (**A**) hardwood xylan (*O*-acetyl-4-*O*-methylglucuronoxylan) and (**B**) softwood xylan (arabino-4-*O*-methylglucuronoxylan) (Sunna and Antranikian, 1997). Numbers indicate the carbon atoms at which substitutions take place. Ac: acetyl group, α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid, α-Araf: α-arabinofuranose.

2.2.2 Enzymology of microbial xylan degradation

2.2.2.1 Enzymes required for complete xylan degradation

The structure of xylan is variable, involving not only linear β-1,4-linked chains of D-xylose, but also branched heteropolysaccharides, its degradation requires the synergistic action of a range of different enzymes (Kormelink et al. 1992; Thomson, 1993; Sunna and Antranikian, 1997; Li et al. 2000; Beg et al. 2001; Subramaniyan and Prema, 2002). Degradation of the β-1,4-xylan backbone requires the action of endo-β-1,4-xylanases (β-1,4-D-xylan xylanohydrolase EC 3.2.1.8) and β-xylosidases (a family 3 glycoside hydrolase, β-1,4-D-xylan xylohydrolase EC 3.2.1.37). The former are generally considered to be those enzymes that hydrolyse the xylan backbone, whereas the latter are those that hydrolyse xylo-oligomers produced through the action of β-xylanases (Wong and Saddler, 1992; Hazelwood and Gilbert, 1993; Faure, 2002). In order to achieve complete degradation of complex substituted xylans, a series of accessory or debranching enzymes are also needed, namely α-D-glucuronidases (EC 3.2.1) (Biely, 1985), α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranosidase EC 3.2.1.55) (Biely, 1985; Jeffries, 1994), and acetylesterases or acetyl xylan esterases (EC 3.1.1.6) (Biely, 1985; Shao and Wiegel, 1995) (Fig. 3). The β-xylanases, β-xylosidases and auxiliary enzymes have been extensively reviewed (Coughlan and Hazelwood, 1993; Jeffries, 1994; Biely and Tenkanen, 1999).

2.2.2.2 Synergistic action of xylanolytic enzymes

Synergy is observed when the amount of products formed by two or more enzymes acting together exceeds the sum of the products formed by these enzymes when acting alone (Coughlan et al. 1993; Puls and Schuseil, 1993; De Vries et al. 2000). The degradation of *O*-acetyl-4-*O*-methylglucuronoxylan to acetic acid, 4-*O*-methylglucuronic acid and D-xylose by purified enzymes of *Trichoderma reesei* required the co-operative action of β-xylanase, β-xylosidase, α-glucuronidase, acetyl esterase and acetyl xylan esterase (Tenkanen et al. 1996). Acetyl xylan esterase, which is active on polymeric xylan, was needed to remove most of the acetyl substituents so that the β-xylanase can efficiently degrade the xylan backbone to form xylo-oligosaccharides. β-Xylosidase further

degrades the xylo-oligosaccharides to D-xylose and substituted xylo-oligosaccharides, carrying residual acetyl or 4-*O*-methylglucuronic acid substituents. These were removed by acetyl esterase and α-glucuronidase, respectively. The remaining xylo-oligosaccharides were cleaved to D-xylose by β-xylosidase.

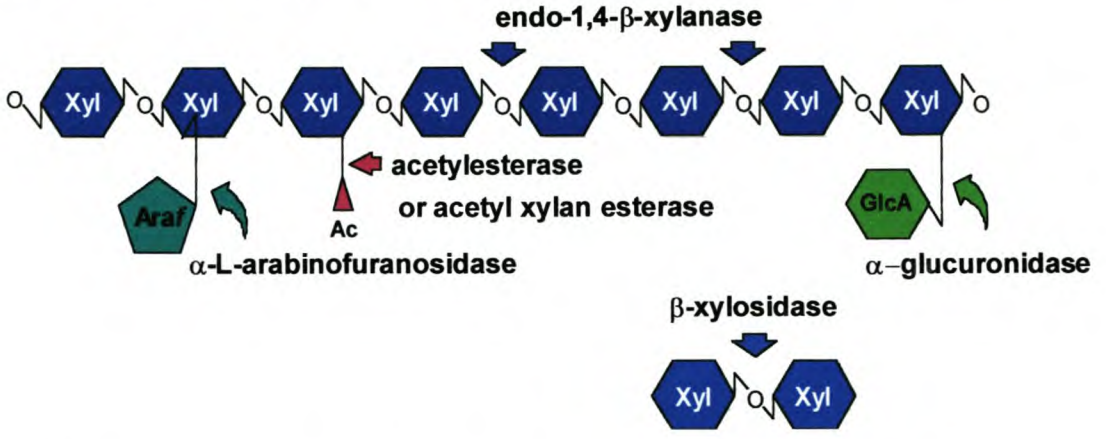


Fig. 3 A hypothetical plant xylan and the sites of attack by microbial xylanolytic enzymes. Xyl, D-xylose; GlcA, 4-*O*-methyl-D-glucuronic acid; Araf, L-arabinofuranose; Ac, acetyl group (Biely, 1985; Sunna and Antraniaken, 1997).

There are different types of synergy s

uch as homeosynergy, heterosynergy, uni- and biproduct synergy and antisynergy (Coughlan et al. 1993). Antisynergy is defined as the action of one enzyme preventing the action of a second enzyme. This phenomenon is unlikely to occur *in vivo*. Homeosynergy is the synergistic or co-operative action between two or more different types of side chain cleaving enzymes, or two or more different types of main chain cleaving enzymes. For example, acetyl groups on adjacent side chains impede the action of α-glucoronidase (Fig. 3). The addition of acetyl xylan esterase would, by removal of acetyl groups, allow α-glucuronidase access to its site of action and would so enhance the amount of α-glucuronic acid released. This would be an example of uniproduct homeosynergy if only the amount of α-glucuronic acid released was enhanced by the action of the two enzymes acting together. If the amounts of both α-glucuronic acid and acetyl groups released were enhanced, this would be an example of biproduct homeosynergy. Homeosynergy would also be observed if mixtures of two main chain

than the sum of the products released by the individual enzymes. In such a synergy the one enzyme often provides the substrate for the second enzyme, or the action of the first enzyme allows the second enzyme greater access to its substrate. Several xylanolytic fungal strains have been reported to produce multiple β-xylanases which may work synergistically to improve the efficiency of xylan degradation (Hrmová et al. 1989; Törrönen et al. 1992; Beg et al. 2001).

Heterosynergy is defined as the synergistic interaction between a side chain cleaving enzyme and a main chain cleaving enzyme (Coughlan et al. 1993; Puls and Schuseil, 1993). A striking example is the heterosynergistic interaction between the main chain and side chain cleaving enzymes of *T. reesei* (Fig. 4) (Tenkanen et al. 1996). Less than 25% of the available D-xylose was released with the action of main chain cleaving enzymes alone, whereas 100% of the available D-xylose was released with the synergistic actions of main and side chain cleaving enzymes. Heterosynergy was also observed between main chain and side chain cleaving enzymes of *Aspergillus niger* when degrading arabinoxylan (Table 1) (De Vries et al. 2000). Degradation of the xylan backbone by β-xylanase and β-xylosidase was influenced most strongly by the action of α-L-arabinofuranosidase and arabinoxylan arabinofuranohydrolase, resulting in a 2.5-fold and 2-fold increase in the release of D-xylose, respectively. Release of 4-*O*-methyl glucuronic acid by α-glucuronidase depended largely on degradation of the xylan backbone by β-xylanase, but was also influenced by other enzymes.

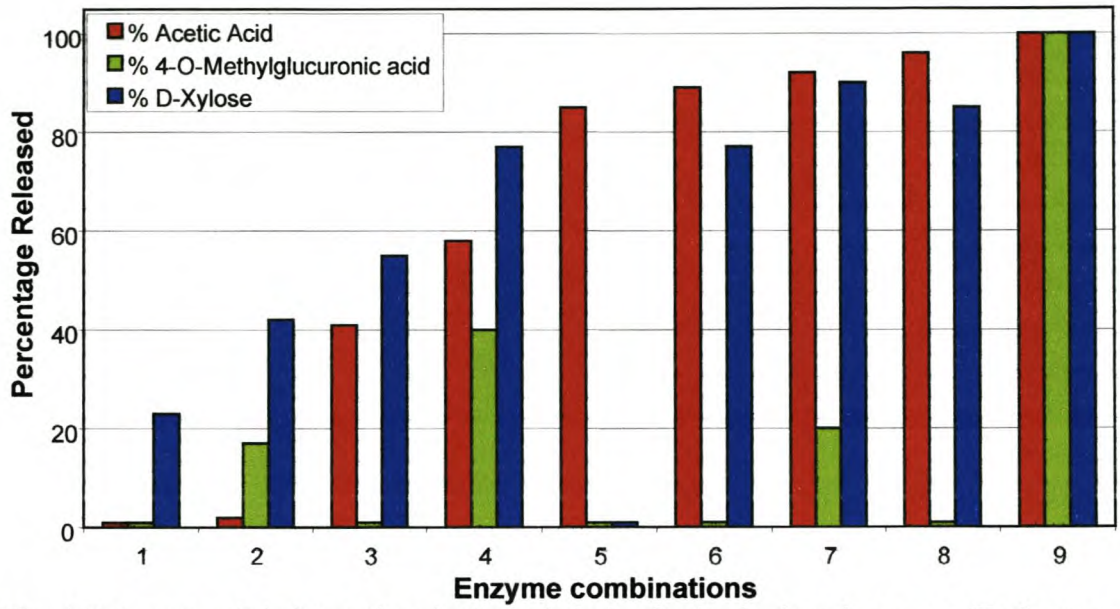


Fig. 4 Heterosynergistic interactions in the hydrolysis of xylan by fungal enzymes (Tenkanen et al. 1996). Acetic acid, 4-*O*-methylglucuronic acid and D-xylose were liberated by the *T. reesei* enzymes β-xylanase II (Xyl), β-xylosidase (βX), α-glucuronidase (Glur), acetyl xylan esterase (AXE) and acetyl esterase (AE). The combinations used were: **1.** Xyl/βX, **2.** Xyl/βX/Glur, **3.** Xyl/βX/AE, **4.** Xyl/βX/Glur/AE, **5.** AXE, **6.** Xyl/βX/AXE, **7.** Xyl/βX/Glur/AXE, **8.** Xyl/βX/AE/AXE, **9.** Xyl/βX/Glur/AE/AXE.

Table 1 The influence of accessory enzymes on the release of D-xylose by *A. niger* β-xylanase and β-xylosidase from water insoluble pentosan (WIP)[a] (De Vries et al. 2000). (Xyl, β-xylanase; βX, β-xylosidase; AbfB, arabinofuranosidase B; AxhA, arabinoxylan arabino-furanohydrolase A; Glur, α-glucuronidase; AgblB, α-galactosidase; LacA, β-galactosidase; FaeA, feruloyl esterase)

Enzyme(s)	Intact WIP	Pretreated WIP[b]
Xyl	4.4	ND[c]
Xyl/AbfB	10.9	6.5
Xyl/AxhA	8.9	4.8
Xyl/AbfB/AxhA/FaeA	16.8	ND[c]
Xyl/βX	52.4	ND[c]
βX	12.6	21.9
βX/AbfB	19.6	45.2
βX/AxhA	23.3	71.6
Xyl/βX/AbfB/AxhA/ Glur/AgblB/LacA/FaeA	98.7	99.6

[a]Values are the percentage of the total amount of D-xylose present in the sample
[b]Pretreated with XlnA
[c]Not determined

2.2.3 β-Xylanase families and domain organisation

The understanding of basic protein structure and its correlation with function has become very important in studies dealing with molecular aspects of an enzyme. However, detailed structural data on enzymes such as β-xylanases is often not available. Furthermore, considerable sequence variability exists among the hemicellulases making the detection of homologies difficult. For this reason various theoretical analytical methods are used to compliment the existing data. Of these methods, hydrophobic cluster analysis and thermostability analysis are of particular interest to basic and industrial applications. Hydrophobic cluster analysis (HCA) is a sensitive method for the comparison of amino acid sequences to derive structural, functional and evolutionary information (Kulkarni et al. 1999). HCA detects homologies between similar three-dimensional structures of proteins, even if they have low sequence identity, implying a structural and functional correlation between these structures. HCA is therefore indispensable in classing related enzymes into families. HCA also provides information on conserved amino acids that are likely to be involved in catalysis.

2.2.3.1 β-Xylanase families

Using HCA and amino acid sequence homology, hydrophobic catalytic domains of cellulases and β-xylanases were first classified into six families (A-F) (Henrissat et al. 1989), then subsequently into 11 families (Gilkes et al. 1991) and later into 45 families including 482 glycosyl hydrolase amino acid sequences (Henrissat and Bairoch, 1993). More recently the available sequences of glycosyl hydrolases was classified into 58 families (Henrissat and Bairoch, 1996; Clarke, 1997). Currently, on the "Carbohydrates-active enzymes" website (Http://afmb.cnrs-mrs.fr/CAZY/families.html) there are 90 glycoside hydrolase families listed and this resource is continually updated. Based on HCA β-xylanases were subdivided into two families, F and G, which are shared by other glycanases. The families F and G are analogous to glycohydrolase families 10 and 11, comprising high and low molecular weight β-xylanases, respectively. The high molecular weight β-xylanases with low pI values belong to glycanase family 10 (formerly family F), while the low molecular weight β-xylanases with high pI values are classified as family 11 (formerly family G) (Subramaniyan and Prema, 2002). Generally

no significant homologies were found between the β-xylanases from the two different families, including the region around the catalytic residues and they have different patterns of protein folding. Both use ion pair catalytic mechanisms and both retain anomeric configuration following hydrolysis (transferase activity) (Jeffries, 1996). Most β-xylanases in family 11 comprise only a catalytic domain and are relatively small molecules (Biely et al. 1997). The larger family 10 β-xylanases are more complex. β-Xylanases from family 10, in contrast to β-xylanases from family 11, are capable of attacking the glycosidic linkage next to the branched D-xylose residue and towards the non-reducing end. While the β-xylanases of family 10 require two unsubstituted xylopyranosyl residues between the branches, β-xylanases of family 11 require three unsubstituted consecutive xylopyranosyl residues (Fig. 5). Family 10 β-xylanases therefore produce smaller oligosaccharides.

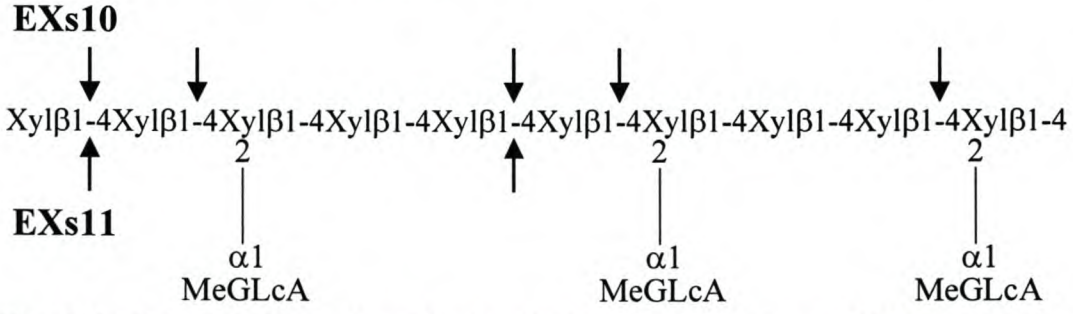


Fig. 5 The linkages that are cleaved by family 10 (EXs10) and family 11 (EXs11) β-xylanases (Biely et al. 1997). In contrast to family 11 β-xylanases, family 10 β-xylanases can cleave glycosidic linkages next to the branch and toward the non-reducing end. Xyl: D-xylose, MeGlc: 4-*O*-methyl-D-glucuronic acid.

2.2.3.2 Domain structure

Domains are clusters of amino acids that represent structural homology motifs, which can be well distinguished from each other on the basis of structural and spatial identity (Kulkarni et al. 1999). At molecular level, the β-xylanase is comprised of functional domains, non-functional domains and linker regions (Hazelwood and Gilbert, 1993; Kulkarni et al. 1999; Subramaniyan and Prema, 2002). Functional domains can be catalytic or substrate binding domains. Generally, β-xylanases contain a single catalytic domain although it was found that the β-xylanase 3 of *Neocallimastix frontalis* contained

two catalytic domains (Durand et al. 1996). Most of the plant cell wall hydrolysing enzymes have carbohydrate binding modules (CBMs) that bind to the substrate polysaccharide. Substrate binding domains are more common in family 10 than family 11 β-xylanases. Furthermore, repeated domains, unrelated to the catalytic domain, are relatively common in bacterial β-xylanases and glucanases, however, little is known about their functions. It was shown that for the thermostable β-xylanase from the thermophilic bacterium *Thermoanaerobacterium saccharolyticum* the intrinsic thermostability of the enzyme correlates to its N-terminal domain (Fontes et al. 1995). The β-xylanase encoded by another thermophilic bacterium *Caldibacillus cellulovorans* also contains an N-terminal thermostabilising domain (Sunna et al. 2000). It is thought that domains that confer thermostability may be common among family 10 β-xylanases from thermophilic organisms. These domains are unique to β-xylanases and have not been observed in numerous other thermophilic endoglucanases characterised to date.

2.2.4 β-Xylanase regulation and production

2.2.4.1 Induction of xylanolytic enzymes

β-Xylanases are usually produced during growth on xylan (Wong and Saddler, 1992; Coughlan and Hazlewood, 1993; Beg et al. 2001). Since soluble xylans are large, heavily substituted molecules, they are not transported into the microbial cell, and therefore are unlikely to be the physiological inducer for the production of β-xylanases (Biely and Petrakova, 1984a). Low molecular mass xylan fragments therefore play a key role in the regulation of β-xylanase synthesis. These may include D-xylose, xylobiose, xylo-oligosaccharides, heterodisaccharides of D-xylose and D-glucose and their positional isomers (Biely and Petrakova, 1984a; Biely and Petrakova, 1984b; Defaye and Guillot, 1992). In most microorganisms, low levels of β-xylanases are formed constitutively and are bound to the mycelial surface, from where they may attack xylan whenever available (Fig. 6). The resulting low molecular mass xylan fragments produced by this β-xylanase attack may enter the cell and promote further β-xylanase biosynthesis by acting at the transcriptional level. Small amounts of D-xylose also induced low levels of β-xylanase production in some organisms such as *Aureobasidium pullulans* (Leathers et al. 1984),

however, in *Cryptococcus albidus* β-xylanase was repressed by D-xylose (Biely, 1985). Uptake of xylobiose is most probably carried out by a general β-linked disaccharide transporter, which also transports cellobiose and sophorose. Upon induction, the levels of transcription and translation of xylanolytic genes increase considerably above that of the basal levels.

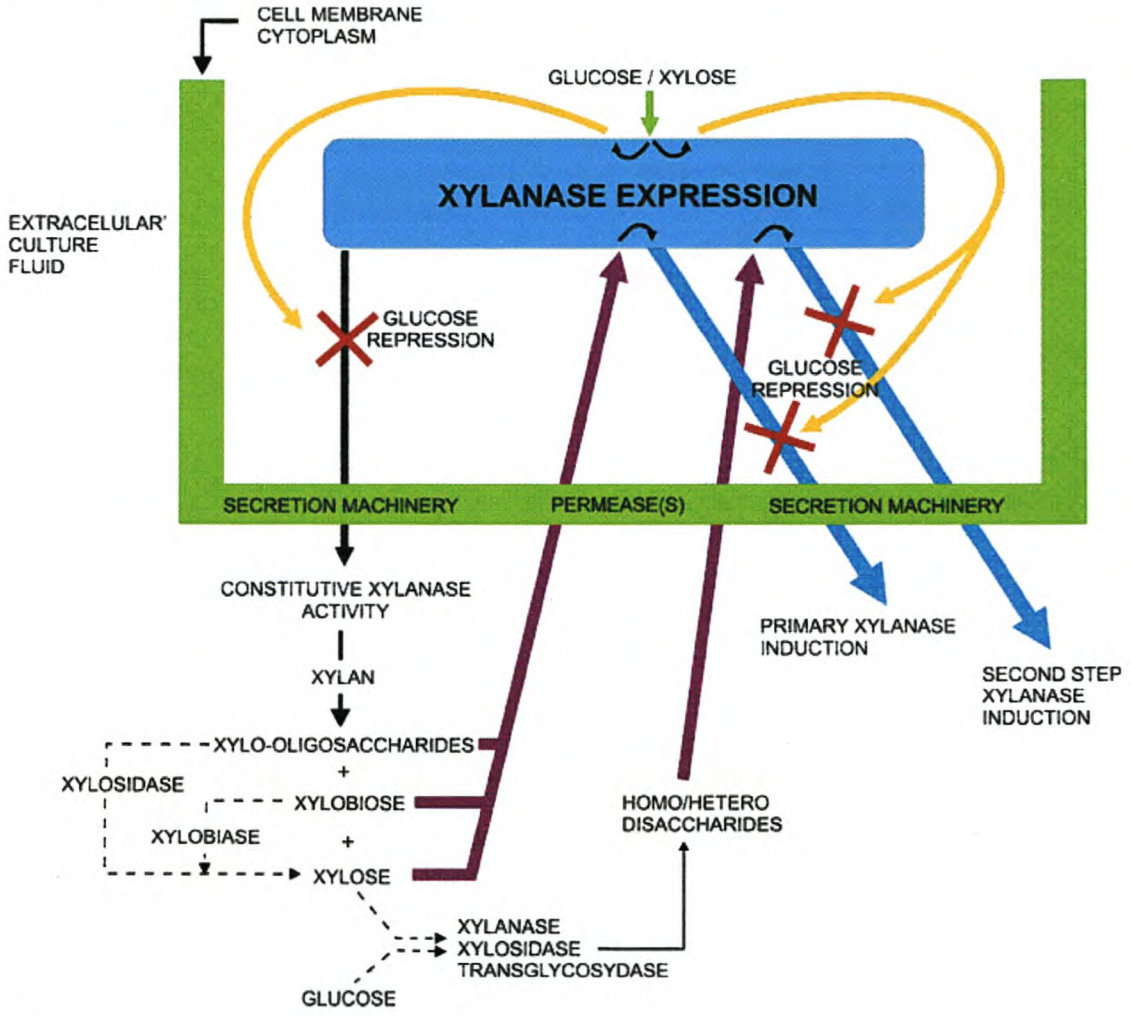


Fig. 6 Hypothetical model of the regulation of β-xylanase biosynthesis (Kulkarni et al. 1999). Constitutive β-xylanases degrade xylan to xylo-oligosaccharides, which are taken up by the cell and induce the transcription of other xylanolytic genes. β-Xylosidases convert xylobiose to D-xylose and may subsequently transglycosylate it to Xylβ1,2Xyl and Glcβ1,2Xyl. These compounds are taken up by the cell and act as additional inducers of the genes encoding xylanolytic enzymes.

2.2.4.2 Catabolite repression

Catabolite repression is a common phenomenon observed in β-xylanase biosynthesis. In the yeast *C. albidus*, when xylan was used as the inducer, cAMP caused a two-fold increase in β-xylanase production (Morosoli et al. 1989). However, cAMP had no effect on the repression caused by D-xylose. It was suggested that a 15-bp nucleotide sequence upstream of the β-xylanase gene may be part of the cAMP regulatory sequence. Biosynthesis of β-xylanases occurs several hours after depletion of the inducer in the medium, in contrast to the synthesis of β-xyloside permease and β-xylosidase, which have very short induction periods (Kulkarni et al. 1999).

2.2.4.3 β-Xylanase production for industrial applications

The basic factors for efficient production of xylanolytic enzymes are the choice of an appropriate inducing substrate and optimum medium composition (Subramaniyan and Prema, 2000). The importance of cellulase-free β-xylanases in the paper and pulp industry (Section 2.2.6.1) has prompted research into the correlation between microbial cellulase and β-xylanase production. Filamentous fungi are particularly interesting producers of β-xylanases because they secrete the enzymes into the culture medium and they produce levels of enzymes that are much higher than those produced by yeasts or bacteria (Biely, 1993). However, fungal β-xylanase production is generally linked to cellulase production. Selective production of β-xylanase is possible for species of *Trichoderma* and *Aspergillus* grown on xylan as sole carbon source (Hrmová et al. 1989; Wong and Saddler, 1992; Haltrich et al. 1996). On cellulose, these strains were shown to produce both cellulases and β-xylanases, however, this may be due to traces of hemicellulose present in the cellulose substrates. Therefore, for some fungi, growing the culture on medium containing xylan as sole carbon source with no cellulose contamination, under a low nitrogen/carbon ratio is one strategy for producing xylanolytic systems free of cellulases. Interestingly, some organisms yielded higher levels of β-xylanase when grown on cellulose than when cultivated on xylan at a similar concentration (Haltrich et al. 1996). To produce an enzyme system for use in the paper and pulp industry in the biobleaching of softwood pulp, the filamentous fungus

Penicillium kloeckeri was found to be one of the best producers of β-mannanases and β-xylanases when grown on medium containing β-mannan and β-xylan as carbon substrates (Farrel et al. 1996).

2.2.4.3.1 Submerged cultivations

For enzyme production in submerged cultivations, the yield of β-xylanases in a fermentation is further governed by other key factors. When β-xylanase fermentation is carried out on complex heterogeneous substrates, various factors have a combined effect on the level of β-xylanase expression (Hrmová et al. 1989; Haltrich et al. 1996; Subramaniyan and Prema, 2000). They include substrate accessibility, rate and amount of release of the xylo-oligosaccharides and quantity of D-xylose released. The released D-xylose acts not only as the carbon source but also as an inhibitor of β-xylanase synthesis in most cases. Generally, the slow release of the inducer molecules and the possibility of the culture filtrate converting the inducer to its non-metabolisable derivative are believed to boost the level of β-xylanase activity. Pre-treatment of the often complex inducing substrates may significantly improve β-xylanase production, as these pre-treatments may be necessary to make the substrate more available to the organism (Haltrich et al. 1996). Furthermore, β-xylanases bind tightly to the substrate, thus a part of the enzyme produced during the fermentation is lost and discarded as bound enzyme, along with the insoluble substrate (Kulkarni, 1999). Metabolic enzymes of the β-xylanase producing microorganism such as proteases also affect the actual yield of the β-xylanases. Proteases are optimally expressed at the end of the exponential phase, and the harvesting time of the β-xylanases must be correlated to the production of these enzymes. Other bioprocess parameters that can affect the activity and productivity of β-xylanase attained in a fermentation process include the pH, temperature and agitation (Wong and Saddler, 1992; Haltrich et al. 1996; Stöllnberger et al. 1996). Shear stresses and mechanical forces on filamentous fungi due to agitation of the growth medium affect β-xylanase production of the culture. Increasing the impeller speed, in some cases, lead to a drastic decrease in β-xylanase production, presumably due to damage to the mycelium.

2.2.4.3.2 Solid-state cultivation

β-Xylanases can also be produced using solid-state fermentation (Haltrich et al. 1996; Subramaniyan and Prema, 2002). Solid state fermentation is the growth of microorganisms on moist substrates in the absence of free flowing water. Water is present in an absorbed or complexed association with the solid matrix of the substrate. These cultivation conditions are especially suitable for the growth of filamentous fungi, which can grow at relatively low water activities. The advantages of solid state fermentation over liquid batch fermentations include smaller volumes of liquid required for recovery of the product, cheap substrate, low cultivation costs and low risk of contamination (Beg et al. 2001). Solid-state fermentations are practical for complex substrates such as agricultural, forestry and food-processing residues and wastes which can be used as inducing substrates for the production of xylanolytic enzymes (Haltrich et al. 1996). These are used in very high concentrations, which depend on their water absorbance capacity. They enhance enzyme production in certain cases without creating some of the problems encountered with submerged fermentations. Pre-treatment of the substrates is necessary in some cases. Most media used for solid-state fermentation contain some mineral salts and in some cases a complex nitrogen source. Solid-state fermentation is performed under static incubation conditions. Another way to enhance β-xylanase production and thus reduce the cost of the enzyme is by isolation of hyper-producing mutants (Subramaniyan and Prema, 2002). Strain improvement in *T. reesei* has increased the cellulase concentration, yield and productivity by a factor of three. *T. reesei* mutants were isolated that were resistant to catabolite repression and certain mutants were also isolated that were more resistant to disruption by stiring.

2.2.5 Fungal xylanolytic systems

Many bacterial and fungal species produce the full complement of enzymes necessary to enable them to utilise xylan as a carbon source (Poutanen et al. 1987; Uffen, 1997). Bacterial species that produce xylanolytic enzymes are reviewed elsewhere (Hazelwood and Gilbert, 1993; Sunna and Antranikian, 1997; Subramaniyan and Prema, 2002).

2.2.5.1 Xylanolytic systems of filamentous fungi

Filamentous fungi that degrade wood components are generally termed as white rot, brown rot or soft rot fungi (Rayner and Boddy, 1988). The term white rot has traditionally been used to describe those microbial decays in which the wood acquires a bleached appearance because it is predominantly the lignin component that is broken down, with the cellulose and hemicellulose components broken down to a lesser extent. The traditional view of brown rot is a decay in which the wood is discoloured brown, becomes friable and ultimately powdery. This friable residue is composed predominantly of modified lignin from which hemicelluloses and cellulose have been selectively removed. Soft-rot fungi degrade cellulose and hemicelluloses, but do not appear to break down lignin significantly. Unlike brown rots and some white rots, a distinguishing feature of soft rots is that the breakdown of cell walls always occurs in the immediate vicinity of the hyphae.

Strains of the soft rot filamentous fungi *Trichoderma* and *Aspergillus* secrete large amounts of efficient xylan-degrading enzymes and are important producers of cellulolytic and hemicellulolytic enzymes for industrial use (Hrmová et al. 1989; Wong and Sadler, 1992; De Vries et al. 2000). Several strains from the genus *Trichoderma* produce multiple β-xylanases that can be divided into three groups (Lappalainen, 1986; Nevalainen et al. 1990; Wong and Sadler, 1992; Biely and Tenkanen, 1999). Various strains produce a pair of low molecular mass β-xylanases having molecular masses of 18-23 kDa. Some strains produce an additional β-xylanase that has a molecular mass of 29-33 kDa. Both of these types of β-xylanases are specific β-xylanases and do not hydrolyses cellulose, however, they do differ in catalytic properties. Several strains of *Trichoderma* were found to produce a third type of xylan depolymerising enzyme of 53-57 kDa. These enzymes have been identified as non-specific endo-1,4-β-glucanases which are components of the cellulolytic rather than the xylanolytic system (Biely, 1985). Grouping of *Trichoderma* β-xylanases on the basis of molecular mass and pI values is in good agreement with the classification of glycosyl hydrolases on the basis of hydrophobic cluster analysis and sequence similarities (Section 2.2.3). The low molecular mass β-xylanases of *Trichoderma* belong to the glycosyl hydrolase family 11.

The complete nucleotide and amino acid sequences of several *Trichoderma* β-xylanases are known. Among these, Törrönen et al. (1992) reported the sequences of the two major β-xylanases of *T. reesei* encoded by the *xyn1* and *xyn2* genes. The two proteins encoded by these genes of 19 kDa and 21 kDa, respectively, have furthermore been crystallised and their tertiary structure determined (Biely and Tenkanen, 1999). These enzymes are small, well-packed molecules consisting mainly of β-sheets (Fig. 7). The β-sheets are twisted forming a cleft where the catalytic site is located. Two glutamic acid residues in the cleft serve as active groups. The loop overhanging the cleft undergoes a conformational change after substrate binding. The enzymes differ in pH optima and affinity for xylan (Törrönen et al. 1992). The two β-xylanases utilise the method of hydrolysis that is associated with the retention of the configurations of the glycosidic linkage, which is in accordance with the fact that both enzymes catalyse glycosyl transfer reactions at high substrate concentrations (Biely et al. 1993; Biely and Tenkanen, 1999). Although species of the genus *Trichoderma* produce all the enzymes required to fully degrade xylan, they are not among the best producers of the β-xylosidases.

Strains of the filamentous fungus *Aspergillus* also secrete large amounts of efficient and industrially important xylan-degrading enzymes (Hrmová et al. 1989; Kormelink et al. 1992; Kormelink et al. 1993). Three β-xylanases were purified from a crude culture filtrate of *Aspergillus awamori* grown on oat straw as carbon source (Kormelink et al. 1992; Kormelink et al. 1993). The optimal pH for the β-xylanases was between pH 4.0 and pH 5.5 and the temperature optima were between 45°C and 55°C. The molecular masses of β-xylanase I, II and III were 39 kDa, 23 kDa and 26 kDa, respectively and they were all able to degrade xylan to xylobiose and xylotriose. A β-xylosidase of 110 kDa was also isolated and its optimal conditions were at pH 6.5 and 70°C. This β-xylosidase is not an exo-type enzyme as it does not release D-xylose from xylan. Xylo-oligosaccharides are however largely degraded by this enzyme with D-xylose as its main end product. β-Xylanases were also isolated from *Aspergillus terreus* and *A. niger* (Hrmová et al. 1989). D-Xylose, xylobiose and xylan initiated the synthesis of these enzymes but not of cellulolytic enzymes. The two β-xylanases and the β-xylosidase of

A. niger are encoded by *xlnB*, *xlnC* and *xlnD*, respectively (Van Peij et al. 1997). The *xlnD* gene encodes the 804 amino acid β-xylosidase with a predicted molecular mass of 85 kDa. The β-xylosidase encoding gene, *xlnD*, was cloned and sequenced (Van Peij et al. 1997). *A. niger* strains in which the *xlnD* gene was disrupted, accumulated mainly xylobiose and xylotriose when grown on xylan and did not show significant β-xylosidase activity in the supernatant, indicating that *xlnD* encodes the major extracellular β-xylosidase. *Aspergillus kawachii* is a fungus used in the fermentation of traditional Japanese spirits, a process carried out under acidic conditions (pH 3.2 – 4.3) (Ito et al. 1992a; Kormelink et al. 1992). The main β-xylanase of *A. kawachii* is encoded by *xynC*. This gene has been successfully cloned and sequenced (Ito et al. 1992b; Crous et al. 1995)

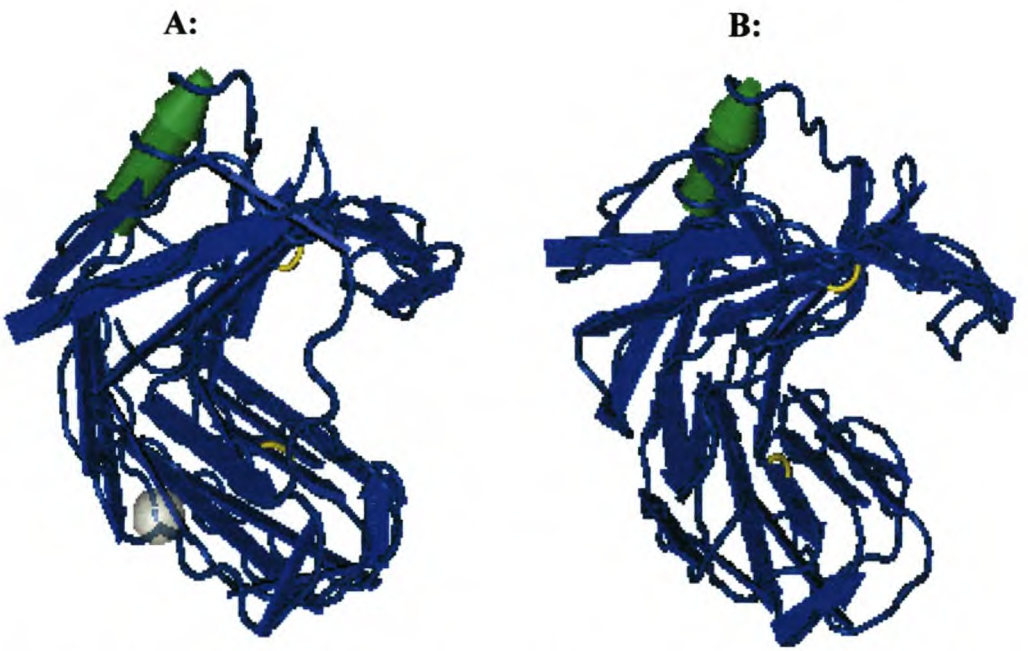


Fig. 7 Ribbon representation of the two main β-xylanases of *T. reesei* (**A**) Xyn1 and (**B**) Xyn2. The two glutamic acid residues in the cleft that serve as active groups are shown in yellow. Despite the great similarity in tertiary structure, the enzymes show differences in catalytic properties, pointing to different substrate binding sites (Biely and Tenkanen, 1999).

2.2.5.2 Naturally occurring xylanolytic yeasts

The production of xylanolytic enzymes is wide-spread among filamentous fungi and bacteria (Hazelwood and Gilbert, 1993; Sunna and Antranikian, 1997). However, this

trait is not as common among naturally occurring yeasts. Biely et al. (1978) tested the ability of 95 strains of yeast and yeast-like organisms, spanning 35 genera, for the ability to grow in media containing D-xylose or xylan as sole carbon source. Of the 54 strains that grew on D-xylose, only 13 strains, of the genera *Aureobasidium*, *Cryptococcus* and *Trichosporon*, could utilise xylan as sole carbon source. These strains were found to secrete xylanolytic enzymes that degraded the xylan in the medium to D-xylose and xylo-oligosaccharides. In addition, it was found that certain strains of the genera *Bullera*, *Candida*, *Geotrichum*, *Pheococcomyces*, *Sporothrix*, *Pichia*, *Pseudozyma* and *Rhodotorula* also hydrolyse xylan (Kremnický et al. 1996; Schäfer et al. 1996; Middelhoven, 1997). Several of these yeast species were isolated from plants growing in arid regions or from termite guts (Schäfer et al. 1996; Middelhoven, 1997). A recent study screened several naturally occurring non-*Saccharomyces* wine yeasts for the production of β-xylanases and other extracellular hydrolytic enzymes (Strauss et al. 2001). It was found that *Candida stellata*, *Candida oleophila* and *Kloeckera apiculata* produced high β-xylanase activities. Table 2 summarises most of the yeast species that have been found to degrade xylan or produce xylanolytic enzymes. It should be noted that not all of the isolated genera and/or species mentioned necessarily degrade xylan or produce a full complement of xylanolytic enzymes. The xylanolytic systems of some of these species have been characterised further and will be discussed in the following section.

2.2.5.2.1 *Aureobasidium pullulans*

Xylan-grown cultures of *A. pullulans* were found to produce low levels of β-xylanase activity (Leathers et al. 1984). However, naturally occurring colour variant strains of *A. pullulans* expressed much higher levels of β-xylanase activity. The β-xylanases from these colour variant strains of *A. pullulans* showed high specific activity and high specificity for xylan. β-Xylanase activity was induced by xylan. β-xylosidase activity was also detected in these strains. The β-xylosidase of *A. pullulans* has been isolated and characterised (Dobberstein and Emeis, 1991). The production of the enzyme was induced by xylan but inhibited by the product of its action, D-xylose. The optimal conditions for the enzyme were at pH 4.5 and 80°C, and it had a high stability over a

wide pH range and at high temperatures. When grown on xylan this yeast produces hemicellullytic enzymes and no cellulase activity, therefore it is especially useful for the production of enzymes for the paper and pulp industry (Section 2.2.6.1) and has been successfully employed in biobleaching procedures (Christov and Prior, 1996; Christov et al. 1997).

Table 2 Naturally occurring xylanolytic yeast species

Yeast	Reference
Aureobasidium sp.	Biely et al. 1978
A. pullulans	Leathers et al. 1984
A. pullulans var. *melanigenum*	Ohta et al. 2001
Bullera sp.	Kremnický et al. 1996
Candida sp.	Schäfer et al. 1996
C. oleophila	Strauss et al. 2001
C. pulcherrima	Strauss et al. 2001
C. shehatae	Lee et al. 1986
C. stellata	Strauss et al. 2001
Cryptococcus sp.	Biely et al. 1978
C. albidus	Leathers et al. 1984
C. adeliae	Gomes et al. 2000
C. flavus	Sunna and Antranikian 1997
C. laurentii	Lee et al. 1987
C. luteolus	Lee et al. 1987
Debaryomyces sp.	Schäfer et al. 1996
Geotrichum sp.	Kremnický et al. 1996
Kloeckera apiculata	Strauss et al. 2001
Pichia sp.	Schäfer et al. 1996
P. stipitis	Lee et al. 1986
Pheococcomyces sp	Kremnický et al. 1996
Pseudozyma sp.	Middelhoven 1997
Rhodotorula sp.	Kremnický et al. 1996
R. glutinis AM-8	Middelhoven 1997
Trichosporon sp.	Biely et al. 1978
T. cutaneum	Hrmová et al. 1984

A. pullulans was found to produce a xylobiose permease transporting β-1,4-xylobiose into the cells from extracellular media (Kremnický and Biely, 1998). The permease is induced by the inducers of the xylanolytic enzyme system, namely D-xylose, β-1,4-xylobiose or during the growth on xylan. The permease is energy dependent, synthesized *de novo* and inhibited by D-glucose. Furthermore, the xylobiose permease appears to transport β-1,4-mannobiose, β-1,4-xylobiose and methyl-β-D-xylopyranoside. An

α-L-arabinofuranosidase was produced by a colour variant strain of *A. pullulans* when grown in liquid culture on oat spelt xylan (Saha and Bothast, 1998a). The enzyme was purified to homogeneity from the culture supernatant. It had a native molecular weight of 210 kDa and was composed of two equal subunits and displayed optimal activity at 75°C and pH 4.0-4.5. The purified α-L-arabinofuranosidase hydrolysed and debranched arabinan, and released L-arabinose from arabinoxylans. However, it was inactive against arabinogalactan. The enzyme was produced when *A. pullulans* was grown in liquid culture on sugar beet arabinan, wheat arabinoxylan, L-arabinose, L-arabitol, D-xylose, xylitol, oat spelt xylan, corn fibre, or arabinogalactan (Saha and Bothast, 1998b). Some *A. pullulans* strains also produce all the enzymes required for complete hydrolysis of wood and plant β-mannans (Kremnický and Biely, 1998).

The gene (*xynA*) encoding *A. pullulans* β-xylanase II was cloned and sequenced (Li and Ljungdahl, 1994). The 895-bp open reading frame encoded a polypeptide of 221 amino acids, including a 34 amino acid putative signal peptide at the aminoterminus. The gene contained a single intron. Southern blot analysis further indicated that the β-xylanase encoding gene was present as a single copy in the genome. mRNA transcripts of the gene were found in cultures grown on D-xylose and xylan as sole carbon source but the transcription was suppressed when the carbon source was D-glucose. The genomic and cDNA copies of a β-xylanase encoding gene of *A. pullulans* var. *melanigenum* were also cloned and sequenced (Ohta et al. 2001). Southern blot analysis indicated that the β-xylanase encoding gene (*xynI*) was present as a single copy in the genome. The DNA region encoding the prepeptide was interrupted by a 59-bp intron. The deduced amino acid sequence showed 94% identity with that of *xynA* and encoded a β-xylanase with an optimal pH of 4.8.

2.2.5.2.2 <u>*Cryptococcus albidus*</u>

Strains of *C. albidus* were found to grow on xylan and secrete low levels of β-xylanases (Biely et al. 1978; Leathers et al. 1984). *C. albidus* was also found to produce significant levels of β-xylosidase and β-xyloside permease (Biely and Petráková, 1984a; Leathers et al. 1984). β-Methylxyloside, a non-metabolisable inducer, was found to induce

β-xylanase production in *C. albidus* but production was repressed by D-xylose (Morosoli et al. 1989). In another study, a series of compounds structurally related to xylan and 1,4-β-xylobiose were tested for induction of the xylan-degrading enzyme system of *C. albidus* (Biely and Petráková, 1984a). The most rapid induction of β-xylanase, β-xylosidase and β-xyloside permease was observed with 1,4-β-xylobiose, but efficient induction was also achieved with different positional isomers. The thioglycoside, 4-thioxylobiose has a highly stimulatory effect on the synthesis of the xylanolytic enzymes of *C. albidus* in the presence of 1,4-β-xylobiose (Defaye et al. 1992). The mature β-xylanase of the yeast is a highly glycosylated enzyme with an apparent molecular mass of 48 kDa (Morosoli et al. 1992b). The β-xylosidase, in contrast, was found to be located in the periplasmic space or in the cytoplasm (Jeffries, 1990). When the β-xylanase was purified and its substrate binding site investigated, it was found that it had four subsites and that the catalytic groups were localised in the centre (Biely et al. 1980; Biely et al. 1981). The β-xylanase encoding gene of *C. albidus* was cloned and sequenced (Boucher et al. 1988; Morosoli et al. 1992a). The genomic copy of the gene contained seven introns within the open reading frame (Moreau et al. 1992).

Using a series of methyl β-D-xylotriosides, the substrate specificity of the *C. albidus* β-xylanase was tested (Vršanská et al. 1990). In addition to the cleavage of β-1,4 linkages, the enzyme could also cleave β-1,3 and β-1,2 linkages adjacent to a β-1,4 linkage. However, the rate of cleavage of β-1,4 linkages was much higher than the rate of cleavage of the other linkages. The enzyme did not attack α-xylosidic linkages. Through transglycosylation and hydrolysis catalysed by β-xylanase and β-xylosidase, β-1,2-xylobiose and β-1,3-xylobiose can be converted into β-1,4-xylobiose (Biely and Petráková, 1984b).

2.2.5.2.3 *Cryptococcus adeliae*

C. adeliae, isolated from Antarctica, is a psychrophilic basidiomycete yeast that shows thermolabile β-xylanase activity (Scorzetti et al. 2000). rDNA comparisons showed that the yeast is closely related to *C. albidus*. After optimisation of the growth media, the

yeast produced 400 nkat/ml β-xylanase activity at 4°C when xylan was the sole carbon source (Gomes et al. 2000). β-Xylosidase and α-L-arabinofuranosidase activities were also detected. Although maximum levels of β-xylanase activity were achieved when xylan was the sole carbon source, appreciable levels of β-xylanase activity were also observed using lignocelluloses such as steamed wheat straw and alkali-treated bagasse as the carbon source. No filter paper cellulase activities were detected. The β-xylanase showed optimal activity at pH 5.0-5.5 with good stability at pH 4-9. Although the enzyme was maximally active at 45°–50°C, it appeared very thermolabile, showing a half-life of just 78 min at 35°C. At 40°–50°C, it lost 71%–95% activity within 5 min. Compared to the β-xylanases from mesophiles and thermophiles, very little is known about the use of cold-active β-xylanases. However, potential biotechnological applications of cold-active β-xylanases together with cellulases, lipases and proteases may be in the digestion of industrial or sewerage wastes and decomposition of agricultural residues at ambient temperatures in cold and temperate climates.

2.2.5.2.4 *Trichosporon cutaneum*

The yeast *T. cutaneum* was shown to grow on xylan as carbon source (Hrmová et al. 1984). However some strains also produced very low levels of cellulases when xylan was the sole carbon source, which is important for the production of cellulase-free β-xylanases for the pulp and paper industry (Liu et al. 1999). Extracellular β-xylanase synthesis in the yeast was inducible (Hrmová et al. 1984; Liu et al. 1999). The enzyme could be induced in washed D-glucose-grown cells by xylan or D-xylose. The induction of β-xylanase by xylan was longer lasting and the final activities were significantly higher compared to induction by D-xylose. Methyl β-D-xylopyranoside, a synthetic analogue of xylobiose, however, was not an inducer of β-xylanase activity in *T. cutaneum*. Furthermore, β-xylanase induction was subject to D-glucose repression.

2.2.5.2.5 *Pichia stipitis*

P. stipitis is able to utilise and ferment the main component sugar of xylan to ethanol. In fact, it is the best D-xylose-fermenting yeasts thus far described (Slininger et al. 1985; Jeffries and Kurtzman, 1994). A xylan-degrading yeast therefore has implications for a

theoretical direct conversion of xylan to ethanol. The metabolism of D-xylose to ethanol by *P. stipitis* and the importance of bioethanol will be discussed further in section 2.3. *P. stipitis* and other non-*Pichia* yeasts that are able to grow on D-xylose were screened for the ability to degrade xylan (Lee at al. 1986). β-Xylanase activity in these yeasts was rare with only 19 xylanolytic strains of 250 strains tested. The activity was localised to *Cryptococcus* species as well as *P. stipitis* and *Candida shehatae*. Two *P. stipitis* strains, CBS 5773 and CBS 5775, converted xylan to ethanol with about 60% of the theoretical yield. The maximum β-xylanase activity that was observed in *P. stipitis* was only 2.5-3% of the activity reported for *Cryptococcus albidus* (Basaran et al. 2001).

The β-xylanase and β-xylosidase produced by *P. stipitis* were purified to homogeneity and characterised (Özcan et al. 1991). Both enzymes are secreted into the culture media upon growth on xylan as sole carbon source. Production of the β-xylanase was induced by xylan but repressed by D-xylose and D-glucose (Gírio et al. 1996). By contrast it was found that the β-xylosidase is synthesised constitutively to a considerable degree (Özcan et al. 1991). These results are in accordance to our results in chapter 4, where we found very little β-xylanase activity, but considerable β-xylosidase activity for strains cultured on D-xylose. The β-xylanase was estimated to be a 43 kDa glycoprotein with a carbohydrate content of about 26% (Özcan et al. 1991). The β-xylosidase was estimated to be 37 kDa by SDS-PAGE analysis. The purified β-xylosidase was also able to hydrolyse aryl-β-D-glucosides. The β-xylanase encoding gene of *P. stipitis* (*xynA*) was cloned from a genomic DNA library and expressed in *Escherichia coli* (Basaran et al. 2001). The 1146 bp gene encoded a 381 amino acid protein including a 20 amino acid putative N-terminal signal sequence and four putative N-linked glycosylation sites. The nucleotide sequence of *xynA* contained no introns and showed homology to family 11 β-xylanases. There is a 59% identity of amino acids between the catalytic domains of the *P. stipitis* β-xylanase and a β-xylanase from *Bacillus circulans*.

2.2.5.3 Heterologous expression of xylanolytic enzymes in yeasts

Several researchers have successfully expressed heterologous xylanolytic genes in a variety of yeast hosts (Moreau et al. 1992; Crous et al. 1995; La Grange et al. 1996; Berrin et al. 2000; La Grange et al. 2001; Fujita et al. 2002).

2.2.5.3.1 *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* cannot degrade xylan nor ferment the main component sugar, D-xylose, to ethanol (La Grange et al. 2001). However, *S. cerevisiae* is capable of high-level fermentation on D-glucose yielding high levels of ethanol and high productivity. Furthermore *S. cerevisiae* also has good tolerance towards high ethanol concentrations and other inhibitors that may be present in the fermentation media (Hahn-Hägerdal et al. 2001). For these reasons it would be a great advantage to be able to use *S. cerevisiae* for xylan to ethanol fermentations. The considerable efforts that have been made in transferring efficient D-xylose fermentation ability to *S. cerevisiae* will be discussed in section 2.3.

Genomic and cDNA copies of the *C. albidus* β-xylanase encoding gene were expressed in *S. cerevisiae* under transcriptional control of its native promoter and the *S. cerevisiae* alcohol dehydrogenase 2 gene (*ADH2*) promoter (Moreau et al. 1992). Secreted β-xylanase activities were relatively low. The functional 48-kDa β-xylanase was secreted in strains expressing the cDNA copy, but the 7 introns interrupting the open reading frame could not be spliced out of the pre-mRNA to form the functional enzyme from the genomic DNA copy of the gene. The *A. pullulans* β-xylanase encoding gene (*xynA*) was expressed as cDNA and genomic DNA copies in *S. cerevisiae* under transcriptional control of the *S. cerevisiae* galactokinase encoding gene (*GAL1*) promoter (Li and Ljungdahl, 1996). Active β-xylanase was detected in the culture supernatant when the cDNA copy was expressed, but expression of the genomic DNA copy containing one intron did not yield β-xylanase activity. Two protein bands of 25 and 27 kDa were detected on SDS-PAGE and western blot analysis, using an anti-XynA antibody. It was shown that that the native XynA signal peptide was more efficient at

promoting secretion of the heterologous β-xylanase than the *S. cerevisiae* invertase (*SUC2*) or mating pheromone α-factor (*MFα1*) secretion signals.

The β-xylanase encoding genes of the filamentous fungi *A. niger*, *A. kawachii* and *T. reesei* were expressed in *S. cerevisiae* (Crous et al. 1995; La Grange et al. 1996; Luttig et al. 1997). In all three cases, cDNA copies of the genes were cloned as it was known than *S. cerevisiae* does not efficiently splice out the introns of heterologous genes (Romanos et al. 1992). The *A. kawachii xynC* was expressed under transcriptional control of the *S. cerevisiae* phosphoglycerate kinase I gene (*PGK1)* promoter and active β-xylanase was secreted by the recombinant yeast strain with optimal activity at below pH 3 and 60°C (Crous et al. 1995). Two β-xylanase encoding genes of *A. niger* were cloned and expressed under transcriptional control of the *S. cerevisiae ADH2* promoter (Luttig et al. 1997). Both enzymes, found to be 92% identical at amino acid level, were successfully secreted and exhibited pH and temperature optima of pH 4 and 60°C, respectively. The cDNA copy of the *T. reesei* β-xylanase II (*xyn2*) was expressed in *S. cerevisiae* under the transcriptional control of the *PGK1* and *ADH2* promoters (La Grange et al. 1996). Efficient secretion of the heterologous β-xylanase was achieved by the native *T. reesei xyn2* secretion signal, a 33-amino acid leader sequence. Optimal conditions for the recombinant enzyme were at pH 6 and 60°C, respectively. The recombinant β-xylanase was 27 kDa in size, as determined by SDS-PAGE. The molecular mass of the mature protein in *T. reesei* was found to be 21 kDa, with virtually no glycosylation. The extra molecular weight of the heterologous Xyn2 protein secreted by *S. cerevisiae* was shown to be the result of hyperglycosylation of the protein. The levels of β-xylanase activity in the culture supernatant when expressing either the *A. niger*, *A. kawachii* or *T. reesei* β-xylanases were shown to be greatly increased when growing the strains in rich culture media after creating *ura3*, *fur1* autoselective strains by disrupting the *S. cerevisiae FUR1* gene (Crous et al. 1995; La Grange et al. 1996; Luttig et al. 1997).

A yeast strain displaying *T. reesei* β-xylanase II (*xyn2*) on the cell-surface was created (Fujita et al. 2002). The fusion gene encoding the mature region of *xyn2* and the C-terminal region (320 amino acid residues from the C-terminal end) of *S. cerevisiae* α-agglutinin was constructed and expressed in *S. cerevisiae* under the transcriptional control of the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (*GPD1*) promoter. Immunofluorescence labeling indicated that Xyn2 was displayed on the cell-surface in the active form. The Xyn2-displaying yeast showed highest β-xylanase activity at pH 5.0 and 40°C, respectively. This differs significantly from the recombinant Xyn2 secreted by *S. cerevisiae* (La Grange et al. 1996). The differences are probably ascribable to the different tertiary structure that resulted from the Xyn2-agglutinin fusion.

The β-xylosidase encoding gene of *Bacillus pumilus* (*xynB*) was cloned from a genomic DNA library and expressed in *S. cerevisiae* under transcriptional control of the *S. cerevisiae ADH2* promoter (La Grange et al. 1997). To promote secretion of the enzyme the gene was fused in reading frame with the *S. cerevisiae* mating pheromone α-factor secretion signal (*MFα1*). Biologically active β-xylosidase was obtained, but remained mostly cell associated with optimal conditions of pH 6.6 and 45-50°C, respectively. When this fusion gene and *T. reesei xyn2* were co-expressed in *S. cerevisiae* under transcriptional control of the *S. cerevisiae ADH2* promoter, a 25% increase in the amount of reducing sugars released from birchwood xylan was obtained, compared to strains expressing β-xylanase alone. However, no D-xylose was produced from birchwood xylan, presumably due to very low β-xylosidase activity. A cDNA copy of the *A. niger* β-xylosidase encoding gene was cloned (La Grange et al. 2001). The 2.4 kb open reading frame encodes a 804 amino acid propeptide. The 778 aminoacid mature protein encoding region was fused in reading frame with the *S. cerevisiae* mating pheromone α-factor secretion signal to ensure secretion from *S. cerevisiae*. β-Xylosidase activity was obtained with optimal conditions of pH 3.2 and 60°C, respectively. When this fusion gene and *T. reesei xyn2* were co-expressed in *S. cerevisiae* under transcriptional control of the *S. cerevisiae ADH2* promoter, high levels of β-xylanase and β-xylosidase activity were obtained in autoselective (*FUR1* disrupted) strains grown in

rich medium. Co-production of these two enzymes allowed this recombinant *S. cerevisiae* strain to degrade birchwood xylan to D-xylose.

2.2.5.3.2 *Kluyveromyces lactis*

The β-xylanase encoding gene of the extreme thermophilic bacterium *Thermotoga* sp. strain FjSS3B.1 was cloned and fused in reading frame with the *K. lactis* killer toxin secretion signal and under control of the *K. lactis LAC4* (β-galactosidase) promoter (Walsh et al. 1998). Correctly processed, unglycosylated β-xylanase was secreted and active on oat spelt xylan with a half-life similar to the native enzyme of 48h at 90°C. The *K. lactis* system could also secrete g/L amounts of largely pure β-xylanase A from *Dictyoglomus thermophilum* in chemostat culture (Bergquist et al. 2002). Thermostable β-xylanases could be of great use in the biobleaching processes in the pulp and paper industry (Section 2.2.6).

A cDNA fragment encoding the "A" catalytic domain of the *N. frontalis* β-xylanase gene (*xyn3*) was cloned by PCR and expressed in *K. lactis* under transcriptional control of the *S. cerevisiae PGK1* promoter (Durand et al. 1999). The gene was successfully expressed and active β-xylanase was secreted by the yeast cells, using the *K. lactis* killer toxin secretion signal. Differently glycosylated forms of the β-xylanase were secreted by the transformants.

2.2.5.3.3 *Pichia pastoris*

A β-xylanase encoding gene from *A. niger* was cloned as a cDNA copy and expressed in *P. pastoris* under control of the *P. pastoris* alcohol oxidase encoding gene (*AOX1*) promoter and the *S. cerevisiae SUC2* invertase secretion signal (Berrin et al. 2000). Methanol induction of the recombinant strains yielded up to 60 mg/L biologically active β-xylanase in synthetic medium. The secreted protein was 19.9 kDa in size and had a pI of 3.5, identical to the native *A. niger* enzyme. Optimal conditions for the recombinant and native forms of the enzyme were pH 3.5 and 50°C, respectively.

2.2.5.3.4 *Pichia stipitis*

The β-xylanase encoding gene of *C. albidus* was expressed as cDNA and genomic DNA copies in the D-xylose fermenting yeast *P. stipitis* under tanscriptional control of the *P. stipitis* xylose reductase encoding gene (*XYL1*) promoter (Morosoli et al. 1992b; Morosoli et al. 1993). The enzyme secreted by strains expressing the cDNA copy was 50 kDa in size, slightly larger than the native and recombinant *S. cerevisiae* enzyme (Moreau et al. 1992), probably reflecting a different glycosylation pattern. The genomic DNA copy of the gene was transcribed by the recombinant strains that carried them but no functional β-xylanase was secreted by these strains. Therefore the seven introns interrupting the open reading frame of the *C. albidus* β-xylanase encoding gene could not be spliced out of the mRNA by *P. stipitis*, as was the case when the genomic DNA copy was expressed in *S. cerevisiae*. The secretion of biologically active β-xylanase in *P. stipitis* enabled the yeast to grow on and ferment xylan as sole carbon source under conditions of low oxygen concentrations. The *P. stipitis ADH2* gene promoter was also used to control expression of the *C. albidus* β-xylanase encoding gene for expression in a *P. stipitis* strain with no native β-xylanase activity (Passoth and Hahn-Hägerdal, 2000). The *ADH2* gene promoter is oxygen regulated and is induced in conditions of low oxygen concentration, thus the conditions needed to induce fermentation in *P. stipitis* (Section 2.3). Biologically active β-xylanase was secreted by this recombinant strain after a shift to hypoxic and anoxic conditions.

In our investigation we expressed the *T. reesei xyn 2* gene under transcriptional control of the inducible *P. stipitis XYL1* gene and the constitutive transketolase encoding *TKL* gene promoters (Chapter 3; Chapter 4). Funtional β-xylanase was secreted under control of the *T. reesei* secretion signal. The molecular size of the recombinant Xyn2 protein produced by *P. stipitis* was 20.7 kDa, which is similar to that of the native *T. reesei* Xyn2 protein. This indicates no or minimal glycosylation of the recombinant secreted protein. The recombinant *xyn2*-expressing strain also yielded twice the amount of biomass yielded by the control strain when cultivated in medium containing 1% birchwood xylan as sole carbon source.

As was stated earlier, xylan, as the second most abundant polysaccharide after cellulose, represents one of the most important targets in the exploitation of renewable carbon resources. Therefore, in the second part of our study we further explored the increase in biomass yield by recombinant *P. stipitis* strains expressing and co-expressing xylanolytic genes (Chapter 4). β-Xylanase encoding genes of *T. reesei* (*xyn2*) and *A. kawachii* (*xynC*) were cloned under transcriptional control of the inducible *P. stipitis XYL1* gene promoter on episomal plasmids. The β-xylosidase encoding gene of *A. niger* (*xlnD*) was cloned as an in reading frame fusion with the *S. cerevisiae MFα1* secretion signal under transcriptional control of the constitutive *P. stipitis TKL* gene promoter on an episomal plasmid. Combinations of the individual β-xylanase encoding genes and β-xylosidase expression cassette were also cloned onto episomal plasmids. All of the plasmids were subsequently transformed to *P. stipitis* and the β-xylanase activities, β-xylosidase activities and growth of the recombinant strains on xylan as sole carbon source were monitored. The strains expressing the *A. kawachii xynC* gene reached the highest maximum levels of β-xylanase activity and the activity was sustained for longer periods than the *T. reesei xyn2* expressing strains, where the levels of activity declined rapidly after reaching a maximum. The decline in the β-xylanase activities of the recombinant *P. stipitis* strains is likely due to a combination of factors. The *XYL1* promoter was induced by D-xylose and the depletion of D-xylose during the time course of the experiment led to a loss of the *XYL1* promoter activity. However, the depletion of D-xylose alone does not explain why the β-xylanase activity already accumulated is lost. The pH of these cultures had dropped to below pH 3, therefore, it was thought that the production of acid proteases might be responsible for the rapid loss of β-xylanase activity in the recombinant *P. stipitis* strains, as was the case for other strains of the genus *Pichia* (Sreekrishna et al. 1997). When *P. stipitis* transformants were patched onto milk plates to screen for extracellular protease activity all transformants showed clearing zones, confirming the presence of extracellular proteases. All strains expressing the *A. niger* (*xlnD*), whether alone or in combination with a heterologous β-xylanase, reached similar levels of β-xylosidase activity, markedly higher than the control strains. The co-

expression of the heterologous β-xylanases and β-xylosidase did not result in significantly lower levels of activity of the two enzymes in comparison with strains where they were expressed separately. The xylanolytic gene products, whether expressed alone or simultaneously, led to an increase in biomass production of the recombinant strains when grown on medium containing 2% birchwood glucuronoxylan as sole carbon source. Simultaneous expression of the *A. kawachii xynC* gene and the *A. niger xlnD* gene led to the highest level of biomass production of any of the recombinant strains, showing a maximum biomass yield improvement of approximately 5-fold over the control strain. It was also noted that the percentage of improvements in biomass yields of the strains co-expressing the xylanolytic genes is more than the sum of the percentages of improvement of the strains expressing the genes individually. This shows the synergistic action of the heterologous xylanolytic enzymes on xylan. These strains represent a step towards the efficient degradation of hemicellulosic materials by yeasts.

The recombinant strains of *P. stipitis* were also grown in a fermentor under conditions of high oxygenation and controlled pH to increase biomass yields from xylan as sole carbon source (Chapter 5). The high levels of oxygenation led to even further increases in biomass yield of the recombinant *P. stipitis* strains grown on 2% birchwood glucuronoxylan (Chapter 5). Cell counts improved on average by 85% over the cell counts attained in shake flasks. However, increases in biomass over the control strain, for cultures grown in the fermentor, remained roughly the same as the increases in biomass yield over the control strain attained in shake flask cultures of the same strains. Interestingly the *P. stipitis* strain co-expressing the *A. kawachii* β-xylanase and the *A. niger* β-xylosidase, the most successful of the recombinant *P. stipitis* strains, yielded ca. 32% of the theoretical maximum biomass yield in both shake flasks and fermentor. This may indicate that the biomass yield of this strain from birchwood glucoronoxylan in minimal media is possibly limited by other factors such as the glucuronic acid side chains, preventing further xylan degradation. The birchwood glucuronoxylan used in this study contained glucuronic acid side chains at a ratio of one per ten xylose residues, thus the degradation of the polymer by the β-xylanases was limited because of the limitations of family 11 β-xylanases (Puls and Schuseil, 1993; Biely et al. 1997) leading to a

significant amount of unutilised D-xylose residues. Therefore, in the case of recombinant strains expressing the recombinant β-xylanase alone 40% of the D-xylose residues would not be utilised. The recombinant β-xylosidase expressed by strains co-expressing the β-xylanase and the β-xylosidase would remove the D-xylose residue adjacent to the substituted residue at the reducing end, resulting in only 30% of the D-xylose residues remaining unutilised. The biomass yield could therefore be increased even further with the co-expression of an α-glucuronidase gene in these strains.

It has previously been shown that some strains of the yeast *P. stipitis* can convert xylan directly to ethanol (Lee et al. 1986; Morosoli et al. 1993). *P. stipitis* has a specific requirement for low oxygenation for ethanol formation (Skoog and Hahn-Hägerdal, 1990; Mahler and Nudel, 2000). Therefore, the recombinant *P. stipitis* strain expressing the *A. kawachii xynC* gene and the *A. niger xlnD* gene (*P. stipitis* [*xynC XLO2*], the strain showing most improvement in biomass production) and the corresponding *P. stipitis* control strain were grown on defined media containing 2% birchwood glucuronoxylan as sole carbon source in shake flasks at low oxygenation to determine ethanol yield (Chapter 5). Maximal ethanol concentrations attained were 1.35 g/L ethanol for the *P. stipitis* [*xynC XLO2*] strain and 0.37 g/L for the *P. stipitis* reference strain. These ethanol levels are lower than those that were attained by a natural isolate of *P. stipitis* grown on 1% larchwood xylan, which reached 1.8 g/L ethanol (Lee et al. 1986). The recombinant *P. stipitis* strain constructed by Morosoli et al. (1993) yielded 1.8 g/L ethanol on 1% xylan. Furthermore, a hybrid strain constructed by fusing *T. reesei* nuclei and *P. stipitis* protoplasts attained 2.3 g/L ethanol on rich medium containing 2% xylan as carbon source. Therefore, although the *P. stipitis* [*xynC XLO2*] recombinant strain showed a significant increase in ethanol yield from xylan as sole carbon source over the reference strain, thus showing the effect of the heterologous β-xylanase and β-xylosidase, the final ethanol yield was relatively low. However, the auxotrophic *P. stipitis* strain used was reported to be a poor fermenter (Yang et al. 1994) and higher ethanol yields may therefore be achieved by expressing the recombinant genes in a better fermenting strain.

2.2.5.4 β-Xylanases of extremophilic origin

Considerable progress has been made in the isolation of extremophilic microorganisms and their successful cultivation in the laboratory (Kulkarni et al. 1999; Subramaniyan and Prema, 2000). Commercial applications of β-xylanases demand identification of highly stable enzymes active under routine handling conditions. The use of biocatalysts has been constrained due to their labile nature under extreme temperature and pH conditions. Many advantages, such as reduced contamination risk and faster reaction rates, have been proposed for the use of thermophiles in biotechnology processes. In general, parameters such as temperature, pH and chemical and enzymatic stability are important considerations for the industrial applicability of any enzyme. One of the ways to identify the industrially suitable β-xylanase preparations is to look for the enzymes from extremophilic microorganisms. The unique framework of enzymes isolated from extreme environments will be the ideal choice to engineer novel proteins with the desired functions suitable for a particular application.

The β-xylanases from thermophilic bacteria such as *Thermotoga maritima* and *Dictyoglomus thermophilum* show an optimum temperature in the range of 65-80°C (Liebl et al. 1996; Bergquist et al. 1996). β-Xylanase from *Dictyoglomus* sp. exhibited a half-life of 80 min at 90°C. The thermostable β-xylanase produced by a thermotolerant *Aspergillus* strain at 37°C showed maximum activity at 80°C (Mendicuti et al. 1997). These enzymes may prove advantageous in the pulp and paper industry, as thermostability is an important consideration (Section 2.2.6.1).

Studies of alkaliphiles have led to the discovery of a variety of enzymes, which exhibit some unique properties (Horikoshi, 1999; Kulkarni et al. 1999; Subramaniyan and Prema, 2000). Biological detergents contain enzymes such as alkaline proteases and/or alkaline cellulases from alkaliphiles. One significant application is the use of the enzyme, cyclodextrin glycosyl transferase, to catalyze the degradation of starch to cyclodextrins. Commercial production became economical only after the discovery of an alkaliphilic *Bacillus* producing cyclodextrin glycosyl transferase with enhanced pH stability. Alkaline β-xylanases have gained importance due to their application for the

development of environmentally friendly technologies used in paper and pulp industries (Section 2.2.6.1). The enzymes are able to hydrolyse xylan that is soluble in alkaline solutions. Many of the β-xylanases produced by alkaliphilic microorganisms such as *Bacillus* sp. with optimum growth at pH 10.0, showed remarkable stability at pH 9-10 but were not highly active above pH 8.0. A *Bacillus* sp. that produces high levels of β-xylanase was recently isolated (Subramaniyan and Prema, 2000). This enzyme has optimal conditions of 50°C and pH 6-8, and shows no cellulase activity above pH 9.

An alkothermophilic actinomycete *Thermomonospora* sp. producing high levels of β-xylanase was isolates from self-heating compost (George et al. 2001). High levels of β-xylanase activity were produced when cultured in shake flasks at pH 9 and 50°C. Lower levels of cellulase, mannanase and β-xylosidase activities were also detected. The β-xylanase was active over a wide range of pH (pH 5-9) and temperature (40-90°C) with optima at pH 7 and 70°C, respectively. The enzyme was stable at a pH range of pH 5-8 and had a half-life of 8 and 4 hours at 60°C and 70°C but only 9 minutes at 80°C.

2.2.6 β-Xylanases in the industry

Basic and applied research on microbial cellulases, hemicellulases and pectinases has not only generated a wealth of scientific knowledge, but has also shown the enormous potential of these enzymes in biotechnology. At present these enzymes are used in the paper and pulp, food, brewing, wine, animal feed, textile and laundry industries as well as in agriculture (Kulkarni et al. 1999; Bhat, 2000; Subramaniyan and Prema, 2002). The demand for these enzymes has grown rapidly and is the driving force for research on these enzymes. Table 3 summarises of the important industrial applications of these enzymes. The utilisation of cellulases and hemicellulases in the conversion of cellulose and hemicellulose to its monomeric sugars for eventual fermentation to bioethanol will be discussed in section 2.3.

2.2.6.1 β-Xylanases in the pulp and paper industry

The mechanical pulping processes such as refining and grinding of the wood raw material lead to pulps with high contents of fines, bulk and stiffness (Bhat, 2000). Although these

fibres can be used to create different grades of papers, the main disadvantage of mechanical pulping is high-energy consumption. Biomechanical pulping using white-rot fungi resulted in substantial energy savings during refining, and improvement in hand-sheet strength properties. Unfortunately these encouraging laboratory results have not been successfully commercialised.

Table 3 The important industrial applications of β-xylanases and other hemicellulases

Industry & Enzyme(s) used	Application	Reference
Paper & Pulp		
cellulases & hemicellulases	Biomechanical pulping De-inking of recycled fibres Improved draining in mills	Bhat, 2000
β-xylanases and other hemicellulases	Biobleaching of pulp, hydrolyses xylan and reprecipitated xylan, lowers chlorine requirement	Subramaniyan and Prema, 2002
Animal Feed		
cellulases & hemicellulases	Partial hydrolysis of lignocellulosic materials improves nutritional quality of feeds for monogastrics and ruminants	Kulkarni, 1999
β-xylanases	Hydrolysis of cereal arabinoxylans improves feed digestion and absorption	Bhat, 2000
Food & Beverage		
Macerating enzymes (cellulases, hemicellulases and pectinases)	Hydrolysis of cell wall components, improves extraction fruit juices and olive oil	MacCabe et al. 2002; Bhat, 2000
β-xylanases	Modification of cereal arabinoxylan improves texture, quality and shelf life of baked products	Poutanen, 1997
Wine		
Macerating enzymes (cellulases, hemicellulases and pectinases)	Hydrolysis of plant cell wall polysaccharides improves colour extraction and stability of wine	Bhat, 2000

The process of lignin removal from chemical pulps to produce bright or completely white pulp is called bleaching (Beg et al. 2001). It is necessary for aesthetic reasons and for improvement of paper properties, because the leftover lignin fraction imparts an undesirable brown colour to the paper. In conventional bleaching harsh reagents such as chlorine or chlorine dioxide are used during acidic bleaching stages to cleave these

linkages. These bleaching chemicals cause severe effluent based problems in the pulp and paper industry, as the effluent often contains toxic and mutagenic substances. Environmental regulations have put a limit on the amount of chlorine that can be used in the bleaching process in the paper and pulp industry (Kulkarni et al. 1999; Beg et al. 2001). β-Xylanases play an important role in debarking, de-inking of recycled fibers and in the purification of cellulose for the preparation of the dissolving pulp. β-Xylanase pretreatment has been reported to lower bleaching chemical consumption and to result in greater final brightness. The enzymatic bleaching works by cleavage of the bonds that link the hemicellulose and lignin fractions and opening the pulp structure, resulting in a lower subsequent chlorine requirement (Subramaniyan and Prema, 2002). β-Mannanases interact synergistically with β-xylanases to improve the bleaching of craft pulps especially from softwoods (Bhat, 2000). β-Xylanases further promote bleaching by hydrolysis of relocated, reprecipitated xylan on the surface of the pulp fibres, resulting in better chemical penetration and thus improving lignin extraction (Kulkarni et al. 1999; Bhat, 2000; Antonopoulos et al. 2001). Reprecipitated xylan creates a physical barrier for the chemical extraction of residual brown lignin and lignin-carbohydrate molecules. Thus treatment with the β-xylanase makes the pulp more permeable and increases porosity for subsequent chemical extraction. However, the use of these enzymes did not lead to a decrease in the mechanical strength of the fibres as they do not degrade cellulose. Alkaline-stable lignin-carbohydrate complexes present in the wood seem to be the major obstacles in the solubilization of the residual lignin. Hemicellulose can selectively hydrolyse polysaccharide chains attached to lignin, thereby decreasing the amounts of chemicals required and yielding high-molecular mass lignin molecules which are currently gaining more importance as a valuable by-product of the paper and pulp industry.

In the process of pulp bleaching, apart from being entirely cellulase free, β-xylanase preparations with high pH and temperature optima are of utmost importance (Subramaniyan and Prema, 2000; Beg et al. 2001). Thermostable β-xylanases from thermophilic organisms have thus been isolated for their potential use in the pulp and paper industry (Bergquist et al. 1996; Gibbs and Bergquist, 1996; Liebl et al. 1996;

Nordberg-Karlsson et al. 1996). Many alkali-tolerant organisms such as *Streptomyces albus* and strains of *Bacillus* produce β-xylanases with pH optima of around pH 9, and have been used for biobleaching (Subramaniyan and Prema, 2000; Antounopoulos et al. 2001). It is also important to produce cellulase-free β-xylanases so as not to negatively influence the strength of the cellulose fibres in the pulp (Stöllnberger et al. 1996). Christov and Prior (1996) found that the yeast *A. pullulans* when grown on D-xylose produces hemicellulytic enzymes with predominantly β-xylanase and β-xylosidase activity and no cellulase activity. The enzymes in a concentrated supernatant preparation were able to selectively hydrolyse the hemicellulose portion of the unbleached pulps. Repeated consecutive enzymatic and alkali-oxygen treatments were successfully applied in a chlorine free bleaching sequence.

The effect of purified family 10 and family 11 β-xylanases as well as acetyl xylan esterases on eucalyptus (*Eucalyptus grandis*) sulphite-dissolving pulp, with regard to their bleaching ability, was recently assessed (Christov et al. 2000). The purified β-xylanases hydrolysed less than 30% of the acetylglucuronoxylan present in the pulp. The enzymes of family 10 produced acetylated xylobiose and xylotriose, whereas acetylated xylobiose was not observed among the products of the family 11 enzymes. The esterases however, were not capable of deacetylating the acetylated aldo-uronic acids generated by the β-xylanases. No additional brightness was observed when β-xylanases were used in conjunction with acetyl xylan esterases, implying that the latter do not play an important role in biobleaching.

Detailed laboratory studies carried out to adapt the enzymatic treatment to existing mill conditions showed that no expensive investment is necessary for full-scale runs, except for the pH adjustment facilities (Kulkarni et al. 1999). Thus, enzymatic pre-treatment is fully compatible with existing industrial equipment. Addition of an enzymatic step to any existing conventional bleaching sequence results in a higher final brightness value of the pulp. The application of cellulases and hemicellulase mixtures for de-inking has been extensively studied but not commercialised (Bhat, 2000; Subramaniyan and Prema, 2002). The two principle approaches for using enzymes in de-inking are the hydrolysis

of soy-based ink carriers by lipase and the release of ink from fibre surfaces by cellulases, hemicellulases and pectinases (Pommier et al. 1990). The main advantage of enzymatic de-inking is the avoidance of using an alkali, thus preventing alkaline yellowing, simplifying the de-inking process, changing the ink particle size distribution and reducing environmental pollution. Cellulase and hemicellulase mixtures have further been used to modify fibre properties with the aim of improving drainage, beatability and runability of the paper mills (Bhat, 2000). Enzymatic treatment is performed before beating of the pulps to improve beatability response or to modify the fibre properties. Addition of cellulases and hemicellulase after beating is done to improve the drainage properties of the pulps, which determine the speed of paper mills. Furthermore, during mechanical pulping, various wood components such as pitch, lignin and hemicellulose are dissolved and released into the drainage (Bhat, 2000). During peroxide bleaching of mechanical pulps other wood components including pectin are also released. These dissolved and colloidal substances cause severe problems in paper mills such as pitch depositions, specs in the paper and decreased de-watering. Cellulase and hemicellulase mixtures are expected to improve the overall performance of paper mills by decreasing the turbidity of pulping filtrates.

2.2.6.2 Hemicellulases and cellulases in the animal feed industry

Undigested fibres increase the viscosity of food in the guts of monogastric animals. The increased viscosity interferes with the penetration of digestive enzymes, absorption of the digested food and may support pathogenic conditions, especially in broiler chicks (Kulkarni et al. 1999; Beg et al. 2001). The use of β-xylanases and other hemicellulases corrects these problems and increases the nutritive value of the feed. Hydrolases are the main class of enzymes used in monogastric feed (Bhat, 2000; Subramaniyan and Prema, 2002). These enzymes are used to eliminate anti-nutritional factors present in grains or vegetables, degrade certain cereal components in order to improve the nutritional value of the feed or to supplement the animals' own digestive enzymes. β-Xylanases and β-glucanases have been successfully used in monogastric diets to hydrolyse non-starchy polysaccharides such as barley β-glucans and arabinoxylans, which cause poor feed conversion rates, slow weight gain and sticky droppings by young animals. Furthermore,

addition of β-xylanases and β-glucanases during feed production also improved digestion and absorption of feed components and led to greater weight gain by broiler chickens and egg laying hens (Bhat, 2000).

There is currently great interest in the use of enzyme preparations with high levels of cellulases and hemicellulases for the improvement of feed utilisation, milk yield and body weight gain by ruminants (Peiji et al. 1997; Bhat, 2000). The successful use of these enzymes depends on their stability in the feed and in the rumen, the ability of the cellulases and hemicellulases to hydrolyse plant cell wall polysaccharides and the ability of the animal to use the reaction products. Because the forage diets of ruminants is generally more complex than that of monogastrics, the results obtained after addition of the enzymes have been inconsistent. It was reported by Beauchemin et al. (1995) that addition of commercial enzyme preparations of cellulases and hemicellulases to a hay diet, improved live weight gain of cattle by as much as 35%, however, other studies observed no significant weight gain (Lewis et al. 1996).

2.2.6.3 Hemicellulases, cellulases and pectinases in food and beverage biotechnology

Hemicellulase, cellulases and pectinases, collectively called macerating enzymes, are used in the extraction and clarification of fruit juices (Bhat, 2000; Beg et al. 2001). The use of macerating enzymes increased yield and process performance without additional capital investment. Macerating enzymes are used after crushing to macerate the fruit pulp either to partial or complete liquefaction, which increases the juice yield, reduces the processing time and improves the extraction of valuable fruit components (MacCabe et al. 2002). Macerating enzymes can also be added after juice extraction for its clarification. Macerating enzymes furthermore improves cloud stability, texture and facilitates easy concentration of nectars and purees. Macerating enzymes are also used in the extraction of olive oil. Furthermore, β-xylanases play a key role in the liquefaction of coffee mucilage for the making of liquid coffee.

β-Xylanases also find application in the baking industry due to the presence of substantial amounts of residual hemicellulose in the raw material (Poutanen, 1997). In bakeries, the

β-xylanases act on the gluten fraction of the dough where β-xylanases presumably hydrolyse arabinoxylan present in the dough, and this facilitates even redistribution of the water content of the bread (Poutanen, 1997; Beg et al. 2001). This significantly improves the desirable texture and loaf volume, and increases the shelf life of the bread (Maat et al. 1992). Arabinases, α-L-arabinofuranosidases, α-L-arabinofuranohydrolases and esterases have also been shown to play important roles in improving the texture, quality and sensory attributes of bakery products, however, a suitable combination of these enzymes has yet to be designed.

2.3 D-XYLOSE UTILISATION AND FERMENTATION

This section will focus mainly on the microbial conversion of D-xylose to ethanol with the focus on yeasts that perform this conversion. The conversion of lignocellulosic feedstocks to commodities such as bioethanol has received a lot of attention in the last three decades. When, in 1973, the OPEC countries reduced their oil production, it triggered a worldwide energy crisis and initiated research into energy that can be obtained from renewable resources (Hahn-Hägerdal et al. 2001; Galbe and Zacchi, 2002). Although the restrictions on oil production have since been relieved, research on bioethanol production from lignocellulose resources has continued because of the renewable nature of this energy and the awareness of global warming as a result of the burning of fossil fuels. Accumulation of CO_2 in the atmosphere is recognised as the major contributor to global warming. Bioethanol used as a replacement for gasoline reduces vehicle CO_2 emissions by 90% (Ward and Sing, 2002). With respect to global warming, ethanol from biomass reduces net CO_2 emissions since fermentation CO_2, produced during ethanol production, is part of the global carbon cycle. Furthermore, blending oxygenates such as ethanol and methyl tertiary butyl ether causes reduction in carbon monoxide levels by improving overall combustion of the fuel (Mielenz, 2001; Ward and Sing, 2002). A disadvantage of ethanol is that it has only 65-69% of the energy density of gasoline. The potential of using lignocellulosic biomass for energy production derives from its position as the most abundant and renewable organic material in the biosphere, accounting for 50% of world biomass (Lynd et al. 1999; Ward and Sing, 2002). Potential biomass feedstocks include agronomic residues such as corn stover,

sugar cane waste (bagasse), wheat or rice straw, forestry and paper mill discards, the paper portion of municipal waste and dedicated energy crops (Mielenz, 2001; Van Wyk, 2001).

For the efficient and economically feasible conversion of lignocellulosic materials to fuels and chemicals D-xylose, as the major sugar component of hardwood and agricultural waste hemicellulose (Fig. 1), needs to be fermented efficiently to ethanol (Jeffries and Shi, 1999, Lynd et al. 1999). Lignocellulose contains five major monosaccharides, the abundance of which varies with the feedstock. They are the hexoses: D-glucose, D-mannose and D-galactose and the pentoses: D-xylose and L-arabinose (Jeffries and Shi, 1999; Mielenz, 2001; Galbe and Zacchi, 2002). Most industrial ethanol fermentations use the yeast *S. cerevisiae*, which has been established through millennia for this purpose and as a result it ferments D-glucose to ethanol with a high yield and productivity. *S. cerevisiae* can, in fact, utilise all the major hexose sugars of lignocellulose. However it cannot utilise the pentose sugars of which D-xylose is generally the most abundant. For this reason several yeast, filamentous fungi and bacterial species have been investigated that can perform this conversion and various efforts have been made to impart D-xylose fermentation to *S. cerevisiae* (Jeffries, 1996; Jeffries and Shi, 1999; Jeffries and Jin, 2000; Hahn-Hägerdal et al. 2001). Firstly, methods used to obtain fermentable monomeric sugars from lignocellulosic feedstocks will be reviewed. Thereafter, fermentation of the sugars obtained by naturally occuring and recombinant organisms will be discussed.

2.3.1 Conversion of biomass to fermentable sugars

As most efficient ethanol producers do not contain efficient cellulose and hemicellulose degrading systems, methods have been developed to release the fermentable, monomeric sugars from their polymers. Non-enzymatic degradation of lignocellulosic materials demands the breakdown of the wood polymers to fermentable monomeric sugars by physical and/or chemical means (Jeffries and Jin, 2000; Galbe and Zacchi, 2002; Ward and Singh, 2002). Ethanol can be obtained from lignocellulosic materials following different strategies, the main features of which are outlined in Fig. 8. All the processes

comprise the same components: hydrolysis of the hemicellulose and cellulose fractions to monomer sugars, fermentation and product recovery and concentration by distillation. The main differences are in the hydrolysis step, which can be performed using dilute acids, concentrated acids or enzymes. The enzymatic procedures employed for converting cellulose into D-glucose have been reviewed elsewhere (Lynd et al. 1999; Mielenz, 2001; Galbe and Zacchi, 2002; Sun and Cheng, 2002; Ward and Sing, 2002) and will not be discussed here. This work focusses on the production and fermentation of the D-xylose fraction of lignocellulosic biomass.

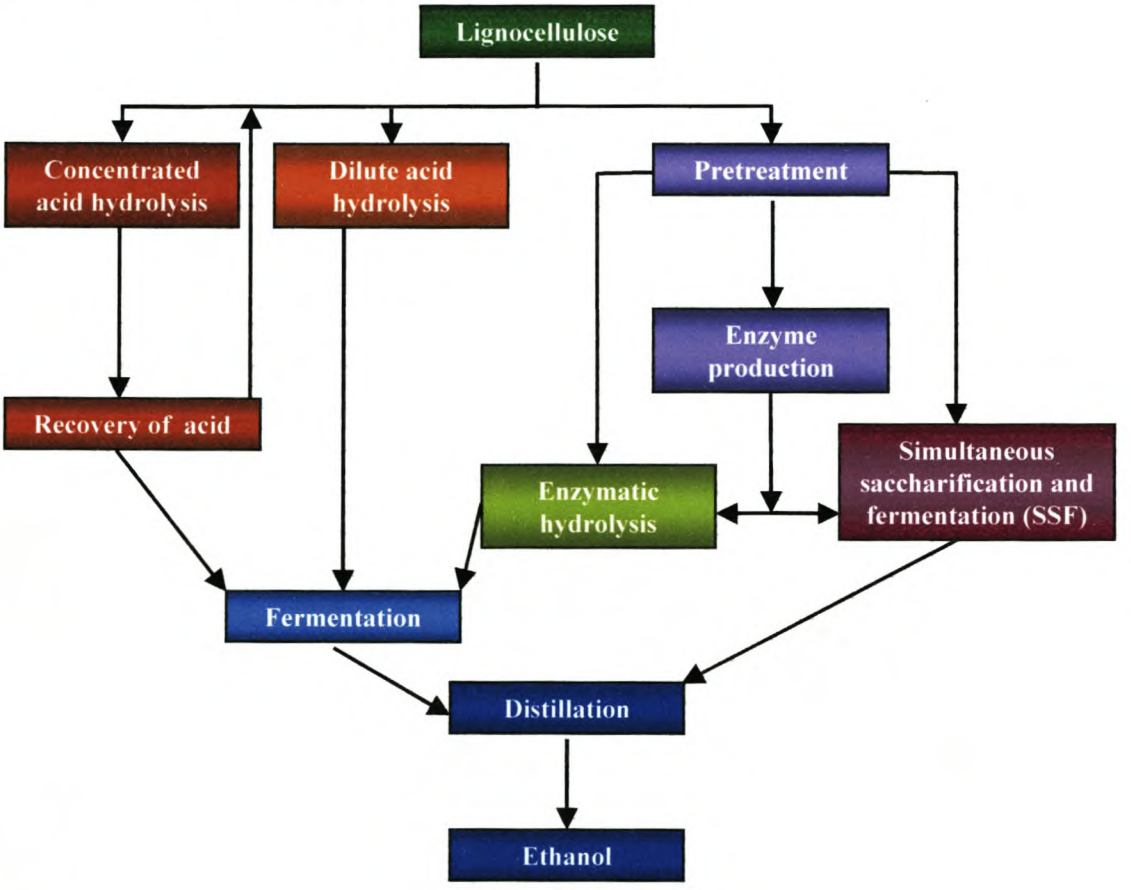


Fig. 8 A summary of different methods of ethanol production from lignocellulosic materials.

2.3.1.1 Acid hydrolysis

As was stated earlier, lignocellulose contains polyphenolic lignin and other "extractables" in addition to the cellulose and hemicellulose fractions (Section 2.1; Zaldivar et al. 2001). Pretreatment processes aim to separate the carbohydrates from the lignin matrix while minimising the chemical destruction of fermentable sugars (Mielenz, 2001; Galbe and Zacchi, 2002). Development of an ideal pretreatment is made difficult by the highly variable composition of lignocellulosic materials. Numerous processes have been developed ranging from hot water and steam explosion treatments, alkaline and solvent pretreatments, to many versions of acid pretreatment. The various methods of pretreatment are summarised in table 4.

Table 4 Various pretreatments methods used in lignocellulose substrate preparation. (Lynd et al. 1999; Mielenz, 2001; Galbe and Zacchi, 2002; Sun and Cheng, 2002; Ward and Sing, 2002)

Pretreatment type	Specific Method
Physical pretreatment:	
Mechanical	Weathering and milling
Irradiation	Gamma radiation, electron beam, photooxidation
Thermal	Steam explosion, hydrothermolysis, boiling, ammonia fibre explosion CO_2 explosion, pyrolysis, moist or dry air heat expansion
Chemical pretreatment:	
Alkaline hydrolysis	Sodium hydroxide, ammonium hydroxide
Acid hydrolysis	Sulphuric, hydrochloric, nitric, phosphoric, maleic
Oxidising agents	Peracetic acid, hydrogen peroxide, sodium (hypo)chloride
Solvents	Ethanol, butanol, phenol, ethylamine acetone, ethylene glycol
Gases	Ammonia, chlorine, nitrous oxide, ozone, sulphur dioxide
Biological pretreatment:	Lignolytic fungi

Acid hydrolysis of lignocellulose has been used for several decades (Zaldivar et al. 2001; Galbe and Zacchi, 2002; Ward and Singh, 2002). This process can be performed using dilute or concentrated sulphuric, hydrochloric, hydrofluoric, phosphoric, nitric or formic acid. Using concentrated acids, the process is run at low temperatures and gives yields of up to 90% of theoretical D-glucose yield. However, the large amounts of acids used causes problems such as equipment corrosion and energy demanding acid recovery. The main advantage of using dilute acid hydrolysis is the relatively low acid consumption. However, the high temperatures that are required to achieve acceptable levels of cellulose

to D-glucose conversion also causes hemicellulose sugar decomposition and equipment corrosion. Sugar degradation products can inhibit the subsequent fermentation process (Larsson et al. 1999a; Mielenz, 2001; Sun and Cheng, 2002; Ward and Sing, 2002). A two-stage process was developed to decrease sugar degradation. In the first hydrolysis stage, the relatively easily hydrolysed hemicellulose fraction is released under comparitively mild conditions. This enables the second, cellulase-degrading acid hydrolysis step to proceed under harsher conditions without degrading the hemicellulose sugars to inhibitory compounds such as furfural, hydroxymethylfurfural and other degradation products. Using such a process, recovery yields of 70-90% of D-xylose, galactose, mannose and L-arabinose have been reported. However, the yield of D-glucose was still low at 50%. A drawback of dilute acid hydrolysis is the dilute sugar streams obtained. A recent innovation is a countercurrent flow-through system, using low sulphuric acid concentrations. This method yields high hydrolysis levels while retaining high sugar concentrations (Mielenz, 2001; Ward and Singh, 2002). It yields 82% hydrolysis of cellulose and near total depolymerisation of xylan while yielding a solution containing about 40 g/L sugar. Research in the field of acid hydrolysis of lignocellulosic materials continues with the following goals: production of high sugar yields, minimisation of corrosive processing, minimisation of requirement for energy intensive mechanical size reduction, minimisation of water addition to avoid dilution of sugars and minimisation of toxic product formation during hemicellulose hydrolysis.

2.3.1.2 Detoxification of acid hydrolysed feedstocks

Toxic compounds formed by acid-hydrolysed lignocellulosic biomass inhibit the subsequent fermentation process (Delgenes et al. 1996; Larsson et al. 1999a; Larsson et al. 1999b; Lynd et al. 1999; Galbe and Zacchi, 2002; Sun and Cheng, 2002; Ward and Sing, 2002). Although *S. cerevisiae* is fairly resistant to these toxic effects, varying with the specific strain, organisms that ferment pentose sugars such as recombinant *E. coli, P. stipitis* and *C. shehatae* are very sensitive to these inhibitors (Delgenes et al. 1996). The main inhibitors formed during steam pretreatment and acid hydrolysis are weak acids, furan derivatives and phenolic compounds. Phenolic compounds appear to be the major inhibitory problem inhibiting both cell growth and ethanol production. Acetic acid

is formed from acetyl groups in hemicellulose during acid hydrolysis as well as during steam pretreatment without addition of mineral acids (Larsson et al. 1999a; Hahn-Hägerdal et al. 2001). Although high concentrations of acetic acid are inhibitory, low amounts can increase the ethanol yield at the expense of biomass yield. The toxicity of the hydrolysate can be removed by optimised overliming with calcium hydroxide (Mielenz, 2001; Sun and Cheng, 2002; Ward and Sing, 2002). Toxicity is removed by the addition of calcium hydroxide to pH 9-10, followed by pH neutralisation to pH 5.5 with sulphuric acid addition, which precipitates the toxic compounds and produces better fermentability. Overliming produced hydrolysates that could be fermented by recombinant *E. coli, P. stipitis* and *C. shehatae* (Section 2.3.5). Enzymatic detoxification has been achieved by treatment of the hydrolysate with the peroxidase and laccase enzymes of *Trametes versicolor*, by selective removal of phenolic monomers. A recombinant *S. cerevisiae* strain was developed with enhanced resistance to phenolic inhibitors by heterologous expression of the laccase encoding gene from *T. versicolor* (Larsson et al. 2001a). Adaptation of *P. stipitis* to lignocellulosic hydrolysate improved growth and fermentability (Nigam, 2001a). A summary of various detoxification procedures is shown in Table 5.

Table 5 Lignocellulose hydrolysate detoxification procedures. (Larsson et al. 1999b; Larsson et al. 2001a; Nigam, 2001a; Sun and Cheng, 2002; Ward and Sing, 2002)

Method of detoxification	Effect of the treatment
Physiochemical:	
Overliming	Reduces acetic acid, furfurals, tannins, terpenes, phenolics, metals
Neutralisation with CoO, NaOH, KOH, activated C	Removal of acetic acid
Ether extraction	Removal of furfurals
Ethyl acetate extraction	Removal of lignin degradation products
Vacuum evaporation	Removal of acetic acid
Steam stripping	Removal of furfurals, phenols and acetic acid
Ion exchange chromatography	Removal of aromatic monomers & dimers, partial removal of acetic acid, furfurals soluble lignin, and metals
Enzymatic	
Laccase and peroxidase	Reduction of phenolics in hydrolysate
Microbial	
Culture adaptation	Increased tolerance to acetic acid by *P. stipitis*, improved ethanol yield and productivity
Genetic	Development of a laccase producing *S. cerevisiae* with enhanced resistance to phenolic inhibitors

2.3.2 Fermentation of lignocellulosic sugars to ethanol

Improvements to the fermentation step involve efficient ethanol producing strain development, a possible combination of saccharification and fermentation and fermentation optimisation (Ward and Singh, 2002). *S. cerevisiae*, the organism most often used in commercial ethanol fermentations, does not ferment the pentose sugar fraction of lignocellulosic biomass (Jeffries and Shi, 1999; Hahn-Hägerdal et al. 2001). For this reason several research efforts have been aimed at the improvement of *S. cerevisiae* and other yeasts as well as bacterial hosts for ethanol production from lignocellulosic biomass sugars. Most work has focussed on the fermentation of D-xylose to ethanol, as this is the most abundant hemicellulosic sugar. The ideal bioethanol producer should possess high ethanol productivity, yield and tolerance; ferment all sugars in the biomass; possess good resistance to phenolic lignin monomers, acetic acid and other inhibitory compounds present in the lignocellulose hydrolysate and produce a synergistic combination of cellulases needed for complete cellulose hydrolysis. A final ethanol concentration of 50-60 g/L is seen as a benchmark for commercial processes (Jeffries, 1985). Characteristics of some potential bioethanol producers are presented in Table 6.

While bacterial fermentations of D-xylose for ethanol production are being commercialised, yeast fermentations hold several advantages over bacterial fermentations (Jeffries and Jin, 2000; Hahn-Hägerdal et al. 2001). Bacteria often produce mixtures of metabolic products, making product recovery difficult and lowering the ethanol yield. Bacteria often possess low ethanol tolerance and are sensitive to the inhibitors produced during hydrolysis of lignocellulosic biomass. However, perhaps most importantly, yeast fermentations are far less susceptible to contamination by bacteria or viruses. Aspects of D-xylose fermentations in bacteria and yeast will be briefly discussed before biotechnological advances in bacteria and yeasts for improved D-xylose fermentations are reviewed.

Table 6 Characteristics of some potential bioethanol-producing microorganisms. (Jeffries, 1983; Jeffries and Jin, 2000; Ward and Sing, 2002)

Organism		Fermentation advantages/disadvantages	Optimal pH	Optimal temperature
Yeast				
S. cerevisiae	☑	Ferments hexose sugars with high yield and productivity, high ethanol tolerance	3-7	30-35°C
	☒	Cannot ferment pentose sugars: engineered for D-xylose fermentation		
C. shehatae	☑	Ferments hexose and pentose sugars	3.5-4.5	28-32°C
	☒	Lack of anaerobic growth, xylitol formation, low ethanol tolerance		
Pachysolen tanophilus	☑	Ferments hexose and pentose sugars	2.5-5	28-32°C
	☒	Xylitol formation, low ethanol tolerance		
P. stipitis	☑	Ferments all sugars in wood hydrolysates, some strains ferment xylan	4-4.5	28-32°C
	☒	Lack of anaerobic growth, low ethanol productivity and tolerance		
Filamentous fungi				
Fusarium oxysporum	☑	Ferments a wide range of sugars, some strains ferment xylan and cellulose	5-6	28-34°C
	☒	Low ethanol productivity and tolerance		
Neurospora crassa	☑	Ferments cellulose and D-xylose to ethanol	5-6	28-37°C
	☒	Low ethanol productivity and tolerance		
Bacteria				
Clostridium thermocellum	☑	Ferments cellulose, D-glucose & D-xylose	4-8	65-70°C
	☒	Low ethanol productivity		
C. aceto-butilicum	☑	Ferments D-xylose	4-8	65-70°C
	☒	Low ethanol productivity		
C. thermohydro-sulfuricum	☑	Ferments arabinose, D-glucose & D-xylose	4.7-8	65-70°C
	☒	Low ethanol productivity		
Zymomonas mobilis	☑	Rapid D-glucose fermentation Engineered for D-xylose fermentation	4-6.5	30-37°C
	☒	Low tolerance to inhibitors, sensitive for contamination		
Klebsiella oxytoca	☑	Rapid D-xylose & cellobiose fermentation Engineered for ethanol production	6-8	30-37°C
	☒	Low tolerance to inhibitors, sensitive for contamination		
E. coli	☑	Ferments D-glucose & D-xylose Engineered for high yield ethanol production	6-8	30-37°C
	☒	Low tolerance to ethanol and inhibitors, sensitive for contamination		

☑ - Advantage
☒ - Disadvantage

2.3.3 D-Xylose transport

Transport across the cell membrane is the first step in the metabolism of D-xylose or any other nutrient, and it can limit the overall rate of utilisation (Jeffries, 1983). Sugar transport can occur by at least three mechanisms: passive diffusion, facilitated diffusion, or active transport. Bacteria generally employ active transport mechanisms for the uptake of D-xylose, whereas transport in yeast and fungi can occur by either facilitated diffusion or active processes. Facilitated diffusion is energy independent and functions well under high sugar concentrations. The active proton symport requires energy but is useful under low sugar concentrations.

2.3.3.1 D-Xylose transport in natural D-xylose utilising yeasts

In yeasts that utilise both D-xylose and D-glucose, these sugars are often taken up by the same transporter system (Kilian and Van Uden, 1988; Hahn-Hägerdal et al. 1994; Boles and Hollenberg, 1996). Natural D-xylose utilising yeasts, such as *C. shehatae*, generally have two kinetically distinct D-xylose transport systems. The low-affinity transporter is generally shared with D-glucose and transport of D-xylose by facilitated diffusion is driven by the concentration gradient across the plasma membrane (Boles and Hollenberg, 1996). The high affinity transporter, a proton symporter, is specific for D-xylose. In *P. stipitis* the presence of a constitutively expressed low affinity proton symport system, which occurs along with the high affinity proton symporter for the transport of D-xylose, has been shown (Kilian and Van Uden, 1988). The low affinity system is strongly inhibited by D-glucose and may therefore be an unspecific D-glucose-proton symporter (Kilian and Van Uden, 1988; Hahn-Hägerdal et al. 2001). Recently, three *P. stipitis* genes encoding low affinity D-glucose transporters have been cloned and sequenced (Weierstall et al. 1999). The D-glucose induced *SUT1* has a K_m of 1.5 mmol/l for D-glucose and a K_m of 149 mmol for D-xylose. The *SUT1* gene product seems to be the major contributor in the low affinity component of D-glucose and D-xylose transport. The products of *SUT2* and *SUT3* have somewhat higher affinities for D-xylose (K_m of 49 mmol/l and 103 mmol, respectively), but they are only fully expressed under completely aerobic conditions and they have a substrate concentration modulated affinity for D-glucose.

2.3.3.2 D-Xylose transport in S. cerevisiae

D-Xylose is taken up in *S. cerevisiae* by means of the D-glucose transporters (Jeffries and Shi, 1999; Hahn-Hägerdal et al. 2001). These are permeases that transport D-xylose by facilitated diffusion and have affinities toward D-xylose that are roughly two orders of magnitude lower than for D-glucose. This leads to competition between the two sugars when they are both present in the medium as they are in lignocellulose hydrolysates. Thus the uptake of D-xylose was severely retarded during co-fermentation of the sugars until D-glucose was depleted (Meinander and Hahn-Hägerdal, 1997; Hahn-Hägerdal et al. 2001). Since *S. cerevisiae* takes up D-xylose with low affinity, the transport limitation could pose a limitation on the flux of D-xylose, at least at low substrate concentrations. It has therefore been suggested that D-xylose uptake in *S. cerevisiae* must be improved in order to construct an efficient D-xylose-fermenting strain. However, there are no known nucleotide sequences of yeast or fungal origin coding for a D-xylose transporter with suitable properties.

2.3.4 D-Xylose metabolism: the conversion of D-xylose to D-xylulose

Once inside the cell, D-xylose is either isomerised or reduced, and subsequently reoxidized to form D-xylulose (Jeffries, 1983). A basic difference seems to exist between prokaryotes and eukaryotes in the initial metabolism of D-xylose: bacteria generally employ an isomerase to convert D-xylose to D-xylulose, whereas yeast and filamentous fungi carry out the same conversion through a two-step reduction and oxidation (Fig. 9).

2.3.4.1 Bacterial D-xylose utilisation

In bacteria the initial step in D-xylose metabolism is the isomerisation of D-xylose to D-xylulose (Hahn-Hägerdal et al. 1994). The enzyme xylose isomerase catalyses this conversion (Fig. 9). The temperature and pH optima for xylose isomerase depend on its source, and range between 50 and 90°C and pH 6.0 and 9.5, respectively (Jeffries, 1983). In some organisms the utilisation of D-xylose is inhibited by the presence of xylitol, due to the inhibition of xylose isomerase by xylitol. In *E. coli*, the genes for D-xylose utilisation are clustered at a single chromosomal location. Such clustering of genes required for D-xylose utilisation is common among bacteria (Schellenberg et al. 1984). The gene that codes for xylose isomerase in *E. coli* has been cloned by complementation

of a xylose isomerase-negative mutant (Lawlis et al. 1984; Schellenberg et al. 1984). The structural gene is 1320 nucleotides in length and codes for a protein of 440 amino acids with a molecular weight of 44 kDa. Almost all xylose isomerases purified and characterised to date are intracellular enzymes.

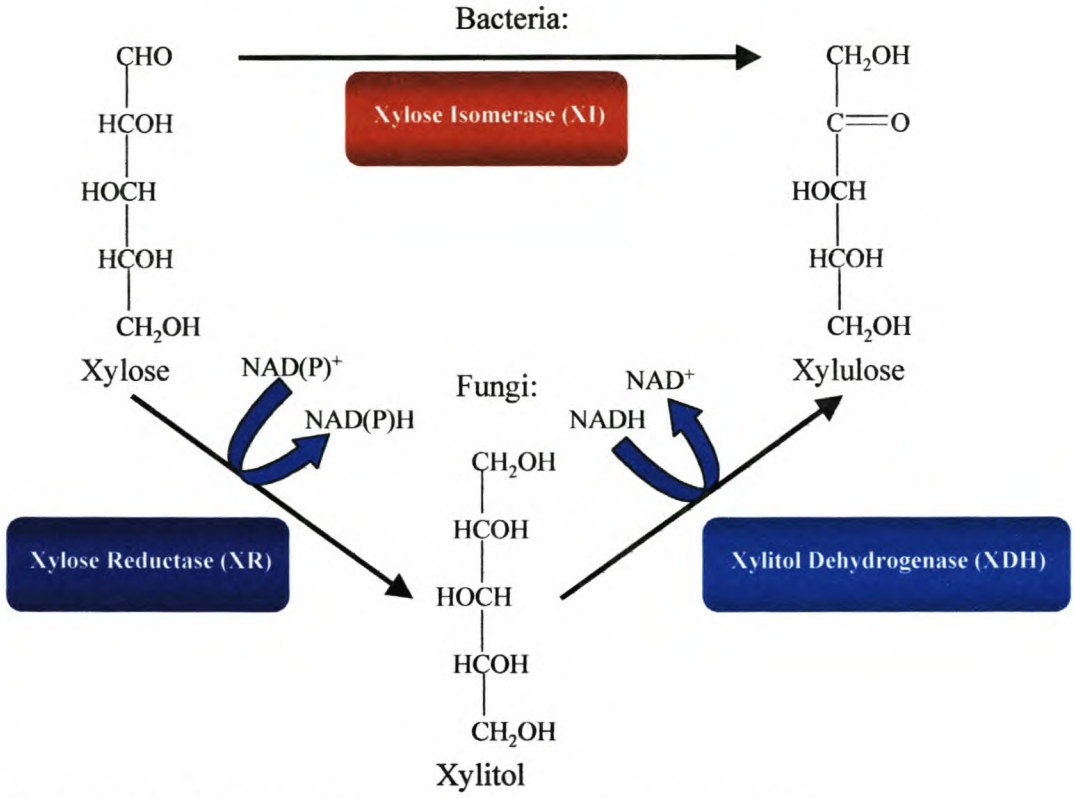


Fig. 9 The microbial pathways used to convert D-xylose into D-xylulose.

Bacteria have been investigated for the production of bioethanol from the sugar mixtures contained in lignocellulosic hydrolysates. Several bacterial species can ferment hexose and pentose sugars to ethanol (Table 6). However, as ethanol is just one of a number of fermentation products produced by enteric bacteria such as *E. coli*, various unwanted byproducts can be formed and ethanol yield can be generally low (Aristidou and Penttilä, 2000). Strategies have been applied to redirect the carbon flux towards ethanol formation.

2.3.4.1.1 *Escherichia coli*

Recent work in the engineering of bacteria for ethanol production has focused on *E. coli* (Aristidou and Penttilä, 2000; Nielsen, 2001; Zaldivar et al. 2001). This is an attractive

host organism for the conversion of renewable resources to ethanol and other useful products because it has the ability to ferment all sugar constituents of lignocellulosic material, it can sustain high glycolytic fluxes both aerobically and anaerobically and it has a reasonable ethanol tolerance of up to 50 g/L. Redirection of glycolytic fluxes to ethanol in *E. coli* was accomplished by transforming this organism with a plasmid containing the *Zymomonas mobilis* genes coding for pyruvate decarboxylase and alcohol dehydrogenase (*pdc* and *adhB*) required to divert pyruvate metabolism to ethanol in an artificial operon, the PET operon (Ohta et al. 1991a; Takahashi et al. 1994). In the recombinant *E. coli*, both of these enzymes were overexpressed to high levels. This led to effective diversion of the carbon flow to ethanol, even in the presence of native fermentation enzymes, such as lactate dehydrogenase. When the recombinant strain was grown on mixtures of sugars typically present in hemicellulose hydrolysates, sequential utilisation was observed with D-glucose consumed first, followed by L-arabinose and D-xylose, to produce near-maximum theoretical yields of ethanol (Takahashi et al. 1994; Zaldivar et al. 2001). Under aerobic conditions, wild-type *E. coli* metabolises pyruvate through pyruvate dehydrogenase and pyruvate formate lyase, with the main products being CO_2 and acetate (Aristidou and Penttilä, 2000; Zaldivar et al. 2001). The apparent K_m for the *Z. mobilis* pyruvate decarboxylase is similar to that of pyruvate dehydrogenase and lower than those of pyruvate formate lyase and lactate dehydrogenase, thereby facilitating acetaldehyde production. NAD^+ regeneration under aerobic conditions primarily results from biosynthesis and from the NADH oxidase that is coupled to the electron transport system. Again, because the apparent K_m for *Z. mobilis* alcohol dehydrogenase is over four-fold lower than that for *E. coli* NADH oxidase, the heterologous alcohol dehydrogenase effectively competes for endogenous pools of NADH, allowing the reduction of acetaldehyde to ethanol. Under anaerobic conditions, wild-type *E. coli* metabolises pyruvate primarily via lactate dehydrogenase and pyruvate formate lyase. However, the apparent K_m values for these two enzymes are eighteen fold and five-fold higher, respectively, than that for *Z. mobilis* pyruvate decarboxylase. Furthermore, the apparent K_m values for primary native enzymes involved in NAD^+ regeneration are also considerably higher in *E. coli* than those of *Z. mobilis* alcohol dehydrogenase. Thus, overexpressed ethanologenic *Z. mobilis* enzymes in *E. coli* can

favourably compete with the native enzymes for pyruvate and redox co-substrates channeling carbon, in the form of pyruvate, to ethanol. Mutations were also introduced to inactivate succinate production. Final ethanol concentrations of 54.4 g/L and 41.6 g/L were obtained from 10% D-glucose and 8% D-xylose, respectively (Ohta et al. 1991a).

Using a recombinant *E. coli* strain containing the PET operon, ethanol could be produced from hardwood and softwood spent sulphite liquors (Lawford and Rosseau, 1993). Spent sulphite liquor is the hemicellulose and lignin containing fraction generated during pulp production by the sulphite process, which is discarded as pulp mill effluent. Conversion efficiencies of sugars varied from 53% to 84% in supplemented spent sulphite liquor media. Ethanol yields of 6 – 13 g/L were obtained.

2.3.4.1.2 *Klebsiella oxytoca*

Ohta et al. (1991b) investigated the expression of the *pdc* and *adh* genes of *Z. mobilis* in a related enteric bacterium, *K. oxytoca*. The native organism has the capability to transport and metabolise cellobiose, thus minimising the need for extracellular additions of cellobiase. As in the case of *E. coli*, it was possible to divert more than 90% of the carbon flow from sugar catabolism away from the native fermentative pathways and toward ethanol. With overexpression of both enzymes, ethanol production was both very rapid and efficient and final ethanol concentrations of 45 g/L for both D-glucose and D-xylose carbon sources were obtained with an efficiency of 0.49 g ethanol/g D-xylose and 0.5 g ethanol/g D-glucose. The maximum volumetric productivity with D-xylose was almost double that obtained with ethanologenic *E. coli* strains.

2.3.4.1.3 *Zymomonas mobilis*

Z. mobilis has been used as a natural fermentative agent in alcoholic beverage production and has been shown to have an ethanol productivity that is superior to that of yeast (Aristidou and Penttilä, 2000; Nielsen, 2001; Zaldivar et al. 2001). Along with high ethanol tolerance it has high ethanol yield, selectivity and specific productivity, as well as tolerance to low pH levels. In D-glucose medium, *Z. mobilis* can achieve ethanol levels of at least 12% (w/v) at yields of up to 97% of the theoretical value. The high yield of this bacterium is attributed to reduced biomass formation during fermentation. When

converting D-glucose to ethanol, this organism produces only 1 mole of ATP per mole of D-glucose through the Entner-Doudoroff pathway compared with 2 moles produced via the more common Embden-Meyerhof-Parnas (EMP) pathway. The metabolically engineered pathway yields only 1 mole of ATP from 1 mole of D-xylose, compared with 5 and 3 moles typically produced through a combination of the pentose phosphate and EMP pathways, respectively. The energy limitation results in a lower biomass formation and, thus, a more efficient conversion of sugar to ethanol. The main drawback of this microorganism is that the wild type can only utilise D-glucose, fructose and sucrose and is thus unable to ferment the widely available pentose sugars. This led to the introduction of a pathway for pentose metabolism in *Z. mobilis*. Early attempts by groups using the xylose isomerase (*xylA*) and xylulokinase (*xylB*) genes from either *Klebsiella* sp. or *Xanthomonas* sp. were met with limited success (Feldmann et al. 1992; Zhang et al. 1995; Zaldivar et al. 2001). This was due to the absence of detectable transketolase and transaldolase activities in *Z. mobilis*, which are necessary to complete a functional pentose metabolic pathway. A chimeric shuttle vector was created that carried two independent operons (Zhang et al. 1995), namely one encoding the *E. coli xylA* and *xylB* genes and the second expressing the *E. coli* transketolase (*tktA*) and transaldolase (*tal*) genes. These operons complete a pentose phosphate pathway that would allow conversion of xylulose-5-phosphate to the fructose-6-phosphate glyceraldehyde-3-phosphate intermediates of the Entner-Doudoroff pathway and subsequently to ethanol. The two operons were expressed successfully in *Z. mobilis*. The recombinant strain was capable of fast growth on D-xylose as the sole carbon source, and, moreover, it efficiently converted D-glucose and D-xylose to ethanol with 86 and 94% of the theoretical yield from D-xylose and D-glucose, respectively. A major drawback of using this bacterial strain at industrial level is that its fermentative activities are greatly reduced by the inhibitors present in lignocellulosic hydrolysates (Delgenes, 1996).

2.3.4.2 Yeast D-xylose utilisation

In most fungi (including yeast) the conversion of D-xylose to D-xylulose involves the action of two enzymes (Fig. 9). Firstly the reduction of D-xylose to xylitol is catalysed by xylose reductase (EC 1.1.1.21) (Jeffries, 1983; Hahn-Hägerdal et al. 1994). The

second reaction, the re-oxidation of xylitol to D-xylulose, is catalysed by xylitol dehydrogenase (EC 1.1.1.9). Xylose reductases from different microorganisms have been characterised and in most cases this enzyme has specificity for NADPH as co-factor. The unspecific aldose reductase from *S. cerevisiae*, which has xylose reductase activity, and the xylose reductase from *Candida utilis* exclusively use NADPH (Bruinenberg et al. 1984; Kuhn et al. 1995). Of the D-xylose utilising yeasts, *P. stipitis*, *C. utilis* and *P. tannophilus*, only *P. stipitis* showed appreciable fermentation of D-xylose to ethanol without xylitol formation. The reason for this may lie in the different cofactor requirements in the initial steps of D-xylose metabolism of this yeast. In *P. stipitis* and *C. shehatae* significant NADH-linked D-xylose reductase could be detected (Bruinenberg et al. 1984; Hahn-Hägerdal et al. 1994). The ratio of specific activity of xylose reductase from *P. stipitis* using NADH and NADPH was 0.65-0.7, regardless of the oxygen tension in the medium (Hahn-Hägerdal et al. 2001). *P. tannophilus* produces two isozymes of xylose reductase of which one can use both NADH and NADPH as co-factor and the other strictly NADPH. The production of these isoforms is oxygen level dependent, with low oxygen concentration favouring production of the enzyme using both co-factors. The equilibrium constant for the reduction of D-xylose to xylitol has been estimated to be 0.575×10^{3} M^{-1} at pH 7, thus favouring xylitol formation. Unlike xylose reductase, all xylitol dehydrogenases studied almost exclusively use NAD^{+} as co-factor, including the open reading frame product that encodes a xylitol dehydrogenase in *S. cerevisiae* (Bruinenberg et al. 1984; Jeffries, 1990; Richard et al. 1999). The equilibrium constant at pH 7 is 6.9×10^{-4} M^{-1}, thus also favouring xylitol formation (Hahn-Hägerdal et al. 2001). A mutant strain of *P. stipitis* was obtained that was unable to grow on D-xylose and L-arabinose, but that could grow on D-arabinitol (Shi et al. 2000). This mutant strain was deficient in xylitol dehydrogenase, D- and L-arabinitol dyhydrogenase and ribitol dehydrogenase. Transforming the mutant with the gene encoding xylitol dehydrogenase (*XYL2*), restored the ability to grow on D-xylose and L-arabinose, suggesting that the D-xylose and L-arabinose metabolic pathways are linked with xylitol as common intermediate. The capacity of the mutant strain to grow on D-arabinitol could proceed through D-ribulose.

2.3.4.3 *Recombinant* S. cerevisiae *for* D-*xylose fermentation*

Since lignocellulosic biomass can contain 10-40% D-xylose, ethanol yield could theoretically be increased by 25% with efficient D-xylose fermentation (Björling and Lindman, 1989). *S. cerevisiae* produces ethanol efficiently from hexoses by the pyruvate decarboxylase/alcohol dehydrogenase system. As was stated earlier, *S. cerevisiae* has the intrinsic limitation of not being able to ferment pentoses such as D-xylose or L-arabinose. However, since the discovery that *S. cerevisiae* can ferment xylulose (Ueng et al. 1981; Jeffries, 1985; Jeppsson et al. 1996; Eliasson et al. 2000a) there has been great interest in the introduction of the heterologous enzyme(s) that catalyse the conversion of D-xylose to xylulose into *S. cerevisiae*. Even though certain types of yeast, such as *P. tannophilus*, *P. stipitis*, or *C. shehatae* are D-xylose fermenting, they have poor ethanol yields and low ethanol tolerance as well as low tolerance, to the inhibitors present in lignocellulose hydrolysates compared with *S cerevisiae* (Jeffries and Jin, 2000).

2.3.4.3.1 Heterologous expression of xylose reductase and xylitol dehydrogenase in *S. cerevisiae*

The xylose reductase (XR) encoding genes from *P. stipitis*, *K. lactis*, and *P. tannophilus*, *Candida guilliermondii* and other species have been cloned (Amore et al. 1991; Billard et al. 1995; Handumrongkul et al. 1998; Aristidou and Penttilä, 2000; Ostergaard et al. 2000). The relative affinity of various xylose reductases for NADH and NADPH vary widely. *S. cerevisiae* has been transformed with the *P. stipitis* genes *XYL1* and *XYL2* coding for xylose reductase and xylitol dehydrogenase (XDH), respectively (Kötter et al. 1990; Amore et al. 1991; Walfridsson et al. 1995). The choice of *P. stipitis* as the donor organism was based on its capability to utilise NADH in the D-xylose reduction step. Attempts to ferment D-xylose to ethanol with these recombinant *S. cerevisiae* strains producing XR/XDH have resulted in low ethanol yield and considerable xylitol by-product formation. When the D-xylose fermentation of recombinant *S. cerevisiae* strains was compared to the fermentative activities of *P. stipitis* it was found that in the absence of respiration, *S. cerevisiae* transformed with *XYL1* and *XYL2* converts about half of the D-xylose present in the medium into xylitol and ethanol in roughly equimolar amounts (Kötter and Ciriacy, 1993). In contrast, *P. stipitis* displayed a homofermentative

conversion of D-xylose to ethanol. This has been ascribed to the unfavourable thermodynamic properties of the reactions and the fact that the first reaction preferably consumes NADPH, whereas the second reaction exclusively produces NADH (Ostergaard et al. 2000). When less NADH is consumed in the XR reaction, then less NAD^+ is available for the XDH reaction thus causing a co-factor imbalance. If the amount of NAD^+ is insufficient, xylitol is produced and excreted (Bruinenberg et al. 1984; Meinander et al. 1996). A metabolic scheme for ethanol production from D-xylose is given in Fig. 10. Measures to circumvent this cofactor imbalance generated in the first two steps of D-xylose metabolism in recombinant strains of *S cerevisiae* producing XR and XDH will be discussed briefly.

Protein engineering has been attempted to alter the co-factor preferences of the XR and XDH enzymes. Inhibition studies of purified *P. stipitis* XR, a dimeric protein consisting of two identical 32 kDa subunits, suggested that histidine and cysteine residues might be involved in co-factor binding (Webb and Lee, 1992). Using site-directed mutagenesis, each of the three cysteine residues, thought to be involved in co-factor binding (Cys^{19}, Cys^{27} and Cys^{130}), were individually changed into serine residues (Zhang and Lee, 1997). The three mutant forms of XR showed activity when expressed in *E. coli*, but only at levels 50–70% lower than that of the wild type. The affinities for D-xylose, NADPH and NADH did not vary significantly and it was concluded that none of the cysteine residues directly participates in the binding of co-factor. Yeast xylose reductases and other aldo-keto reductases contain a conserved binding motif for NADPH (Ile-Pro-Lys-Ser) (Kostrzynska et al. 1998). It has been suggested that the 2'-phosphate group of NADPH binds to the lysine residue. When this group (Lys^{270}) was changed into a methionine residue, using site-specific mutagenesis, the resulting enzyme lost 80–90% of its specific activity and the affinity for D-xylose decreased by more than ten-fold. The affinity for NADPH decreased, but remained constant for NADH. There are, to date, no reports of the expression of any of the mutated forms of XR in *S. cerevisiae*.

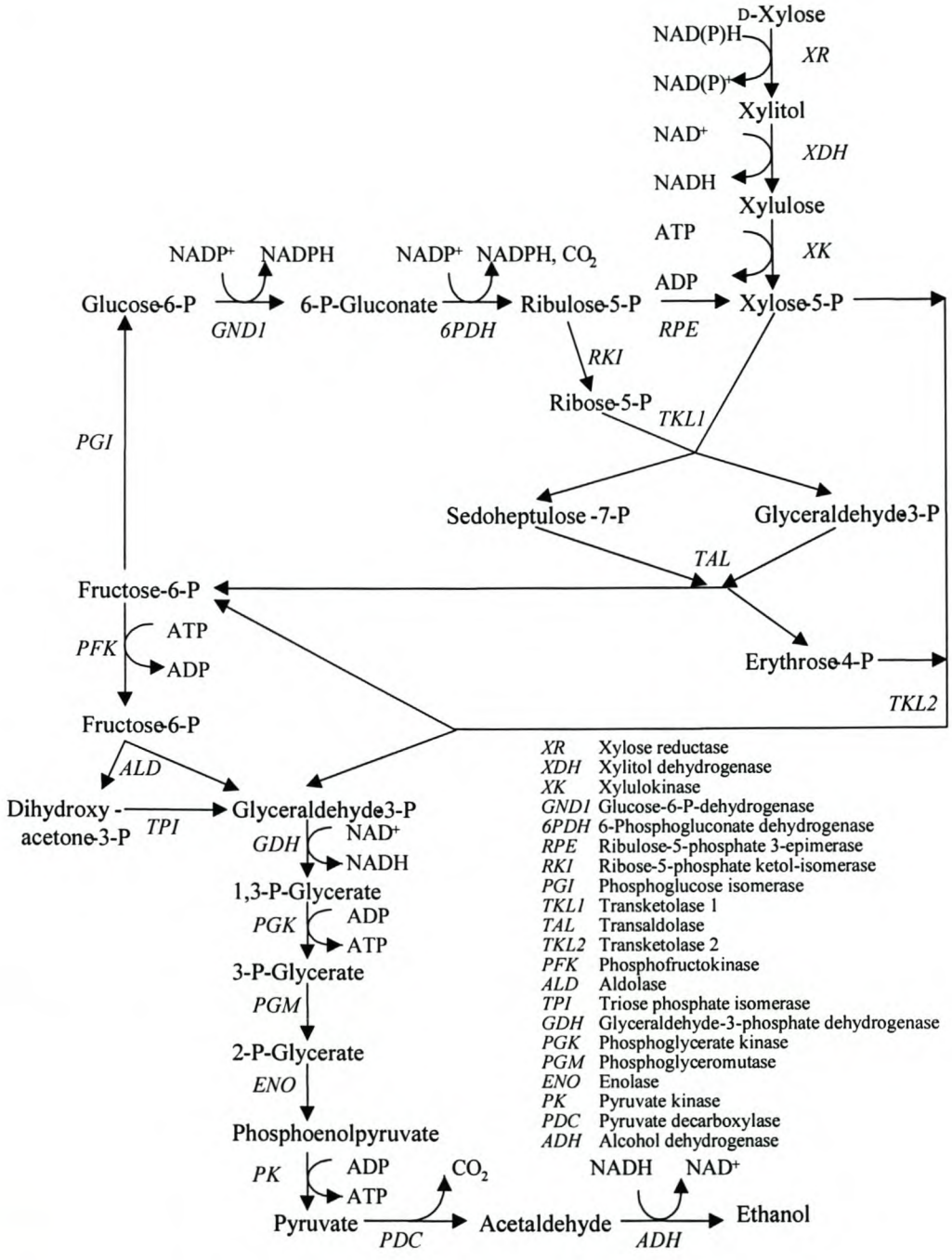


Fig. 10 A metabolic scheme for ethanol formation from D-xylose (Wahlbom, 2000).

Attempts have also been made to alter the co-factor specificity of XDH towards $NADP^+$ instead of NAD^+. Through sequence analysis of XDH, a coenzyme-binding domain, conserved in most examined NAD^+-dependent dehydrogenases was localised (Metzger and Hollenberg, 1995). In the coenzyme-binding domain, a characteristic βαβ-fold is

centered around a glycine motif also containing conserved aspartate and lysine/arginine residues. The aspartate residue and lysine/arginine residue are responsible for the interaction with the adenine ribose of NAD^+. It was found that the steric properties of the aspartate residue and the repulsion between the negatively charged groups of the phosphate of $NADP^+$ and the carboxyl group of aspartate prevent binding of $NADP^+$. When the aspartate residue (Asn^{207} or Asn^{210}) was changed to a glycine residue, the specificity for NAD^+ decreased. The specific activities of the two mutated XDHs decreased to 35% and 47% of that of the unaltered enzyme, respectively. Although steric and electrostatic hindrances were avoided through this substitution, the affinity for $NADP^+$ remained unchanged. The putative recognition sequence of an $NADP^+$-dependent alcohol dehydrogenase of *Thermoanaerobium brockii* was introduced into XDH from *P. stipitis* (Metzger and Hollenberg, 1995). The recombinant enzyme showed a specific activity of 31% of that of the unaltered enzyme, and the affinity for NAD^+ decreased dramatically whereas the affinity for $NADP^+$ remained unchanged. The mutated *XYL2* gene could still mediate growth of *S. cerevisiae* transformants on D-xylose minimal-medium plates when expressed together with the *P. stipitis XYL1* gene.

To compensate for the unfavourable equilibrium constants of XR and XDH, recombinant *S. cerevisiae* strains with higher XDH activity than XR activity were constructed (Walfridsson et al. 1997). The *XYL1* and *XYL2* genes were placed under control of the *S. cerevisiae ADH1* and *PGK1* promoters in different constructions to attain a range of activities. Product formation was studied in strains with ratios of XR:XDH enzyme activities varying from 17.5 to 0.06. The native genes encoding transaldolase and transketolase were also overexpressed. The strain with the highest XR:XDH ratio produced xylitol with a yield of 0.82 g xylitol/g D-xylose, whereas no xylitol was formed by the strain with the lowest ratio. The strain with a XR:XDH ratio of 0.06 consumed 3.25 g/L D-xylose and formed no xylitol and less glycerol and acetic acid, but more ethanol compared with the strains with higher XR:XDH ratios.

In yeasts, pentose sugars enter metabolism through the pentose phosphate pathway (Fig. 10), where it has been suggested that xylulokinase and transaldolase limit the flux of

carbon to glycolysis in *S. cerevisiae* (Senac and Hahn-Hägerdal, 1991; Hahn Hägerdal et al. 2001). The gene, *XKS1*, encoding xylulokinase (XK) (catalysing the phosphorylation of xylulose to xylulose 5-phosphate in *S. cerevisiae*), has been cloned by complementation of an *E. coli* mutation and sequenced (Ho and Chang, 1989; Rodriguez-Peña et al. 1998). During xylulose fermentation, overexpression of *XKS1* increased the ethanol yield by 85% and resulted in near theoretical ethanol yields (Eliasson et al. 2000a; Ostergaard et al. 2000). In a theoretical approach to optimising the levels of XR and XDH and also XK, a kinetic model including the three enzymes was constructed (Eliasson et al. 2001). The XR/XDH/XK ratio was found to determine whether carbon accumulated in a xylitol pool or was further utilised for ethanol production in recombinant *S. cerevisiae* strains. Based on reported kinetic data for the three enzymes, the optimal XR/XDH/XK ratio was determined to be 1:≥10:≥4 for minimal xylitol formation. The steady-state level of intermediary xylitol also depended greatly on intermediary NADH and NAD^+ levels. Xylitol formation was found to decrease with decreasing XR/XDH ratio, while ethanol formation increased. Overproduction of XK enhanced the specific D-xylose consumption. A stable D-xylose-utilising recombinant strain was constructed using this information (Eliasson et al. 2000b). The *XYL1* and *XYL2* genes from *P. stipitis*, and the endogenous *XKS1* gene of *S. cerevisiae*, encoding xylulokinase, were integrated into the chromosomal *HIS3* locus of *S. cerevisiae*. The strain expressed XR, XDH and XK activities of 0.4 to 0.5, 2.7 to 3.4, and 1.5 to 1.7 U/mg, respectively and was stable for more than 40 generations in continuous fermentations. Anaerobic ethanol formation from D-xylose by recombinant *S. cerevisiae* was demonstrated for the first time. However, the strain grew on D-xylose only in the presence of oxygen. A metabolic flux model was constructed for this strain comprising the most important reactions during anaerobic metabolism of D-xylose and D-glucose (Wahlbom et al. 2001). The model was used to calculate the intracellular fluxes of the strain grown anaerobically at various dilution rates. The specific uptake of D-xylose was found to increase with the D-xylose concentration in the feed and with increasing dilution rate. The model showed that the flux through the reaction from ribulose-5-phosphate to xylulose-5-phosphate was very low under all cultivation conditions. Interestingly, it was also found that addition of electron acceptors such as acetoin and acetaldehyde that are

reduced in NADH coupled reactions stop the excretion of xylitol (Wahlbom and Hahn-Hägerdal, 2002). Furfural, present in lignocellulose hydrolysates also reduced xylitol formation.

The control of the expression level of XK may be crucial. This kinase consumes ATP at the beginning of a metabolic pathway and metabolic modeling has suggested that high, uncontrolled activity of such an enzyme leads to "substrate-programmed death" where the cell is depleted of ATP at a faster rate than ATP is regenerated (Hahn-Hägerdal et al. 2001). In fact, the D-xylose consumption and ethanol formation rates were higher in a strain where XK was chromosomally integrated and thus present at a lower gene dosage (Wahlbom et al. 2001), than in a strain where XK was expressed from a multicopy plasmid (Johansson et al. 2001).

The gene encoding xylulokinase in *P. stipitis* (*XYL3*) was recently cloned and characterised (Jin et al. 2002). Disruption of this gene resulted in loss of xylulokinase activity although ribulokinase activity was still present. The disruption mutant could not produce ethanol from D-xylose but it was still assimilated slowly, with xylitol and arabinitol formed as products. It was therefore suggested that *P. stipitis* might have another pathway for D-xylose assimilation. Heterologous expression of *XYL3* in *S. cerevisiae*, under control of its own promoter, resulted in an increase in D-xylulose consumption in the recombinant *S. cerevisiae*.

Accumulation of the non-oxidative pentose phosphate pathway intermediate sedoheptulose 7-phosphate (Fig. 10) has been observed in D-xylose- and xylulose fermenting yeasts (Senac and Hahn-Hägerdal, 1991; Johansson, 2001). This has been ascribed to insufficient transaldolase activity compared to glyceraldehyde-3-phosphate dehydrogenase activity. Overexpression of the endogenous *TAL1* gene encoding transaldolase enhanced aerobic growth of a recombinant strain of *S. cerevisiae* expressing *XYL1* and *XYL2* from *P. stipitis*, but not overproducing XK (Walfridsson et al. 1995; Ostergaard et al. 2000). Overexpression of *TKL1* encoding transketolase did not influence growth, but overexpression of both *TAL1* and *TKL1* improved aerobic growth

even more. The overexpression of *TAL1* and *TAL1/TKL1* did not influence ethanol formation under the experimental conditions employed. When *GND1* encoding the enzyme gluconate-6-phosphate dehydrogenase in the oxidative part of the pentose phosphate pathway was deleted, the ethanol yield from xylulose increased by 30% (Eliasson et al. 2000a). This was ascribed to reduced carbon dioxide formation and could be as a result of decreased flux through the oxidative pentose phosphate pathway. It was recently shown that a decreased flux through the NADPH producing oxidative pentose phosphate pathway led to an increase in the ethanol yield and a decrease in the xylitol yield (Jeppsson et al. 2002). NADPH for the XR reaction is provided either by the oxidative part of the pentose phosphate pathway or by acetate formation from acetaldehyde. Alternatively, XR may use a greater fraction of NADH. Also in the pentose phosphate pathway, the flow of carbon in the reaction catalysed by the enzyme ribulose-5-phosphate epimerase was shown to be very low when analysed using a stoichiometric model (Wahlbom et al. 2001). The deletion of the gene encoding this enzyme, *RPE1*, was conditionally lethal for growth on xylulose (Eliasson et al. 2000a). This adds to the evidence that the non-oxidative pentose phosphate pathway is the main D-xylose metabolic pathway. Hexose phosphates are required for induction of the ethanologenic enzymes, pyruvate decarboxylase and alcohol dehydrogenase, as well as for inactivation of the gluconeogenic fructose-1,6-bisphosphatase (Thevelein, 1994). In xylulose-fermenting cells of *S. cerevisiae*, fructose 1,6-bisphosphate levels were almost an order of magnitude lower than in D-glucose-fermenting cells (Senac and Hahn-Hägerdal, 1991). In strains with deleted trehalose synthesis, fructose-6-phosphate accumulated intracellularly compared with parental strains. In these mutated strains the yield of ethanol from xylulose increased by 15% and 20%, respectively (Eliasson et al. 2000a).

It has been suggested that the inability of pentose sugars to support anaerobic growth in both natural D-xylose fermenting yeasts such as *P. stipitis* and recombinant D-xylose metabolising yeasts is a result of a reduced yield of ATP from pentose metabolism (Jeffries and Shi, 1999). However, the yield of ATP per mole of carbon is the same for pentose and hexose metabolism (Hahn-Hägerdal et al. 2001). Therefore it was thought

that it is rather the rate of pentose utilisation that limits the rate of ATP generation during anaerobic metabolism. Under anaerobic conditions, the D-xylose flux was 2.2 times lower than the D-glucose flux in recombinant D-xylose-utilising *S. cerevisiae* (Eliasson et al. 2000b). However, when a strain was constructed that overexpressed the *P. stipitis XYL1* and *XYL2* as well as the *S. cerevisiae XKS1* and the entire non-oxidative pentose phosphate pathway encoding genes (*RPE1*, *RKI1*, *TAL1* and *TKL1*) (Johansson and Hahn-Hägerdal, 2002), the strain fermented D-xylose at the same rate as the control (Johansson, 2001). This would seem to suggest that the non-oxidative pentose phosphate pathway enzymes do not control the rate of D-xylose fermentation in recombinant *S. cerevisiae*.

The development of D-xylose-fermenting strains will probably require industrial isolates of *S. cerevisiae* as genetic hosts, as they often show higher tolerance to the inhibitors present in the lignocellulosic substrates and could possibly contain a better developed pentose phosphate pathway. However, genetic breeding in industrial prototrophic yeast strains is restricted by the fact that these yeast strains are usually diploid, polyploid or aneuploid and often sporulate poorly. Furthermore, such strains are prototrophic and cannot be transformed using commonly applied auxotrophic markers. An ideal gene transfer system for industrial yeast strains requires the absence of bacterial plasmid nucleotide sequences in the transformant, stable inheritance of the transformed gene, a high transformation efficiency, a wide application to taxonomically diverse industrial yeast strains, as well as a dominant selectable marker. Dominant selective markers, such as resistance against a toxic compound, have been used successfully, for instance resistance to G418/geneticin, sulphometuron methyl (SMM) and zeocin (Casey et al. 1992; Romanos et al. 1992; Johansson and Hahn-Hägerdal, 2002). The introduction of a heterologous D-xylose-utilising pathway into any *Saccharomyces* sp. provides for a selection system whereby the transformation with genes encoding D-xylose metabolising enzymes would allow selection for growth on D-xylose as the sole carbon source. Ho et al. (1998) have reported the construction of a recombinant *Saccharomyces* strain expressing the genes *XYL1* and *XYL2* from *P. stipitis*, and overexpressing the xylulokinase encoding gene *XKS1* from *S. cerevisiae*. The parental yeast strain,

Saccharomyces 1400, is a fusion product of *Saccharomyces diastaticus* and *Saccharomyces uvarum*. Transformants were obtained through selection for growth on D-xylose. Transformants exhibited high ethanol and temperature tolerance and a high fermentation rate. The recombinant *Saccharomyces* strain 1400 was able to ferment D-xylose to ethanol in complex media.

Random mutagenesis has been used to improve the D-xylose utilisation of recombinant *S. cerevisiae* (Tantirungkij et al. 1994) and natural D-xylose fermenting yeast strains (Jeffries, 1984). A diploid industrial strain was transformed with an integrative plasmid carrying the *XYL1* and *XYL2* genes of *P. stipitis* and the *XKS1* gene of *S. cerevisiae* to create a strain *S. cerevisiae* TMB 3399 (Wahlbom, 2002). This strain was subjected to chemical mutagenesis by ethyl methylsulphonate after which mutants were screened for CO_2 production in Durham tubes, growth in defined D-xylose medium, ethanol productivity and the activity of enzymes involved in D-xylose utilization. The best mutant strain, *S. cerevisiae* TMB 3400, was selected and showed a five-fold increase in maximum specific growth rate on D-xylose compared to *S. cerevisiae* TMB 3399. The ethanol yield and maximum specific productivity were also higher in the mutated strain and are among the highest reported for any recombinant *S. cerevisiae* strain. Micro-arrays showed that mRNA levels of the genes *HXT5* (encoding a hexose transporter) *XKS1* (encoding xylulokinase) as well as *SOL3*, *GND1*, *TKL1* and *TAL1*, encoding enzymes of the oxidative and non-oxidative pentose phosphate pathway, were, among others, upregulated in the *S. cerevisiae* TMB 3400 strain compared to the *S. cerevisiae* TMB 3399 strain when grown in a D-glucose and D-xylose mixture.

2.3.4.3.2 Heterologous expression of D-xylose isomerase in *S. cerevisiae*

The co-factor imbalance generated by the first two enzymes in the yeast D-xylose utilisation pathway could be circumvented if the conversion of D-xylose to xylulose was catalysed using the prokaryotic enzyme xylose isomerase, as it does not require redox co-factors (Fig. 9). Many attempts to introduce the one-step pathway of D-xylose metabolism by cloning the gene coding for xylose isomerase from bacteria such as *E. coli*, *Bacillus subtilis*, *Actinoplanes missouriencis* and *Clostridium thermosulfurogenes*

(Amore et al. 1989; Moes et al. 1996) in *S. cerevisiae* were unsuccessful even though specific mRNA was present. This was attributed to the inactivity of the heterologous enzyme, which was present in large amounts as mostly insoluble protein in the recombinant host cell. Subsequently, the *Thermus thermophilus xylA* xylose isomerase encoding gene was cloned and expressed in *S. cerevisiae* under the control of the yeast *PGK1* promoter (Walfridsson et al. 1996). The low levels of ethanol that were produced from D-xylose and the poor ethanol yield were attributed to two factors. Firstly, the recombinant D-xylose isomerase showed the highest activity at 85°C with a specific activity of 1.0 U/mg protein and only trace activity of 0.04 U/mg protein at mesophilic temperatures. Secondly, a major by-product was xylitol, primarily due to the action of the unspecific aldose reductase of *S. cerevisiae*, encoded by the *GRE3* gene (Kuhn et al. 1995; Träff et al. 2002). Xylitol formation leads to a loss of carbon and inhibits xylose isomerase. Reduced xylitol formation from D-xylose in *gre3* mutants of *S. cerevisiae* suggests that Gre3p is the major D-xylose reducing enzyme in *S. cerevisiae* (Träff et al. 2002). Cell extracts from the *gre3* deletion mutant showed no detectable xylose reductase activity. In a recent study, the *GRE3* gene of *S. cerevisiae* was deleted and the *T. thermophilus xylA* gene and *S. cerevisiae* xylulokinase encoding gene were overexpressed in the resulting strain (Träff et al. 2001). The deletion resulted in a 50% reduction in the xylitol formation during anaerobic fermentation of 5% D-xylose and 2% D-glucose. The xylitol that was still formed was probably a result of the action of the unspecific sugar dehydrogenase present in *S. cerevisiae* with xylitol dehydrogenase activity (Richard et al. 1999). To increase the specific activity of the *T. thermophilus* xylose isomerase at physiological temperatures the *xylA* gene was subjected to extensive random mutagenesis using an error-prone PCR method (Lönn et al. 2002). Three promising mutants were identified that had increased specific activity when expressed in *E. coli*. The optimal pH level of the mutants was at pH 7, similar to the wild type enzyme but the mutants were active over a broader pH range. The mutants showed up to nine times higher catalytic rate constants for D-xylose compared with the wild-type enzyme at 60°C. Furthermore, the mutants were less inhibited by xylitol.

2.3.5 Fermentation of lignocellulosic hydrolysates by naturally occurring and recombinant yeasts

Several researchers have tested the ability of available yeast species, both natural isolates and recombinants to ferment hydrolysates of a variety of lignocellulosic feedstock sources. The ethanol yields and productivities vary greatly due to the varying methods used to produce and detoxify the hydrolysates, the species and strains used and the inhibitors present in the hydrolysates. Studies have also investigated possible coculture processes to simultaneously ferment D-glucose and D-xylose using different organisms. However, in a study to ascertain the compatibility of *S. cerevisiae* and *P. stipitis* strains, several problems were identified (Laplace et al. 1992). Some *P. stipitis* strains showed killer activity, inhibiting the growth of *S. cerevisiae*. Furthermore, as D-glucose will be fermented by both yeasts before *P. stipitis* ferments D-xylose, and *P. stipitis* is more sensitive to the inhibitory effects of ethanol, the effectiveness of possible cofermentation is limited.

2.3.5.1 Pichia stipitis *and* Candida shehatae

Naturally occurring strains of *P. stipitis* and *C. shehatae* were tested for their abilities to ferment the monomeric forms of the sugars present in wood to ethanol (Parekh and Wayman, 1986). *P. stipitis* was found to produce ethanol from the monomeric sugars and cellobiose (Parekh and Wayman, 1986; Jeffries, 1990). It was also shown that *P. stipitis* can ferment D-xylose to ethanol using corn steep liquor, a by-product of corn starch processing, as only source of nitrogen, amino acids and vitamins, with ethanol yieds that compared favourably to those attained using more expensive sources of nutrients (Amartey and Jeffries, 1994). Bagasse, the solid residue after extraction of the sugarcane juice, is mostly utilised for producing steam and electricity required for the sugar cane processing plant (Van Zyl et al. 1988). As a process waste, bagasse has traditionally been burnt in low efficiency boilers to produce modest amounts of energy and to limit the disposal problem. Because of its high carbohydrate content, relatively low lignin content and its availability as an industrial waste product, bagasse is a particularly appropriate substrate for bioconversion to ethanol. *P. stipitis* could ferment the D-xylose and D-glucose fraction of acid hydrolysed bagasse after neutralisation of the

hydrolysate to pH 6.5 with $Ca(OH)_2$. The volumetric ethanol productivity was still considerably less than that achieved with a semisynthetic medium containing a sugar composition similar to the hydrolysate due to the inhibitory effects of acetic acid and lignin derivatives in the acid hydrolysate. It was recently shown that in a continuous culture fermentation using sugarcane bagasse hydrolysate, *P. stipitis* could utilise 97% of the reducing sugars in the feed and produce levels of 46 g/L ethanol (Yang et al. 2000).

When thirty strains of D-xylose fermenting yeasts were tested for their ability to produce ethanol from the pentose and hexose sugars in spent sulphite liquor, *P. stipitis* and *C. shehatae* strains showed the most promise (Björling and Lindman, 1989). *P. stipitis* showed the best ethanol yield. Recently, a total of 43 strains of *P. stipitis* and *C. shehatae* were tested for their ability to ferment partially deacidified wood hydrolysates (Sreenath and Jeffries, 2000). The starting sugar concentrations, pH and presence of inhibitors such as acetic acid and furfural differed between the batches of hydrolysates. The lag phase for growth and fermentation depended on the concentration of inhibitors in the hydrolysate and on the capacity of the specific yeast strain to resist them. The ethanol production rates and yields attained were higher with some strains of *C. shehatae*, with *C. shehatae* FPL-Y-049 producing up to 34 g/L ethanol. Using recycled cells of this strain reduced the fermentation lag period and increased final ethanol concentration, presumably because of adaptation of the strain to the inhibitory environment of the hydrolysate medium. All the D-glucose, mannose, galactose and D-xylose in the wood hydrolysates was consumed during fermentation by the *C. shehatae* FPL-Y-049 strain, however, L-arabinose was not fermented. Addition of 10 mg/L zinc to the acid hydrolysate did not affect peak ethanol production but did increase rates of sugar utilisation and ethanol production because of the high activity of zinc-dependent alcohol dehydrogenase in the fermentative patway.

Ethanol production from wheat straw hemicellulose hydrolysate by *P. stipitis* was evaluated (Nigam, 2001b). Treatment by boiling and overliming with $Ca(OH)_2$ significantly improved the ethanol yield and ethanol productivity of the hydrolysate. Adaptation of the yeast to the hydrolysate further increased the yield and productivity and

up to 20 g/L ethanol was produced. The effects of the aforementioned inhibitory compounds present in acid hydrolysates were tested by adding them individually or in combination to simulated medium in similar concentrations to those found in hydrolysates. Their addition resulted in reduction in ethanol yield and productivity, with acetic acid seemingly having the greatest effect when added individually. Addition of the inhibitors in combinations compounded their effect. A mutant strain of *P. stipitis* was also developed through adaptation on hardwood acid hydrolysate to have increased tolerance of acetic acid and other hydrolysate inhibitors (Nigam, 2001a). The adapted strain, capable of fermenting both hexoses and pentoses in the hydrolysate, showed shorter fermentation time, increased tolerance to acid compounds and could ferment at lower pH. The ethanol yield and productivity were increased by 1.6- and 2.1-fold, respectively. About 15 g/L ethanol could be produced from medium containing 30% hardwood prehydrolysate. Water-hyacinth (*Eichhornia crassipes*), which is a widely prevalent aquatic weed, constitutes a potential biomass resource for various uses. Its high hemicellulose content of 30–55% of dry weight can provide hemicellulosic sugars for bioconversion to fuel ethanol (Nigam, 2002). Water-hyacinth hemicellulose acid hydrolysate was used as a substrate for ethanol production using *P. stipitis* NRRL Y-7124. The fermentability of the hydrolysate was improved considerably by boiling and overliming up to pH 10.0 with solid $Ca(OH)_2$, in combination with sodium sulphite. Treatment of the hydrolysate improved the amount of sugar utilised and the ethanol yield. The fermentation was very effective at an aeration rate of 0.02 v/v/m, at 30°C and pH 6.0 (reducing the effect of leftover acetic acid) where up to 18 g/L ethanol was produced. L-Arabinose was not fermented but assimilated. The presence of acetic acid in the hydrolysate decreased the ethanol yield and productivity considerably.

2.3.5.2 Native and recombinant S. cerevisiae *strains*

Hydrolysis of lignocellulosic feedstocks can be catalysed chemically or by cellulolytic enzymes (Section 2.3.1). Since enzymatic hydrolysis has several advantages over acid hydrolysis, it is a very promising method for saccharification (Ward and Singh, 2002). However, when enzymatic hydrolysis is the method of choice, lignocellulosic materials need to be pre-treated to make the cellulose macromolecules accessible for the enzymes.

Sugarcane bagasse was pre-treated by steam explosion at 205°C and 215°C and hydrolysed with cellulolytic enzymes (Martin et al. 2002). The hydrolysates were subjected to enzymatic detoxification by treatment with the phenoloxidase laccase, which removed approximately 80% of the phenolic compounds. Subsequent chemical detoxification by overliming partially removed the phenolic compounds, but also other fermentation inhibitors such as acetic acid, furfural and 5-hydroxy-methyl-furfural. The hydrolysates were fermented with *S. cerevisiae* strain ATCC 96581, isolated from a spent sulphite liquor fermentation plant and the recombinant D-xylose-utilising *S. cerevisiae* laboratory strain TMB 3001 (Eliasson et al. 2000b), which over-expressed xylulokinase activity and expressed the xylose reductase and xylitol dehydrogenase encoding genes of *P. stipitis*. The fermentative performance of the lab strain in undetoxified hydrolysate was better than the performance of the industrial strain (Martin et al. 2002). An almost two-fold increase of the specific ethanol productivity of the recombinant strain in the detoxified hydrolysates, compared to the undetoxified hydrolysates, was observed. The ethanol yield in the fermentation of the hydrolysate detoxified by overliming was 0.18 g/g dry bagasse, whereas it reached only 0.13 g/g dry bagasse in the undetoxified hydrolysate. Fermentation of the hydrolysate media supplemented with other nutrients with starting sugar concentrations of 22 g/L D-glucose and 9 g/L D-xylose, yielded up to 12 g/L ethanol. Whereas D-glucose was completely utilised, only partial D-xylose utilisation with low xylitol formation was observed. L-Arabinose was not utilised. Slow D-xylose consumption may be a result of the competitive inhibition of D-xylose transport by D-glucose.

Ethanol productivity by the non-recombinant strain, *S. cerevisiae* ATCC 96581, in continuous fermentation of an enzymatic hydrolysate of spruce was increased 4.6 times by employing cell recycling (Palmqvist et al. 1998). The maximum growth rate of *S. cerevisiae* ATCC 96581, adapted to fermentation of spent sulphite liquor, was 7 times higher in spent sulphite liquor of hardwood than the maximum growth rate of bakers' yeast. Cell growth in the hydrolysates was strongly influenced by pH as this changed the efficacy of acetic acid as an inhibitor.

A recombinant, heterologous laccase producing strain of *S. cerevisiae* was constructed expressing the laccase encoding gene of *Trametes versicolor* (Larsson et al. 2001a). This strain was able to ferment a dilute acid spruce hydrolysate at a faster rate than the reference strain accompanied by a removal of the low molecular mass aromatic compounds present in the hydrolysate. Another approach to alleviate the inhibition problem was to overexpress the gene encoding phenylacrylic acid decarboxylase (*PAD1*) catalysing a decarboxylation step, by which aromatic carboxylic acids are converted to the corresponding vinyl derivatives in *S. cerevisiae* (Larsson et al. 2001b). Overexpression of *S. cerevisiae* phenylacrylic acid decarboxylase resulted in an improved growth rate and ethanol productivity in the presence of ferulic acid, cinnamic acid and in a dilute acid hydrolysate of spruce. Transformants overexpressing *PAD1* could convert ferulic and cinnamic acid at a faster rate than a control transformant. This enabled faster growth under both aerobic and oxygen-limited conditions.

2.3.5.3 Commercial xylitol production by yeasts

The five carbon alcohol xylitol, the product of D-xylose reduction, is used as a natural sweetener because of its dental cavity reducing properties (Hallborn et al. 1994; Lee et al. 2000; Granström et al. 2001). It is an anticariogenic sweetener also suitable for use by diabetics. Xylitol is currently manufactured by catalytic reduction of highly purified D-xylose solutions. The biotechnological production of xylitol by microorganisms and/or enzymes from substrates such as hemicellulose hydrolysates could be a cheaper alternative and could provide a natural product with superior organoleptic characteristics. *P. tannophilus*, *Debaryomyces hansenii* and *C. guilliermondii* have received great attention as the most promising natural xylitol producing yeasts (Handumrongkul et al. 1998; Sene et al. 2001). Oxygen supply has a strong effect on xylitol production. At low oxygen transfer rates, the respiratory chain can not oxidise the excess NADH, thus preventing the oxidation of xylitol to xylulose, leading to xylitol accumulation. Xylitol production is also regulated by pH, temperature, starting D-xylose level, presence of other sugars and inoculum level (du Preez et al. 1986a; du Preez et al. 1986b). Batch xylitol production from concentrated sugarcane bagasse hydrolysate by *C. guilliermondii* was performed by progressively adapting the cells to the medium (Sene et al. 2001). Samples

were analysed to monitor sugar consumption; xylitol, ethanol and CO_2 production and cell growth. Both xylitol yield and volumetric productivity remarkably increased with the number of adaptations, demonstrating that the more adapted the cells, the better the capacity of the yeast to reduce D-xylose to xylitol in hemicellulose hydrolysates. The xylose reductase encoding gene of *C. guilliermondii* was cloned and heterologously expressed in *Pichia pastoris* (Handumrongkul et al. 1998). Functional xylose reductase was expressed in induced conditions and xylitol formation from D-xylose was shown.

Recombinant *S. cerevisiae* strains that express the *XYL1* gene of *P. stipitis* (Amore et al. 1991; Liden et al. 1996) could conceivably reduce D-xylose to xylitol and not metabolise it further. When such transformants were grown in media containing D-xylose and D-glucose as co-substrates, conversion yields were close to 1 g xylitol/g D-xylose (Hallborn et al. 1994; Thestrup and Hahn-Hägerdal, 1995; Lee et al. 2000). As D-glucose caused inhibition of D-xylose uptake, maximum xylitol productivity was achieved in D-glucose limited fed-batch fermentations with high D-xylose:D-glucose ratios. The D-glucose co-substrate also provided co-factor regeneration. When different co-substrates were used, the transport system of D-xylose was inhibited leading to varying yields of xylitol (Meinander and Hahn-Hägerdal, 1997; Govinden et al. 2001). Xylitol production was most rapid in batch cultivations when co-substrate was still present and in continuous fermentations under co-substrate limited conditions.

2.3.6 D-Xylose metabolism: differences between *P. stipitis* and *S. cerevisiae*

Several differences have been discovered between *S. cerevisiae* and *P. stipitis*, the best studied natural D-xylose fermenting yeast. Some of these differences have practical implications at industrial level, whereas others are differences at molecular level which might help us understand the different efficiencies by which D-xylose is metabolised by *P. stipitis* and recombinant *S. cerevisiae*.

2.3.6.1 Differences in robustness of the yeasts

Although growth generally stops before ethanol production stops, the maximal amount of ethanol produced by an organism is, among other things, limited by its ethanol tolerance (Du Preez et al. 1989; Meyrial et al. 1995). For *P. stipitis*, growth was inhibited by 30-35

g/L ethanol but ethanol production continued up to around 47 g/L at 30°C. The maximal ethanol concentration before the onset of inhibition was increased at lower temperatures (Hahn-Hägerdal et al. 1994). In order to avoid ethanol inhibition, a fermentation was carried out with continuous gas stripping (Dominguez et al. 2000). This led to higher D-xylose consumption rates and ethanol productivity. *S. cerevisiae*, which has been adapted through millennia in ethanolic fermentations, can produce concentrations of up to 150 g/L ethanol from hexose sugars with productivities at least five times those of *P. stipitis* (Eliasson, 2000; Johansson, 2001). The ethanol tolerance is also at least twice that of *P. stipitis* as it can grow in ethanol concentrations of up to 112 g/L (Sa-Correia and Van Uden, 1983; Luong, 1985). A high ethanol concentration affects cells by inhibition of their ATPases. *S. cerevisiae* has unique machinery that gives it higher ethanol tolerance than *P. stipitis*. *S. cerevisiae* can tolerate a loss in activity of its ATPase of up to 70%, while *P. stipitis* stops growing after a 40% loss (Meyrial et al. 1995; Jeffries and Shi, 1999). Also, growth of *S. cerevisiae* in the presence of ethanol reduces the effect of ethanol on proton influx, an adaptation phenomenon not seen in *P. stipitis*.

A strain suitable for industrial processes must be able to withstand the inhibitors present in the lignocellulosic hydrolysate such as weak acids, furans and phenolic compounds (Palmqvist et al. 1999a; Larsson et al. 2001a). *P. stipitis* was strongly affected by inhibitors, especially acetic acid (Björling and Lindman, 1989). The effect of acetic acid is pH dependent. At pH 5.1, 0.8 g/L caused 50% inhibition of ethanol formation rate, but at pH 6.5 the same inhibition was only reached at 13.8 g/L. *P. stipitis* was adapted to tolerate higher concentrations of acetic acid and other inhibitory components in acid hydrolysates (Nigam, 2001a). The adapted strain showed shorter fermentation time, better tolerance to acetic acid and improved ethanol yield and productivity. However, even adapted strains of *P. stipitis* are inhibited far more strongly by acetic acid than *S. cerevisiae* (Palmqvist et al. 1999b).

2.3.6.2 Differences in respiration and fermentation

As stated earlier, in *P. stipitis* D-xylose is transported into the cell by one of two constitutively expressed transport systems, one of high affinity (specific for D-xylose) and one of low affinity (transports D-xylose and D-glucose) (Kilian and Van Uden, 1988). Anaerobic conditions may result in a low uptake of D-xylose due to a shortage of energy for active transport. As stated earlier, D-xylose is taken up in *S. cerevisiae* by means of the D-glucose transporters (Jeffries and Shi, 1999; Hahn-Hägerdal et al. 2001). These are permeases that transport D-xylose by facilitated diffusion and have affinities that are roughly two orders of magnitude lower for D-xylose than D-glucose. Poor D-xylose uptake is one reason for the limited D-xylose fermentation by recombinant strains of *S. cerevisiae* as D-xylose competes with D-glucose for these transporters.

As was discussed in section 2.3.4, the pentose phosphate pathway of *S. cerevisiae* seems to be underdeveloped and is in fact used mostly to generate anabolic metabolites. During xylulose and D-glucose cofermentation studies it was shown that xylulose is metabolised at a much lower rate by *S. cerevisiae* than *P. stipitis*, indicating lower flux through the *S. cerevisiae* pentose phosphate pathway (Yu et al. 1995; Jeppsson et al. 1996). It has been shown that *P. stipitis* consumes up to 50% of its D-glucose through the pentose phosphate pathway, whereas *S. cerevisiae* consumes only roughly 2.5% of the D-glucose through this pathway (Fig. 11) (Metzger and Hollenberg, 1994; Zaldivar et al. 2002).

In the Crabtree-positive yeast *S. cerevisiae*, respiration is down-regulated by high D-glucose concentrations and oxygen limitation (Thevelein, 1994; Zitomer and Lowry, 1992). When excess D-glucose is present, several regulatory mechanisms result in the switch from respiratory to fermentative metabolism even under aerobic conditions (the Crabtree effect). In the Crabtree negative yeast *P. stipitis*, however, fermentation was not induced by high sugar concentrations, but was inactivated by aerobic conditions (Passoth et al. 1996). The ethanologenic enzymes of *S. cerevisiae* are induced by intracellular concentrations of certain metabolites or by high flux (Boles et al. 1993). For example, full induction of pyruvate decarboxylase requires elevated levels of hexose phosphates and C_3-metabolites. During D-glucose fermentation the flux through glycolysis is very

high. Due to the low flux during D-xylose fermentation by recombinant strains of *S. cerevisiae*, the intracellular concentrations of certain metabolites may result in insufficient induction or activation of ethanologenic enzymes, leading to lower ethanol yields due to shuttling of pyruvate into the tricarboxylic acid cycle under aerobic conditions. D-Xylose fermentation by *P. stipitis* is strongly affected by the prevailing levels of dissolved oxygen (Skoog and Hahn-Hägerdal, 1990). Under fully aerobic conditions no ethanol is formed as only biomass is generated. With decreasing oxygen supply, less biomass is produced as ethanol yield increases, but below a certain level ethanol production rate and yield is very low and accompanied by xylitol formation.

In *P. stipitis* pyruvate decarboxylase and alcohol dehydrogenase activities increased when the supply of oxygen became limited (Cho and Jeffries, 1998; Lu et al. 1998b; Passoth et al. 1996). Changes in intracellular concentrations of metabolites were not correlated to the onset of fermentation. Thus the activation of pyruvate decarboxylase and alcohol dehydrogenase activities in *P. stipitis* was not mediated by a glycolytic signal but rather by a change in the redox balance or heme content (Cho and Jeffries, 1999). It was suggested that oxygen is important in maintaining the co-factor balance and that it has an indirect effect through formation of functional mitochondria (Bruinenberg et al. 1984; Skoog and Hahn-Hägerdal, 1990). Optimal oxygen levels for ethanol production with *P. stipitis* are below 1% and thus difficult to quantify or maintain accurately and impractical to maintain at industrial level. Contrary to the situation in *S. cerevisiae*, respiration was not repressed by oxygen limitation in *P. stipitis*, rather, a higher respiratory capacity was found under oxygen limited conditions (Passoth et al. 1996). Cyanide insensitive respiration (CIR) is likely to account for the increase since it was found to be induced under oxygen limited conditions (Eliasson, 2000).

Whether pyruvate undergoes decarboxylation to acetaldehyde catalysed by pyruvate decarboxylase or oxidative decarboxylation to acetyl-CoA by pyruvate dehydrogenase depends on substrate affinities and regulation of the activities of the two enzymes (Passoth et al. 1996). In *S. cerevisiae* and *P. stipitis*, pyruvate dehydrogenase has a ten-fold higher affinity for pyruvate than does pyruvate decarboxylase. At low intracellular

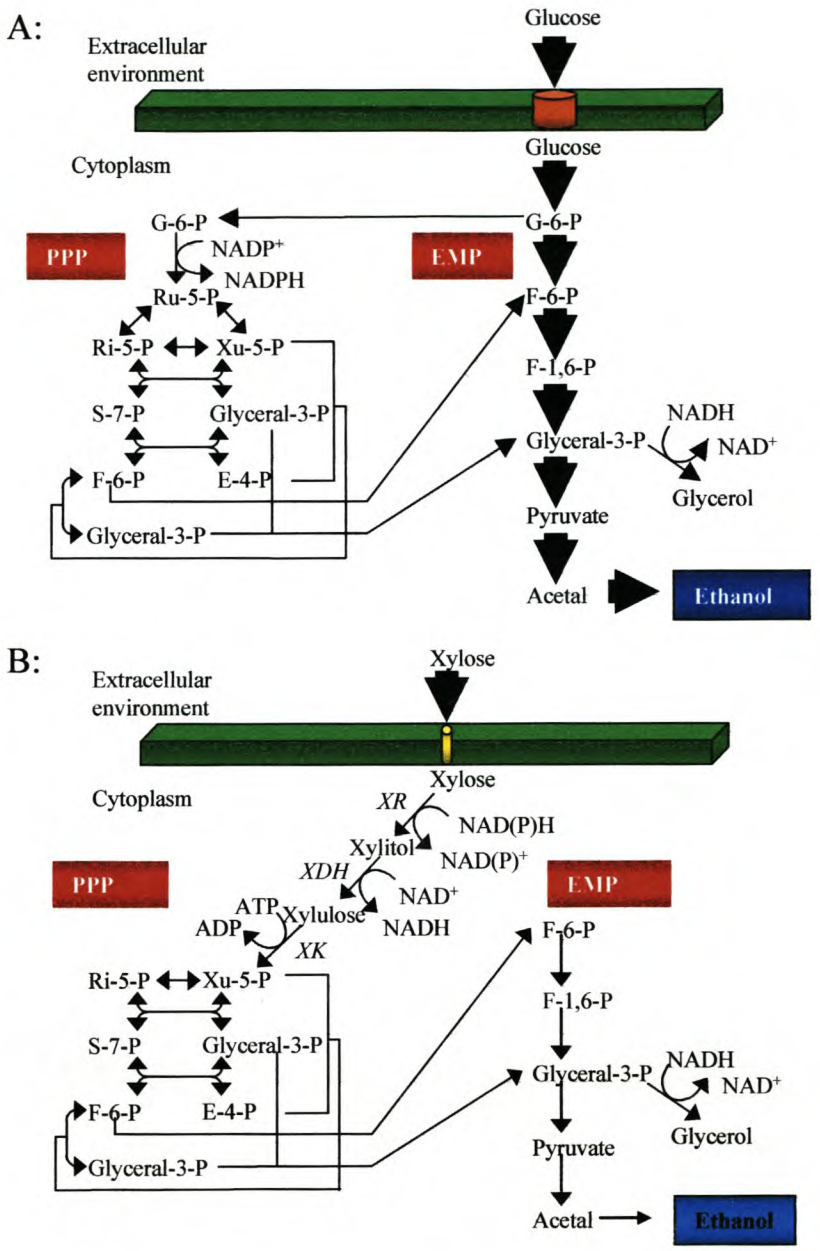


Fig. 11 Overview of metabolic pathways that lead to ethanol production from (**A**) D-glucose and (**B**) D-xylose in yeast (Zaldivar et al. 2002). PPP: pentose phosphate pathway, EMP: Embden-Meyerhof-Parnas pathway (glycolysis), Ru5P: ribulose-5-phosphate, Ri5P ribose-5-phosphate, Xu5P: xylulose-5-phosphate, S7P: sedoheptulose-7-phosphate, F6P: fructose-6-phosphate, Glyceral3P: glyceraldehyde-3-phosphate, Acetal: acetaldehyde, G6P: glucose-6-phosphate, XR: xylose reductase, XDH: xylitol dehydrogenase, XK: xylulokinase. $NADP^+$, NADPH, NAD^+, NADH and ATP co-factors are shown. The sizes of the arrows have been scaled according to flux distribution.

concentrations, pyruvate will be metabolised mainly via pyruvate dehydrogenase, but at high concentrations the pyruvate decarboxylase reaction dominates. Whereas the *S. cerevisiae* pyruvate dehydrogenase is repressed by D-glucose, it was found to be constitutively expressed in *P. stipitis* (Passoth et al. 1996). The pyruvate dehydrogenase activity of *P. stipitis* is relatively high, but the increase in NADH concentration due to a decrease in oxygen concentration inhibits pyruvate dehydrogenase. The constant expression of pyruvate dehydrogenase in *P. stipitis* enables the cells to respond rapidly to respiratory metabolism.

The *S. cerevisiae* electron transport chain, present in the inner membrane of the mitochondria, differs somewhat from that described for mammalian cells. At least two rotenone-insensitive NADH dehydrogenases have been found (Kitajima-Ihara and Yagi, 1998; Luttik et al. 1998). An cytosolic NADH dehydrogenase allows *S. cerevisiae* to oxidise excess NADH formed in the cytosol. The other rotenone-insensitive NADH dehydrogenase is directed towards the mitochondrial matrix. For *P. stipitis* the picture is less clear since rotenone insensitive NADH dehydrogenase exists, but the classical mammalian complex I is probably also present (Lighthelm et al. 1988; Shi et al. 2002). Furthermore, *P. stipitis* has a tricarboxylic acid independent mechanism for oxidation of reducing equivalents produced in the cytosol, suggesting the existence of an cytosolic rotenone-insensitive NADH dehydrogenase (Eliasson, 2000).

2.3.6.2.1 Cyanide-insensitive respiration

In higher plants, yeasts, filamentous fungi and some bacteria cyanide-insensitive respiration has been observed where the terminal oxidase is not cytochrome c oxidase but the so called alternative oxidase (Jeppsson et al. 1995; Li et al. 1996). *P. stipitis* possesses this alternative oxidase whereas *S. cerevisiae* does not. The alternative oxidase branches from the electron transport chain at the level of ubiquinone and thereby looses the coupling sites connected to complexes III and IV (Fig. 12). The alternative oxidase is sensitive to the inhibitor salicyl hydroxamic acid (SHAM), hence it is sometimes referred to as SHAM-sensitive respiration.

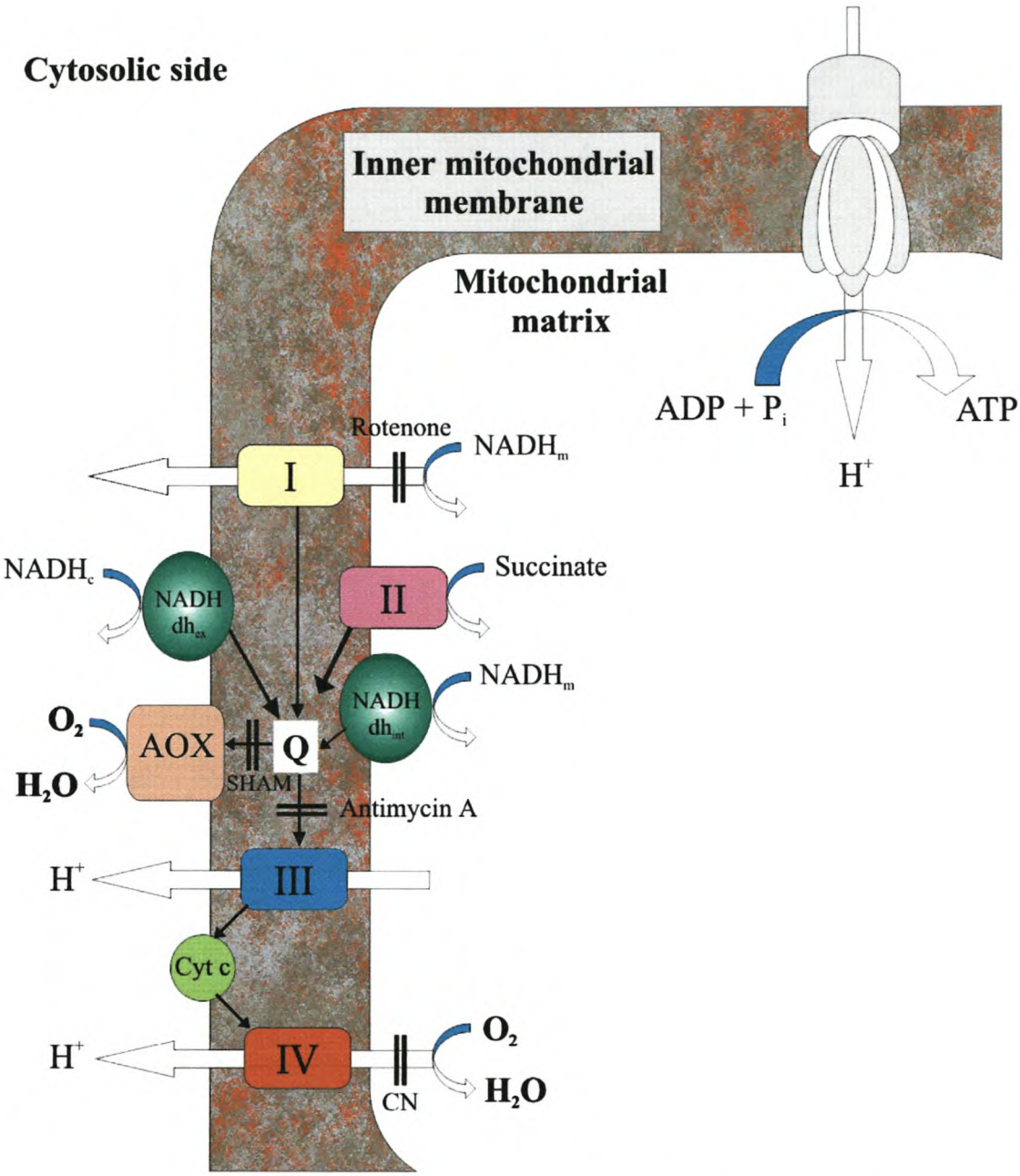


Fig. 12 The redox components present in the electron transport chain of *P. stipitis* (Jeffries and Shi, 1999; Eliasson, 2000; Shi et al. 2002). **I**: rotenone sensitive NADH dehydrogenase, **II**: succinate dehydrogenase, **III**: cytochrome reductase, **IV**: cytochrome c oxidase, AOX: alternative oxidase, NADH dh_{int}: internal NADH dehydrogenase, NADH dh_{ex}: cytosolic (external) NADH dehydrogenase, m: mitochondrial, c: cytosolic.

The alternative oxidase donates electrons directly to oxygen to form water. Because the alternative oxidase is not a proton pump and two of the three sites of proton translocation are bypassed, lower energy will be produced by this pathway via the linkage to the NADH dehydrogenase complex (Shi et al. 1999). The alternative oxidases of *P. stipitis* and *Neurospora crassa* were found to be monomeric in contrast to the plant alternative oxidases, which are dimeric (Umbach and Siedow, 2000). Plant alternative oxidases also contained a regulatory domain that was absent in the fungal alternative oxidase sequences. Furthermore, the alternative oxidases of *P. stipitis* and *N. crassa* were not stimulated by the α-keto acids that stimulated plant alternative oxidases.

The alternative pathway has been suggested to act as overflow when the cytochrome pathway becomes saturated. This is supported by the fact that cyanide-insensitive respiration increases when the cytochrome pathway is blocked by respiratory inhibitors (Moore and Siedow, 1991; Li et al. 1996). The amount of alternative oxidase protein determines the potential capacity of the alternative pathway, however other regulatory phenomena determine to what extent alternative oxidase is used in respiration. Partitioning of electrons between cytochrome oxidase and alternative oxidase depends on the redox state of the ubiquinone pool, which varies with different mitochondrial substrates and with ADP abundance. Alternative oxidase is only involved when the flux to the mitochondria is high or the cytosolic phosphorylation potential large enough to block cytochrome oxidase. The activation state of the alternative oxidase also plays a role in its involvement.

Little is known about the function of the alternative oxidase but a role in redox balancing has been suggested in *P. stipitis* where it is thought to act as redox sink, reoxidising surplus NADH to NAD^+ during D-xylose fermentation (Jeppsson et al. 1995). The addition of the inhibitor SHAM was followed by a decrease in ethanol production, whereas cyanide addition did not alter ethanol production. Thus the occurrence of cyanide-insensitive respiration in *P. stipitis* was suggested as explanation for high ethanol yields from D-xylose fermentation by this yeast. *P. tannophilus* and *C. shehatae* exhibit no alternative oxidase activity and yield significant amounts of xylitol during D-xylose

fermentation (Jeppsson et al. 1995). The redox sink hypothesis during D-xylose fermentation has been confirmed when the expression of the alternative oxidase was analysed with monoclonal antibodies and the activity of the pathway at different levels of oxygenation was determined (Eliasson, 2000). Cultivation conditions leading to highest ethanol productivity and lowest xylitol yield corresponded with highest activity of the alternative oxidase pathway.

The importance of this pathway was further investigated in *P. stipitis* by deletion of the cytochrome c encoding gene (Shi et al. 1999). The mutant strain showed 50% lower growth rates on fermentable sugars than the parental strain and was unable to grow on glycerol. The mutant strain yielded a 21% higher ethanol yield than the parental strain during growth in oxygen limited conditions on D-xylose. These results thus indicated a shift from growth to ethanol production with increasing alternative oxidase involvement in respiration. It would seem that the ability to shift between cytochrome oxidase and alternative oxidase provides the yeast with a flexible system for balancing the carbon metabolism, allowing it to respond to rapid changes in carbon availability and demand for energy and reducing equivalents.

Recently Shi et al. (2002) reported the cloning of the gene encoding the alternative oxidase of *P. stipitis*, *PsSTO1*. The gene was disrupted in *P. stipitis* to study its physiological role and it was found that no isoforms exist. Levels of cytochromes b, c, c_1 and $a.a_3$ were unchanged in the sto1-Δ mutant. The mutant strain stopped growth on D-xylose after 116 h while the parental strain continued to grow, indicating that the mutant had lost the ability to cope with the low oxygen environment after losing the alternative oxidase. The alternative oxidase may play a role in scavenging residual oxygen when cytochrome oxidase is suppressed under oxygen limited conditions, allowing electron transfer and sustaining respiratory flux. When the *PsSTO1* gene was expressed in *S. cerevisiae*, no respiratory growth on ethanol was observed, but an increased oxygen consumption rate was observed, thus suggesting that the alternative oxidase is not proton motive. It can accept electrons from the non-proton-translocating independent NADH dehydrogenases or succinate dehydrogenase complex. The

connection of alternative oxidase to these dehydrogenases can form a system that is not linked to ATP synthesis. Furthermore, it was observed that in *P. stipitis*, rotenone blocked oxygen uptake in D-glucose but not D-xylose fermenting cells. This indicated that wild type cells employ complex I during D-glucose metabolism, but bypass it during D-xylose metabolism and thus only 2 proton translocating sites associated with ATP synthesis are functioning during D-xylose utilisation. If rotenone insensitive NADH dehydrogenases link to the alternative oxidase it would form a system that is not linked to ATP synthesis. Such a system may serve to regulate the redox network in Crabtree-negative species as well as dealing with carbon excess conditions.

2.4 *PICHIA STIPITIS*: D-XYLOSE FERMENTING HOST FOR HETEROLOGOUS GENE EXPRESSION

2.4.1 Introduction to heterologous gene expression in yeast

Yeasts are an attractive eukaryotic host system for the expression of heterologous proteins (Hadfield et al. 1993; Sudberry, 1996). Heterologous eukaryotic proteins are best produced in an eukaryotic host system. Reasons for this are, amongst others, the intracellular organisation of eukaryotes, which ensures posttranslational occurrences, such as correct tertiary structure formation, glycosylation and secretion. Although insect and mammalian cell expression systems can produce proteins that are correctly processed after translation, problems such as low product yield and difficulty in growing the host cells hamper industrial level production of recombinant proteins using these host systems. In addition to being able to perform posttranslational processes, yeasts have a short generation time, therefore they can grow to high cell densities over a short period of time. The most intensely studied yeast system is *S. cerevisiae* (Romanos et al. 1992). It is the most highly characterised eukaryotic organism and its complete genomic DNA sequence is known. Many diverse genes have been successfully expressed in this host. For a more comprehensive review of foreign gene expression in *S. cerevisiae*, refer to Romanos et al. (1992) or Hadfield et al. (1993). In many ways however, *S. cerevisiae* is a sub-optimal host for the production of heterologous proteins (Gellissen and Hollenberg, 1997; Hollenberg and Gellissen, 1997). These drawbacks include the lack of very strong, tightly regulated promoters, hyperglycosylation of secreted foreign proteins and the need

for a fed-batch type fermentation process to attain high cell densities (Mendoza-Vega et al. 1994; Vasavada, 1995). Since yeast systems are not without problems and because yeasts species encompass a broad spectrum of useful physiological characteristics, such as the strong and inducible promoter available in methylotropic yeasts, new yeast expression systems are constantly being developed (Hiep et al. 1993; Piredda and Gaillardin, 1994; Sudberry, 1994; Faber et al. 1995; Müller et al. 1998).

P. stipitis and the related yeast *C. shehatae* are the best D-xylose fermenting yeasts described thus far (Du Preez and Prior, 1985; Hahn-Hägerdal et al. 1994; Jeffries and Kurtzman, 1994). The vegetative cells of *P. stipitis* are spheroidal to ovoidal and occur singly or in pairs (Hansen and Kurtzman, 2000). When grown as Dalmau plate culture on corn meal agar, pseudohyphae with occasional blastospores are formed, although true hyphae are never formed. The yeast does possess a sexual cycle and thus formation of ascospores may occur, which is preceded by a parent cell and bud or independent cells conjugating. The asci formed contain two hat-shaped spores that are released soon after formation. The electrophoretic karyotype of *P. stipitis* reveals a nuclear genome of at least six chromosomes (Passoth et al. 1992). UV-irradiation of four wild type strains of *P. stipitis* resulted in auxotrophic mutants being found among the survivors at a frequency of 1.3 %, a fairly high frequency, comparable to results described for haploid yeasts (Samsonova et al. 1989; Melake et al. 1996). The abundance of the phenotypes correlated with the number of genes responsible for the appropriate biosynthetic pathways in *S. cerevisiae*. The relatively high number of mutants generated, as well as the broad spectrum of genes influenced, suggested that the strains tested were haploid. Furthermore it was found that the wild type strains sporulated after growth on malt extract medium, forming asci containing two hat-shaped spores. Thus it could be concluded that *P. stipitis* is a haploid and homothallic yeast (Melake et al. 1996). Conjugation was followed immediately by sporulation, however if hybrids are transferred to a complete medium before meiosis started, they were mitotically stable and did not sporulate. Strains of this yeast can therefore exist vegetatively as haploids but change to a poor medium induces fusion, karyogamy and meiosis, thus a perfect homothallic life cycle.

As lignocellulose is such an abundant substrate, and *P. stipitis* grows well on D-xylose, D-glucose and the other sugars available in lignocellulose hydrolysates, it is a potential host for the conversion of cheap substrates to various recombinant products if a suitable host-vector system is established. Some features of *P. stipitis*, such as the xylan degrading ability of some strains, D-xylose transport, D-xylose metabolism and fermentation of lignocellulosic hydrolysates have already been discussed in sections 2.2 and 2.3.

2.4.2 Genetic transformation of *P. stipitis*: Vectors, markers and promoters

An episomal vector used to transform a specific host must contain at least a replication origin, used by the host DNA polymerase to initiate replication of the plasmid, and a selectable marker to allow discrimination between transformants and non-transformants (Sambrook et al. 1989). The origin of replication of an intrinsic episomal plasmid would be the most desirable if high copy number recombinant plasmids for a specific yeast strain are to be constructed (Ho et al. 1991). Upon attempts to isolate plasmid DNA from *P. stipitis*, the only circular DNA found corresponded to mitochondrial DNA, which is thus the only extrachromosomal DNA found in *P. stipitis* strains tested. There are therefore no obvious replicators available to create extrachromosomal expression vectors. A plasmid vector was constructed containing the *S. cerevisiae* 2μ replicon and the kanamycin resistance gene and was maintained episomally once introduced into *P. stipitis* (Ho et al. 1991). Plasmids identical to this vector could subsequently be isolated from the transformants, proving that the vector could be introduced and maintained episomally, although transformation frequencies were low. The yeast cells were transformed using both the $CaCl_2$-PEG-protoplast method and by direct electroporation of intact *P. stipitis* cells. A different approach was to use the yeast/*E. coli* shuttle vector pJH-S, containing the *ARS1* replication origin cloned from the yeast *Schwanniomyces occidentalis* and *HIS3* gene cloned from *P. stipitis* (Morosoli et al. 1992b). Although this vector was found to be relatively stable, transformation frequencies were low. More recently, an episomal vector transformation system for a *P. stipitis* tryptophan auxotroph was developed using the autonomous replicating

sequence of *S. occidentalis* and the *S. cerevisiae TRP5* gene (Piontek et al. 1998). *P. stipitis* was successfully transformed with this plasmid and expression of a heterologous cellulase encoding gene under control of the *P. stipitis XYL1* gene promoter and the *S. cerevisiae* (*ADH1*) gene promoter was shown. The *XYL1* promoter was found to be inducible by D-xylose and more successful in expression of the heterologous gene in *P. stipitis* than the *S. cerevisiae ADH1* gene promoter.

A transformation system for *P. stipitis* was described that is probably the most efficient and stable of the systems mentioned (Yang et al. 1994). It was based on the *P. stipitis* oroditine-5'phosphate decarboxylase (*URA3*) encoding gene and an autonomously replicating sequence (*ARS2*). First, spontaneous *P. stipitis ura3* auxotrophs were isolated by their ability to grow on media containing the metabolic analogue 5-fluoroorotic acid (FOA). The strain *P. stipitis* TJ26 had an absolute requirement for uracil, with very low reversion mutation frequencies. The *P. stipitis URA3* gene was isolated from a genomic library after probing with a *S. cerevisiae URA3* probe. The functional *P. stipitis ARS2* sequence was cloned from genomic DNA by plasmid rescue. The resulting plasmid, pJM6, containing the *URA3* and *ARS2* sequences could be maintained for over 50 generations under selective pressure in minimal media after being introduced into *P. stipitis* by electroporation. The copy numbers were estimated to be between 8 to 10 copies of plasmid per nucleus. Integrating vectors were constructed containing only the cloned *URA3* gene and flanking sequences. After such transformations, Southern analysis confirmed that integration had taken place in the resultant transformants. Contrary to the occurence in *S. cerevisiae*, it was found that as many as 80% of these integration occurrences had taken place at nonhomologous sites within the genome. Site directed gene disruption is therefore more difficult to achieve in *P. stipitis* than in *S. cerevisiae*. This has also been found in subsequent studies where targeted gene disruption was attempted (Cho and Jeffries, 1999; Shi et al. 1999; Shi et al. 2002). It was found that although targeted integration was successful, to isolate a transformant where the targeted integration had taken place, several transformants had to be screened by Southern blot or PCR analysis. A *P. stipitis* double auxotrophic strain, deficient in both *URA3* and the gene encoding β-isopropylmalate dehydrogenase (*LEU2*) has since been

constructed along with episomal plasmids containing either functional *URA3* or *LEU2* genes (Lu et al. 1998a). Double auxotrophs are useful because they allow the subsequent transformation of different genes.

The transformation system used in this study (Chapter 3–5) is based on the transformation system developed by Yang et al. (1994). The Ura3$^-$ auxotrophic strain *P. stipitis* TJ26 and the basic plasmid, pJM6, was kindly provided to us by Prof. T.W. Jeffries of the Forest Products Laboratory, Madison, Wisconsin, USA. The basic vectors used in this study were created by cloning the *P. stipitis URA3* and *ARS2* sequences from genomic DNA by means of PCR, using the published sequence information, and cloning the sequences into the pBluescript vector as described in chapter 3. Transformation was done using the lithium acetate/dimethylsulphoxide method (Hill et al. 1991). In our investigation of the heterologous expression system of *P. stipitis*, we started by testing the activity of native and heterologous promoter in this yeast (Chapter 3). The transcriptional control of two native promoters and one heterologous promoter, and the production of a heterologous reporter protein (β-xylanase II from *T. reesei*) from these promoters was evaluated in *P. stipitis* cultivated on D-xylose and D-glucose as carbon sources. Xylose reductase is the first enzyme in the D-xylose assimilation pathway of *P. stipitis* (Amore et al. 1991) and the gene encoding xylose reductase (*XYL1*) is induced in the presence of D-xylose (Webb and Lee, 1992). The *XYL1* promoter was therefore cloned and evaluated as an inducible promoter in *P. stipitis*, since the use of inducible promoters is preferable for the high-level production of heterologous proteins (Romanos et al. 1992). Transketolase is an enzyme central to the pentose phosphate pathway, therefore the transketolase encoding gene (*TKL*) should be constitutively expressed, as *P. stipitis* catabolises most of its carbohydrates, including hexose sugars, through the pentose phosphate pathway (Jeffries and Kurtzman, 1994; Metzger and Hollenberg, 1994). The *TKL* promoter was therefore chosen as a strong, constitutively expressed promoter for heterologous expression in *P. stipitis*. The *PGK1* promoter is a strong constitutive promoter of *S. cerevisiae* that can be induced to a level of expression that constitutes 4 to 10% of total soluble protein under certain growth conditions (Hadfield et al. 1993). The open reading frame of the *T. reesei xyn2* gene was fused to the *P. stipitis*

XYL1 promoter, the *P. stipitis TKL* promoter and the *S. cerevisiae PGK1* promoter DNA sequences on episomal plasmids, respectively. After transformation of the plasmids into *P. stipitis*, the *XYL1* promoter was shown to be inducible in the presence of D-xylose, as *xyn2* transcription and β-xylanase activity could be measured when the recombinant strain was cultivated on D-xylose, but not when it was cultivated on D-glucose. *TKL* promoter expression was found to be constitutive when either D-glucose or D-xylose was used as sole carbon source. The recombinant β-xylanase was successfully secreted by its native *T. reesei* secretion signal. The *PGK1* promoter did not promote *xyn2* transcription in *P. stipitis*.

2.4.3 Genes and sequences cloned from *P. stipitis*

In contrast to *S. cerevisiae*, where the complete genomic DNA sequence is known, relatively few genes have been cloned from *P. stipitis*. As most research on the yeast species focuses on the inherent ability to ferment D-xylose to ethanol without significant xylitol formation, most genes that have been cloned encode products that are involved in D-xylose metabolism. A common feature of the genes seems to be that, as with *S. cerevisiae*, and in contrast to yeasts such as *C. albidus*, *P. stipitis* genes do not contain intron sequences. Table 7 summarises most of the genes and sequences that have been cloned from *P. stipitis*.

Table 7 Genes and sequences cloned from *P. stipitis*.

Gene/Sequence	Product encoded	Reference(s)
XYL2	D-Xylitol dehydrogenase	Kötter et al. 1990
XYL1	D-Xylose reductase	Amore et al. 1991
TKT/TKL	Transketolase	Metzger and Hollenberg 1994
URA3	Orotidine-5'-phosphate decarboxylase	Yang et al. 1994
ARS2	Autonomous replicating sequence	Yang et al. 1994
ARDH	D-Arabinitol dehydrogenase	Hallborn et al. 1995
PsADH1 & PsADH2	Alcohol dehydrogenase	Cho and Jeffries, 1998; Passoth et al. 1998
LEU2	β-Isopropylmalate dehydrogenase	Lu et al. 1998a
PsPDC1 & PsPDC2	Pyruvate decarboxylase	Lu et al. 1998b
PsCYC1	Cytochrome c	Shi et al. 1999
SUT1-3	D-Glucose transporters	Weierstall et al. 1999
XYNA	β-Xylanase	Basaran et al. 2001
XYL3	D-Xylulokinase	Jin et al. 2002
PsSTO1	SHAM-sensitive terminal oxidase	Shi et al. 2002

The *URA3*, *LEU2* and *ARS2* sequences used in the construction of transformation vectors for *P. stipitis* were discussed in section 2.4.1. The genes or gene products involved in D-xylose metabolism (*SUT1-3*, *XYL1*, *XYL2*, *XYL3*, *TKL*, *PsADH1&2*, *PsPDC1&2*, *CYC1* and *PsSTO1*) were discussed in sections 2.3.4 and 2.3.6. Some strains of *P. stipitis* produce an extracellular β-xylanase encoded by the *XYNA* gene, as was discussed in section 2.2.4, along with other natural β-xylanase producing yeasts.

2.4.4 Heterologous gene expression in *P. stipitis*

The successful heterologous expression of β-xylanase encoding genes in *P. stipitis* (Morosoli et al. 1992a; Passoth and Hahn-Hägerdahl, 2000; Chapter 3; Chapter 4) was discussed in section 2.2.5. Secretion of active β-xylanase was attained by expression of the *C. albidus*, *T. reesei* or *A. kawachii* β-xylanase encoding genes under transcriptional control of the *XYL1*, *TKL* or *PsADH2* promoters of *P. stipitis*.

In an attempt to increase ethanol productivity in *P. stipitis*, the *XYL1* gene was overexpressed (Dahn et al. 1996). The *P. stipitis XYL1* gene was inserted into an autonomously replicating multicopy plasmid and transformed to *P. stipitis* TJ26. Aerobic growth on D-xylose medium led to a 1.8 fold increase in xylose reductase activity in the recombinant strain, compared to the reference strain. However, it was found that the xylose reductase activities were similar under oxygen limitation. Furthermore, it was found that ethanol production was not improved but rather decreased in the recombinant strain. Thus it would seem that negative effects owing to the multicopy plasmid countered any benefit for ethanol production by overexpression of *XYL1* or that this step is not rate limiting during ethanol production from D-xylose. The multicopy plasmid could, for example, lead to slow growth, altered metabolism and decreased carbohydrate utilisation.

P. stipitis grows rapidly without ethanol production under fully aerobic conditions, and it ferments D-glucose or D-xylose under oxygen-limited conditions, but it stops growing within one generation under anaerobic conditions (Prior et al. 1989; Du Preez, 1994).

The dependence of *P. stipitis* on oxygen for growth and its constitutive respiratory activity deminishes ethanol yields. Furthermore, the need for controlled oxygenation increases the process and control costs. Expression of *S. cerevisiae URA1* in *P. stipitis* enabled rapid anaerobic growth in minimal defined medium containing D-glucose when essential lipids were present (Shi and Jeffries, 1998). *URA1* encodes a dihydroorotate dehydrogenase (catalyses the conversion of dihydroorotate to orotate in the pyrimidine biosynthesis pathway) that uses fumarate as an alternative electron acceptor to confer anaerobic growth. Initial *P. stipitis* transformants grew and produced 32 g/L ethanol from 78 g/L D-glucose. Cells produced even more ethanol at higher rates following two anaerobic serial subcultures. Control strains were incapable of growing anaerobically and showed only limited fermentation. *P. stipitis* cells bearing the heterologous *URA1* were viable in anaerobic D-xylose medium for long periods, and supplemental D-glucose allowed cell growth, but D-xylose alone could not support anaerobic growth even after serial anaerobic subculture on D-glucose. These data imply that *P. stipitis* can grow anaerobically using metabolic energy generated through fermentation, but that it exhibits fundamental differences in cofactor selection and electron transport with D-glucose and D-xylose metabolism. The proton symport transport system for assimilation of D-xylose may also be a rate-limiting factor in anaerobic growth of *P. stipitis* on D-xylose.

2.4.5 Hybridisations with *P. stipitis* to improve growth and ethanol productivity

Hybridisation by means of protoplast fusion has been used with success to improve yeasts of industrial importance (Gupthar and Garnett, 1987). Using this technique, sexual barriers, preventing unrelated species from mating, may be obviated, facilitating total or partial exchange of genetic components. Auxotrophic strains of *P. stipitis* and *C. shehatae* were fused through polyethylene glycol induced protoplast fusion. Prototrophic partial hybrid strains were obtained with promising ethanol fermenting capabilities. DNA-DNA binding experiments of these hybrids showed that the nucleus contained mostly *P. stipitis* DNA, and it was suggested that certain *C. shehatae* genes were integrated rather than whole chromosomes (Selebano et al. 1993). It was proposed that strains more suitable to industrial processes might be attained using fusion techniques. Aneuploid strains of *P. stipitis* were created by fusing different *P. stipitis*

strains (Gupthar, 1987). It was thought that an increased gene dosage of the D-xylose metabolising genes could lead to increases in rates and yields of ethanol production. However, D-xylose fermentation was not significantly improved in these strains. In an attempt to produce an ethanol tolerant, D-xylose fermenting strain, a *S. cerevisiae* strain was hybridised with strains of *P. stipitis* and *C. shehatae* (Gupthar, 1992). Mononucleate fusants were obtained, but these dissociated into a mixture of parental type segregants. *P. stipitis* and *C. shehatae* type segregants failed to acquire high ethanol tolerance but expressed other novel *S. cerevisiae* properties, such as assimilation of certain sugars, suggesting that karyogamy was impaired after internuclear gene transfer. Recently, hybrids of the nuclei of filamentous fungi and *P. stipitis* protoplasts were made to attain a strain that could degrade xylan and ferment the D-xylose obtained (Vasquez et al. 1997). Nuclei were isolated from the mycelium of *Fusarium moniliforme* and *T. reesei* and fused with protoplasts of a non-xylan degrading *P. stipitis* strain. Hybrids were morphologically yeast-like and able to hydrolyse xylan. Their chromosomal pattern was similar to that of the parental yeast strain in a clamped homogenous field electrophoresis (CHEF) analysis. β-Xylanase activity in one of the hybrids reached 350 nkat/ml. Maximum ethanol concentrations of 2.3 g/L could be attained from medium containing 20 g/L xylan as carbon source.

2.4.6 Yeast extracellular proteases

Proteolytic enzymes catalyse the cleavage of peptide bonds in other proteins (Ogrydziak, 1993). Proteases are degradative enzymes, which catalyse the total or partial hydrolysis of proteins. Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals and microorganisms. Rao et al. (1998) have reviewed microbial proteases, and this overview will only be concerned with the extracellular proteases secreted by some yeast species. Functions of proteases in organisms include their role in protein turnover, sporulation, germination of spores, as well as highly specific and selective modifications of proteins, such as activation of zymogenic forms of enzymes by limited proteolysis and transport of secretory proteins across the membranes. Proteases are also secreted by some organisms to degrade proteins so that amino acids may be taken up when their abundance becomes

limited or as nitrogen source. Secreted proteases also have an impact on the stability and concentration of secreted heterologous proteins. Proteases are classified as exopeptidases (including aminopeptidases and carboxypeptidases) or endopeptidases (including serine proteases, aspartic proteases, cysteine/thiol proteases and metalloproteases) (Rao et al. 1998).

2.4.6.1 Industrial applications of proteases

Proteases are significant with respect to their applications in both physiological and commercial fields. The value of the worldwide sales of industrial enzymes was already estimated at $1 billion in 1998 (Rao et al. 1998). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. Table 8 summarises the important industrial applications of proteases.

Table 8 Industrial applications of proteases (Rao et al. 1998; Gupta et al. 2002)

Industry	Application
Laundry and detergents	Removal of protein containing stains from fabric
Leather industry	Selective hydrolysis of noncollagenous skin constituents and swelling of the hide
Food industry	
Dairy industry	Milk coagulating enzymes for cheese production
Baking industry	Modification of gluten in wheat flour reduces mixing time and increases loaf volume
Soy products	Preparation of soy sauce and other soy products
Debittering of protein hydrolysates	Improving tastes of the hydrolysates used in health and infant foods
Synthesis of aspartame	Proteases are used to produce aspartame, an artificial sweetener
Pharmaceutical industry	Developing therapeutic agents such as digestive aids
Waste management	Solubilising proteinaceous waste to lower biological oxygen demand
Photographic industry	Bioprocessing of used film for silver recovery
Silk industry	Silk degumming
Other applications	Important role in research

2.4.6.2 Fungal proteases

Fungi secrete a wide variety of proteases, for example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases (Ogrydziak, 1993; Rao et al. 1998). Fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and heat tolerance than do the bacterial enzymes. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable

between pH 2.5 and 6.0. They are particularly useful in the cheese making industry due to their pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents such as EDTA. Fungal alkaline proteases are also used in food protein modification.

There are several examples of yeasts producing extracellular proteases (Ogrydziak, 1993). Although it has been shown that few *S. cerevisiae* strains secrete significant amounts of proteases, the *PEP4* gene of *S. cerevisiae*, which encodes an aspartyl protease implicated in the posttranslational regulation of the yeast vacuolar hydrolases, has been cloned and studied (Ogrydziak, 1993; Rao et al. 1998). The nucleotide sequence was deduced and the predicted amino acid sequence showed substantial homology to that of the aspartyl protease family. Aberrant proteolytic processing of heterologously expressed proteins in *S. cerevisiae* has been avoided by using *PEP4* deficient strains in some cases (Romanos et al. 1992). *Candida albicans* and *Candida tropicalis* are the medically important opportunistic pathogens causing infections in immunocompromised patients (Hube and Naglik, 2001; Zaugg et al. 2001). Their secreted proteolytic activity is considered to be a major virulence factor. The deduced amino acid sequence of the acid protease from *C. tropicalis* shows similarity to the amino acid sequence of the pepsin family (Tongi et al. 1991). The aspartyl protease gene from various *C. albicans* strains was cloned and sequenced (Hube and Naglik, 2001). The genes encoding secreted aspartic proteases in *C. albicans* (the *SAP1*, *SAP2*, *SAP3*, *SAP4*, *SAP5* and *SAP6* genes) constitute a multigene family. Evidence was also obtained for the existence of secreted aspartic protease multigene families in *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii* (Zaugg et al. 2001). The amino acid sequence of an acid extracellular protease from *Yarrowia lipolytica* deduced from the nucleotide sequence revealed a putative 17-aa pre-peptide, a 27-aa pro-peptide and a 353-aa mature protein (Rao et al. 1998). Acid extracellular protease showed homology to proteases of several fungal genera. The transcription of both acid extracellular protease and the alkaline extracellular protease genes in *Y. lipolytica* was regulated by the pH of the culture (Glover et al. 1997; Gonzalez-Lopez et al. 2002). Protease synthesis is, in most cases, tightly regulated and

responds to a combination of environmental stimuli, including nutrient availability, ambient pH and temperature.

P. pastoris, a methylotrophic yeast, has been used successfully to produce a number of industrially important proteins and enzymes (Romanos et al. 1992; Hollenberg and Gellissen, 1997; Gellissen, 2000). However, proteases produced by this yeast lead to instability of secreted heterologous proteins, which impacts negatively on the levels of protein produced. It was shown that altering of the medium pH (controlling the medium pH to pH 6) or addition of casamino acids could enhance stability of the secreted product (Sreekrishna et al. 1997). Product stability was also enhanced using strains that were deficient in the protease encoding genes *PEP4*, *PRB1* or *PRC* (Eckart and Bussineau, 1996).

In our study it was found that the heterologous β-xylanase activity of the *P. stipitis* transformants was transient, dropping of to levels close to zero nkat/ml soon after reaching maximum activity levels (Chapter 3; Chapter 4). It was argued that the reduction in β-xylanase activity was due to the presence of an extracellular protease. It was also shown that recombinant *P. stipitis* strains secreted an extracellular protease by zone formation of the strains on milk plates (Chapter 4). Subsequently, the recombinant strains of *P. stipitis* were grown in a fermentor under conditions of high oxygenation and controlled pH levels (controlled to pH 6) to assess the stability of heterologous β-xylanase activity (Chapter 5). The heterologous β-xylanase activity was not stabilised by controlling the pH level in the fermentor to pH 6 with addition of NaOH, showing that the proteolytic activity in the supernatant was probably not limited to acid proteases. After depletion of the initial D-xylose in the media, new, but transient β-xylanase activity could be induced by the addition of D-xylose to the fermentor. The proteolytic activity in the culture supernatant had an optimum activity at pH 7 and 60°C. Research on the type of protease secreted by *P. stipitis* and the regulation thereof, as well as cloning of the gene(s) encoding the protease activity and isolation of protease-deficient *P. stipitis* strains, will be important for the efficient production of secreted heterologous proteins by this yeast.

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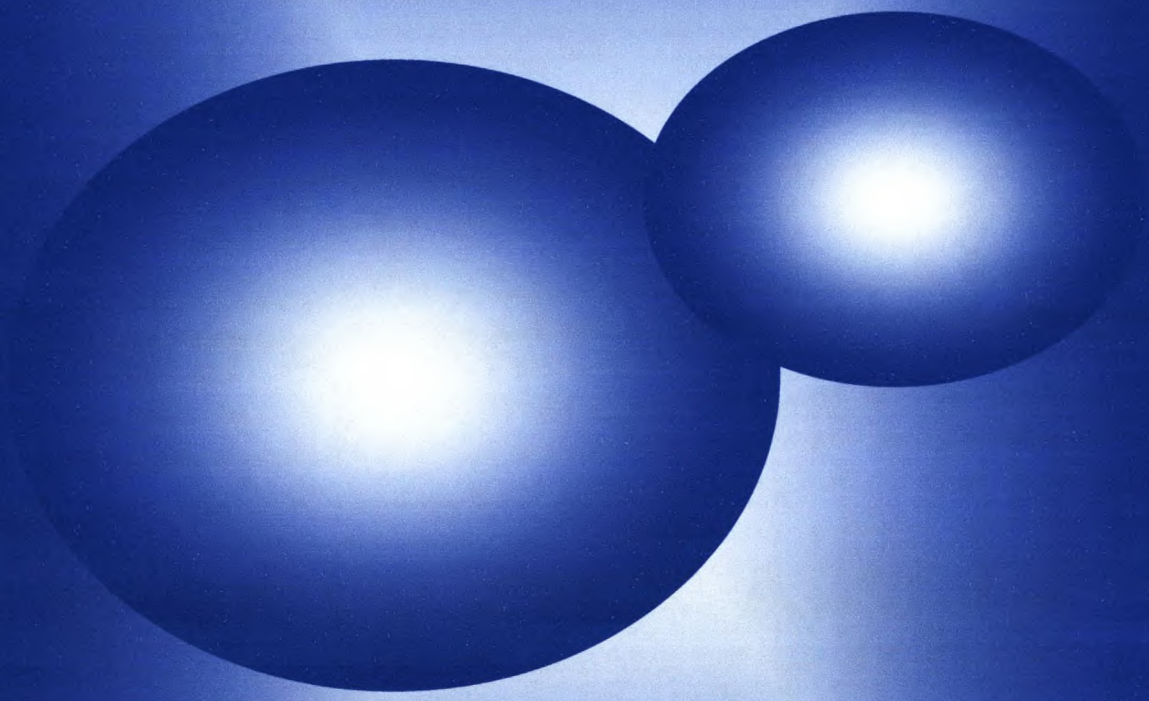
Chapter 3
Paper 1



Differential expression of the *Trichoderma reesei* β-xylanase II (*xyn2*) gene in the xylose fermenting yeast *Pichia stipitis*

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3 DIFFERENTIAL EXPRESSION OF THE *TRICHODERMA REESEI* β-XYLANASE II (*XYN2*) GENE IN THE XYLOSE FERMENTING YEAST *PICHIA STIPITIS*

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3.1 ABSTRACT

The transcriptional control of two native promoters and one heterologous promoter and the production of a heterologous protein from these promoters were evaluated in the xylose fermenting yeast *Pichia stipitis* cultivated on xylose and glucose as carbon sources, using the β-xylanase II *xyn2* gene of *Trichoderma reesei*. The *xyn2* gene open reading frame was fused to the *P. stipitis* xylose reductase gene (*XYL1*) promoter, the *P. stipitis* transketolase gene (*TKL*) promoter and the *Saccharomyces cerevisiae* phosphoglycerate kinase gene (*PGK1*) promoter DNA sequences on episomal plasmids. The plasmids were transformed into *Pichia stipitis* and gene expression and β-xylanase production monitored. The *XYL1* promoter was shown to be inducible in the presence of xylose, as *xyn2* transcription and β-xylanase activity could be measured when the recombinant strain was cultivated on xylose but not when it was cultivated on glucose. *TKL* promoter expression was found to be constitutive when either glucose or xylose was used as sole carbon source. The *PGK1* promoter did not promote *xyn2* transcription in *P. stipitis*. The molecular size of the recombinant Xyn2 protein produced by *P. stipitis* was 20.7 kDa, which is similar to that of the native *T. reesei* Xyn2 protein. This indicates no or minimal glycosylation of the recombinant protein. The recombinant *xyn2*-expressing strain also yielded twice the amount of biomass yielded by the control strain when cultivated in medium containing 1% birchwood xylan as sole carbon source.

Pichia stipitis *xylanase* *heterologous expression* *TKL* *XYL1*

3.2 INTRODUCTION

Yeasts are attractive eukaryotic host systems for the expression of heterologous eukaryotic proteins since yeasts provide the subcellular machinery for the posttranslational processing often required by these proteins. The most intensely studied yeast expression system is that of *S. cerevisiae* (Romanos et al. 1992). In many respects, however, *S. cerevisiae* is a sub-optimal host for the production of heterologous proteins. One of the disadvantages is the lack of very strong, tightly regulated promoters, which are essential for high-level foreign protein production. A second disadvantage is improper post-translational processing of secreted proteins, such as hyperglycosylation. Extra high-mannose carbohydrate structures can interfere with the secondary or tertiary structure of the protein or with its functionality and in some cases have been shown to be immunoreactive (Hadfield et al. 1993; Gairin et al. 1991).

The yeast *P. stipitis* and the related *Candida shehatae* are the best xylose-fermenting yeasts thus far described (Jeffries and Kurtzman, 1994). D-Xylose is the predominant pentose sugar in hemicellulose, the second most abundant renewable carbon source in nature. The xylose content of hemicellulose can vary from 17% of the total dry weight of woody angiosperms (hardwoods) to 31% in herbacious angiosperms, such as the residues from agricultural crops (Jeffries and Jin, 2000). Furthermore, lignocellulosic waste liquors, such as spent sulfite liquor, provide abundant resources of both hexoses and pentoses (Lawford and Rousseau, 1993). *P. stipitis* therefore has the potential of producing pharmaceutically and industrially important proteins from these abundant substrates.

Ho et al. (1991) previously described the transformation of *P. stipitis*, and an efficient transformation system based on the *P. stipitis* orotidine-5'-phosphate decarboxylase (*URA3*) gene was later established (Yang et al. 1994). The *Cryptococcus albidus* xylanase gene was successfully expressed in *P. stipitis* using the native xylose reductase encoding gene (*XYL1*) promoter (Morosoli et al. 1993) and the oxygen regulated alcohol dehydrogenase (*PsADH2*) promoter (Passoth and Hahn-Hägerdal, 2000). Both reports showed the successful production of the heterologous endo-1,4- β-xylanase in *P. stipitis*.

The xylose reductase (*XYL1*) gene of *P. stipitis* has been shown to be induced in the presence of xylose (Webb and Lee, 1992; Morosoli et al. 1993). In contrast, expression of the transketolase (*TKL*) gene is thought to be constitutive as *P. stipitis* catabolises most of its carbohydrates, including hexose sugars, through the pentose phosphate pathway (Jeffries and Kurtzman, 1994; Metzger and Hollenberg, 1994). The *S. cerevisiae PGK1* gene promoter has been employed successfully in the constitutive expression of several genes in *S. cerevisiae* (Romanos et al. 1992). In this study we explored the possibility that this promoter could also direct expression of genes in *P. stipitis*.

The *T. reesei xyn2* gene encodes a β-1,4-xylanase able to degrade xylan to xylo-oligosaccharides. This enzyme can readily be detected by several enzyme assays. The *xyn2* gene was previously successfully expressed in *S. cerevisiae* (La Grange et al. 1996; La Grange et al. 2000). The gene encodes a xylanase precursor containing a native pro-sequence that directs secretion in both *T. reesei* and *S. cerevisiae*. The mature *T. reesei* protein has a molecular mass of 20.7 kDa (Törrönen et al. 1992). In this report the *T. reesei xyn2* gene is employed as reporter gene to explore the expression capabilities of the native xylose reductase gene (*XYL1*) promoter, the native transketolase gene (*TKL*) promoter and the heterologous *S. cerevisiae* phosphoglycerate kinase gene (*PGK1*) promoter in the yeast *P. stipitis*.

3.3 MATERIALS AND METHODS

3.3.1 Media and culture conditions

P. stipitis and *S. cerevisiae* were cultivated on YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or selective synthetic (SC) medium [containing 20 g/L glucose, 1.7 g/L yeast nitrogen base without amino acids (Difco) (pH 6) and all the required growth factors except uracil (SC^{-URA})]. Solid media contained 2% agar. Yeasts were routinely cultured in a 250-ml Erlenmeyer flasks containing 100 ml medium at 30°C on a rotary shaker at 150 rpm.

3.3.2 Microbial strains and plasmids

The genotypes and sources of the yeasts and bacterial strains, as well as the plasmids that were constructed and used in this study, are summarized in Table 1.

Table 1 Microbial strains and plasmids

Strain/Plasmid	Genotype	Source/Reference
Yeast strains:		
Pichia stipitis TJ26	*ura3*	Yang et al. 1994
P. stipitis TJ26:		
pJM6[a]	*ura3/[URA3]*	This work
pRDH7[b]	*ura3/[URA3 PGK1$_P$-xyn2-PGK1$_T$]*	This work
pRDH12[c]	*ura3/[URA3 XYL1$_P$-xyn2-XYL1$_T$]*	This work
pRDH14[d]	*ura3/[URA3 TKL$_P$-xyn2-XYL1$_T$]*	This work
Saccharomyces cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	This laboratory
S. cerevisiae Y294: pDLG5	*ura3/[URA3 ADH2$_P$-xyn2-ADH2$_T$]*	La Grange et al. 1996
Bacterial strain:		
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1hsdR17 supE44 relA1 lac*[F' *proAB lacI^{q}ZΔM15 Tn10* (Tet^r)][c]	Stratagene
Plasmids:		
pBluescript II SK(+)	*bla*	Stratagene
pDLG6	*bla URA3 PGK1$_P$-xyn2-PGK1$_T$*	La Grange et al. 1996
pDLG5	*bla URA3 ADH2$_P$-xyn2-ADH2$_T$*	La Grange et al. 1996
pJM6	*bla URA3*	Yang et al. 1994
pRDH7	*bla URA3 PGK1$_P$-xyn2-PGK1$_T$*	This work
pRDH12	*bla URA3 XYL1$_P$-xyn2-XYL1$_T$*	This work
pRDH14	*bla URA3 TKL$_P$-xyn2-XYL1$_T$*	This work

[a] *P. stipitis* TJ26 (pJM6) was designated *P. stipitis* (VECT)
[b] *P. stipitis* TJ26 (pRDH7) was designated *P. stipitis* (*PGK1-xyn2*)
[c] *P. stipitis* TJ26 (pRDH12) was designated *P. stipitis* (*XYL1-xyn2*)
[d] *P. stipitis* TJ26 (pRDH14) was designated *P. stipitis* (*TKL-xyn2*)

3.3.3 Plasmid construction and transformation

Standard protocols were followed for DNA manipulations (Sambrook et al. 1989). Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Tautz and Renz (1983). Restriction endonucleases, T4 DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I were purchased from Roche Molecular Biochemicals and used as recommended by the manufacturer. The basic vectors used in this study were created by cloning the DNA elements encoding the *P. stipitis XYL1* promoter, *XYL1* terminator, *TKL* promoter, *ARS2* and *URA3* from *P. stipitis* genomic DNA using standard PCR amplification. Table 2 details the primers and annealing

temperatures used in this study (restriction enzyme sites are underlined). Fig. 1 summarises the plasmids that were used in this study. After PCR amplification and restriction endonuclease digestion the DNA fragments were purified from agarose gels and sequentially cloned into pBluescript II SK(+). The *XYL1* promoter was cloned as a 0.335-kb *Sac*I/*Xba*I fragment into pBluescript. The *XYL1* terminator was subsequently cloned as a 0.385-kb *Hin*dIII/*Sal*I fragment into the resulting plasmid to yield pRDH8 (containing the *XYL1* promoter/terminator cassette). The *URA3* fragment was cloned as a 1.46-kb *Sal*I fragment into the compatible *Xho*I site of pRDH8 to yield pRDH9 and finally the *ARS2* fragment was cloned as a 1.15-kb *Sal*I fragment into the unique *Sal*I site of pRDH9 to yield pRDH10 (Fig. 1). The *XYL1* promoter was excised from pRDH10 with a *Sac*I/*Xba*I endonuclease digest and after purification of the vector the *TKL* promoter was cloned as a 0.35 kb *Sac*I/*Xba*I fragment into the vector to yield pRDH13. The *T. reesei xyn2* gene was cloned as a 0.78-kb *Xba*I/*Bgl*II fragment from pDLG5 (La Grange et al. 1996) into the *Xba*I/*Bam*HI sites of pRDH10 to yield pRDH12 and into the *Xba*I/*Bam*HI sites of pRDH13 to yield pRDH14. The $PGK1_P$-*xyn2*-$PGK1_T$ cassette was cloned as a 2.8-kb *Hin*dIII fragment from pDLG6 (La Grange et al. 1996) into the unique *Hin*dIII site of pJM6 to yield pRDH7. DNA transformation of *P. stipitis* was performed using the lithium acetate dimethylsulfoxide (DMSO) method described by Hill et al. (1991).

3.3.4 Screening for β-xylanase activity

Transformants were screened for xylan degrading ability by patching on SC^{-URA} medium containing 0.2% of 2-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB)-xylan (Sigma) containing either 2% glucose or 2% xylose as carbon source (Biely et al. 1988).

3.3.5 Enzyme activity assays

Endo-β-1,4-xylanase activity was assayed according to the method described by Bailey et al. (1992) with 1% birchwood glucuronoxylan (Carl Roth) as the substrate at 50°C. Appropriate dilutions of the cell free culture solution in 50 mM sodium citrate buffer (pH5.0) were used as the enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid method described by Miller et al (1960).

Table 2 PCR primers

Gene/fragment and annealing temperature used	Primers	Source of DNA sequence
P. stipitis XYL1 promoter 43°C	XYLPL (*Sac*I restriction site underlined) 5'-GATAGAGCTCGCGGCCGCCCACAGACACTAATTG-3' XYLPR (*Xba*I restriction site underlined) 5'-GAGCTCTAGAAGTATAGTTGTATAGAAAAG-3'	Amore et al. 1991
P. stipitis XYL1 terminator 43°C	PXYLTL (*Hin*dIII restriction site underlined) 5'-GATCAAGCTTAGGTTGCTTTATAGAGAG-3' PXYLTR (*Sal*I restriction site underlined) 5'-GATCGTCGACGATGCAGAAGTAGTTTTG-3'	Amore et al. 1991
P. stipitis TKL promoter 55°C	PTKLL(*Sac*I restriction site underlined) 5'-GTACGAGCTCCCAGAGTTCATGCTACTCAAC-3' PTKLR (*Xba*I restriction site underlined) 5'-GTACTCTAGAGGCAAATGGAAGTCTGG-3'	Amore et al. 1991
P. stipitis URA3 50°C	PURAL (*Sal*I restriction site underlined) 5'-GATCGTCGACTTGTCTGAGAAGAA-'3 PURAR (*Sal*I restriction site underlined) 5'-GATCGTCGACCTACAATGCATCAGCTAC-3'	Yang et al. 1994
P. stipitis ARS2 50°C	PARSL (*Sal*I restriction site underlined) 5'-GATCGTCGACGAATTCAGTATAGGATATGG-3' PARSR (*Sal*I restriction site underlined) 5'-GATCGTCGACGTGTCTACAAGGTCAGAAG-3'	Yang et al. 1994

3.3.6 Northern blot analysis

RNA was isolated from shake flask cultures (SC^{-URA} containing either 2% glucose or 2% xylose) of *P. stipitis* (VECT), *P. stipitis* (*PGK1*$_P$*-xyn2*), *P. stipitis* (*XYL1*$_P$*-xyn2*), and *P. stipitis* (*TKL*$_P$*-xyn2*) at 24 h after inoculation. RNA isolations and Northern hybridisations were carried out as described by Sambrook et al. (1989). DNA probes were prepared by labelling a 0.78-kb *xyn2* DNA fragment from pDLG5 and a 1.5-kb *S. cerevisiae* 5S rDNA fragment with [α-^{32}P]dATP, using the Random Primed DNA Labelling Kit (Roche Molecular Biochemicals) according to the manufacturers instructions. The ^{32}P-labelled 5S rDNA fragment was used to probe the 5S rRNA levels as internal standard in the Northern blots.

3.3.7 Protein preparation and gel electrophoresis

The supernatants of 200-ml cultures of *P. stipitis* (VECT) and *P. stipitis* (*TKL-xyn2*) at 48 h were separated from the cellular mass by centrifugation for 5 min at 4,000 x g. The supernatants were filtered and concentrated in a Diaflo Ultrafilter PM10 concentrator (Amicon) and subsequently precipitated with 2 volumes of ice cold acetone for 1 h.

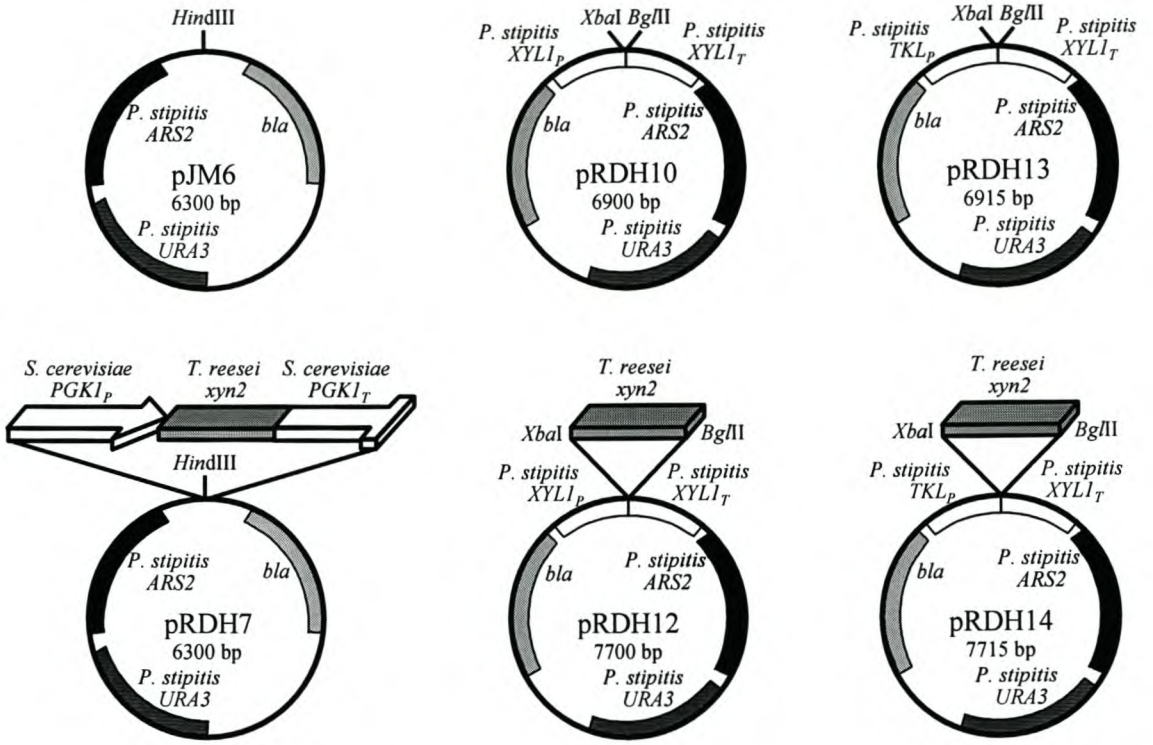

Fig. 1 Schematic representation of the plasmids used in this study. The *P. stipitis* autonomous replicating sequence (*ARS2*) is responsible for episomal replication of the plasmid and the *P. stipitis* orotidine-5'-phosphate decarboxylase (*URA3*) used as selectable marker. $XYL1_P$, $XYL1_T$, $TKL1_P$, $PGK1_P$, $PGK1_T$ represents the *P. stipitis* xylose reductase gene promoter, *P. stipitis* xylose reductase gene terminator, *P. stipitis* transketolase gene promoter, *S. cerevisiae* phosphoglycerate kinase gene promoter and *S. cerevisiae* phosphoglycerate kinase gene terminator, respectively.

Protein fractions were resuspended in 50 of HEPES buffer [20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; pH 8.0), 5mM EDTA (pH 8.0), 7 mM β-mercaptoethanol] after centrifugation at 13,500 x g for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide was performed by the method of Laemmli (1970). Protein fractions were boiled for 3 min and applied to the gel. The purified Xyn2 protein of *T. viride* (Sigma) was used as a positive control. Proteins were visualised by Coomassie brilliant blue staining (Sambrook et al. 1989).

3.3.8 Growth of *P. stipitis* transformants on xylan

P. stipitis (VECT) and *P. stipitis* (*TKL-xyn2*) were inoculated from precultures in the stationary growth phase into 50 ml SC^{-URA} medium containing 1% birchwood glucuronoxylan (Sigma) as the sole carbon source. Three cultures of each strain were inoculated simultaneously. Samples were periodically taken over a 45 h period and yeast cells in the media were counted in triplicate on a haemocytometer.

3.4 RESULTS

3.4.1 Expression of the *xyn2* gene in *P. stipitis*

P. stipitis TJ26 was transformed with episomal vectors expressing the *xyn2* gene of *T. reesei* under the transcriptional control of the *P. stipitis XYL1* and *TKL* promoters, as well as the *S. cerevisiae PGK1* promoter. Transformation frequency of the recombinant plasmids did not vary greatly and was generally $1x10^3$ colonies per μg DNA. Transformants were confirmed by Southern blot hybridisation using a α-^{32}P labelled 0.78-kb *xyn2* fragment as probe (results not shown). Northern analysis of the mRNA produced by the recombinant *P. stipitis* transformants showed that the levels of *xyn2* mRNA formed differed significantly between the recombinant *P. stipitis* strains under different growth conditions (Fig. 2A, B). The *P. stipitis* (VECT) control strain (lanes 1 and 2) produced no *xyn2* mRNA, as expected. The *P. stipitis* (*PGK1-xyn2*) strain (lanes 3 and 4) however also produced no detectable *xyn2* mRNA when cultivated in either xylose or glucose containing medium, showing that the *S. cerevisiae* promoter is not active in *P. stipitis*. The *P. stipitis* (*XYL1-xyn2*) strain (lanes 5 and 6) exhibited a low level of *xyn2* mRNA transcripts when the strain was grown in medium containing glucose, however the amount of transcripts increased in medium containing xylose, suggesting induction of the *XYL1* promoter. In the *P. stipitis* (*TKL-xyn2*) strain (lanes 7 and 8) *xyn2* mRNA was found when the strain was cultivated on either glucose or xylose as carbon source, suggesting constitutive basal expression of the *TKL* promoter. However, mRNA levels were higher when the carbon source was xylose indicating further induction on this carbon source.

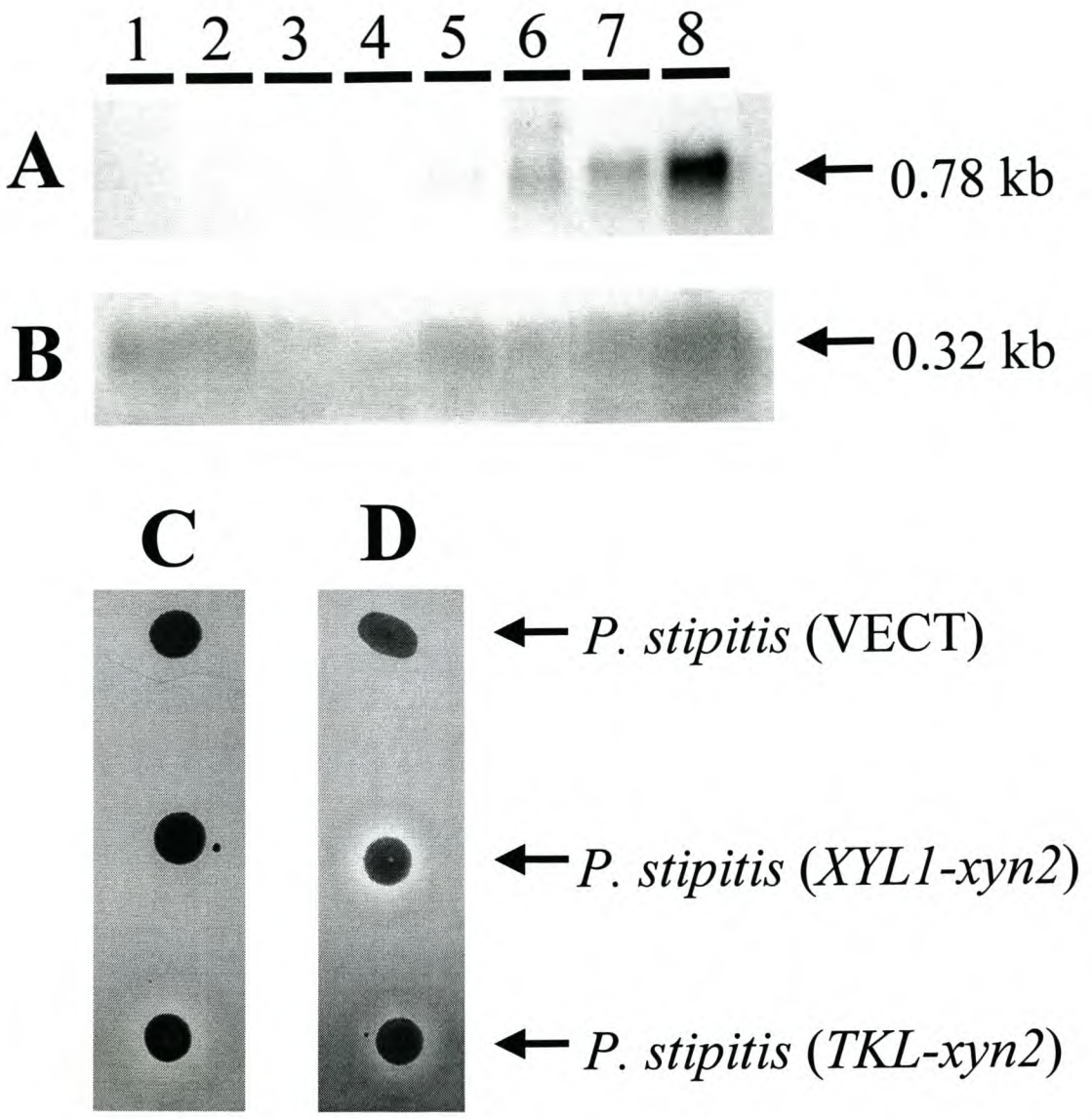

Fig. 2 (**A, B**) Northern blot analysis of total RNA isolated from *P. stipitis* (VECT) (lanes 1 and 2), *P. stipitis* (*PGK1-xyn2*) (lanes 3 and 4), *P. stipitis* (*XYL1-xyn2*) (lanes 5 and 6) and *P. stipitis* (*TKL-xyn2*) (lanes 7 and 8) at 24 h after inoculation. Lanes 1, 3, 5 and 7 represent samples grown on glucose and lanes 2, 4, 6 and 8 represent samples grown on xylose. A 0.78-kb *xyn2* and a 1.5-kb *S. cerevisiae* 5S rDNA fragment was used as α-^{32}P labelled probes in panels **A** and **B**, respectively. Molecular sizes are indicated in kilobases. (**C, D**) Recombinant β-xylanase producing *P. stipitis* strains. SC medium with 0.2% RBB-xylan supplemented with (**C**) 2% glucose or (**D**) 2% xylose as the sole carbon source was patched with *P. stipitis* (VECT), *P. stipitis* (*XYL1-xyn2*) and *P. stipitis* (*TKL-xyn2*). Colonies degrading RBB-xylan are surrounded by pale clearing zones. The plates were photographed after 30 h of incubation at 30°C.

3.4.2 Screening for β–xylanase activity

The *P. stipitis* (VECT) control strain showed no clearing zone formation on RBB-xylan plates containing either xylose or glucose as sole carbon source as no β-xylanase is produced (Fig. 2C, D). The *P. stipitis* (*XYL1-xyn2*) strain showed a clearing zone on the RBB-xylan plate containing xylose but not on the RBB-xylan plate containing glucose, confirming induction of the *XYL1* promoter by xylose. The *P. stipitis* (*TKL-xyn2*) strain showed clearing zone formation on RBB-xylan plates containing xylose and plates containing glucose, indicating constitutive expression from this promoter.

3.4.3 β-xylanase activity

The β-xylanase producing yeast strains were analysed for their ability to secrete biologically active β-xylanase over a period of 90 h (Fig 3A, B). *P. stipitis* (VECT), *P. stipitis* (*XYL1-xyn2*) and *P. stipitis* (*TKL-xyn2*) were cultured on double strength SC^{-URA} medium containing either 5% glucose or 5% xylose. The pH of the cultures was measured prior to cultivation and after 90 h of growth. Only basal levels of β-xylanase activity was produced by the *P. stipitis* (VECT) control strain throughout the 90-h incubation period. The β-xylanase activity of the *P. stipitis* (*XYL1-xyn2*) strain cultured on glucose also remained at basal levels for the 90-h period, but when cultured on xylose the activity reached a maximum level of 143 nkat/ml after 60 h, indicating that the *XYL1* promoter was induced in the presence of xylose. The β-xylanase activity of the *P. stipitis* (*TKL-xyn2*) strain cultured on glucose reached a maximum activity level of 154 nkat/ml after 36 h. When cultured on xylose the maximum activity level of 156 nkat/ml was reached after 48 h. Therefore, expression from the *TKL* promoter is constitutive on media containing either glucose or xylose.

3.4.4 SDS-PAGE analysis of recombinant protein

SDS-PAGE analysis of extracellular protein fractions of the recombinant yeast strains (Fig. 4) showed a distinctive protein species of 20.7 kDa in the extracellular fraction of the *P. stipitis* (*TKL-xyn2*) strain (lane 2) cultivated on xylose, which was absent in the *P. stipitis* (VECT) control strain (lane 1) and thus should represent β-xylanase. This

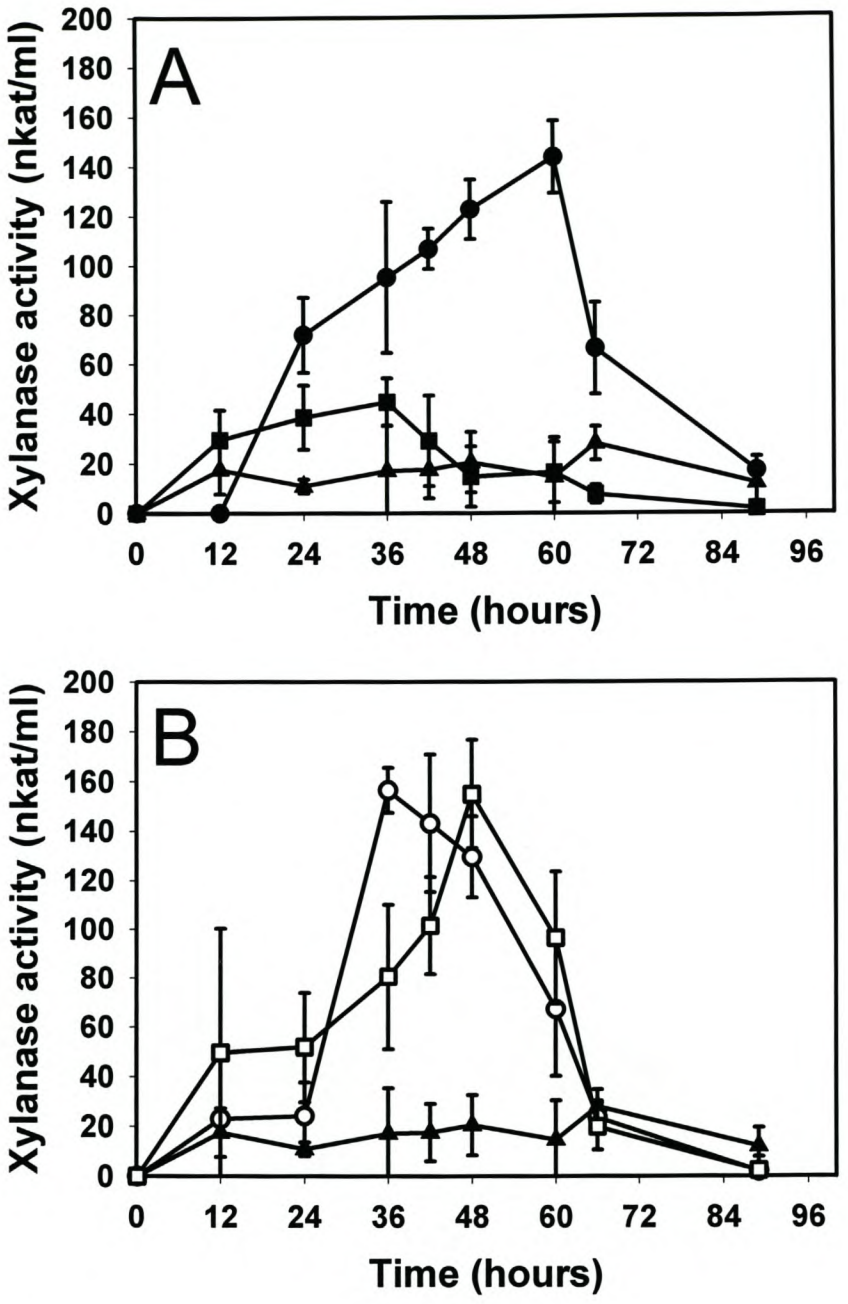

Fig. 3 Time course of β-xylanase produced by (**A**) *P. stipitis* (VECT) (▲) and *P. stipitis* (*XYL1-xyn2*) (filled symbols) and (**B**) *P. stipitis* (VECT) (▲) and *P. stipitis* (*TKL-xyn2*) (open symbols) on glucose (■, □) and xylose (●, ○) as carbon source. Activity is expressed in katals/ml, with 1 katal being the amount of enzyme needed to produce 1 mol of reducing sugar from birchwood xylan per second under the conditions of the assay (Bailey et al. 1992).

corresponds to the size of the native *T. reesei* xylanase (Törrönen et al. 1992), suggesting a very low level of glycosylation. The purified *T. viride* xylanase used as reference (lane 5) has a molecular size of 22.0 kDa (Törrönen et al. 1992). In lane 4 the extracellular protein fraction of the recombinant *S. cerevisiae* (pDLG5) strain showed a distinctive protein species of 27 kDa which was absent in the *S. cerevisiae* control strain (lane 3). This result corresponds to that obtained by La Grange et. al (1996), who showed that the increased size was due to hyperglycosylation of the secreted xylanase.

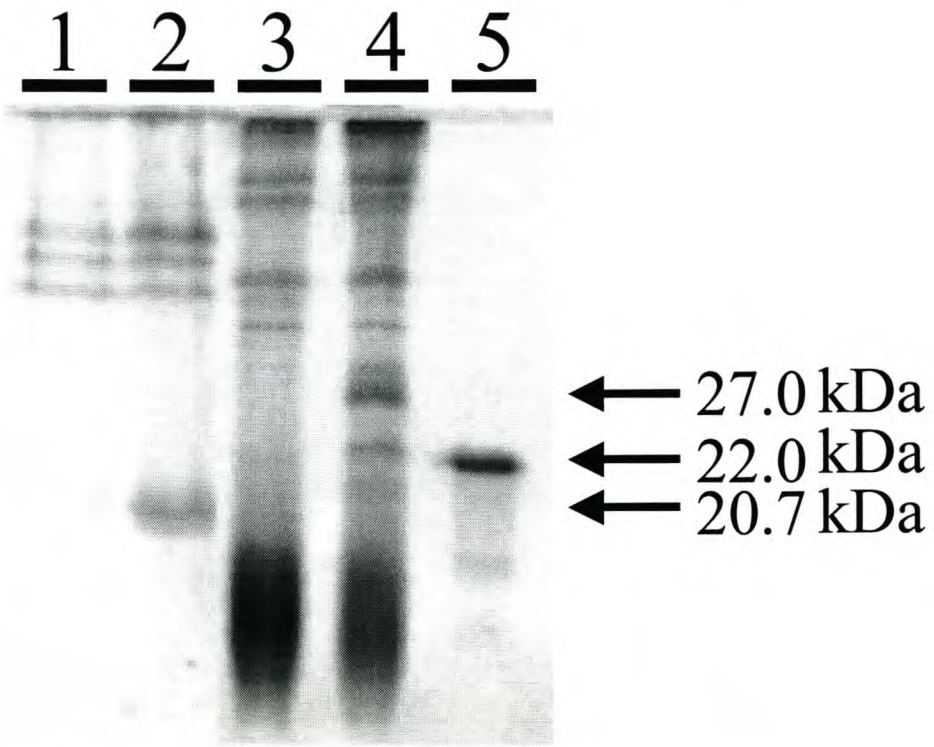

Fig. 4 SDS-PAGE of extracellular protein fractions of *P. stipitis* (VECT) (lane 1) and *P. stipitis* (*TKL-xyn2*) (lane 2) and of *S. cerevisiae* Y294 (VECT) (lane 3) and *S. cerevisiae* Y294 (*XYN2*) (lane 4) (La Grange et al. 2000). Lane 5 contains the 22 kDa xylanase purified from *Trichoderma viride* (Sigma).

3.4.5 Growth of recombinant strains on birchwood xylan as sole carbon source

It has been shown that various *P. stipitis* strains can utilize xylan as sole carbon source (Özcan et al. 1991). The *P. stipitis* (VECT) strain and *P. stipitis* (*TKL-xyn2*) strain were tested for growth on SC minimal medium containing 1% birchwood xylan as sole carbon

source. The *P. stipitis* (*TKL-xyn2*) strain showed a significant increase in biomass production on this substrate when compared to the control strain (Fig. 5).

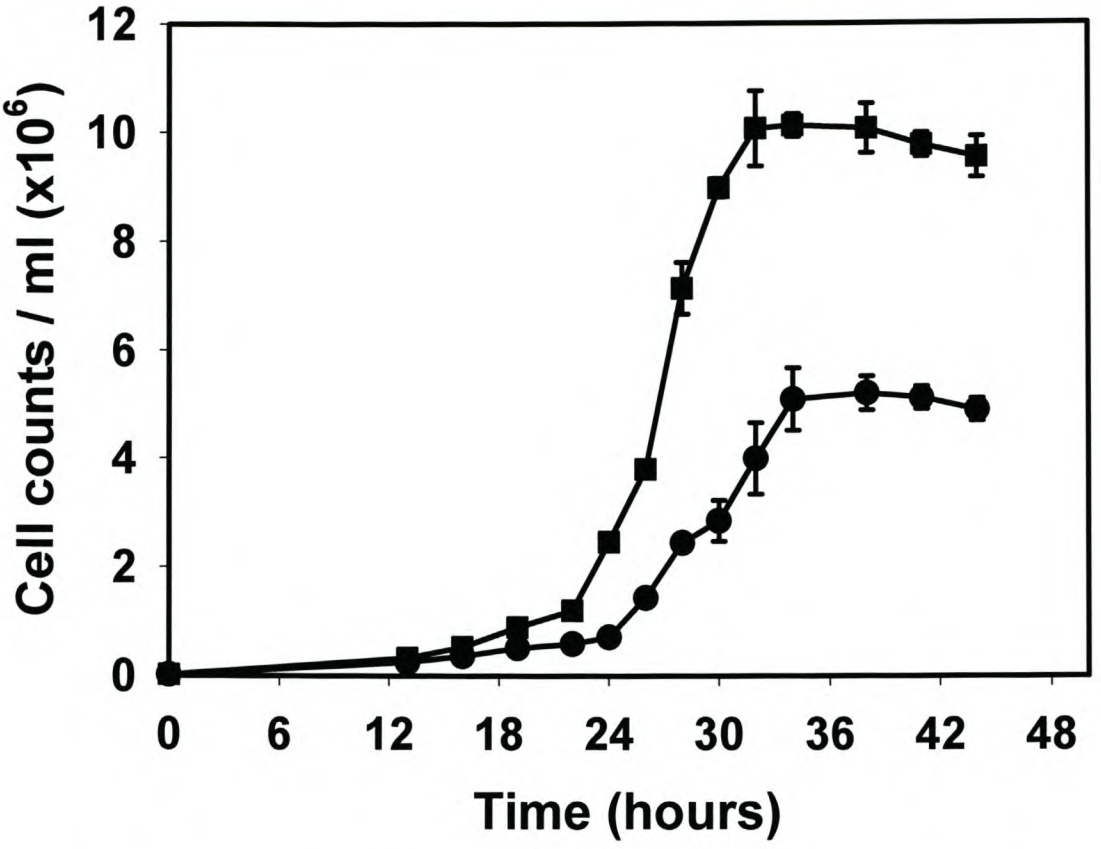

Fig. 5 Growth curve of recombinant *P. stipitis* strains, *P. stipitis* (VECT) (●) and *P. stipitis* (*TKL-xyn2*) (v) on SC medium containing 1% birchwood xylan as sole carbon source.

3.5 DISCUSSION

DNA fragments encoding the *P. stipitis XYL1* promoter, *P. stipitis TKL* promoter and the *S. cerevisiae PGK1* promoter were fused to the *xyn2* gene of *T. reesei* and tested for their ability to promote expression of a heterologous β-xylanase in *P. stipitis*. Xylose reductase is the first enzyme in the xylose assimilation pathway of *P. stipitis* (Amore et al. 1991) and the gene encoding xylose reductase (*XYL1*) is induced in the presence of xylose (Webb and Lee, 1992). The *XYL1* promoter was therefore cloned and evaluated as an inducible promoter in *P. stipitis*, since the use of inducible promoters is preferable for the high-level production of heterologous proteins (Romanos et al. 1992). Transketolase

is an enzyme central to the pentose phosphate pathway, therefore the transketolase gene (*TKL*) should be constitutively expressed as *P. stipitis* catabolises most of its carbohydrates, including hexose sugars, through the pentose phosphate pathway (Jeffries and Kurtzman, 1994; Metzger and Hollenberg, 1994). The *TKL* promoter was therefore chosen as a strong, constitutively expressed promoter for heterologous expression in *P. stipitis*. The *PGK1* promoter is a strong constitutive promoter of *S. cerevisiae* that can be induced to a level of expression that constitutes 4 to 10% of total soluble protein under certain growth conditions (Hadfield et al. 1993). The *PGK1* gene encodes an enzyme that is central in the conserved glycolysis pathway, suggesting the possibility that its promoter sequence would also promote gene expression in *P. stipitis*. The gene encoding the *T. reesei* β-xylanase (*xyn2*), previously cloned and successfully expressed in *S. cerevisiae* (La Grange et al. 1996), was fused downstream of these promoter DNA sequences and transformed to *P. stipitis*. The *xyn2* gene thus provided us with a marker to simultaneously monitor activity of the promoter elements and secretion of the heterologous protein product in *P. stipitis*.

Expression from the *XYL1*, *TKL* and *PGK1* promoters was evaluated on both the mRNA (Fig. 2) and enzyme production (Fig. 3) level. The *P. stipitis* (*XYL1-xyn2*) strain exhibited elevated mRNA levels and β-xylanase activity when cultured on medium containing xylose but not on medium where glucose was the sole carbon source, confirming that *XYL1* promoter expression is induced by xylose or repressed by glucose. Conversely, in the *P. stipitis* (*TKL-xyn2*) strain, *xyn2* mRNA and β-xylanase activity was present both when the yeast was cultivated on glucose or xylose as carbon source, indicating constitutive expression on both carbon sources. However, higher induction was observed at 24 h with cultivation on xylose, corresponding with optimal xylanase production between 24h and 36h (Fig. 3B). The *P. stipitis* (*PGK1-xyn2*) strain showed no *xyn2* mRNA production when cultivated on either glucose or xylose as sole carbon source and also no clearing zone formation on RBB-xylan plates (not shown). The *S. cerevisiae PGK1* promoter is therefore considered not active in *P. stipitis*.

The β-xylanase activities produced by all the recombinant *P. stipitis* strains dropped sharply after maximum activities were obtained (Fig. 3A, B). The pH of these cultures was at pH 5 prior to inoculation. When the pH was measured after 90 hours of growth it had dropped to between pH 2.5 and pH 3. The production of acid proteases might have been responsible for the rapid loss of β-xylanase activity in the recombinant *P. stipitis* strains. This is in accordance with the loss of enzymatic activity for secreted enzymes in the related yeast *P. pastoris* due to protease activity (Sreekrishna et al. 1997) and the observation that many yeast species secrete significant amounts of proteases (Ogrydziak 1993). Maintaining the culture pH above 5.0 may maintain or improve the levels of β-xylanase activity observed.

The *XYL1* gene was successfully overexpressed in *P. stipitis* yielding a yeast strain with increased xylose reductase activity (Dahn et al. 1996). The xylose reductase activity was further elevated under oxygen limitation conditions, suggesting that the *XYL1* promoter might be further induced under these conditions. Using the *XYL1* promoter (Morosoli et al. 1993), and the *PsADH2* promoter (Passoth and Hahn-Hägerdal 2000), successful expression of the *C. albidus* xylanase in *P. stipitis* yielding low levels of β-xylanase activity was obtained. In this study the increased expression levels of *T. reesei xyn2* allowed assessments of the expression levels of the promoter fragments tested as well as giving insights into the post-translational processing of heterologously expressed proteins in *P. stipitis*. The 20.7-kDa heterologous Xyn2 produced in *P. stipitis* corresponded to the molecular size of the native *T. reesei* Xyn2, indicating very low levels of glycosylation (Fig. 4). The heterologous Xyn2 produced in *S. cerevisiae* has a molecular size of 27.5 kDa because of higher levels of glycosylation (La Grange et al. 2000). These results suggested that proteins secreted from *P. stipitis* are not hyperglycosylated as they are in *S. cerevisiae*. Reduced glycosylation during secretion of a heterologous protein is advantageous as many hyperglycosylated secreted enzymes produced in *S. cerevisiae* are inactive or present altered antigenicity (Romanos et al. 1992)

The additional production of recombinant β-xylanase II in *P. stipitis*, a yeast known to produce low xylanase activity when cultured on xylan as sole carbon source (Özcan et al.

1991), should improve its growth on xylan as sole carbon source. The growth of the *P. stipitis* (VECT) control strain in medium containing 1% birchwood xylan as sole carbon source was a result of the low level of native xylanase activity and the residual reducing sugars available in the medium (Fig. 5). We have shown that the *P. stipitis* (*TKL-xyn2*) strain produced significantly more biomass over the *P. stipitis* (VECT) control strain when cultured in medium containing 1% birchwood xylan as sole carbon source (Fig. 5). This was most likely due to higher β-xylanase activity or improved hydrolysis of xylan by the *P. stipitis* (*TKL-xyn2*) strain.

3.6 ACKNOWLEDGEMENTS

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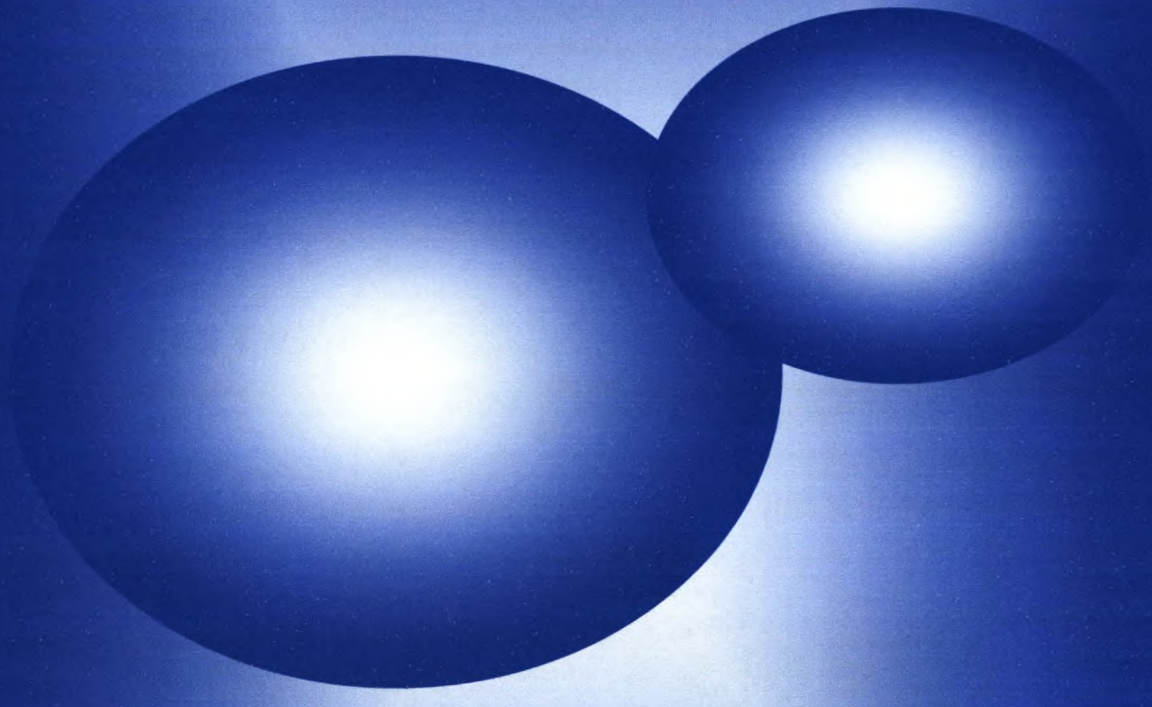
Chapter 4
Paper 2



Enhanced xylan degradation and utilization by *Pichia stipitis* overproducing fungal xylanolytic enzymes

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ENHANCED XYLAN DEGRADATION AND UTILIZATION BY *PICHIA STIPITIS* OVERPRODUCING FUNGAL XYLANOLYTIC ENZYMES

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4.1 ABSTRACT

β-Xylanase encoding genes of *Trichoderma reesei* (*xyn2*) and *Aspergillus kawachii* (*xynC*) were cloned as cDNA copies under transcriptional control of the inducible *Pichia stipitis* xylose reductase gene (*XYL1*) promoter on episomal plasmids pRDH12 and pRDH16, respectively. A cDNA copy of the β-xylosidase encoding gene of *Aspergillus niger* (*xlnD*) was cloned as an in reading frame fusion with the *Saccharomyces cerevisiae MFα1* secretion signal under transcriptional control of the constitutive *P. stipitis* transketolase (*TKL*) gene promoter on an episomal plasmid (pRDH21). Combinations of the individual β-xylanase encoding genes and β-xylosidase expression cassette were also cloned onto episomal plasmids (pRDH22 & pRDH26). All of the plasmids were subsequently transformed to *P. stipitis* TJ26 and the β-xylanase activity, β-xylosidase activity and growth of the recombinant strains on xylan as sole carbon source were monitored. The strains expressing the *A. kawachii xynC* gene reached the highest maximum levels of β-xylanase activity and the activity was sustained for a longer period than the *T. reesei xyn2* expressing strains where the levels of activity declined rapidly after reaching a maximum. All recombinant strains as well as the control strain were shown to produce extracellular protease activity. The strains expressing the *A. niger* (*xlnD*) reached similar levels of β-xylosidase activity, markedly higher than the control strains. The recombinant xylanolytic enzymes, whether produced alone or simultaneously, lead to an increase in biomass production of the recombinant strains when grown on medium containing xylan as sole carbon source. Simultaneous expression of the *A. kawachii xynC* gene and the *A. niger* (*xlnD*) gene gave the highest level of biomass production of any of the recombinant strains.

4.2 INTRODUCTION

Xylan, the major hemicellulose component in plant cell walls, is the most abundant polysaccharide after cellulose [1]. This makes the main constituent sugar of xylan, D-xylose, the second most abundant renewable monosaccharide in nature [2]. The heterogenous xylan polymer consists of a main chain of β-1,4-linked D-xylose residues. The C-2 and C-3 positions of these D-xylose moieties can be substituted with L-arabinofuranose or 4-*O*-methyl glucaronic acid residues, or they can be esterified with acetic acid. Furthermore, the L-arabinofuranosyl residues in the side chains can be esterified with ferulic and *p*-coumaric acid [3,4]. Therefore, complete degradation of this complex structure requires the synergistic action of a range of different enzymes. The main xylanolytic activities are catalysed by endo-β-1,4-xylanases, which hydrolyse β-1,4-bonds between D-xylose residues in the main chain yielding xylo-oligosaccharides. β-D-Xylosidases hydrolyse xylo-oligosaccharides to D-xylose. However, α-L-arabinofuranosidase, α-glucuronidase, acetyl (xylan) esterase, ferulic and *p*-coumaric acid esterase all have specific cooperative functions in the complete degradation of xylan [4].

Many bacterial and fungal species produce the full complement of enzymes necessary to enable them to utilise xylan as a carbon source [5]. Strains of the fungi *Trichoderma* and *Aspergillus* secrete large amounts of efficient xylan-degrading enzymes [6-8]. *Trichoderma reesei* is a filamentous, mesophilic fungus known for its cellulolytic and xylanolytic activities [4,9]. This fungus secretes two major β-1,4-endoxylanases encoded by *xyn1* and *xyn2* [10]. The *xyn2* gene encodes a 21 kDa enzyme that represents 50% of the total xylanolytic activity of *T. reesei* cultivated on xylan. Members of the genus *Aspergillus* are also efficient producers of cellulolytic and xylanolytic enzymes [5,9]. The two β-1,4-endoxylanases and the β-xylosidase of *Aspergillus niger* are encoded by *xlnB*, *xlnC* and *xlnD*, respectively [3]. The *xlnD* gene encodes the 804 amino acid β-xylosidase with a predicted molecular mass of 85 kDa. *Aspergillus kawachii* is a fungus used in the fermentation of traditional Japanese spirits, a process carried out under

acidic conditions (pH 3.2 – 4.3) [11,12]. The main β-1,4-endoxylanase of *A. kawachii* is encoded by *xynC*. This gene has been successfully cloned and sequenced [13,14]

The yeast *Saccharomyces cerevisiae* has been used extensively for the production of ethanol and has also been established as a host for the expression of heterologous proteins of biotechnological interests [15]. Several xylanolytic genes have been successfully expressed in the *S. cerevisiae* [14,16,17]. A *S. cerevisiae* strain has been constructed that co-expresses the *T. reesei xyn2* gene and the *A. niger xlnD* gene [17]. This strain effectively degrades birchwood xylan to D-xylose, however, the main disadvantage of *S. cerevisiae* is that it cannot utilise or ferment D-xylose, the main component of xylan.

The yeast *Pichia stipitis* and the related *Candida shehatae* are the best xylose-fermenting yeasts thus far described [18]. It has also been shown that various *P. stipitis* strains can utilise xylan as sole carbon source [19,20]. The β-1,4-endoxylanase encoding gene of *P. stipitis*, *xylA*, was cloned and successfully expressed in *Escherichia coli* [20]. However, xylan-degrading *P. stipitis* strains isolated produce very low levels of xylanolytic enzymes. In this study we have worked toward enhancing the xylan degrading ability of *P. stipitis* for the potential direct conversion of xylan to ethanol. This was effected by expressing the *T. reesei xyn2*, the *A. kawachii xynC* and the *A.niger xlnD* genes separately in *P. stipitis*. Furthermore, the *T. reesei xyn2* and the *A.niger xlnD* genes, as well as the *A. kawachii xynC* and the *A.niger xlnD* genes, were co-expressed. The β-xylanase activity, β-xylosidase activity and growth of the recombinant strains on xylan as sole carbon source were evaluated.

4.3 MATERIALS AND METHODS

4.3.1 Media and culture conditions

P. stipitis was cultivated on YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or selective synthetic (SC) medium [containing 20 g/L glucose or xylose, 1.7 g/L yeast nitrogen base without amino acids (Difco) (pH 6) and all the required growth factors except uracil (SC^{-URA})]. Solid media contained 20 g/L agar. Yeasts were

routinely cultured in 250-ml Erlenmeyer flasks containing 100 ml medium at 30°C on a rotary shaker at 150 rpm.

4.3.2 Microbial strains and plasmids

The genotypes and sources of the yeast and bacterial strains, as well as the plasmids that were constructed and used in this study, are summarised in Table 1.

4.3.3 Plasmid construction and transformation

Standard protocols were followed for DNA manipulations [23]. Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Tautz and Renz [24]. Restriction endonucleases and T4 DNA ligase were purchased from Roche Molecular Biochemicals and used as recommended by the manufacturer. The construction of two inducible expression vectors, containing the *XYL1* promoter sequences, pRDH10 and pRDH12, was described previously [22]. The constitutive expression vector pRDH20 was created by cloning the DNA elements encoding the *P. stipitis TKL* promoter, *TKL* terminator, *ARS2* and *URA3* from *P. stipitis* genomic DNA using PCR amplification and inserting them into pBluescriptII SK(+). DNA was amplified in 50-µl reaction mixtures using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) according to the manufacturers instructions, with a Perkin Elmer GeneAmp® PCR System 2400 (The Perkin-Elmer Corporation, 761 Main Avenue, Norwalk, Connecticut 06859). Denaturation, and polymerisation were carried out for 1 min at 94°C, and 1 min at 72°C, respectively for 30 cycles. Annealing was done at the temperatures stated in table 2 for 30 seconds. The primers for the amplification of the *P. stipitis ARS2* and *URA3* were described previously [22]. Table 2 details the primers and annealing temperatures used in this study (restriction enzyme sites are underlined). Fig. 1 summarises the plasmids that were used in this study. The *A. kawachii xynC* gene was cloned as a 0.65-kb *Xba*I/*Bam*HI fragment from pJC3 [14] into the *Xba*I/*Bam*HI sites of pRDH10 to yield pRDH16. For the construction of pRDH20 the PCR amplification products were digested with restriction endonucleases and the DNA fragments were purified from agarose gels and sequentially cloned into pBluescript II SK(+). The *TKL* promoter was cloned as a 0.36-kb *Sac*I/*Xba*I fragment into pBluescript. The *TKL* terminator was subsequently cloned as a 0.15-kb *Hin*dIII/*Sal*I

fragment adjacent to the *TKL* promoter region to yield pRDH18 (containing the *TKL* promoter/terminator cassette). The *URA3* fragment was cloned as a 1.46-kb *Sal*I fragment into the compatible *Xho*I site of pRDH18 to yield pRDH19 and finally the *ARS2* fragment was cloned as a 1.15-kb *Sal*I fragment into the unique *Sal*I site of pRDH19 to yield pRDH20. The *A. niger xlnD* gene was previously fused in reading frame with the *S. cerevisiae MFα1* secretion signal [17]. The fused product was designated *XLO2*. This fusion gene was cloned as a 2.62-kb *Xba*I/*Bam*HI fragment from pDLG55 into the *Xba*I/*Bam*HI sites of pRDH20 to yield pRDH21. The TKL_P-*XLO2*-TKL_T cassette was cloned as a 3.15-kb *Apa*I fragment from pRDH21 into the unique *Apa*I site of pRDH12 to yield pRDH22 and into the unique *Apa*I site of pRDH16 to yield pRDH26. DNA transformation of *P. stipitis* was performed using the lithium acetate dimethylsulfoxide (DMSO) method described by Hill et al. [26].

Table 1 Microbial strains and plasmids

Strain/Plasmid	Genotype	Source/Reference
Yeast strains:		
Saccharomyces cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	This laboratory
Pichia stipitis TJ26	*ura3*	[21]
P. stipitis TJ26: pJM6[a]	*ura3/[URA3]*	[22]
pRDH12[b]	*ura3/[URA3* $XYL1_P$-*xyn2*-$XYL1_T$*]*	[22]
pRDH16[c]	*ura3/[URA3* $XYL1_P$-*xynC*-$XYL1_T$*]*	This work
pRDH21[d]	*ura3/[URA3* TKL_P-*XLO2*-TKL_T*]*	This work
pRDH22[e]	*ura3/[URA3* $XYL1_P$-*xyn2*-$XYL1_T$ TKL_P-*XLO2*-TKL_T*]*	This work
pRDH26[f]	*ura3/[URA3* $XYL1_P$-*xynC*-$XYL1_T$ TKL_P-*XLO2*-TKL_T*]*	This work
Bacterial strain:		
Escherichia coli XL1-Blue MRF'	*Δ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[*F' proAB* $lacI^qZ\Delta M15$ *Tn10* (Tet^r)]	Stratagene
Plasmids:		
pbluescript II SK(+)	*bla*	Stratagene
pDLG55	*bla URA3* $ADH2_P$-*XLO2*-$ADH2_T$	[17]
pJC3	*bla URA3* $PGK1_P$-*xynC*-$PGK1_T$	[14]
pJM6	*bla URA3*	[21]
pRDH12	*bla URA3* $XYL1_P$-*xyn2*-$XYL1_T$	[22]
pRDH16	*bla URA3* $XYL1_P$-*xynC*-$XYL1_T$	This work
pRDH21	*bla URA3* TKL_P-*XLO2*-TKL_T	This work
pRDH22	*bla URA3* $XYL1_P$-*xyn2*-$XYL1_T$ TKL_P-*XLO2*-TKL_T	This work
pRDH26	*bla URA3* $XYL1_P$-*xynC*-$XYL1_T$ TKL_P-*XLO2*-TKL_T	This work

[a] *P. stipitis* TJ26 (pJM6) was designated *P. stipitis* [VECT]
[b] *P. stipitis* TJ26 (pRDH12) was designated *P. stipitis* [*xyn2*]
[c] *P. stipitis* TJ26 (pRDH16) was designated *P. stipitis* [*xynC*]
[d] *P. stipitis* TJ26 (pRDH21) was designated *P. stipitis* [*XLO2*]
[e] *P. stipitis* TJ26 (pRDH22) was designated *P. stipitis* [*xyn2 XLO2*]
[f] *P. stipitis* TJ26 (pRDH26) was designated *P. stipitis* [*xynC XLO2*]

Table 2 PCR primers

Gene/fragment and annealing temperature used	Primers	Source of DNA sequence
A. kawachii xynC 60°C	XYNCL (*Xba*I restriction site underlined) 5'- GACTTCTAGACATGAAGGTCACTGCGG -3' XYNCR (*Bam*HI restriction site underlined) 5'- GACTGGATCCCCCTTAAGAGGAGATCGTG -3'	[14]
P. stipitis TKL promoter 55°C	TKLPL (*Sac*I and *Apa*I restriction sites underlined) 5'- GACTGAGCTCGGGCCCCCAGAGTTCATGCTACTCAAC -3' PTKLR (*Xba*I restriction site underlined) 5'-GTACTCTAGAGGCAAATGGAAGTCTGG-3	[25]
P. stipitis TKL terminator 55°C	TKLTL(*Hin*dIII restriction site underlined) 5'- GACTAAGCTTTTCAAGTTTTGGTTCTAGC -3' TKLTR (*Sal*I and *Apa*I restriction sites underlined) 5'- GACTGTCGACGGGCCCTATTCCATATTTTTCTGAACT -3'	[25]
S. cerevisiae MFα1 *A niger xlnD* fusion (*XLO2*) 60°C	MFXLNL (*Xba*I restriction site underlined) 5'- TGACTCTAGAATGAGATTTCCTTCAATTTTTAC -'3 MFXLNR (*Bam*HI restriction site underlined) 5'- TGACGGATCCTACTTTCTACTCCTTCCCAGG -3'	[17]

4.3.4 Screening for β-xylanase and β-xylosidase activity

Transformants containing fungal β-1,4-endoxylanase encoding genes were screened for xylan-degrading ability by patching on SC^{-URA} medium containing 0.2% 2-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB)-xylan (Sigma) containing 20 g/L xylose as carbon source [27]. Transformants expressing *A. niger* β-xylosidase were screened by patching on SC^{-URA} medium containing 1 mM *p*-nitrophenyl-β-D-xyloside (PNPX) (Sigma) [28].

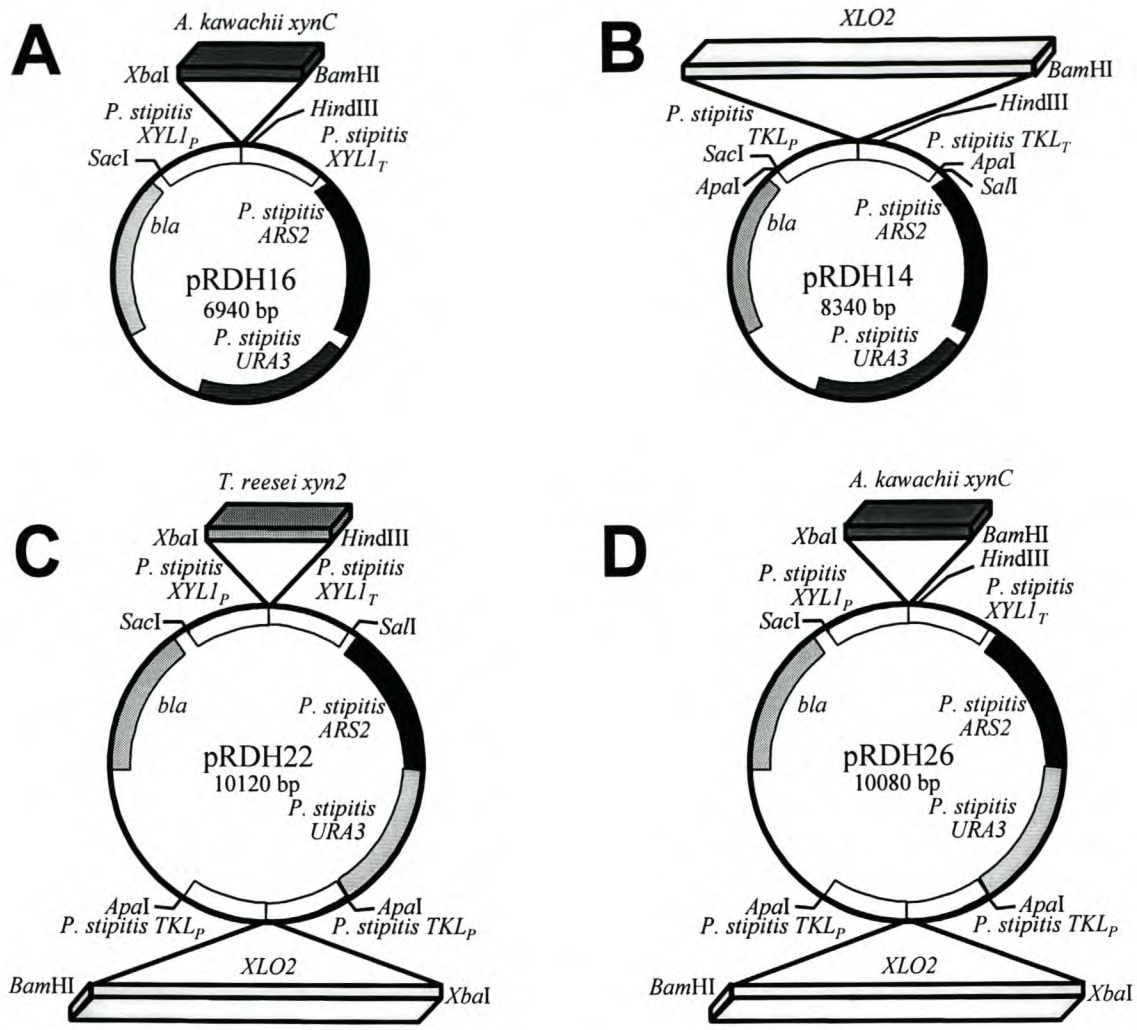

Fig. 1 Schematic representation of the plasmids used in this study. The *P. stipitis* autonomous replicating sequence (*ARS2*) is responsible for episomal replication of the plasmid and the *P. stipitis* orotidine-5'-phosphate decarboxylase (*URA3*) is used as selectable marker. $XYL1_P$, $XYL1_T$, $TKL1_P$, $TKL1_T$, represents the *P. stipitis* xylose reductase gene promoter and terminator sequences as well as the *P. stipitis* transketolase gene promoter and terminator sequences, respectively.

4.3.5 β-Xylanase and β-xylosidase activity assays

β-Xylanase and β-xylosidase producing cultures were grown in 50 ml double strength SC^{-URA} medium containing 40 g/L xylose as carbon source. Three cultures of every transformant were inoculated and all enzyme activity determinations were done in triplicate. Samples were periodically taken over a 120 h period. Endo-β-1,4-xylanase

activity was assayed according to the method described by Bailey et al. [29] with 1% birchwood glucuronoxylan (Roth) as the substrate at 50°C for 5 minutes. Appropriate dilutions of the cell free culture solution in 50 mM sodium citrate buffer (pH5.0 for transformants containing the *T. reesei xyn2* gene and pH4.0 for transformants containing the *A. kawachii xynC* gene) were used as the enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid (DNS) method described by Miller et al. [30]. The β-xylanase activity of the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains was measured at pH 4, this is higher than the optimum pH level of the *A. kawachii xynC* gene product [14]. The higher pH level was used since the DNS assay employed for β-xylanase activity measurement becomes unreliable below pH 4 because of spontaneous substrate hydrolysis. The β-xylosidase activity was quantified using the chromophoric substrate *p*-nitrophenyl-β-D-xyloside (PNPX) [28]. PNPX was used at a concentration of 5 mM. The supernatant with intact cells was used as source of β-xylosidase for the activity determination assays. All activities were expressed in katals per millilitre; one katal is the amount of enzyme needed to produce 1 mol of reducing sugar (or D-xylose equivalent) from birchwood xylan (or chromophoric substrate) per second [29].

4.3.6 Analysis of extracellular protease activity

All *P. stipitis* transformants were screened for extracellular protease activity by patching on SC^{-URA} medium containing 20 g/L skim milk powder and 20 g/L xylose as carbon source [31].

4.3.7 Growth of *P. stipitis* transformants on xylan

P. stipitis transformants were inoculated from precultures in the stationary growth phase into 50 ml double strength SC^{-URA} medium containing 20 g/L birchwood glucuronoxylan (Sigma) as the sole carbon source. The *P. stipitis* [VECT] strain was also grown on double strength SC^{-URA} medium with 20 g/L xylose as the carbon source in order to set a reference of the growth of *P. stipitis*. Three cultures of each strain were inoculated simultaneously. Samples were periodically taken over a 110 h period and yeast cells in the media were counted in triplicate on a haemocytometer.

4.4 RESULTS

4.4.1 Expression of the *xyn2*, *xynC* and *XLO2* genes in *P. stipitis*

P. stipitis TJ26 was transformed with episomal vectors expressing the *xyn2* gene of *T. reesei* under the transcriptional control of the inducible *P. stipitis XYL1* promoter, the *xynC* gene of *A. kawachii* under the control of the *P. stipitis XYL1* promoter as well as the *XLO2* fusion gene under the control of the constitutive *P. stipitis TKL* promoter. Furthermore, an episomal vector containing both the $XYL1_P$-*xyn2*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes and an episomal vector containing the $XYL1_P$-*xynC*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes were transformed to *P. stipitis* TJ26. The transformation frequency of the recombinant plasmids did not vary greatly and was generally $1x10^3$ colonies per μg DNA. Transformants were confirmed by Southern blot hybridisation using either a α-^{32}P labelled 0.78-kb *xyn2* fragment, a α-^{32}P labelled 0.65-kb *xynC* fragment or a α-^{32}P labelled 2.2-kb *xlnD* fragment as DNA probes (results not shown).

4.4.2 Screening for β-xylanase and β-xylosidase activity

The *P. stipitis* [VECT] and *P. stipitis* [*XLO2*] strains showed no clearing zone formation on the RBB-xylan plates after 30 hours incubation at 30°C (Fig. 2). This was expected, as these strains do not overexpress a fungal xylanase. The *P. stipitis* [*xyn2*], *P. stipitis* [*xyn2 XLO2*], *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains showed clearing zone formation on the RBB-xylan plate, confirming endoxylanase production. The *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] strains showed no yellow zone formation on the SC^{-URA} plates containing *p*-nitrophenyl-β-D-xyloside (not shown) as these strains do not produce significant amounts of β-xylosidase. However, the *P. stipitis* [*XLO2*] *P. stipitis* [*xyn2 XLO2*] *P. stipitis* [*xynC XLO2*] showed yellow zone formation on the SC^{-URA} plates containing *p*-nitrophenyl-β-D-xyloside (not shown).

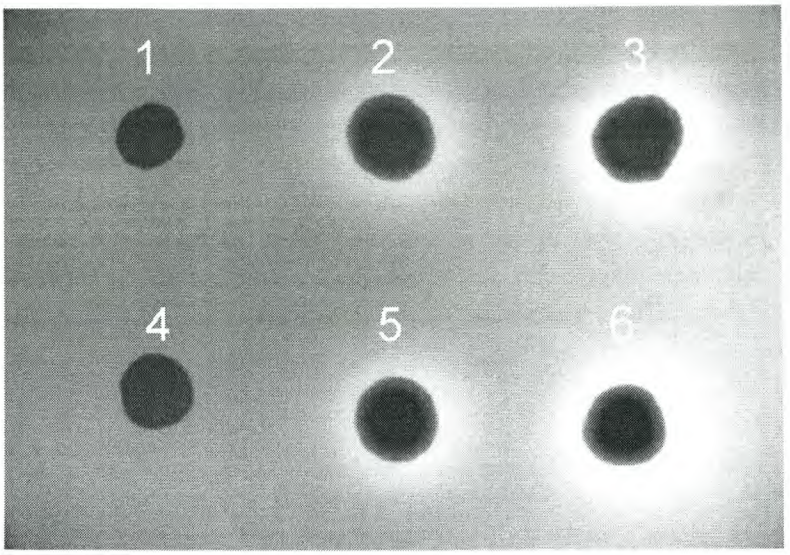

Fig. 2 Recombinant β-xylanase producing *P. stipitis* strains. SC^{-URA} medium with 0.2% RBB-xylan supplemented with 20 g/L xylose as the carbon source was patched with (1) *P. stipitis* [VECT], (2) *P. stipitis* [*xyn2*], (3) *P. stipitis* [*xyn2 XLO2*] (4) *P. stipitis* [*XLO2*] (5) *P. stipitis* [*xynC*] and (6) *P. stipitis* [*xynC XLO2*]. Colonies degrading RBB-xylan are surrounded by pale clearing zones. The plates were photographed after 30 h of incubation at 30°C.

4.4.3 β-xylanase activity

The β-xylanase producing yeast strains were analysed for their ability to secrete biologically active β-xylanase over a period of 120 h (Fig. 3). *P. stipitis* [VECT], *P. stipitis* [*xyn2*], *P. stipitis* [*xynC*], *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] were cultured on double strength SC^{-URA} medium containing 40 g/L xylose. Only basal levels of β-xylanase activity were produced by the *P. stipitis* [VECT] control strain and the *P. stipitis* [*XLO2*] strain throughout the 120-h incubation period. The *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains showed maximal levels of β-xylanase activity of 136.7 ± 5.6 nkat/ml and 128.1 ± 5.3 nkat/ml respectively after 48 h of incubation. After 48 h there was a decline in the level of activity and the β-xylanase activity of these strains had dropped to levels close to zero at 120 h of incubation. The *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains showed maximal levels of β-xylanase activity of 171.8 ± 8.7 nkat/ml and 165.1 ± 2.3 nkat/ml respectively after 50 h of incubation. The levels of β-xylanase activity produced by these strains are

significantly higher than the levels produced by the *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains. Furthermore, although the β-xylanase activity of the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains declined after 50 h, the levels were still above 50 nkat/ml at the 120 h point. The pH of the cultures was measured periodically throughout the 120-h period and was found to have dropped from pH 6 to pH 2.5 (Fig. 3).

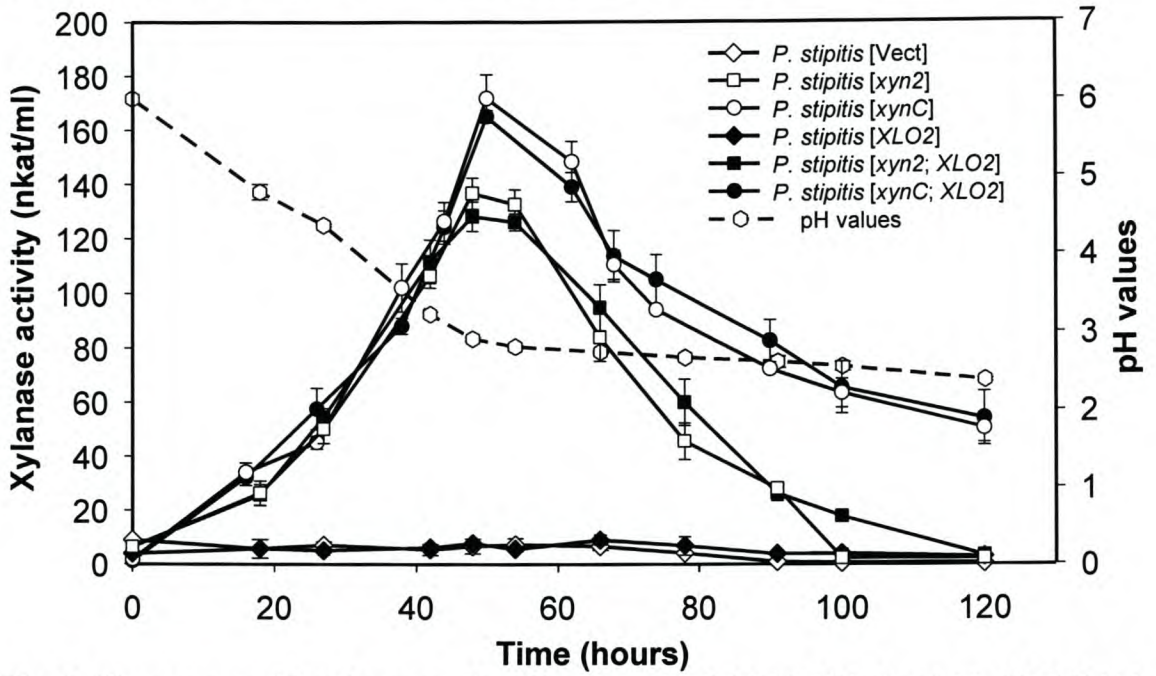

Fig. 3 Time course of β-xylanase produced by *P. stipitis* [VECT] (◇), *P. stipitis* [*XLO2*] (♦), *P. stipitis* [*xyn2*] (□), *P. stipitis* [*xyn2 XLO2*] (■), *P. stipitis* [*xynC*] (○) and *P. stipitis* [*xynC XLO2*] (●), on 40 g/L xylose as carbon source. The average pH level of the cultures are given as a dashed line. Activity is expressed in katals/ml, with 1 katal representing the amount of enzyme needed to produce 1 mol of reducing sugar from birchwood xylan per second under the conditions of the assay [29].

4.4.4 β-xylosidase activity

The β-xylosidase producing yeast strains were analysed for their ability to produce biologically active β-xylosidase over a period of 120 h (Fig. 4). These assays were done simultaneously with the β-xylanase assays, using the same cultures. The β-xylosidase activity of the *P. stipitis* [VECT] control strain, the *P. stipitis* [*xyn2*] strain and the *P. stipitis* [*xynC*] strain remained at low levels (≤ 0.04 nkat/ml) throughout the 120 h incubation period. The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC*

XLO2] strains showed maximal levels of β-xylosidase activity of 0.132 ± 0.007 nkat/ml, 0.113 ± 0.005 nkat/ml and 0.124 ± 0.003 nkat/ml, respectively, after 90 h of incubation. The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] strains showed levels of β-xylosidase activity of 0.092 ± 0.004 nkat/ml, 0.090 ± 0.004 nkat/ml and 0.110 ± 0.006 nkat/ml, respectively, after 120 h of incubation. Although the β-xylosidase activity declined after 90 h of incubation, the decline was not as dramatic as was observed with the β-xylanase activity.

4.4.5 Extracellular protease activity

All the recombinant *P. stipitis* strains showed clearing zone formation on the 20 g/L skim milk plates after 30 hours incubation at 30°C, confirming the production of extracellular proteases in these strains (Fig. 5). Proteinase K solution (Roche) was spotted on the plate to serve as positive control and a laboratory strain of *S. cerevisiae* served as extracellular protease negative control.

4.4.6 Growth of recombinant strains on birchwood xylan as sole carbon source

It has been shown that various *P. stipitis* strains can utilise xylan as sole carbon source [19,22]. The *P. stipitis* recombinant strains were tested for growth on double strength SC^{-URA} minimal medium containing 20 g/L birchwood glucuronoxylan as the sole carbon source. The amount of yeast growth was monitored over a 110 h period by means of cell counts (Fig. 6). Cell counts were used as an indication of the biomass production. The biomass yield of *P. stipitis* [VECT] on medium containing 20 g/L xylose as sole carbon source was also monitored to determine maximum *P. stipitis* growth. The maximum cell counts achieved by the different recombinant strains grown

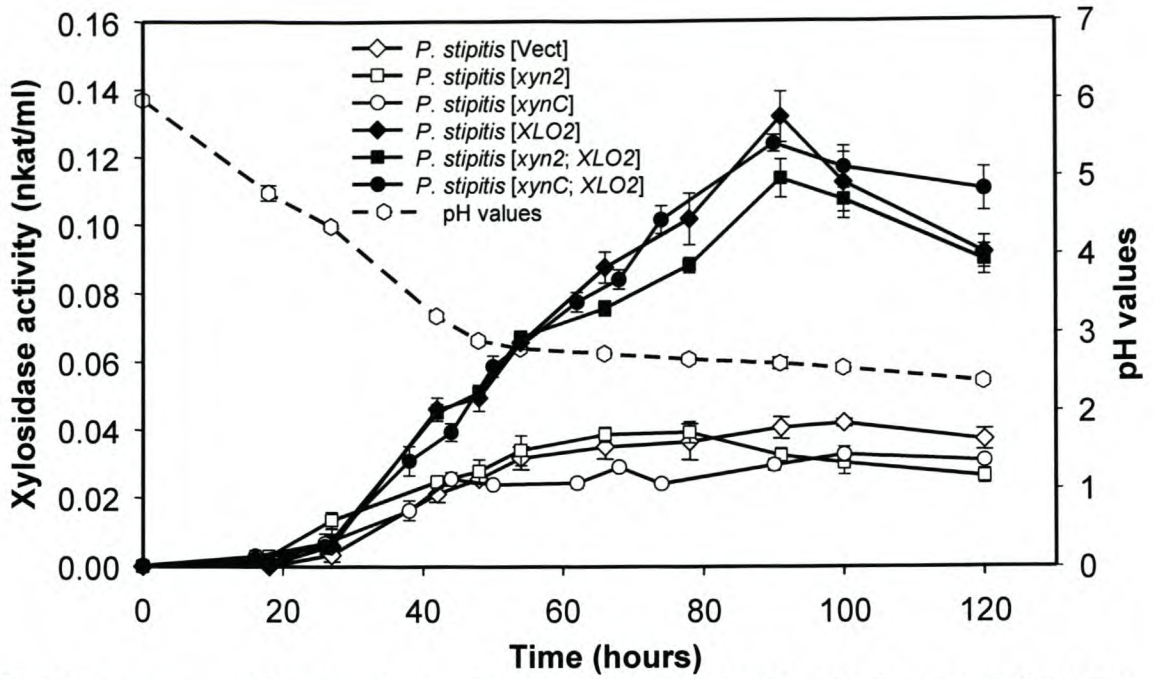

Fig. 4 Time course of β-xylosidase produced by *P. stipitis* [VECT] (◇), *P. stipitis* [*XLO2*] (♦), *P. stipitis* [*xyn2*] (□), *P. stipitis* [*xyn2 XLO2*] (■), *P. stipitis* [*xynC*] (○) and *P. stipitis* [*xynC XLO2*] (●), on 40 g/L xylose as carbon source. The average pH level of the cultures are given as a dashed line. Activity is expressed in katals/ml, with 1 katal representing the amount of enzyme needed to produce 1 mol of reducing sugar equivalent from the chromophoric substrate (PNPX) per second.

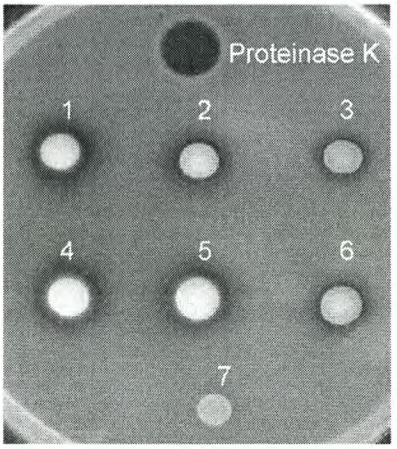

Fig. 5 Extracellular protease producing *P. stipitis* strains. SC^{-URA} medium with 20 g/L skim milk powder supplemented with 20 g/L xylose as the carbon source was patched with (1) *P. stipitis* [VECT], (2) *P. stipitis* [*xyn2*], (3) *P. stipitis* [*xyn2 XLO2*], (4) *P. stipitis* [*XLO2*], (5) *P. stipitis* [*xynC*] (6) *P. stipitis* [*xynC XLO2*], and (7) *S. cerevisiae* Y294. The plates were photographed after 30 h of incubation at 30°C. Proteinase K solution (Roche) was spotted on the plate to serve as positive control 3 hours before the plates were photographed.

on xylan, as well as the percentage of biomass yield achieved with regard to *P. stipitis* [VECT] grown on xylose and the percentage of improvement of the recombinant strains over *P. stipitis* [VECT] grown on xylose are given in Table 3. The heterologous expression of either *XLO2*, *xyn2* or *xynC* in *P. stipitis* clearly results in increased biomass yields on xylan. When *XLO2* is co-expressed with either *xyn2* or *xynC*, a further significant increase in biomass production on xylan was observed. The co-expression of *XLO2* and *A. kawachii xynC* gave the highest increase of biomass yield on xylan, approximately 27% higher than observed for the parental strain.

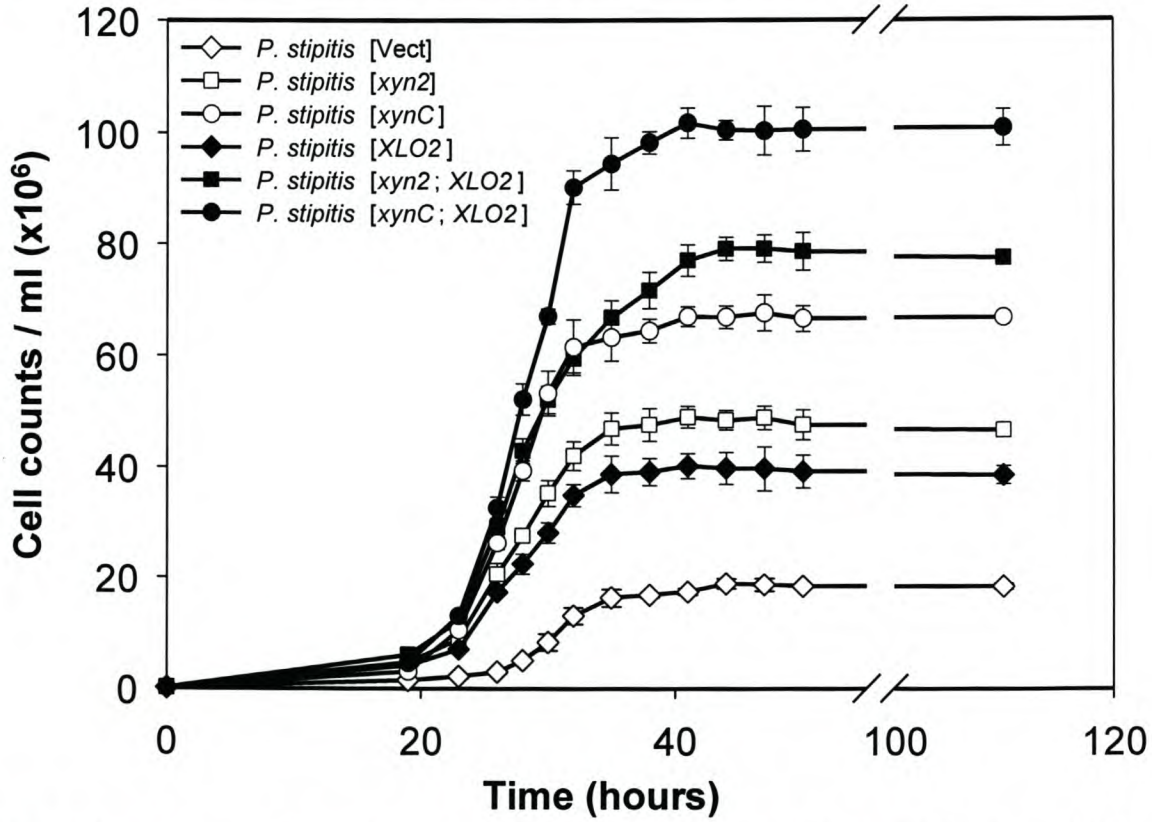

Fig. 6 Growth curve of recombinant *P. stipitis* strains, *P. stipitis* [VECT] (◇), *P. stipitis* [*XLO2*] (♦), *P. stipitis* [*xyn2*] (□), *P. stipitis* [*xyn2 XLO2*] (■), *P. stipitis* [*xynC*] (○) and *P. stipitis* [*xynC XLO2*] (●), on double strength SC^{-URA} medium containing 20 g/L birchwood xylan as sole carbon source.

4.5 DISCUSSION

Xylan, as the major hemicellulose component in plant cell walls, and thus the second most abundant polysaccharide after cellulose, represents one of the most important targets in the exploitation of renewable carbon resources. Therefore, for efficient

Table 3 Maximum levels of biomass achieved by recombinant *P. stipitis* strains

Recombinant strain	Maximum cell count achieved on 20 g/L xylan (10^6 cells per ml)	Percentage of theoretical maximum cell count*	Percentage of improvemen over control strain
P. stipitis [VECT]	18.9 ± 0.87	6.1 ± 0.28	(control strain)
P. stipitis [*XLO2*]	40.3 ± 2.22	13.0 ± 0.72	6.9 ± 0.72
P. stipitis [*xyn2*]	49.1 ± 1.94	15.8 ± 0.63	9.7 ± 0.63
P. stipitis [*xynC*]	67.6 ± 3.21	21.8 ± 1.04	15.7 ± 1.04
P. stipitis [*xyn2 XLO2*]	79.1 ± 2.09	25.5 ± 0.67	19.4 ± 0.67
P. stipitis [*xynC XLO2*]	102 ± 2.66	32.8 ± 0.86	26.7 ± 0.86

*Maximum theoretical cell count was taken as the maximum cell count achieved when *P. stipitis* [VECT] was grown on 2% xylose as sole carbon source (3.10E+08 cells/ml).

utilization of xylan as renewable resource, the main component of xylan, D-xylose, must be utilized efficiently. The yeasts *P. stipitis* and *C. shehatae* are the best xylose-fermenting yeasts known and it has been shown that some *P. stipitis* strains can utilise xylan as sole carbon source [18-20]. In this study we have worked towards enhancing the xylan degrading ability of *P. stipitis*. We have shown successful co-expression of heterologous genes in *P. stipitis* and that the over-expression of fungal xylanolytic enzymes results in increased biomass production when the yeast was grown on xylan as sole carbon source. The *T. reesei xyn2* and *A. kawachii xynC* β-xylanase encoding genes were cloned under transcriptional control of the *P. stipitis* xylose reductase gene (*XYL1*) promoter that was previously shown to be induced in the presence of xylose [22,32]. The *A. niger* β-xylosidase encoding gene (*xlnD*) fused in reading frame with the *S. cerevisiae* *MFα1* secretion signal (designated *XLO2*) [17] was placed under transcriptional control of the constitutive *P. stipitis* transketolase encoding gene (*TKL*) promoter. Recombinant strains with combinations of the $XYL1_P$-*xyn2*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes and the $XYL1_P$-*xynC*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes were also generated. All vectors were transformed to *P. stipitis* TJ26 and maintained episomally under auxotrophic selection.

When β-xylanase activities of the recombinant *P. stipitis* strains were determined quantitatively, the *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains reached maximum activity levels of 136.7 ± 5.6 nkat/ml and 128.1 ± 5.3 nkat/ml respectively, but dropped to levels close to zero after 120 h (Fig. 3). In contrast, the *P. stipitis* [*xynC*] and *P. stipitis*

[*xynC XLO2*] strains reached maximum activity levels of 171.8 ± 8.7 nkat/ml and 165.1 ± 2.3 nkat/ml respectively, and remained at activity levels above 50 nkat/ml after 120 h of incubation. The β-xylanase activity of the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains was measured at pH 4 which is higher than the optimum pH level of the *A. kawachii* XynC β-xylanase [13]. The values achieved are therefore likely to underestimate the actual β-xylanase activity achieved. The decline in the β-xylanase activities of the recombinant *P. stipitis* strains is likely due to a combination of factors. The *XYL1* promoter is induced by D-xylose and the depletion of xylose during the time course of the experiment will lead to a loss of *XYL1* promoter induction. It was found that when xylose was added to the heterologous β-xylanase expressing cultures at 120 h a marked increase in β-xylanase activity was observed (data not shown). However, the depletion of D-xylose alone could not explain why the β-xylanase activity already accumulated was lost. When *P. stipitis* transformants were patched onto milk plates to screen for extracellular protease activity all transformants showed clearing zones, confirming the presence of extracellular proteases (Fig. 5). This is in accordance with the loss of enzymatic activity for secreted enzymes in the related yeast *P. pastoris* due to protease activity [33] and the observation that many yeast species secrete significant amounts of proteases [34]. The synthesis of extracellular proteases by the recombinant *P. stipitis* strains explains the loss of β-xylanase activity already accumulated. The *A. kawachii xynC* expressing strains retained more β-xylanase activity, probably because the *A. kawachii xynC* gene product was more stable at the lower pH level. This might be because the fungus *A. kawachii* naturally occurs in low pH environments and its β-xylanase may therefore be more resistant to acid protease degradation [11,14].

When β-xylosidase activities of the recombinant *P. stipitis* strains were determined quantitatively, the *P. stipitis* [VECT], the *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] strains showed similar low levels of β-xylosidase activity attributed to native *P. stipitis* β-xylosidase activity (Fig. 4). The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] strains achieved distinctly higher levels of β-xylosidase activity of 0.132 ± 0.007 nkat/ml, 0.113 ± 0.005 nkat/ml and 0.124 ± 0.003 nkat/ml, respectively.

Furthermore, there was no sudden decline of the β-xylosidase activity as was observed with the β-xylanase activity. This was likely due to the fact that most of the β-xylosidase activity was found to be cell associated and not free in the supernatant [17]. Proteins in the supernatant seem to be more vulnerable to protease degradation, as is the case with β-xylanase. It is notable that the co-expression of the heterologous β-xylanases and β-xylosidase did not result in significantly lower levels of activity of the two enzymes in comparison with strains where they were expressed separately, as was observed in *S. cerevisiae* [17,35].

The additional production of recombinant β-xylanases in *P. stipitis*, a yeast known to produce low xylanase activity when cultured on xylan as sole carbon source [19], enhanced its growth on xylan as sole carbon source [22]. In our study the recombinant strains over-expressing fungal xylanolytic enzymes show a dramatic increase in biomass formation on xylan as sole carbon source (Fig. 6, Table 3). The expression of the *XLO2* gene alone also resulted in a marked increase of 6.9 ± 0.72% in biomass formation over the control strain. This could be attributed to very weak native *P. stipitis* β-xylosidase activity, as is the case with the native β-xylanase activity [20]. The production of either of the fungal β-xylanases resulted in higher cell counts, with the expression of *A. kawachii xynC* yielding about 16% more and of *T. reesei xyn2* about 10% more biomass than the control strain. During co-expression of the β-xylanases and the β-xylosidase, the simultaneous action of the two heterologous xylanolytic enzymes gave further improvements in biomass yields. The *P. stipitis* [*xynC XLO2*] strain showed the maximum biomass yield improvement of about 27% and the *P. stipitis* [*xyn2 XLO2*] strain showed about 20% improvement over the *P. stipitis* [VECT] control strain. It is likely that the *P. stipitis* [*xynC*] and the *P. stipitis* [*xynC XLO2*] strains produce higher biomass yields from xylan as sole carbon source than the *T. reesei xyn2* expressing strains because of the higher β-xylanase activity observed in these strains (Fig. 4) and the higher stability of the *xynC* gene product. It is interesting to note that the percentage of improvements in biomass yields of the strains co-expressing the xylanolytic genes is more than the sum of the percentages of improvement of the strains expressing the genes

individually. This showed the synergistic action of the heterologous xylanolytic enzymes on xylan. The production of heterologous enzymes by the recombinant *P stipitis* strains might account for lower than expected biomass yields as energy and therefore carbon is required for heterologous protein production. Furthermore, the birchwood glucoronoxylan used contained a certain amount of glucoronic acid side chains. The degradation of the polymer by the xylanases expressed was therefore limited because of the limitations of family 11 xylanases, they are incapable of attacking the glycosydic linkage next to the side chain and towards the non-reducing end, thus requiring three unsubstituted consecutive xylopyranosyl residues for cleavage [36]. The biomass yield could therefore be further increased with the co-expression of an α-glucuronidase gene. In this study we have created recombinant *P. stipitis* strains expressing fungal xylanolytic enzymes that are more efficient in degrading xylan than native *P. stipitis* strains. These strains represent a step towards the efficient degradation and utilisation of hemicellulosic materials by ethanol-producing yeasts.

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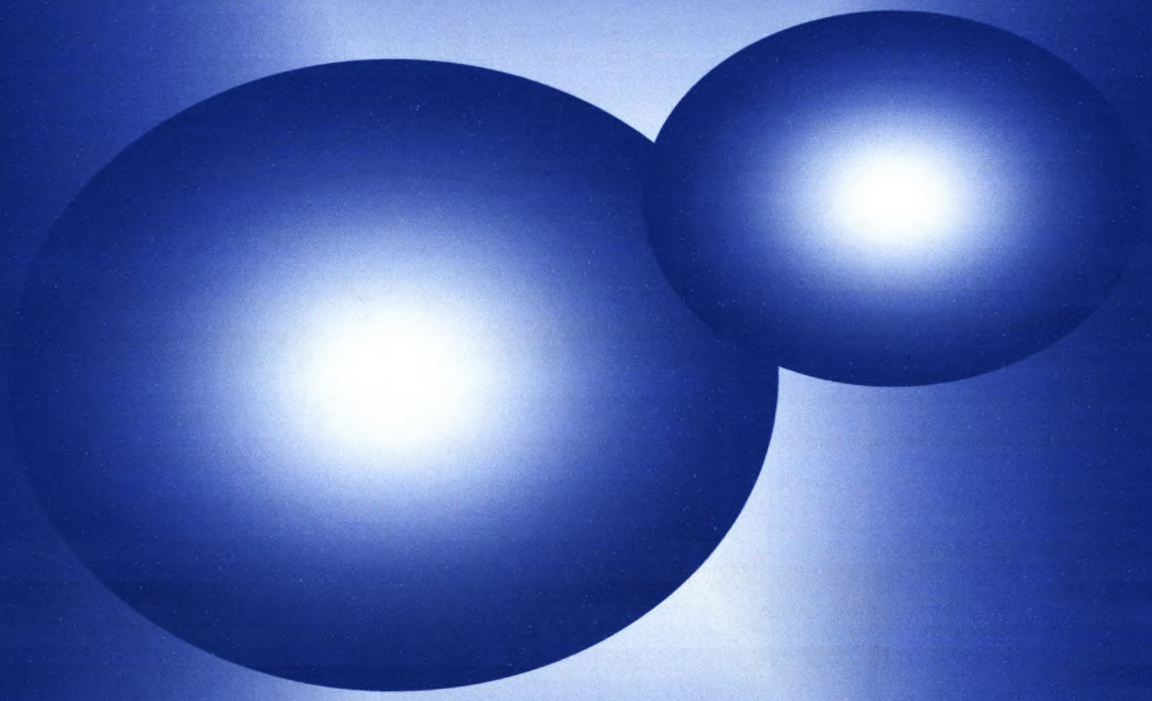
Chapter 5
Paper 3



Xylan degradation and β-xylanase expression of recombinant *Pichia stipitis* strains in a fermentor

In preparation

XYLAN DEGRADATION AND β-XYLANASE EXPRESSION OF RECOMBINANT *PICHIA STIPITIS* STRAINS IN A FERMENTOR

R. Den Haan, J.L. Snoep and W.H. Van Zyl

5.1 ABSTRACT

Recombinant strains of the D-xylose fermenting yeast *Pichia stipitis* were grown in a fermentor under conditions of high oxygenation and controlled pH levels to assess the stability of heterologous β-xylanase activity and increase biomass yields from xylan as sole carbon source. In a previous study, recombinant *P. stipitis* strains over-expressing fungal xylanolytic enzymes showed a significant increase in biomass formation on xylan as sole carbon source in shake flasks (Chapter 4). In the fermentor, high levels of oxygenation led to even further increases in biomass yield of the recombinant *P. stipitis* strains. Cell counts improved on average by 85% over the cell counts attained in shake flasks. However, increases in biomass yield over the control strain remained similar to those attained in shake flask cultures of the same strains. The *P. stipitis* [*xynC XLO2*] strain, the most successful of the recombinant *P. stipitis* strains, yielded approximately 3.2-fold more biomass than the *P. stipitis* [VECT] reference strain. Interestingly, the *P. stipitis* [*xynC XLO2*] strain yielded ca. 32% of the theoretical maximum cell count yield in both shake flasks and fermentor, indicating that the biomass yield of this strain is possibly limited by other factors. Ethanol could be produced directly from xylan as sole carbon source by this recombinant strain. The *P. stipitis* [*xynC XLO2*] strain showed a 3.6-fold increase in ethanol yield over the reference strain and produced a maximum ethanol concentration of 1.35 ± 0.12 g/L. The heterologous β-xylanase activity was not stabilized by controlling the pH level in the fermentor to pH 6, showing that the proteolytic activity in the supernatant was not limited to acid proteases. After depletion of the initial D-xylose in the media, new, but transient, β-xylanase activity was induced by the addition of D-xylose to the fermentor. The proteolytic activity in the medium had an optimum activity at 60°C and pH 7.

5.2 INTRODUCTION

Xylan, the major hemicellulose component in hardwood, is a heterogenous wood polymer that consists of a main chain of β-1,4-linked D-xylose residues that can contain side chains of L-arabinofuranose, 4-*O*-methyl glucuronic acid residues and acetic acid (Van Peij et al. 1997; Tenkanen et al. 1996). Furthermore, the L-arabinofuranosyl residues in the side chains can be esterified with ferulic and *p*-coumaric acid. Therefore, complete degradation of this complex polymer requires the synergistic action of a range of different enzymes. Many bacterial and fungal species produce the full range of enzymes required to enable them to utilise xylan as a carbon source (Uffen, 1997). Xylan is the most abundant polysaccharide after cellulose (Lappalainen, 1986), making the main constituent sugar of xylan, D-xylose, the second most abundant renewable monosaccharide in nature (Jeffries and Jin, 2000).

Xylan can be utilised by conversion into useful commodities such as bioethanol. It has been shown that one step degradation and fermentation would make bioethanol production more economically feasible (Lynd et al. 1999; Hahn-Hägerdal et al. 2001). Several yeast species such as *Aureobasidium* sp., *Cryptococcus* sp. and *Trichosporon* sp. are able to degrade xylan (Biely et al. 1978; Hrmová et al. 1984; Liu et al. 2002). However these yeast species do not show high ethanol productivity and/or tolerance to the toxic effects of ethanol. The yeast *Saccharomyces cerevisiae* has, in contrast, been used extensively for the production of ethanol and has also been established as a host for the expression of heterologous proteins of biotechnological interest (Romanos et al. 1992; Aristidou and Penttilä, 2001). *S. cerevisiae* has been engineered to degrade xylan to D-xylose by expression of xylanolytic genes in the yeast (Crous et al. 1995; La Grange et al. 1996; La Grange et al. 2001). However, the main disadvantage of *S. cerevisiae* is that it cannot utilise or ferment D-xylose. Considerable efforts have been made to enable *S. cerevisiae* to utilise D-xylose (Ho et al. 1998; Eliasson et al. 2000; Hahn-Hägerdahl et al. 2001). However, high level D-xylose utilisation and ethanol formation in *S. cerevisiae* remains a significant challenge.

The yeast *Pichia stipitis* and the related yeast *Candida shehatae* are the best D-xylose-fermenting yeasts thus far described (Prior et al. 1989; Jeffries and Kurtzman, 1994). Furthermore, various *P. stipitis* strains can utilise xylan as sole carbon source (Özcan et al. 1991; Basaran et al. 2001). However, xylan-degrading *P. stipitis* strains produce very low levels of xylanolytic enzymes. Xylan grown cultures of these *P. stipitis* strains produced only 2.5-3% of the β-xylanase activity reported for xylan grown cultures of *Cryptococcus albidus*. In previous studies, we have worked toward enhancing the xylan degrading ability of *P. stipitis* for the potential direct conversion of xylan to ethanol (Chapter 3; Chapter 4). This was effected by expressing the β-xylanase encoding *Trichoderma reesei xyn2* and the *Aspergillus kawachii xynC* genes, as well as the β-xylosidase encoding *Aspergillus niger xlnD* gene, separately and in combination, in *P. stipitis*. In the present work we have studied the biomass yield of these xylan-degrading strains grown on xylan as sole carbon source in a fermentor. Higher biomass yields can generally be attained by yeasts grown in a fermentor where pH level can be kept at the optimum level for growth and the culture can be maintained under high levels of dissolved oxygen by stirring and sparging the culture with air (Du Preez et al. 1986; Du Preez 1994). Possible direct conversion of xylan to ethanol by the recombinant *P. stipitis* strains was also studied.

It was suggested in previous studies that the loss of recombinant β-xylanase activity of *P. stipitis* recombinant strains was the result of degradation of the β-xylanase by a native protease (Chapter 3; Chapter 4). Several yeast species, including members of the genera *Candida*, *Yarrowia* and *Pichia* produce extracellular proteases (Ogrydziak 1993; Glover et al. 1997; Sreekrishna et al. 1997; Rao et al. 1998; Zaugg et al. 2001). Extracellular proteases are commonly secreted when the nitrogen source and/or amino acids in the growth media becomes limited. The type of protease secreted is often dependent on the pH of the growth media, as in *Yarrowia lipolytica* where an acid or alkaline extracellular protease is secreted depending on the culture pH (Glover et al. 1997). In this study, we tested the stability of the secreted extracellular β-xylanase of recombinant *P. stipitis* strains by growing the yeast in a fermentor and controlling the pH level to pH 6. This was done as it was previously found that the pH levels in shake flask cultures had

dropped to pH 2.5 (Chapter 3). We therefore wanted to test whether an acid protease was responsible for the degradation of the recombinant β-xylanase. Furthermore, the pH and temperature optima of the proteolytic activity secreted by the recombinant strains of *P. stipitis* was determined.

5.3 MATERIALS AND METHODS

5.3.1 Microbial strains, media and culture conditions

P. stipitis was cultivated on YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or selective synthetic (SC) medium [containing 20 g/L glucose, 20 g/L D-xylose or 20 g/L birchwood glucuronoxylan (Roth) as stated, 1.7 g/L yeast nitrogen base without amino acids (Difco) (pH 6) and all the required growth factors except uracil (SC^{-URA})]. Solid media contained 20 g/L agar. Yeasts were routinely cultured in 125-ml Erlenmeyer flasks containing 50 ml medium at 30°C on a rotary shaker at 150 rpm. For controlled batch fermentations the yeast strains were cultured in double strength SC^{-URA} medium in a Bioflo 110 1-L fermentor (New Brunswick Scientific, Edison, NJ, USA). Antifoam was added as required to prevent foam formation (Silicone antifoaming agent, BHD Laboratory Supplies, Poole, England). Cultivations were set up at 30°C with a stirring speed of 500 rpm. The culture pH level was controlled to pH 6.0 by addition of 2 M NaOH, unless otherwise stated. The fermentor was sparged with air at 1 L min^{-1}. The genotypes and sources of the yeast strains used in this study are summarised in Table 1.

5.3.2 Growth of *P. stipitis* transformants on xylan

P. stipitis transformants were inoculated from precultures in the stationary growth phase into 1 L double strength SC^{-URA} medium containing 20 g/L birchwood glucuronoxylan (Roth) as the sole carbon source in a Bioflo 110 1-L fermentor (New Brunswick Scientific, Edison, NJ, USA) and cultivated as stated above. The *P. stipitis* [VECT] strain was also grown on double strength SC^{-URA} medium with 20 g/L D-xylose as the carbon source to act as reference of *P. stipitis* growth on D-xylose. The maximum cell count achieved by this strain (in the stationary phase) grown on 20 g/L D-xylose was taken as a theoretical maximum cell count and was used to compare with the cell counts achieved by the recombinant strains grown on 20 g/L xylan in the fermentor Three

cultures of each strain were inoculated. Samples were periodically taken over a 72-h period and yeast cells in the media were counted in triplicate on a haemocytometer.

Table 1 Microbial strains

Strain/Plasmid	Genotype	Source/Reference
Yeast strains:		
Saccharomyces cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	This laboratory
Pichia stipitis TJ26:		
pJM6[a]	*ura3/[URA3]*	Chapter 3
pRDH12[b]	*ura3/[URA3 XYL1$_P$-xyn2-XYL1$_T$]*	Chapter 3
pRDH16[c]	*ura3/[URA3 XYL1$_P$-xynC-XYL1$_T$]*	Chapter 4
pRDH21[d]	*ura3/[URA3 TKL$_P$-XLO2-TKL$_T$]*	Chapter 4
pRDH22[e]	*ura3/[URA3 XYL1$_P$-xyn2-XYL1$_T$ TKL$_P$-XLO2-TKL$_T$]*	Chapter 4
pRDH26[f]	*ura3/[URA3 XYL1$_P$-xynC-XYL1$_T$ TKL$_P$-XLO2-TKL$_T$]*	Chapter 4

[a] *P. stipitis* TJ26 (pJM6) was designated *P. stipitis* [VECT]
[b] *P. stipitis* TJ26 (pRDH12) was designated *P. stipitis* [*xyn2*]
[c] *P. stipitis* TJ26 (pRDH16) was designated *P. stipitis* [*xynC*]
[d] *P. stipitis* TJ26 (pRDH21) was designated *P. stipitis* [*XLO2*]
[e] *P. stipitis* TJ26 (pRDH22) was designated *P. stipitis* [*xyn2 XLO2*]
[f] *P. stipitis* TJ26 (pRDH26) was designated *P. stipitis* [*xynC XLO2*]

5.3.3 Ethanologenic fermentations in shake flasks

P. stipitis [VECT] and *P. stipitis* [*xynC XLO2*] transformants were precultured in 100 ml SC^{-URA} medium containing 20 g/L D-xylose as carbon source until the stationary phase. The cells were then washed 3 times with sterile distilled water. The precultures were used to inoculate 25 ml double strength SC^{-URA} medium containing 20 g/L birchwood glucuronoxylan (Roth) as sole carbon source in 125 ml shake flasks to a cell density of 10^9 cells per ml. Three shake flask cultures of each strain were inoculated and incubated at 30°C on a rotary shaker at 100 rpm. Samples were periodically taken over a 120-h period. Samples were centrifuged for 5 min at 13 000 g and filtered through a 0.45 μm filter after which ethanol levels were determined using the Roche ethanol determination kit as prescribed by the manufacturer.

5.3.4 β-Xylanase activity assays

The recombinant β-xylanase producing strains, *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*], and the control strain *P. stipitis* [VECT] were grown in 1 L double strength SC^{-URA} medium containing 40 g/L D-xylose as carbon source in a Bioflo 110 1-L fermentor (New Brunswick Scientific, Edison, NJ, USA) as described above. Three cultures of every

strain were inoculated and all enzyme activity determinations were done in triplicate. After 62 h of growth D-xylose was added to the culture to a final concentration of 30 g/L D-xylose. Samples were periodically taken over a 94 h period and growth was monitored by measurement of optical density at 600 nm. Endo-β-1,4-xylanase activity was assayed according to the method described by Bailey et al. (1992) with 1% birchwood glucuronoxylan (Roth) as the substrate at 50°C for 5 minutes. Appropriate dilutions of the cell free culture solution in 50 mM sodium citrate buffer (pH 5.0 for transformants containing the *T. reesei xyn2* gene and pH 4.0 for transformants containing the *A. kawachii xynC* gene) were used as the enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid (DNS) method described by Miller et al. (1960). The β-xylanase activity of the *P. stipitis* [*xynC*] strain was measured at pH 4, which is higher than the optimum pH level of the *A. kawachii xynC* gene product (Crous et al. 1995). All activities were expressed in katals per millilitre; one katal is the amount of enzyme needed to produce 1 mol of reducing sugar from birchwood xylan per second (Bailey et al. 1992).

5.3.5 Screening for extracellular protease activity

The recombinant *P. stipitis* strains were screened for extracellular protease activity by patching them on solid SC^{-URA} medium containing 20 g/L skim milk powder and 20 g/L D-xylose as carbon source (Mattern et al. 1992). The supernatants of 1-L fermentor grown cultures of *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] at 94 h were separated from the cellular mass by centrifugation for 10 min at 13,000 g. The supernatants were filtered through a 0.22 µm filter (Millipore) and concentrated in a Diaflo Ultrafilter PM10 concentrator (Amicon) to a final volume of 10 ml. 5 µl of each concentrated supernatant was spotted on the skim milk plates to detect extracellular protease activity.

5.3.6 Protease activity assays

Protease activity was assayed using the synthetic substrate azocasein (Sigma) (Long et al. 1981). One unit of protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0.1 at 440 nm in 30 minutes. Activities were expressed as

percentages of the maximum activity attained. Each sample was assayed in triplicate. The optimum conditions for the activity and stability of the protease activity in the culture supernatant concentrates were determined by assaying the enzyme activity at temperatures between 20°C and 80°C and pH values between 2 and 10.

5.4 RESULTS

5.4.1 Growth of *P. stipitis* transformants on birchwood xylan as sole carbon source

It was shown previously that various *P. stipitis* strains can utilize xylan as sole carbon source and that the expression of fungal xylanolytic genes increased the biomass yields attained by *P. stipitis* (Özcan et al. 1991; Chapter 3; Chapter 4). The *P. stipitis* recombinant strains were tested for growth on double strength SC^{-URA} minimal medium containing 20 g/L birchwood glucuronoxylan as the sole carbon source, under conditions of high oxygenation in a fermentor. The amount of biomass produced was monitored over a 72 h period by means of cell counts (Fig. 1). The biomass yield of *P. stipitis* [VECT] on medium containing 20 g/L D-xylose as sole carbon source was also monitored in the fermentor under the same conditions to determine maximum *P. stipitis* biomass yield. The maximum cell counts achieved by the different recombinant strains grown on xylan, as well as the percentage of biomass yield achieved with regard to *P. stipitis* [VECT] grown on D-xylose and the percentage of improvement of the recombinant strains over *P. stipitis* [VECT] grown on D-xylose are given in Table 2. The heterologous expression of either *XLO2*, *xyn2* or *xynC* in *P. stipitis* clearly results in increased biomass yields on xylan, as was seen previously (Chapter 4). When *XLO2* is co-expressed with either *xyn2* or *xynC*, a further significant increase in biomass production on xylan was observed. The co-expression of *XLO2* and *A. kawachii xynC* gave the highest increase of biomass yield on xylan, approximately 22% higher than observed for the parental strain.

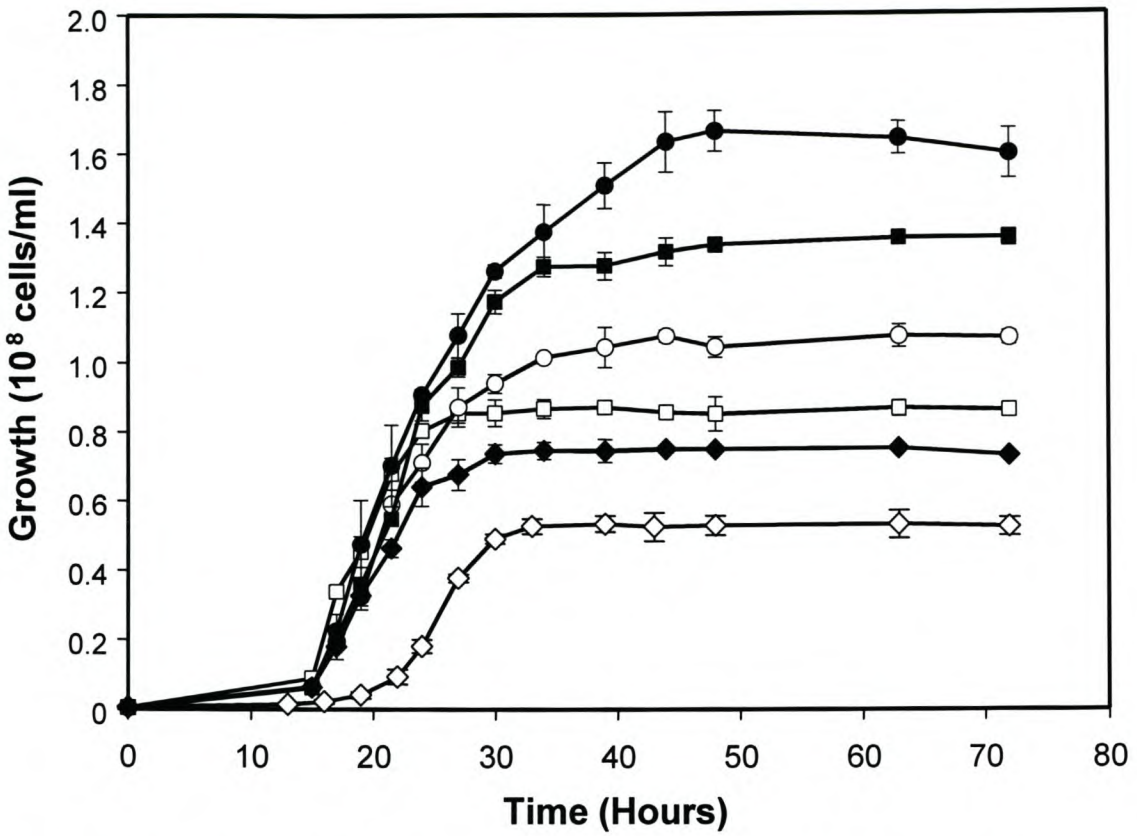


Fig. 1 Growth curve of recombinant *P. stipitis* strains. *P. stipitis* [VECT] (◇), *P. stipitis* [*XLO2*] (♦), *P. stipitis* [*xyn2*] (□), *P. stipitis* [*xyn2 XLO2*] (■), *P. stipitis* [*xynC*] (○) and *P. stipitis* [*xynC XLO2*] (●), were grown on double strength SC^{-URA} medium containing 20 g/L birchwood xylan as sole carbon source, in the fermentor at high oxygenation.

Table 2 Maximum cell counts achieved by recombinant *P. stipitis* strains grown in the fermentor

Recombinant strain	Maximum cell count achieved on 20 g/L xylan (10^6 cells per ml)	Percentage of theoretical maximum cell count*	Percentage of improvement over control strain
P. stipitis [VECT]	52.4 ± 4.1	10.1 ± 0.8	(control strain)
P. stipitis [*XLO2*]	74.9 ± 0.9	14.4 ± 0.2	4.3 ± 0.2
P. stipitis [*xyn2*]	87.0 ± 1.5	16.7 ± 0.3	6.6 ± 0.3
P. stipitis [*xynC*]	107.3 ± 1.8	20.6 ± 0.3	10.6 ± 0.3
P. stipitis [*xyn2 XLO2*]	135.3 ± 0.9	26.1 ± 0.2	15.9 ± 0.2
P. stipitis [*xynC XLO2*]	166.5 ± 5.9	32.0 ± 1.1	21.9 ± 1.1

*Maximum theoretical cell count was taken as the maximum cell count achieved when *P. stipitis* [VECT] was grown on 20 g/L D-xylose as sole carbon source in bioreactor (5.2E+08 cells/ml).

5.4.2 Ethanol production by *P. stipitis* transformants from birchwood xylan

It was shown previously that apart from growing on xylan as sole carbon source, some strains of *P. stipitis* can produce ethanol from xylan (Lee et al. 1986; Morosoli et al. 1993). *P. stipitis* [VECT] and *P. stipitis* [*xynC XLO2*] were grown in shake flasks, with high cell counts ensuring low oxygenation, to determine ethanol production of the recombinant strains. The amounts of ethanol produced under these conditions are given in Fig. 2. The reference strain, *P. stipitis* [VECT] was only able to produce an amount of 0.37 ± 0.07 g/L ethanol from xylan. The *P. stipitis* [*xynC XLO2*] strain, in contrast, produced up to 1.35 ± 0.12 g/L ethanol. Most of the ethanol was produced within the first 24 h of the fermentation.

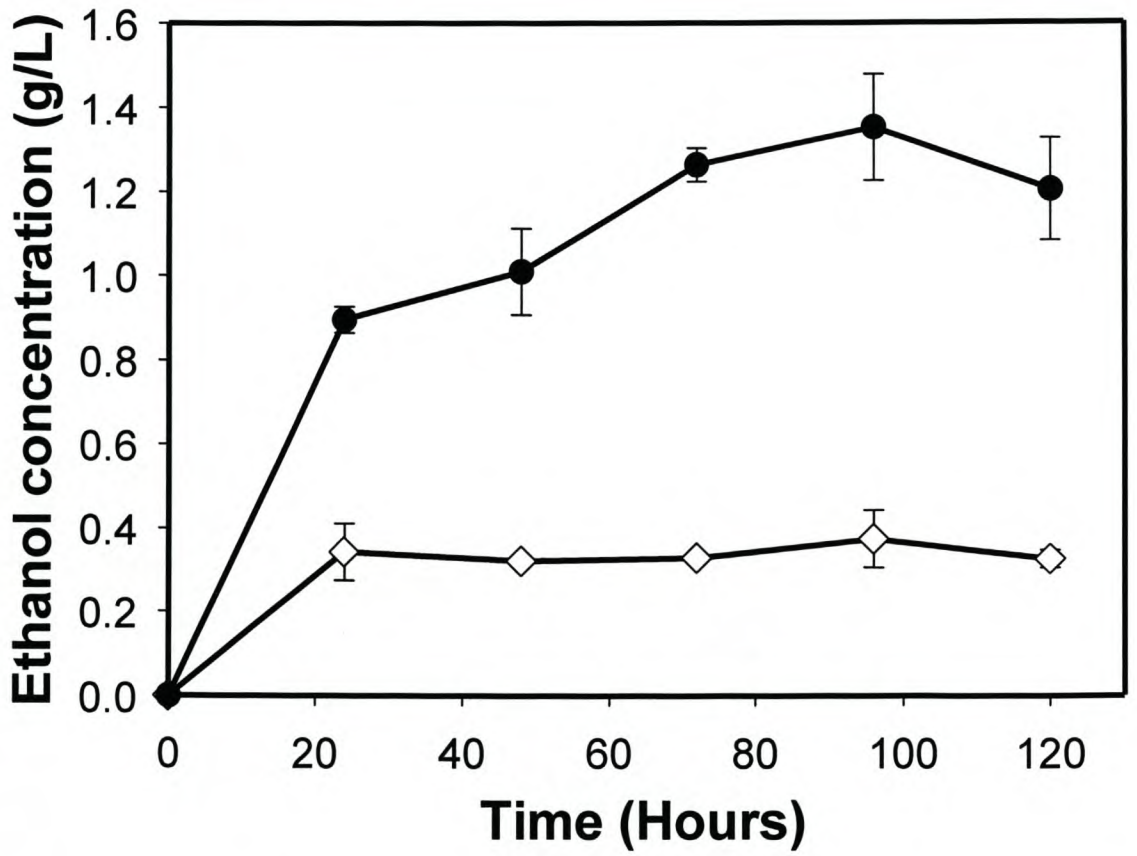


Fig. 2 Ethanol production by recombinant *P. stipitis* strains. *P. stipitis* [VECT] (◇) and *P. stipitis* [*xynC XLO2*] (●) were grown on on double strength SC^{-URA} medium containing 20 g/L birchwood xylan as sole carbon source. Ethanol determinations were done using the Roche ethanol determination kit as instructed by the manufacturer.

5.4.3 β-xylanase activity

The heterologous β-xylanase producing yeast strains, *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*], and the reference strain *P. stipitis* [VECT], were analysed for their ability to secrete biologically active β-xylanase (Fig. 3A) and growth (Fig. 3B) over a period of 94 h. *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] were cultured on double strength SC^{-URA} medium containing 40 g/L D-xylose in a fermentor where the pH of the culture was controlled to pH 6. Only basal levels of β-xylanase activity were produced by the *P. stipitis* [VECT] control strain throughout the 94-h incubation period. The *P. stipitis* [*xyn2*] strain showed a maximal level of β-xylanase activity of 142.3 ± 3.3 nkat/ml after 21 h of incubation. After 21 h there was a decline in the level of activity and the β-xylanase activity of these strains had dropped to levels close to zero at 62 h of growth in the fermentor. The *P. stipitis* [*xynC*] showed a maximal level of β-xylanase activity of 226.7 ± 7.5 nkat/ml after 21 h of incubation. The level of β-xylanase activity produced by this strain is significantly higher than the levels produced by the *P. stipitis* [*xyn2*] strain, however the level of β-xylanase activity also declined to a level close to zero at 62 h of growth. After 62 h of growth the D-xylose in the media was depleted (Fig. 3C) and D-xylose was added to the medium to a final concentration of 30 g/L. This led to a rapid increase in secreted β-xylanase activity for the *P. stipitis* [*xyn2*] strain to a maximum level of 104.0 ± 12.6 nkat/ml and for the *P. stipitis* [*xynC*] strain to a maximum level of 138.8 ± 10.3 nkat/ml after 66 h of growth (4 h after the D-xylose addition). Thereafter β-xylanase activity declined and reached levels close to zero nkat/ml at 94 h for both strains. After 94 h the D-xylose in the medium was again depleted (Fig. 3C).

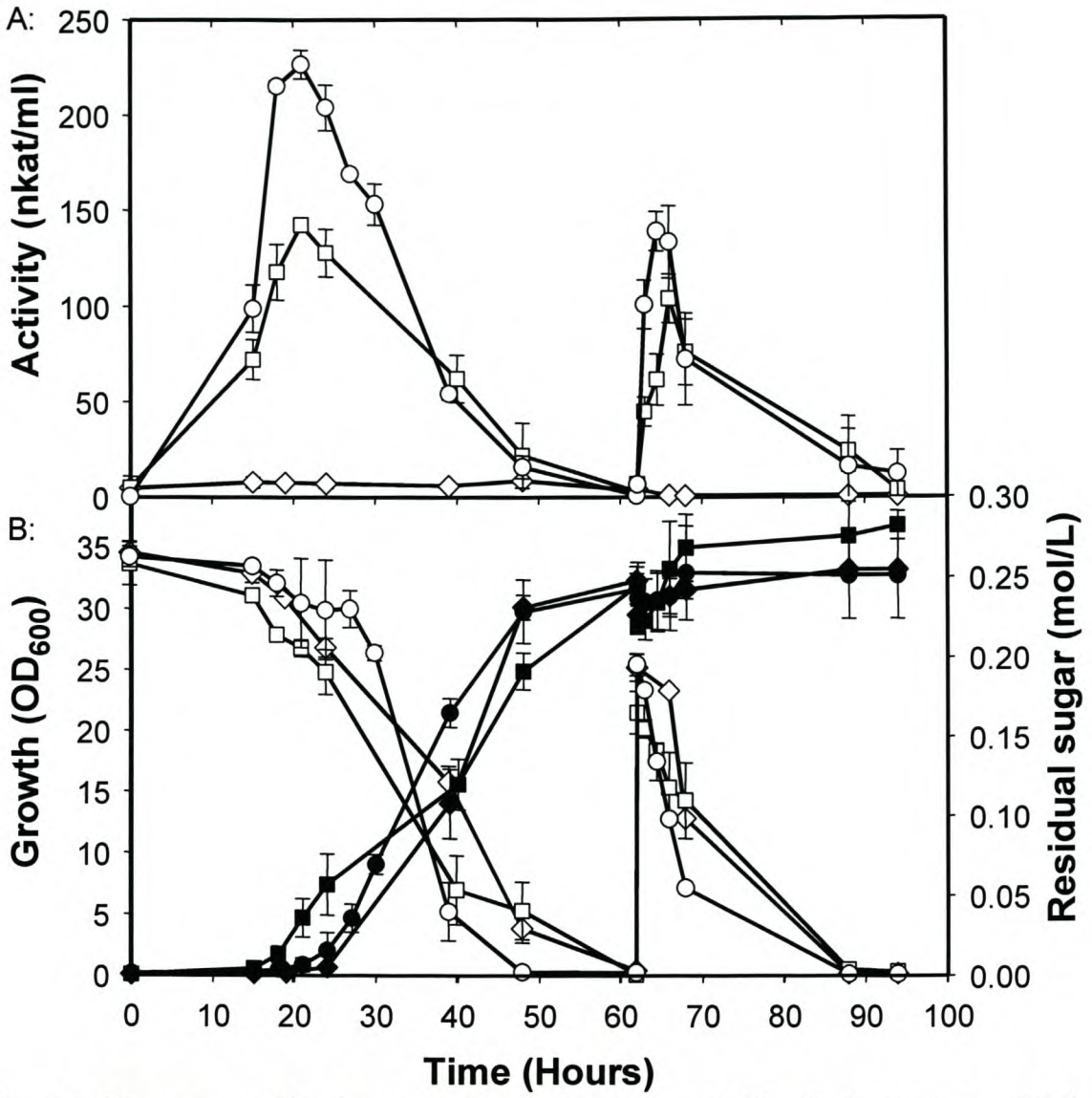


Fig. 3 A: Time course of β-xylanase produced by *P. stipitis* [VECT] (◇), *P. stipitis* [*xyn2*] (□), and *P. stipitis* [*xynC*] (○) on 40 g/L D-xylose as carbon source. After 62 h D-xylose was added to the medium to a final concentration of 30 g/L. The pH level of the cultures was kept at pH 6 with addition of 2 M NaOH. Activity is expressed in katals/ml, with 1 katal representing the amount of enzyme needed to produce 1 mol of reducing sugar from birchwood xylan per second under the conditions of the assay (Bailey et al. 1992). **B:** Growth of *P. stipitis* [VECT] (♦), *P. stipitis* [*xyn2*] (■) and *P. stipitis* [*xynC*] (●) was monitored by measurement of the optical density of the culture at 600 nm. Time course of background reducing sugar in the medium as measured by the dinitrosalicylic acid assay for the *P. stipitis* [VECT] (◇), *P. stipitis* [*xyn2*] (□) and *P. stipitis* [*xynC*] (○) fermentations.

5.4.4 Extracellular protease activity

The recombinant *P. stipitis* strains, *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] showed clearing zone formation on the 20 g/L skim milk plates after 30 hours of incubation at 30°C, confirming the production of extracellular proteases in these strains (Fig. 4). Proteinase K solution (Roche) was spotted on the plate to serve as positive control and a protease-deficient laboratory strain of *S. cerevisiae* (Y294) served as extracellular protease negative control. Furthermore, concentrates of the culture supernatants from the fermentor, were also spotted on the skim milk plates to detect protease activity in the supernatant. Extracellular protease concentrates were prepared by ultrafiltration with the Amicon filtration system from the supernatants of cultures of *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] and *S. cerevisiae* Y294 grown in the fermentor, controlled at pH 6. Final protein concentrations of ca. 150 µg/ml were attained. All protein concentrates of *P. stipitis* cultures showed clearing zone formation on the milk plates, confirming the presence of proteases. A concentrate of a *S. cerevisiae* Y294 culture, serving as negative control, showed no clearing zone formation on the milk plates. To determine the optimum conditions for the protease activity in the culture supernatant, the enzyme activity in the concentrates was assayed at temperatures between 20°C and 80°C and pH values between 2 and 10. The optimal conditions for the protease activity were found to be at 60°C and pH 7 respectively (Fig. 5).

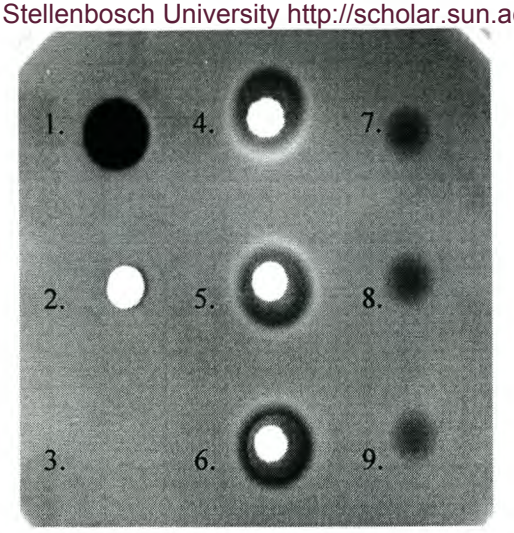


Fig. 4 Extracellular protease producing *P. stipitis* strains. SC^{-URA} medium with 20 g/L skim milk powder supplemented with 20 g/L glucose as the carbon source was patched with (1) Proteinase K solution (Roche), (2) *S. cerevisiae* Y294, (3) concentrated supernatant of *S. cerevisiae* Y294, (4) *P. stipitis* [VECT], (5) *P. stipitis* [*xyn2*], (6) *P. stipitis* [*xynC*], (7) concentrated supernatant of *P. stipitis* [*xynC*] (8) concentrated supernatant of *P. stipitis* [*xynC*] (9) concentrated supernatant of *P. stipitis* [*xynC*] The plates were photographed after 30 h of incubation at 30°C. Proteinase K solution (Roche) was spotted on the plate to serve as positive control 3 hours before the plates were photographed.

5.5 DISCUSSION

The additional production of recombinant xylanolytic enzymes in *P. stipitis*, a yeast known to produce low xylanolytic activity when cultured on xylan as sole carbon source, enhanced its growth on xylan as sole carbon source (Lee et al. 1986; Özcan et al. 1993; Chapter 3; Chapter 4). In our study, the recombinant strains over-expressing fungal xylanolytic enzymes previously showed a dramatic increase in biomass formation on xylan as sole carbon source in shake flasks (Chapter 4). In the fermentor, high levels of oxygenation led to even further increases in biomass yield of the recombinant *P. stipitis* strains (Table 2, Fig. 1), cell counts improved on average by 85% over the cell counts attained in shake flasks. The higher cell counts may also be due to the fact that the culture was grown in a controlled pH environment, resulting in less strain on the cells. However, increases in biomass over the control strain remained similar to the increases in biomass yield over the control strain attained in shake flask cultures of the same strains (Chapter 4). The *P. stipitis* [*xynC XLO2*] strain, the most successful of the recombinant *P. stipitis* strains, yielded approximately 3.2-fold more

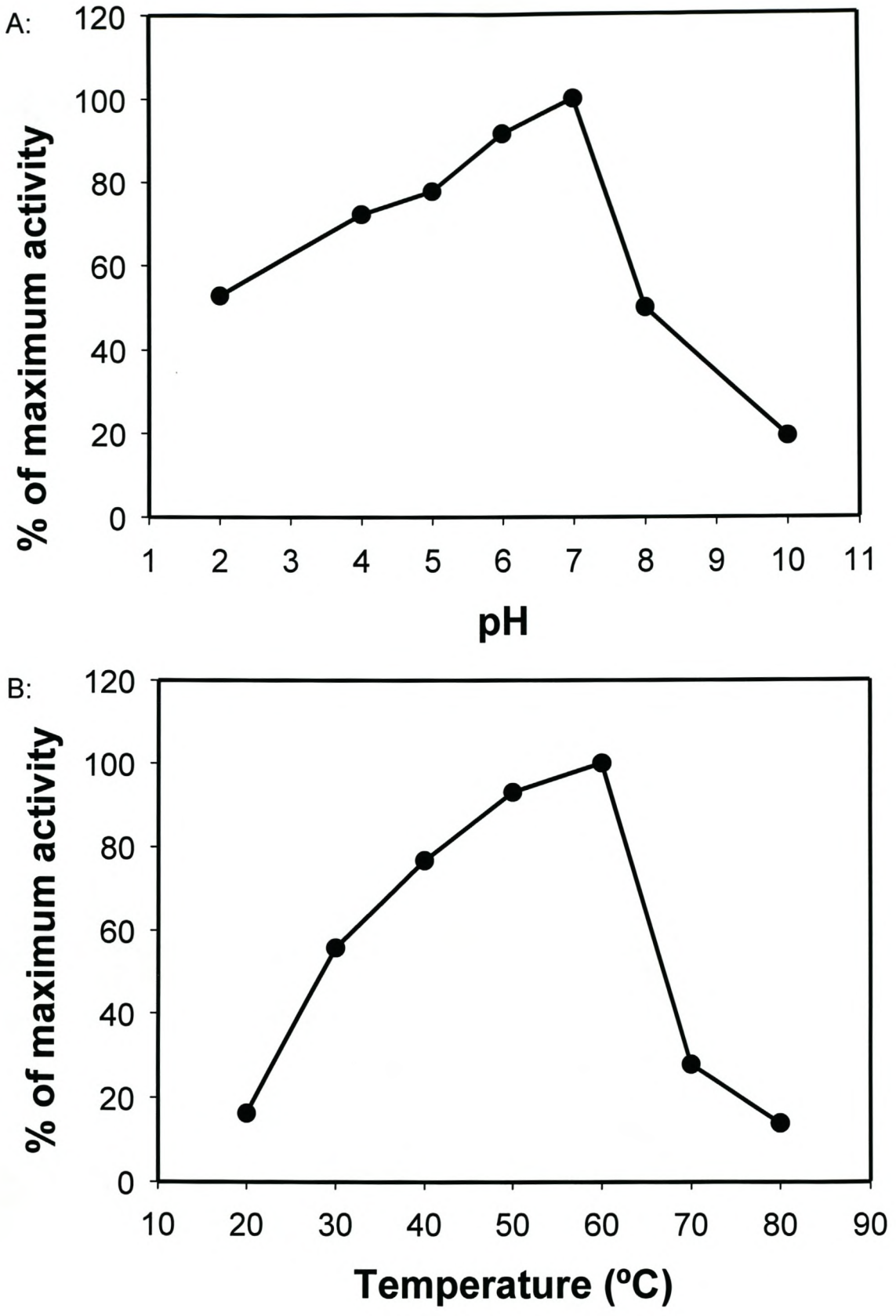


Fig. 5 The effect of (**A**) temperature and (**B**) pH on the proteolytic activity in the culture supernatant concentrates.

biomass than the *P. stipitis* [VECT] control strain. Interestingly, the *P. stipitis* [*xynC XLO2*] strain yielded ca. 32% of the theoretical maximum cell count in both shake flasks and fermentor, indicating that biomass yield in this strain is possibly limited by other factors. The birchwood glucuronoxylan used contained glucuronic acid side chains at a ratio of one per ten xylose residues, thus the degradation of the polymer by the β-xylanases was limited because of the limitations of family 11 β-xylanases (Puls and Schuseil, 1993; Biely et al. 1997). Family 11 β-xylanases are incapable of attacking the glycosydic linkage next to the side chain and towards the non-reducing end, thus requiring three unsubstituted consecutive xylopyranosyl residues for cleavage. This would lead to a significant amount of unutilised D-xylose residues. Therefore, in the case of recombinant strains expressing the β-xylanase alone (*P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*]) 40% of the D-xylose residues would not be utilised. In the case of recombinant strains co-expressing the β-xylanase and the β-xylosidase (*P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*]) the heterologous β-xylosidase can remove the D-xylose residue adjacent to the substituted residue at the reducing end. This results in only 30% of the D-xylose residues remaining unutilised. The biomass yield could therefore be increased even further with the co-expression of an α-glucuronidase gene in these strains.

It was previously shown that some strains of the yeast *P. stipitis* can convert xylan directly to ethanol (Lee et al. 1986; Morosoli et al. 1993). *P. stipitis* has a specific requirement for low oxygenation for ethanol formation (Skoog and Hahn-Hägerdal, 1990) and as such *P. stipitis* [VECT] and *P. stipitis* [*xynC XLO2*] (as the strain showing the most improvement in biomass yield) were grown in shake flasks at low oxygenation to determine ethanol production of the recombinant strains. The amounts of ethanol produced and the cell counts under these conditions were determined over a 120-h period (Fig. 2). The *P. stipitis* [*xynC XLO2*] strain showed significant improvement in ethanol productivity of approximately 3.6-fold over the *P. stipitis* [VECT] control strain, presumably because of the action of the heterologous xylanolytic enzymes that liberate more D-xylose from the xylan polymer to be available for ethanol formation. However, these ethanol levels are lower than those that were attained by a natural isolate of

P. stipitis grown on 10 g/L larchwood xylan, which reached 1.8 g/L ethanol (Lee et al. 1986). The recombinant *P. stipitis* strain constructed by Morosoli et al. (1993) also yielded 1.8 g/L ethanol on 10 g/L xylan. Furthermore, a hybrid strain constructed by fusing *T. reesei* nuclei and *P. stipitis* protoplasts attained 2.3 g/L ethanol on rich medium containing 20 g/L xylan as carbon source. Therefore, although the *P. stipitis* [*xynC XLO2*] recombinant strain showed a significant increase in ethanol yield from xylan as sole carbon source over the control strain, the final ethanol yield was relatively low. However, the auxotrophic *P. stipitis* strain used to construct these strains was reported to be a poor fermenter (Yang et al. 1994) and higher ethanol yields may therefore be achieved by expressing the recombinant genes in a better fermenting strain. Furthermore, as the buffering cappasity of the medium used in our study was low, a drop in the pH level would lead to increased stess on the yeast cell which would also decrease the ethanol yield. We have therefore shown in this study that the successful expression of xylanolytic genes in *P. stipitis* leads to an increase in biomass and ethanol yield of the resulting recombinant strain(s) grown on xylan as sole carbon source.

In earlier studies, recombinant strains of *P. stipitis* were created that expressed the *T. reesei xyn2* β–xylanase encoding gene (*P. stipitis* [*xyn2*]) and the *A. kawachii xynC* β-xylanase encoding gene (*P. stipitis* [*xynC*]) under transcriptional control of the *P. stipitis* xylose reductase gene (*XYL1*) promoter (Chapter 3; Chapter 4). In these studies it was found that the heterologous β-xylanase activity was transient, dropping of to levels close to zero nkat/ml soon after reaching maximum activity levels. It was argued that although D-xylose, the inducer of the *XYL1* promoter was depleted and therefore no longer able to induce the expression of the heterologous β-xylanase, the reduction in β-xylanase activity that was already present had to be due to the presence of an extracellular protease. It was also shown that recombinant *P. stipitis* strains secreted an extracellular protease by zone formation on milk plates (Chapter 4). The pH of these cultures in shake flask experiments was found to have dropped to below pH 2.5. It was therefore thought that the protease secreted by these recombinant *P. stipitis* strains may be an acid protease and that protease secretion may be averted by maintaining the culture pH at a level close to neutral. In this study we have grown the recombinant *P. stipitis*

strains in a fermentor and controlled the pH level by addition of NaOH to pH 6. Maximal β-xylanase activity levels of 142.3 ± 3.3 nkat/ml and 226.7 ± 7.5 nkat/ml for the *P. stipitis* [*xyn2*] and the *P. stipitis* [*xynC*] strains, respectively, was attained after 21 h of growth. However, the levels of β-xylanase activity still declined to levels close to zero nkat/ml after reaching maximal levels (Fig. 3A). The presence of extracellular proteases secreted by the *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and the *P. stipitis* [*xynC*] strains, and proteolytic activity in the supernatants of fermentor cultures were confirmed by spotting the cultures and supernatant concentrates onto milk plates (Fig. 4). Therefore, the proteolytic activity was not merely limited to acid protease(s). Several yeast species are known to produce extracellular proteases including species of the genus *Pichia* (Ogrydziak 1993; Glover et al. 1997; Sreekrishna et al. 1997; Rao et al. 1998; Zaugg et al. 2001). Yeast species such as *Y. lipolytica* and *Candida albicans* secrete different extracellular proteases over a wide range of acidic and alkali pH values, therefore, suppressing acid protease activity will not necessarily alleviate extracellular proteolysis (Glover et al. 1997; Hube and Naglik 2001).

The optimal temperature and pH conditions of the proteolytic activity in the culture supernatant was further characterized by assaying protease activity of the supernatant concentrates over a range of temperature and pH values (Fig. 5A and 5B). The optimal conditions for the proteolytic activity were at 60°C and pH 7, respectively. However, the proteolytic activity was still relatively high at far lower temperature and pH values with over 40% of the maximal activity still present at 30°C and over 60% of the maximal activity still present at pH 3. Proteolytic activity can therefore not easily be alleviated by changes in the growth conditions as such conditions would make it very difficult for the yeast to grow and ferment (Prior et al. 1989; Du Preez 1994). Extracellular proteases are often secreted when the nitrogen source and/or amino acids in the growth media become limited (Ogrydziak 1993; Rao et al. 1998). Therefore growth of the recombinant *P. stipitis* strains in continuous culture at carbon limited conditions instead of nitrogen limited conditions might give further insight into the conditions leading to secretion of proteases by these strains. The recombinant Xyn2 β-xylanase secreted by *P. stipitis* [*xyn2*] was previously shown not to be hyperglycosylated as it's size corresponded to the

native *T. reesei* Xyn2, which is not highly glycosylated (Chapter 3; Törrönen et al. 1992). The low levels of glycosylation of the heterologous enzymes may lead to an increased sensitivity to the proteases in the supernatant.

After 62 h of growth of the recombinant *P. stipitis* strains in the fermentor, the 40 g/L D-xylose that was initially present in the medium was depleted. D-Xylose was again added to the media up to a final concentration of 30 g/L. This led to a rapid induction of the *XYL1* promoter and increase in secreted β-xylanase activity for the *P. stipitis* [*xyn2*] strain to a maximum level of 104.0 ± 12.6 nkat/ml, and for the *P. stipitis* [*xynC*] strain to a maximum level of 138.8 ± 10.3 nkat/ml 4 h after the addition of D-xylose. Thereafter β-xylanase activity again declined and reached levels close to zero nkat/ml at 94 h for both strains. This result shows that new recombinant β-xylanase production stoped as a result of D-xylose depletion but resumed with addition of the inducer of the *XYL1* promoter, D-xylose. Research on the type of protease secreted by *P. stipitis*, the regulation thereof, as well as cloning of the gene(s) encoding extracellular protease(s) and isolation of protease deficient *P. stipitis* strains, will be important for the efficient production of secreted heterologous proteins by this yeast.

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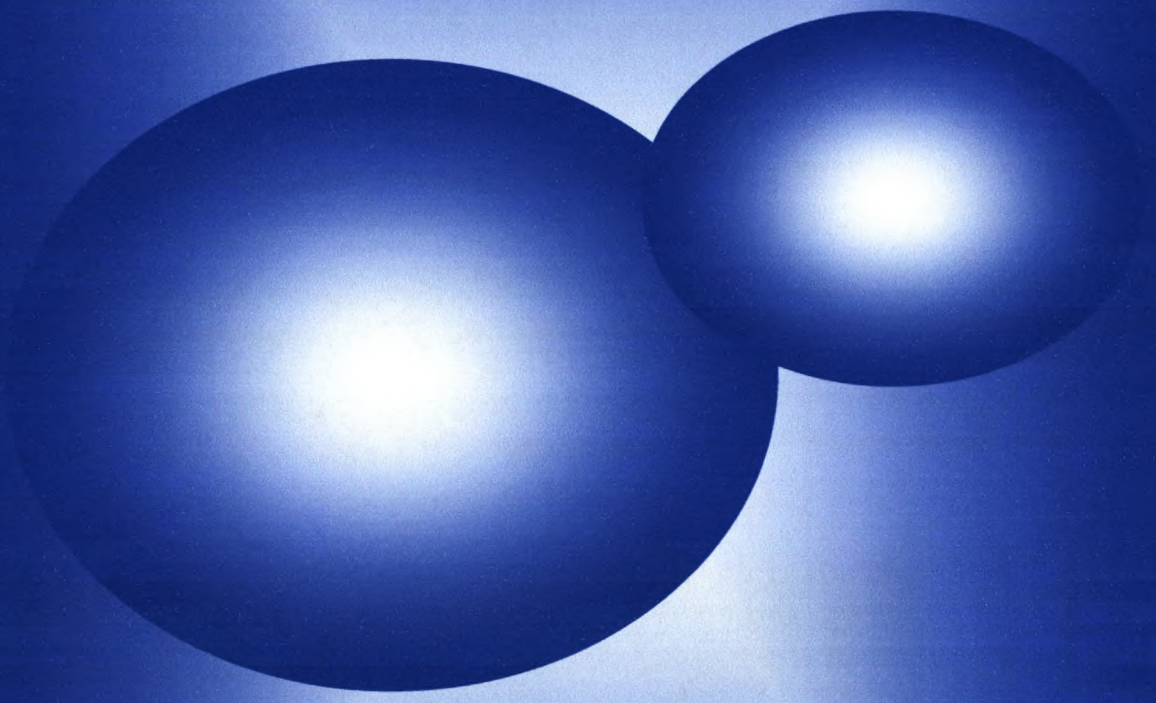
Chapter 6



General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

For millennia man has used the solar energy, contained in the chemical bonds of biomass, by burning it as fuel or eating plants as nutritional energy. More recently humans have exploited the fossilised biomass in the form of coal and oil, representing a more condensed source of energy as fuel. However, as it takes millions of years to convert biomass into coal and oil these fossil fuels are not renewable in the timeframe that they are used in. Plant biomass, in comparison, represents the only probable, sustainable resource of organic fuels and significant efforts are underway to harness this potential energy source. Xylan, the major hemicellulose present in plant biomass, can represent a third of the total carbohydrate content and is the most abundant polysaccharide after cellulose (Lappalainen, 1986). If biomass-based processes of producing commodities such as ethanol are to be cost effective, bioconversion of the xylan component is as important as that of cellulose (Lynd et al. 1999).

The heterogenous xylan polymer requires the synergistic action of a range of main chain and side chain cleaving enzymes for complete and efficient degradation (Thomson, 1993). The solubility of xylan is influenced by the substituents in that solubility is directly proportional to the number of substituents. The frequency and composition of substituents in xylan differ depending on the plant origin. The production of xylanolytic enzymes is wide-spread among filamentous fungi and bacteria (Hazelwood and Gilbert, 1993; Sunna and Antranikian, 1997). Strains of the filamentous fungi *Trichoderma* and *Aspergillus* secrete large amounts of efficient xylan-degrading enzymes, and are important producers of cellulolytic and hemicellulolytic enzymes for industrial use (Hrmová et al. 1989; De Vries et al. 2000). However, the ability to degrade xylan is not as common among naturally occurring yeasts. Xylan-grown cultures of the yeasts *Aureobasidium pullulans* and *Cryptococcus albidus* were found to produce β-xylanase activity (Leathers et al. 1984).

Pichia stipitis and the related yeast *Candida shehatae* are the best D-xylose fermenting yeasts described thus far (Du Preez and Prior, 1985; Jeffries and Kurtzman, 1994). A xylan-degrading strain of *P. stipitis* therefore has implications for a theoretical direct

conversion of xylan to the biocommodity ethanol. *P. stipitis* and other yeasts that are able to grow on D-xylose were screened for the ability to degrade xylan (Lee at al. 1986). β-Xylanase activity in these yeasts was rare. The maximum β-xylanase activity that was observed in *P. stipitis* was only 2.5-3% of the activity reported for *C. albidus* (Basaran et al. 2001). Production of the β-xylanase was induced by xylan but repressed by D-xylose and D-glucose (Gírio et al. 1996). By contrast, it was found that the β-xylosidase is synthesised constitutively to a considerable degree (Özcan et al. 1991). These results are in accordance with our results in chapter 4, where we found very little β-xylanase activity, but considerable β-xylosidase activity for strains cultured on D-xylose. The β-xylanase encoding gene of *C. albidus* was expressed as cDNA and genomic DNA copies in the D-xylose fermenting yeast *P. stipitis* under transcriptional control of the *P. stipitis* xylose reductase encoding gene (*XYL1*) promoter (Morosoli et al. 1992; Morosoli et al. 1993). The enzyme secreted by strains expressing the cDNA copy was 50 kDa in size, slightly larger than the native enzyme, probably reflecting a different glycosylation pattern.

The yeast *Saccharomyces cerevisiae* is capable of high-level fermentation on D-glucose yielding high levels of ethanol and high productivity. Furthermore, *S. cerevisiae* also has good tolerance towards high ethanol concentrations and other inhibitors that may be present in the fermentation media (Hahn-Hägerdal et al. 2001). For these reasons it would be a great advantage to be able to use *S. cerevisiae* for xylan to ethanol fermentations. The β-xylanase encoding genes of the filamentous fungi *Aspergillus niger*, *Aspergillus kawachii* and *Trichoderma reesei* were successfully expressed in *S. cerevisiae* (Crous et al. 1995; La Grange et al. 1996; Luttig et al. 1997). Furthermore, a cDNA copy of the *A. niger* β-xylosidase encoding gene was cloned (La Grange et al. 2001). The 778 amino acid mature protein encoding region was fused in reading frame with the *S. cerevisiae* mating pheromone α-factor secretion signal to ensure secretion from *S. cerevisiae*. When this fusion gene and the *T. reesei xyn2* were co-expressed in *S. cerevisiae* under transcriptional control of the *S. cerevisiae ADH2* promoter, high levels of β-xylanase and β-xylosidase activity were obtained in autoselective (*FUR1*

disrupted) strains grown in rich medium. Co-production of these two enzymes allowed this recombinant *S. cerevisiae* strain to degrade birchwood xylan to D-xylose. However, *S. cerevisiae* cannot degrade xylan nor ferment the main component sugar, D-xylose, to ethanol (La Grange et al. 2001). Considerable efforts have been made to introduce efficient D-xylose fermentation ability into *S. cerevisiae*. However, high-level ethanol production by *S. cerevisiae* from D-xylose as sole carbon source remains a significant challenge. In this study, we have attempted to enhance the xylanolytic ability of *P. stipitis*, a yeast that is considered an efficient D-xylose fermenter.

6.1 CLONING OF NATIVE PROMOTER AND TERMINATOR SEQUENCES.

The transformation system used in this study (Chapter 3–5) is based on the transformation system developed by Yang et al. (1994). The basic vectors were created by cloning the *P. stipitis URA3* and *ARS2* sequences from genomic DNA by means of PCR, using the published sequence information, and cloning the sequences into the pBluescript vector as described in chapter 3 to create a *P. stipitis*/*Escherichia coli* shuttle vector. In our investigation of the heterologous expression system of *P. stipitis*, we started by testing the transcriptional control of two native promoters and one heterologous promoter, and the production of a heterologous protein from these promoters in *P. stipitis* cultivated on D-xylose and D-glucose as carbon sources. DNA fragments encoding the *P. stipitis* xylose reductase gene (*XYL1*) promoter, *P. stipitis* transketolase encoding gene (*TKL*) promoter and the *S. cerevisiae* phosphoglycerate kinase gene (*PGK1*) promoter were fused to the β-xylanase encoding *xyn2* gene of *T. reesei* (used as a reporter gene) and tested for their ability to promote expression of the heterologous β-xylanase in *P. stipitis*. Xylose reductase is the first enzyme in the D-xylose assimilation pathway of *P. stipitis* (Amore et al. 1991) and the *XYL1* gene is induced in the presence of D-xylose (Webb and Lee, 1992). The *XYL1* promoter was therefore cloned and evaluated as an inducible promoter for foreign gene expression in *P. stipitis*, since the use of inducible promoters is preferable for the high-level production of heterologous proteins (Romanos et al. 1992). Transketolase is an enzyme central to the pentose phosphate pathway, therefore the *TKL* gene should be constitutively expressed in *P. stipitis* as this yeast catabolises most of its carbohydrates, including

hexose sugars, through the pentose phosphate pathway (Jeffries and Kurtzman, 1994; Metzger and Hollenberg, 1994). The *TKL* promoter was therefore chosen as a strong, constitutively expressed promoter for heterologous expression in *P. stipitis*. The *PGK1* promoter is a strong constitutive promoter of *S. cerevisiae* that can be induced to a level of expression that constitutes 4 to 10% of total soluble protein under certain growth conditions (Romanos et al. 1992). After transformation of the plasmids into *P. stipitis*, the *XYL1* promoter was shown to be inducible in the presence of D-xylose, as *xyn2* transcription and β-xylanase activity could be measured when the recombinant strain was cultivated on D-xylose, but not when it was cultivated on D-glucose. *TKL* promoter expression was found to be constitutive when either D-glucose or D-xylose was used as sole carbon source. The recombinant β-xylanase was successfully secreted by its native *T. reesei* secretion signal. The *PGK1* promoter did not promote *xyn2* transcription in *P. stipitis*. Therefore, the native promoters of the *XYL1* and *TKL* genes emerged as good candidates for the transcriptional control of foreign genes expressed in *P. stipitis*.

6.2 THE EXPRESSION AND CO-EXPRESSION OF FUNGAL β-XYLANASES AND A β-XYLOSIDASE IN *P. STIPITIS*.

When the *T. reesei xyn2* gene was expressed under transcriptional control of the inducible *P. stipitis XYL1* and the constitutive transketolase encoding *TKL* promoters, funtional β-xylanase was secreted under control of the *T. reesei* secretion signal. The molecular size of the recombinant Xyn2 protein produced by *P. stipitis* was 20.7 kDa, which is similar to that of the native *T. reesei* Xyn2 protein. This indicates no or minimal glycosylation of the recombinant secreted protein. The recombinant *xyn2*-expressing strain also yielded twice the amount of biomass yielded by the control strain when cultivated in medium containing 1% birchwood xylan as sole carbon source. Therefore, in the second part of our study we further explored the increase in biomass yield by recombinant *P. stipitis* strains expressing and co-expressing xylanolytic genes (Chapter 4). β-Xylanase encoding genes of *T. reesei* (*xyn2*) and *A. kawachii* (*xynC*) were cloned under transcriptional control of the inducible *P. stipitis XYL1* gene promoter on episomal plasmids. The β-xylosidase encoding gene of *A. niger* (*xlnD*) was cloned as an in reading frame fusion with the *S. cerevisiae MFα1* secretion signal under

transcriptional control of the constitutive *P. stipitis TKL* gene promoter on an episomal plasmid. Combinations of the individual β-xylanase encoding genes and β-xylosidase expression cassette were also cloned onto episomal plasmids. All of the plasmids were subsequently transformed to *P. stipitis* and the β-xylanase activities, β-xylosidase activities and growth of the recombinant strains on xylan as sole carbon source were monitored. When β-xylanase activities of the recombinant *P. stipitis* strains were determined quantitatively, the *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains reached maximum activity levels of 136.7 ± 5.6 nkat/ml and 128.1 ± 5.3 nkat/ml, respectively, but dropped to levels close to zero after 120 h. In contrast, the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains reached maximum activity levels of 171.8 ± 8.7 nkat/ml and 165.1 ± 2.3 nkat/ml, respectively, and remained at activity levels above 50 nkat/ml after 120 h of incubation. The reduction of β-xylanase activity could be ascribed to the depletion of D-xylose, the inducer of the *XYL1* promoter, in the medium and the production of extracellular protease activity by *P. stipitis*. To ascertain the stability of the heterologous β-xylanase activity, the cultures were grown in a fermentor where the pH level was controlled to pH 6. Maximal β-xylanase activity levels of 142.3 ± 3.3 nkat/ml and 226.7 ± 7.5 nkat/ml for the *P. stipitis* [*xyn2*] and the *P. stipitis* [*xynC*] strains, respectively, were attained after 21 h of growth. However, the levels of β-xylanase activity still declined to levels close to zero nkat/ml after reaching maximal levels. The presence of extracellular proteases secreted by the *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and the *P. stipitis* [*xynC*] strains, and proteolytic activity in the supernatants of fermentor cultures were confirmed by spotting the cultures and supernatant concentrates onto milk plates. Therefore, the proteolytic activity was not merely limited to acid protease(s). The proteolytic activity in the medium had an optimum activity at 60°C and pH 7.

When β-xylosidase activities of the recombinant *P. stipitis* strains grown in shake flasks were determined quantitatively, the *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] strains showed similar low levels of β-xylosidase activity attributed to native *P. stipitis* β-xylosidase activity. The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and

P. stipitis [*xynC XLO2*] strains achieved distinctly higher levels of β-xylosidase activity of 0.132 ± 0.007 nkat/ml, 0.113 ± 0.005 nkat/ml and 0.124 ± 0.003 nkat/ml, respectively. It is notable that the co-expression of the heterologous β-xylanases and β-xylosidase did not result in significantly lower levels of activity of the two enzymes in comparison with strains where they were expressed separately, as was observed in *S. cerevisiae* (La Grange et al. 2000; La Grange et al. 2001).

6.3 GROWTH AND ETHANOL PRODUCTION OF RECOMBINANT XYLANOLYTIC *P. STIPITIS* STRAINS ON XYLAN AS SOLE CARBON SOURCE.

The additional production of recombinant β-xylanases in *P. stipitis*, a yeast known to produce low xylanase activity when cultured on xylan as sole carbon source (Özcan et al. 1991), enhanced its growth on xylan as sole carbon source. In our study the recombinant strains over-expressing fungal xylanolytic enzymes show a dramatic increase in biomass formation on xylan as sole carbon source (Chapter 4; Chapter 5). The expression of the *XLO2* gene alone also resulted in a marked increase of 6.9 ± 0.72% in biomass formation over the control strain in shake flask cultures. This could be attributed to weak native *P. stipitis* β-xylosidase activity, as is the case with the native β-xylanase activity (Basaran et al. 2001). The production of either of the fungal β-xylanases resulted in higher biomass yields, with the expression of *A. kawachii xynC* yielding about 16% more and of *T. reesei xyn2* about 10% more biomass than the control strain. During co-expression of the β-xylanases and the β-xylosidase, the simultaneous action of the two heterologous xylanolytic enzymes gave further improvements in biomass yields. The *P. stipitis* [*xynC XLO2*] strain showed the maximum biomass yield improvement of about 27%, and the *P. stipitis* [*xyn2 XLO2*] strain showed about 20% improvement over the *P. stipitis* [VECT] control strain. It is likely that the *P. stipitis* [*xynC*] and the *P. stipitis* [*xynC XLO2*] strains produce higher biomass yields from xylan as sole carbon source than the *T. reesei xyn2* expressing strains because of the higher β-xylanase activity observed in these strains and the higher stability of the *xynC* gene product. When these strains were grown in a fermentor, high levels of oxygenation led to even further increases in cell yield of the recombinant *P. stipitis* strains (Chapter 5). Cell counts improved on average

by 85% over the cell counts attained in shake flasks. However, increases in biomass over the control strain remained similar to the increases in biomass yield over the control strain attained in shake flask cultures of the same strains. The *P. stipitis* [*xynC XLO2*] strain, the most successful of the recombinant *P. stipitis* strains, yielded approximately 3.2-fold more biomass than the *P. stipitis* [VECT] control strain on xylan as sole carbon source.

Interestingly, the *P. stipitis* [*xynC XLO2*] strain yielded ca. 32% of the theoretical maximum biomass yield in both shake flasks and fermentor, indicating that biomass yield in this strain is possibly limited by other factors. The birchwood glucuronoxylan used contained glucuronic acid side chains at a ratio of one glucuronic acid molecule per ten xylose residues, thus the degradation of the polymer by the β-xylanases was limited because of the limitations of family 11 β-xylanases (Puls and Schuseil, 1993; Biely et al. 1997). Family 11 β-xylanases are incapable of attacking the glycosydic linkage next to the side chain and towards the non-reducing end, thus requiring three unsubstituted consecutive xylopyranosyl residues for cleavage. This would lead to a significant amount of unutilised D-xylose residues. Therefore, in the case of recombinant strains expressing the β-xylanase alone up to 40% of the D-xylose residues would not be utilised. In the case of recombinant strains co-expressing the β-xylanase and the β-xylosidase the heterologous β-xylosidase can remove the D-xylose residue adjacent to the substituted residue at the reducing end. This results in only approximately 30% of the D-xylose residues remaining unutilised. The biomass yield could therefore be increased even further with the co-expression of an α-glucuronidase gene in these strains. It is also interesting to note that the percentage of improvements in biomass yields of the strains co-expressing the xylanolytic genes is more than the sum of the percentages of improvement of the strains expressing the genes individually. This showed the synergistic action of the heterologous xylanolytic enzymes on xylan.

It has been shown that some strains of the yeast *P. stipitis* can convert xylan directly to ethanol (Lee et al. 1986; Morosoli et al. 1993). *P. stipitis* has a specific requirement for

low oxygenation for ethanol formation (Skoog and Hahn-Hägerdal, 1990) and as such, *P. stipitis* [VECT] and *P. stipitis* [*xynC XLO2*] (as the strain showing the most improvement in biomass yield) were grown in shake flasks at low oxygenation to determine ethanol production of the recombinant strains. The *P. stipitis* [*xynC XLO2*] strain showed significant improvement in ethanol productivity of approximately 3.6-fold over the *P. stipitis* [VECT] control strain, yielding 1.35 g/L ethanol. The increase is presumably due to the action of the heterologous xylanolytic enzymes that liberate more D-xylose from the xylan polymer to be available for ethanol formation. However, in previous studies higher ethanol levels were obtained using *P. stipitis* strains on xylan as sole carbon source. A natural isolate of *P. stipitis* grown on 1% larchwood xylan, attained 1.8 g/L ethanol (Lee et al. 1986). The recombinant *P. stipitis* strain constructed by Morosoli et al. (1993) also yielded 1.8 g/L ethanol on 1% xylan. Therefore, although the *P. stipitis* [*xynC XLO2*] recombinant strain showed a significant increase in ethanol yield from xylan as sole carbon source over the control strain, the final ethanol yield was relatively low. However, other stress factors present, because of the dissimilarity in medium composition, could also lead to differences in ethanol yield. Furthermore, it was reported that the auxotrophic *P. stipitis* strain used to construct these strains is a poor fermenter (Yang et al. 1994) and higher ethanol yields may therefore be achieved by expressing the recombinant genes in a better fermenting strain. We have therefore shown in this study that the successful expression of xylanolytic genes in *P. stipitis* leads to an increase in biomass and ethanol yield of the resulting recombinant strains grown on xylan as sole carbon source.

6.4 CONCLUSIONS

From the data presented in this study the following can be concluded:

1. The *XYL1* and *TKL* promoter and terminator sequences of *P. stipitis* were cloned and reporter gene expression using these promoters was shown to be inducible by D-xylose (*XYL1*) and constitutive, respectively, depending on the carbon source.

2. The *T. reesei xyn2*, the *A. kawachii xynC* and the *A. niger xlnD* (as a fusion with the *S. cerevisiae* mating factor secretion signal) genes were successfully expressed

and coexpressed in *P. stipitis*. Heterologous β-xylanase and β-xylosidase production was shown.

3. Extracellular β-xylanase activity was reduced by the action of secreted proteases produced by *P. stipitis*.

4. Biomass yield from xylan as sole carbon source was improved in the recombinant *P. stipitis* strains producing heterologous xylanolytic enzymes.

5. The recombinant *P. stipitis* strains producing heterologous xylanolytic enzymes also showed an improvement in ethanol production from xylan as sole carbon source.

In this study we have shown the potential of the yeast *P. stipitis* as heterologous expression system. We have created recombinant *P. stipitis* strains expressing fungal xylanolytic enzymes that are more efficient in degrading xylan than native *P. stipitis* strains. These strains represent a step towards the efficient degradation and utilisation of hemicellulosic materials by ethanol-producing yeasts.

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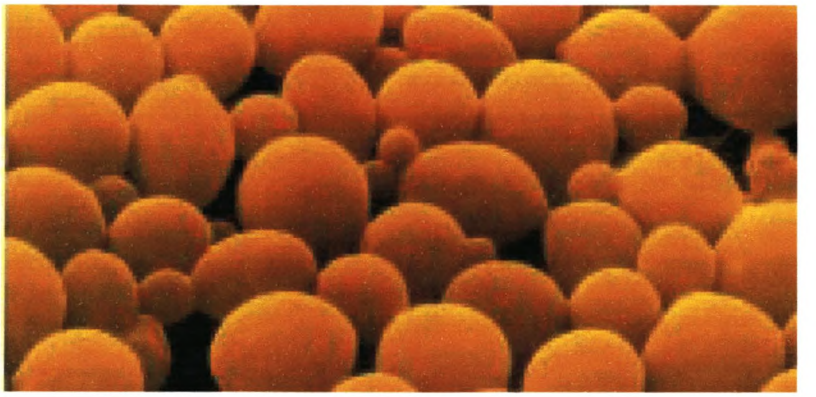
7 APPENDIX



Provisional Patent

DrG REF 607014spec

TITLE OF INVENTION

Method for enhancing xylan degrading ability.

FIELD OF INVENTION

The present relates to a method for enhancing xylan degrading ability.

More particularly, the invention relates to a method for enhancing xylan degrading ability of *Pichia stipitis*.

BACKGROUND TO INVENTION

Xylan, the major hemicellulose component in plant cell walls, is the most abundant polysaccharide after cellulose (Lappalainen, 1986). This makes the main constituent sugar of xylan, D-xylose, the second most abundant renewable monosaccharide in nature (Jeffries and Jin, 2000). The heterogenous xylan polymer consists of a main chain of β-1,4 linked D-xylose residues. The C-2 and C-3 positions of these D-xylose moieties can be substituted with L-arabinofuranose or 4-*O*-methyl glucaronic acid residues, or they can be esterified with acetic acid. Furthermore, the L-arabinofuranosyl residues in the side chains can be esterified with ferulic and *p*-coumaric acid (Van Peij et al. 1997; Tenkanen et al. 1996). Therefore, complete degradation of this complex structure requires the synergistic action of a range of different enzymes. The main xylanolytic activities are catalysed by endo-β-1,4 xylanases, which hydrolyse β-1,4 bonds between D-xylose residues in the main chain yielding xylo-oligosaccharides. β-D-Xylosidases hydrolyse xylo-oligosaccharides to D-xylose. However, α-L-arabinofuranosidase, α-glucuronidase, acetyl (xylan) esterase, ferulic and *p*-coumaric acid esterase all have specific cooperative functions in the complete degradation of xylan (Tenkanen et al. 1996).

Many bacterial and fungal species produce the full complement of enzymes necessary to enable them to utilise xylan as a carbon source (Uffen, 1997). Strains of the fungi *Trichoderma* and *Aspergillus* secrete large amounts of efficient xylan-degrading enzymes (Wong and Sadler, 1992; De Vries et al. 2000; Hrmova et al. 1989). *Trichoderma reesei* is a filamentous, mesophilic fungus known for its cellulolytic and xylanolytic activities (Haltrich et al. 1996; Tenkanen et al. 1996). This fungus secretes two major β-1,4 endoxylanases encoded by *xyn1* and *xyn2* (Törrönen et al. 1992). The *xyn2* gene encodes

a 21 kDa enzyme that represents 50% of the total xylanolytic activity of *T. reesei* cultivated on xylan. Members of the genus *Aspergillus* are also efficient producers of cellulolytic and xylanolytic enzymes (Uffen, 1997; Haltrich et al. 1996). The two β-1,4 endoxylanases and the β-xylosidase of *Aspergillus niger* are encoded by *xlnB*, *xlnC* and *xlnD*, respectively (Van Peij et al. 1997). The *xlnD* gene encodes the 804 amino acid β-xylosidase with a predicted molecular mass of 85 kDa. *Aspergillus kawachii* is a fungus used in the fermentation of traditional Japanese spirits, a process carried out under acidic conditions (pH 3.2 – 4.3) (Ito et al. 1992a; Kormelink et al. 1992). The main β-1,4 endoxylanase of *A. kawachii* is encoded by *xynC*. This gene has been successfully cloned and sequenced (Ito et al. 1992b; Crous et al. 1995)

The yeast *Saccharomyces cerevisiae* has been used extensively for the production of ethanol and has also been established as a host for the expression of heterologous proteins of biotechnological interests (Romanos et al. 1992). Several xylanolytic genes have been successfully expressed in the *S. cerevisiae* (La Grange et al. 1996; La Grange et al. 2001; Crous et al. 1995). A *S. cerevisiae* strain has been constructed that co-expresses the *T. reesei xyn2* gene and the *A. niger xlnD* gene (La Grange et al. 2001). This strain effectively degrades birchwood xylan to D-xylose, however, the main disadvantage of *S. cerevisiae* is that it cannot utilise or ferment D-xylose, the main component of xylan.

The yeast *Pichia stipitis* and the related *Candida shehatae* are the best xylose-fermenting yeasts thus far described (Jeffries and Kurtzman, 1994). It has also been shown that various *P. stipitis* strains can utilise xylan as sole carbon source (Özcan et al. 1991; Basaran et al. 2001). The β-1,4 endoxylanase encoding gene of *P. stipitis*, *xylA*, was cloned and successfully expressed in *Escherichia coli* (Basaran et al. 2001). However, xylan-degrading *P. stipitis* strains isolated produce very low levels of xylanolytic enzymes.

It is an object of the invention to suggest a method for enhancing xylan degrading ability which will assist in overcoming the aforesaid problems.

SUMMARY OF INVENTION

According to the invention, a method of enhancing xylan degrading ability of *Pichia stipitis* includes the step of expressing *Trichoderma reesei (xyn2) gene*, *Aspergillus kawachii (xynC)* gene and *Aspergillus niger (xlnD)* gene separately in *Pichia stipitis*.

Also according to the invention, a method of expressing *Trichoderma reesei (xyn2) gene*, *Aspergillus kawachii (xynC)* gene and *Aspergillus niger (xlnD)* gene in *Pichia stipitis* includes the step of expressing *Trichoderma reesei (xyn2) gene*, *Aspergillus kawachii (xynC)* gene and *Aspergillus niger (xlnD)* gene separately in *Pichia stipitis.*

Yet further according to the invention, a method of enhancing xylan degrading ability of *Pichia stipitis* includes the step of co-expression of heterologous genes in *Pichia stipitis.*

Yet further according to the invention, a method of cloning genes, includes the step of cloning in combination individual β-xylanase encoding genes and β-xylosidase encoding genes.

Yet further according to the invention, a method of conversion of xylan to ethanol includes any one of the aforementioned methods.

Yet further according to the invcention, a recombinant Pichia stipitis strain, which is capable of expressing fungal xylanolytic enxymes.

The method may include over-expression of fungal xylanolytic enzymes.

The *Trichoderma reesei* (*xyn2*) gene may be expressed by cloning β-Xylanase encoding genes under transcriptional control of inducible *Pichia stipitis* xylose reductase (*XYL1*) gene promoter on episomal plasmids pRDH12.

The *Aspergillus kawachii* (*xynC*) gene may be expressed by cloning β-Xylanase encoding genes under transcriptional control of inducible *Pichia stipitis* xylose reductase (*XYL1*) gene promoter on episomal plasmids pRDH16.

The *Aspergillus niger* (*xlnD*) gene may be expressed by cloning β-xylosidase encoding gene of *Aspergillus niger* (*xlnD*) in reading frame fusion with *Saccharomyces cerevisiae MFα1* secretion signal under transcriptional control of constitutive *Pichia stipitis* transketolase (*TKL*) gene promoter on episomal plasmids (pRDH21).

The method may include generation of recombinant strains with combinations of the $XYL1_P$-*xyn2*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes and the $XYL1_P$-*xynC*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes.

Vectors may be transformed to *P. stipitis* TJ26 and maintained episomally.

The cloning in combination of the individual β-xylanase encoding genes and β-xylosidase encoding genes may occur onto episomal plasmids pRDH22 and/or pRDH26.

The plasmids may be subsequently transformed to *Pichia stipitis* TJ26.

Xylan may be the sole carbon source.

Xylose may be added to heterologous β-xylanase expressing cultures.

The *Trichoderma reesei xyn2* and the *Aspergillus niger xlnD* genes may be co-expressed.

The *Aspergillus kawachii xynC* and the *Aspergillus niger xlnD* genes may be co-expressed.

The β-xylanase activity, β-xylosidase activity and growth of recombinant strains may be monitored.

The fungal β-xylanase may be held at pH 3 for increased activity.

Biomass yield may be increased by expression of α-glucuronidase.

The method and/or Pichia stipitis strain may be used in degradation and utilisation of hemicellulosic materials.

In the specification the term P. Stipitis refers to Pichia stipitis, A. kawachii refers to Aspergillus kawachii, A. niger refers to Aspergillus niger, S. cerevisiae refers to Saccharomyces cerevisiae, and T. reesei refers to Trichoderma reesei.

BRIEF DESCRIPTION OF DRAWINGS

The invention will now be described by way of example with reference to the accompanying schematic drawings.

In the drawings there is shown in:

Figure 1: Schematic representation of plasmids used in the invention. The *P. stipitis* autonomous replicating sequence (*ARS2*) is responsible for episomal replication of the plasmid and the *P. stipitis* orotidine-5'-phosphate decarboxylase (*URA3*) is used as selectable marker. $XYL1_P$, $XYL1_T$, $TKL1_P$, $TKL1_T$, represent the *P. stipitis* xylose reductase gene promoter and terminator sequences as well as the *P. stipitis* transketolase gene promoter and terminator sequences, respectively;

Figure 2: Recombinant β-xylanase producing *P. stipitis* strains. SC^{-URA} medium with 0.2% RBB-xylan supplemented with 2% xylose as the carbon source was patched with (1) *P. stipitis* [VECT], (2) *P. stipitis* [*xyn2*], (3) *P. stipitis* [*xyn2 XLO2*] (4) *P. stipitis* [*XLO2*] (5) *P. stipitis* [*xynC*] and (6) *P. stipitis* [*xynC XLO2*]. Colonies degrading RBB-xylan are surrounded by

pale clearing zones. The plates were photographed after 30 h of incubation at 30°C;

Figure 3: Time course of β-xylanase produced by *P. stipitis* [VECT], *P. stipitis* [*xyn2*], *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*], *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*], on 4% xylose as carbon source. Activity is expressed in katals/ml, with 1 katal representing the amount of enzyme needed to produce 1 mol of reducing sugar from birchwood xylan per second under the conditions of the assay (Bailey et al. 1992);

Figure 4: Time course of β-xylosidase produced by *P. stipitis* [VECT], *P. stipitis* [*xyn2*], *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*], *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] on 4% xylose as carbon source. Activity is expressed in katals/ml, with 1 katal representing the amount of enzyme needed to produce 1 mol of reducing sugar equivalent from the chromophoric substrate (PNPX) per second;

Figure 5: Extracellular protease producing *P. stipitis* strains. SC^{-URA} medium with 2% skim milk powder supplemented with 2% xylose as the carbon source was patched with (1) *P. stipitis* [VECT], (2) *P. stipitis* [*xyn2*], (3) *P. stipitis* [*xyn2 XLO2*], (4) *P. stipitis* [*XLO2*], (5) *P. stipitis* [*xynC*] (6) *P. stipitis* [*xynC XLO2*], and (7) *S. cerevisiae* Y294. The plates were photographed after 30 h of incubation at 30°C. Proteinase K solution (Roche) was spotted on the plate to serve as positive control 3 hours before the plates were photographed; and

Figure 6: Growth curve of recombinant *P. stipitis* strains, *P. stipitis* [VECT], *P. stipitis* [*xyn2*], *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*], *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] on double strength SC^{-URA} medium containing 2% birchwood xylan as sole carbon source.

DETAILED DESCRIPTION OF DRAWINGS

The invention will now be described by way of example with reference to the accompanying schematic drawings and experiment.

EXPERIMENT

(a) Media and culture conditions

P. stipitis was cultivated on YPD medium (1% yeast extract, 2% peptone, 2% glucose) or selective synthetic (SC) medium [containing 2% glucose or xylose, yeast nitrogen base without amino acids (Difco) (pH 6) and all the required growth factors except uracil (SC^{-URA})]. Solid media contained 2% agar. Yeasts were routinely cultured in 250-ml Erlenmeyer flasks containing 100 ml medium at 30°C on a rotary shaker at 150 rpm.

(b) Microbial strains and plasmids

Table 1 summarises the genotypes and sources of the yeast and bacterial strains, as well as the plasmids that were constructed and used in the experiment.

Table 1 Microbial strains and plasmids

Strain/Plasmid	Genotype	Source/Reference
Yeast strains:		
Saccharomyces cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	
Pichia stipitis TJ26	*ura3*	Yang et al. 1994
P. stipitis TJ26:		
pJM6[a]	*ura3/URA3*	Den Haan & Van Zyl 2001
pRDH12[b]	*ura3/URA3 $XYL1_P$-xyn2-$XYL1_T$*	Den Haan & Van Zyl 2001
pRDH16[c]	*ura3/URA3 $XYL1_P$-xynC-$XYL1_T$*	
pRDH21[d]	*ura3/URA3 TKL_P-XLO2-TKL_T*	
pRDH22[e]	*ura3/URA3 $XYL1_P$-xyn2-$XYL1_T$ TKL_P-XLO2-TKL_T*	
pRDH26[f]	*ura3/URA3 $XYL1_P$-xynC-$XYL1_T$ TKL_P-XLO2-TKL_T*	
Bacterial strain:		
Escherichia coli XL1-Blue MRF'	*Δ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]*	Stratagene
Plasmids:		
pbluescript II SK(+)	*bla*	Stratagene
pDLG55	*bla URA3 $PGK1_P$-xyn2-$PGK1_T$*	La Grange et al. 2001
pJC3	*bla URA3 $PGK1_P$-xynC-$PGK1_T$*	Crous et al. 1995
pJM6	*bla URA3*	Yang et al. 1994
pRDH12	*bla URA3 $XYL1_P$-xyn2-$XYL1_T$*	Den Haan & Van Zyl 2001
pRDH16	*bla URA3 $XYL1_P$-xynC-$XYL1_T$*	
pRDH21	*bla URA3 TKL_P-XLO2-TKL_T*	
pRDH22	*bla URA3 $XYL1_P$-xyn2-$XYL1_T$ TKL_P-XLO2-TKL_T*	
pRDH26	*bla URA3 $XYL1_P$-xynC-$XYL1_T$ TKL_P-XLO2-TKL_T*	

[a] *P. stipitis* TJ26 (pJM6) was designated *P. stipitis* [VECT]
[b] *P. stipitis* TJ26 (pRDH12) was designated *P. stipitis* [*xyn2*]
[c] *P. stipitis* TJ26 (pRDH16) was designated *P. stipitis* [*xynC*]

[d] *P. stipitis* TJ26 (pRDH21) was designated *P. stipitis* [*XLO2*]
[e] *P. stipitis* TJ26 (pRDH22) was designated *P. stipitis* [*xyn2 XLO2*]
[f] *P. stipitis* TJ26 (pRDH26) was designated *P. stipitis* [*xynC XLO2*]

(c) Plasmid construction and transformation

Standard protocols were followed for DNA manipulations (Sambrook et al. 1989). Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Tautz and Renz (1983). Restriction endonucleases and T4 DNA ligase were purchased from Roche Molecular Biochemicals and used as recommended by the manufacturer. The construction of two inducible expression vectors, containing the *XYL1* promoter sequences, pRDH10 and pRDH12, was described previously (Den Haan and Van Zyl, 2001). The constitutive expression vector pRDH20 was created by cloning the DNA elements encoding the *P. stipitis TKL* promoter, *TKL* terminator, *ARS2* and *URA3* from *P. stipitis* genomic DNA using PCR amplification and inserting them into pBluescriptII SK(+). DNA was amplified in 50-µl reaction mixtures using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) according to the manufacturers instructions, with a Perkin Elmer GeneAmp® PCR System 2400 (The Perkin-Elmer Corporation, 761 Main Avenue, Norwalk, Connecticut 06859). Denaturation and polymerisation were carried out for 1 min at 94°C and 1 min at 72°C, respectively for 30 cycles. The primers for the amplification of the *P. stipitis ARS2* and *URA3* were described previously (Den Haan and Van Zyl, 2001). Table 2 details the primers and annealing temperatures used in this study (restriction enzyme sites are underlined).

Table 2 PCR primers

Gene/fragment and annealing temperature used	**Primers**	**Source of DNA sequence**
A. kawachii xynC 60°C	XYNCL (*Xba*I restriction site underlined) 5'- GACTTCTAGACATGAAGGTCACTGCGG -3' XYNCR (*Bam*HI restriction site underlined) 5'- GACTGGATCCCCCTTAAGAGGAGATCGTG -3'	Crous et al. 1995
P. stipitis TKL promoter 55°C	TKLPL (*Sac*I and *Apa*I restriction sites underlined) 5'- GACTGAGCTCGGGCCCCCAGAGTTCATGCTACTCAAC -3' PTKLR (*Xba*I restriction site underlined) 5'-GTACTCTAGAGGCAAATGGAAGTCTGG-3	Amore et al. 1991
P. stipitis TKL terminator 55°C	TKLTL(*Hin*dIII restriction site underlined) 5'- GACTAAGCTTTTCAAGTTTTGGTTCTAGC -3' TKLTR (*Sal*I and *Apa*I restriction sites underlined) 5'- GACTGTCGACGGGCCCTATTCCATATTTTTCTGAACT -3'	Amore et al. 1991
S. cerevisiae MFα1 *A niger xlnD* fusion (*XLO2*)	MFXLNL (*Xba*I restriction site underlined) 5'- TGACTCTAGAATGAGATTTCCTTCAATTTTTAC -'3 MFXLNR (*Bam*HI restriction site underlined)	La Grange et al. 2001

60°C	5'- TGACGGATCCTACTTTCTACTCCTTCCCAGG -3'	

Fig. 1 summarises the plasmids that were used in the invention.

The *A. kawachii xynC* gene was cloned as a 0.65-kb *Xba*I/*Bam*HI fragment from pJC3 (Crous et al. 1995) into the *Xba*I/*Bam*HI sites of pRDH10 to yield pRDH16. For the construction of pRDH20 the PCR amplification products were digested with restriction endonucleases and the DNA fragments were purified from agarose gels and sequentially cloned into pBluescript II SK(+). The *TKL* promoter was cloned as a 0.36-kb *Sac*I/*Xba*I fragment into pBluescript. The *TKL* terminator was subsequently cloned as a 0.15-kb *Hin*dIII/*Sal*I fragment adjacent to the *TKL* promoter region to yield pRDH18 (containing the *TKL* promoter/terminator cassette). The *URA3* fragment was cloned as a 1.46-kb *Sal*I fragment into the compatible *Xho*I site of pRDH18 to yield pRDH19 and finally the *ARS2* fragment was cloned as a 1.15-kb *Sal*I fragment into the unique *Sal*I site of pRDH19 to yield pRDH20.

The *A. niger xlnD* gene was previously fused in reading frame with the *S. cerevisiae* *MFα1* secretion signal (La Grange et al. 2001). The fused product was designated *XLO2*. This fusion gene was cloned as a 2.62-kb *Xba*I/*Bam*HI fragment from pDLG55 into the *Xba*I/*Bam*HI sites of pRDH20 to yield pRDH21. The TKL_P-*XLO2*-TKL_T cassette was cloned as a 3.15-kb *Apa*I fragment from pRDH21 into the unique *Apa*I site of pRDH12 to yield pRDH22 and into the unique *Apa*I site of pRDH16 to yield pRDH26. DNA transformation of *P. stipitis* was performed using the lithium acetate dimethylsulfoxide (DMSO) method described by Hill et al. (1991).

(d) Screening for β-xylanase and β-xylosidase activity

Transformants containing fungal β-1,4 endoxylanase encoding genes were screened for xylan-degrading ability by patching on SC^{-URA} medium containing 0.2% 2-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB)-xylan (Sigma) containing 2% xylose as carbon source (Biely et al. 1988). Transformants expressing *A. niger* β-xylosidase were screened by patching on SC^{-URA} medium containing 1 mM *p*-nitrophenyl-β-D-xyloside (PNPX) (Sigma) (La Grange et al. 1997).

(e) β-Xylanase and β-xylosidase activity assays

β-Xylanase and β-xylosidase producing cultures were grown in 50 ml double strength SC^{-URA} medium containing 4% xylose as carbon source. Three cultures of every

transformant were inoculated and all enzyme activity determinations were done in triplicate. Samples were periodically taken over a 120 h period. Endo-β-1,4-xylanase activity was assayed according to the method described by Bailey et al. (1992) with 1% birchwood glucuronoxylan (Roth) as the substrate at 50°C for 5 minutes. Appropriate dilutions of the cell free culture solution in 50 mM sodium citrate buffer (pH5.0 for transformants containing the *T. reesei xyn2* gene and pH4.0 for transformants containing the *A. kawachii xynC* gene) were used as the enzyme source. The β-xylanase activity of the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains was measured at pH 4, this is higher than the optimum pH level of the *A. kawachii xynC* gene product (Crous et al. 1995). The higher pH level was used because the DNS assay employed for β-xylanase activity measurement becomes unreliable below pH 4 because of spontaneous substrate hydrolysis. The amount of released sugar was determined by the dinitrosalicylic acid (DNS) method described by Miller et al. (1960). The β-xylosidase activity was quantified using the chromophoric substrate *p*-nitrophenyl-β-D-xyloside (PNPX) (La Grange et al. 1997). PNPX was used at a concentration of 5 mM. The supernatant with intact cells was used as source of β-xylosidase for the activity determination assays. All activities were expressed in katals per millilitre; one katal is the amount of enzyme needed to produce 1 mol of reducing sugar (or D-xylose equivalent) from birchwood xylan (or chromophoric substrate) per second (Bailey et al. 1992).

(f) Analysis of extracellular protease activity

All *P. stipitis* transformants were screened for extracellular protease activity by patching on SC^{-URA} medium containing 2% skim milk powder and 2% xylose as carbon source (Mattern et al. 1992).

(g) Growth of P. stipitis transformants on xylan

P. stipitis transformants were inoculated from precultures in the stationary growth phase into 50 ml double strength SC^{-URA} medium containing 2% birchwood glucuronoxylan (Sigma) as the sole carbon source. The *P. stipitis* [VECT] strain was also grown on double strength SC^{-URA} medium with 2% xylose as the carbon source in order to set a reference of the growth of *P. stipitis*. Three cultures of each strain were inoculated simultaneously. Samples were periodically taken over a 110 h period and yeast cells in

the media were counted in triplicate on a haemocytometer. The medium was at pH 6 at inoculation and the pH was monitored over the 110-h period.

(h) Expression of the xyn2, xynC and XLO2 genes in P. stipitis

P. stipitis TJ26 was transformed with episomal vectors expressing the *xyn2* gene of *T. reesei* under the transcriptional control of the inducible *P. stipitis XYL1* promoter, the *xynC* gene of *A. kawachii* under the control of the *P. stipitis XYL1* promoter as well as the *XLO2* fusion gene under the control of the constitutive *P. stipitis TKL* promoter. Furthermore, an episomal vector containing both the $XYL1_P$-*xyn2*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes and an episomal vector containing the $XYL1_P$-*xynC*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes were transformed to *P. stipitis* TJ26. The transformation frequency of the recombinant plasmids did not vary greatly and was generally $1x10^3$ colonies per μg DNA. Transformants were confirmed by Southern blot hybridisation using either a α-^{32}P labelled 0.78-kb *xyn2* fragment, a α-^{32}P labelled 0.65-kb *xynC* fragment or a α-^{32}P labelled 2.2-kb *xlnD* fragment as DNA probes.

(i) Screening for β-xylanase and β-xylosidase activity

The *P. stipitis* [VECT] and *P. stipitis* [*XLO2*] strains showed no clearing zone formation on the RBB-xylan plates after 30 hours incubation at 30°C (Fig. 2). This was expected, as these strains do not overexpress a fungal xylanase. The *P. stipitis* [*xyn2*], *P. stipitis* [*xyn2 XLO2*], *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains showed clearing zone formation on the RBB-xylan plate, confirming endoxylanase production. The *P. stipitis* [VECT], *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] strains showed no yellow zone formation on the SC^{-URA} plates containing *p*-nitrophenyl-β-D-xyloside as these strains do not produce significant amounts of β-xylosidase. However, the *P. stipitis* [*XLO2*] *P. stipitis* [*xyn2 XLO2*] *P. stipitis* [*xynC XLO2*] showed yellow zone formation on the SC^{-URA} plates containing *p*-nitrophenyl-β-D-xyloside.

(j) β-xylanase activity

The β-xylanase producing yeast strains were analysed for their ability to secrete biologically active β-xylanase over a period of 120 h (Fig 3). *P. stipitis* [VECT], *P. stipitis* [*xyn2*], *P. stipitis* [*xynC*], *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] were cultured on double strength SC^{-URA} medium containing 4% xylose.

Only basal levels of β-xylanase activity were produced by the *P. stipitis* [VECT] control strain and the *P. stipitis* [*XLO2*] strain throughout the 120-h incubation period. The *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains showed maximal levels of β-xylanase activity of 136.7 ± 5.6 nkat/ml and 128.1 ± 5.3 nkat/ml respectively after 48 h of incubation. After 48 h there was a decline in the level of activity and the β-xylanase activity of these strains had dropped to levels close to zero at 120 h of incubation. The *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains showed maximal levels of β-xylanase activity of 171.8 ± 8.7 nkat/ml and 165.1 ± 2.3 nkat/ml respectively after 50 h of incubation. The levels of β-xylanase activity produced by these strains are significantly higher than the levels produced by the *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains. Furthermore, although the β-xylanase activity of the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains declined after 50 h, the levels were still above 50 nkat/ml at the 120 h point. The pH of the cultures was measured periodically throughout the 120-h period and was found to have dropped from pH 6 to pH 2.5 (Fig 3).

(k) β-xylosidase activity

The β-xylosidase producing yeast strains were analysed for their ability to produce biologically active β-xylosidase over a period of 120 h (Fig 4). These assays were done simultaneously with the β-xylanase assays, using the same cultures. The β-xylosidase activity of the *P. stipitis* [VECT] control strain, the *P. stipitis* [*xyn2*] strain and the *P. stipitis* [*xynC*] strain remained at low levels (≤ 0.04 nkat/ml) throughout the 120 h incubation period. The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] strains showed maximal levels of β-xylosidase activity of 0.132 ± 0.007 nkat/ml, 0.113 ± 0.005 nkat/ml and 0.124 ± 0.003 nkat/ml respectively after 90 h of incubation. The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] strains showed levels of β-xylosidase activity of 0.092 ± 0.004 nkat/ml, 0.090 ± 0.004 nkat/ml and 0.110 ± 0.006 nkat/ml respectively after 120 h of incubation. Although the β-xylosidase activity declined after 90 h of incubation, the decline was not as dramatic as was observed with the β-xylanase activity.

(l) Extracellular protease activity

All the recombinant *P. stipitis* strains showed clearing zone formation on the 2% skim milk plates after 30 hours incubation at 30°C, confirming the production of extracellular proteases in these strains (Fig. 5). Proteinase K solution (Roche) was spotted on the plate to serve as positive control and a laboratory strain of *S. cerevisiae* served as extracellular protease negative control.

(m) Growth of recombinant strains on birchwood xylan as sole carbon source

It has been shown that various *P. stipitis* strains can utilise xylan as sole carbon source (Özcan et al. 1991; Den Haan and Van Zyl, 2001). The *P. stipitis* recombinant strains were tested for growth on double strength SC^{-URA} minimal medium containing 2% birchwood glucuronoxylan as the sole carbon source. The amount of biomass produced was monitored over a 110 h period by means of cell counts (Fig. 6). The biomass yield of *P. stipitis* [VECT] on medium containing 2% xylose as sole carbon source was also monitored to determine maximum *P. stipitis* growth. The maximum cell counts achieved by the different recombinant strains grown on xylan, as well as the percentage of biomass yield achieved with regard to *P. stipitis* [VECT] grown on xylose and the percentage of improvement of the recombinant strains over *P. stipitis* [VECT] grown on xylose are given in table 3. The heterologous expression of either *XLO2*, *xyn2* or *xynC* in *P. stipitis* clearly results in increased biomass yields on xylan. When *XLO2* is co-expressed with either *xyn2* or *xynC*, a further significant increase in biomass production on xylan was observed. The co-expression of *XLO2* and *A. kawachii xynC* gave the highest increase of biomass yield on xylan. In all cultures the pH of the medium was found to have decreased to pH 3 at 110 h.

Conclusion

Xylan, as the major hemicellulose component in plant cell walls, and thus the second most abundant polysaccharide after cellulose, represents one of the most important targets in the exploitation of renewable carbon resources. Therefore, for efficient utilization of xylan as renewable resource, the main component of xylan, D-xylose, must be utilized efficiently. The yeasts *P. stipitis* and *C. shehatae* are the best xylose-fermenting yeasts known and it has been shown that some *P. stipitis* strains can utilise xylan as sole carbon source (Jeffries and Kurtzman, 1994; Özcan et al. 1991; Basaran et al. 2001). Thus, the method in accordance to the invention enhances the xylan degrading

ability of *P. stipitis*. It is shown that successful co-expression of heterologous genes in *P. stipitis* and that the over-expression of fungal xylanolytic enzymes results in increased biomass production when the yeast was grown on xylan as sole carbon source. The *T. reesei xyn2* and *A. kawachii xynC* β-xylanase encoding genes were cloned under transcriptional control of the *P. stipitis* xylose reductase gene (*XYL1*) promoter that was previously shown to be induced in the presence of xylose (Webb and Lee, 1992; Den Haan and Van Zyl, 2001). The *A. niger* β-xylosidase encoding gene (*xlnD*) fused in reading frame with the *S. cerevisiae MFα1* secretion signal (designated *XLO2*) (La Grange et al. 2001) was placed under transcriptional control of the constitutive *P. stipitis* transketolase encoding gene (*TKL*) promoter. Recombinant strains with combinations of the $XYL1_P$-*xyn2*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes and the $XYL1_P$-*xynC*-$XYL1_T$ and TKL_P-*XLO2*-TKL_T expression cassettes were also generated. All vectors were transformed to *P. stipitis* TJ26 and maintained episomally.

When β-xylanase activities of the recombinant *P. stipitis* strains were determined quantitatively, the *P. stipitis* [*xyn2*] and *P. stipitis* [*xyn2 XLO2*] strains reached maximum activity levels of 136.7 ± 5.6 nkat/ml and 128.1 ± 5.3 nkat/ml respectively, but dropped to levels close to zero after 120 h (Fig. 3). In contrast, the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains reached maximum activity levels of 171.8 ± 8.7 nkat/ml and 165.1 ± 2.3 nkat/ml respectively, and remained at activity levels above 50 nkat/ml after 120 h of incubation. The β-xylanase activity of the *P. stipitis* [*xynC*] and *P. stipitis* [*xynC XLO2*] strains was measured at pH 4 which is higher than the optimum pH level of the *A. kawachii* XynC β-xylanase (Ito et al. 1992b). The values achieved are therefore likely to underestimate the actual β-xylanase activity achieved. The decline in the β-xylanase activities of the recombinant *P. stipitis* strains is likely due to a combination of factors. The *XYL1* promoter is induced by D-xylose and the depletion of xylose during the time course of the experiment will lead to a loss of *XYL1* promoter induction. It was found that when xylose was added to the heterologous β-xylanase expressing cultures at 120 h a marked increase in β-xylanase activity was observed. However, the depletion of D-xylose alone does not explain why the β-xylanase activity already accumulated is lost. When *P. stipitis* transformants were patched onto milk plates to screen for extracellular

protease activity all transformants showed clearing zones, confirming the presence of extracellular proteases (Fig. 5). This is in accordance with the loss of enzymatic activity for secreted enzymes in the related yeast *P. pastoris* due to protease activity (Sreekrishna et al. 1997) and the observation that many yeast species secrete significant amounts of proteases (Ogrydziak, 1993). The synthesis of extracellular proteases by the recombinant *P. stipitis* strains explains the loss of β-xylanase activity already accumulated. The *A. kawachii xynC* expressing strains retained more β-xylanase activity, probably because the *A. kawachii xynC* gene product was more stable at the lower pH level. This might be because the fungus *A. kawachii* naturally occurs in low pH environments and its β-xylanase may therefore be more resistant to acid protease degradation (Crous et al. 1995; Ito et al. 1992a).

When β-xylosidase activities of the recombinant *P. stipitis* strains were determined quantitatively, the *P. stipitis* [VECT], the *P. stipitis* [*xyn2*] and *P. stipitis* [*xynC*] strains showed similar low levels of β-xylosidase activity attributed to native *P. stipitis* β-xylosidase activity (Fig 4). The *P. stipitis* [*XLO2*], *P. stipitis* [*xyn2 XLO2*] and *P. stipitis* [*xynC XLO2*] strains achieved distinctly higher levels of β-xylosidase activity of 0.132 ± 0.007 nkat/ml, 0.113 ± 0.005 nkat/ml and 0.124 ± 0.003 nkat/ml, respectively. Furthermore, there was no sudden decline of the β-xylosidase activity as was observed with the β-xylanase activity. This was likely due to the fact that most of the β-xylosidase activity was found to be cell associated and not free in the supernatant (La Grange et al. 2001). Proteins in the supernatant seem to be more vulnerable to protease degradation, as is the case with β-xylanase. It is notable that the co-expression of the heterologous β-xylanases and β-xylosidase did not result in significantly lower levels of activity of the two enzymes in comparison with strains where they were expressed separately, as was observed in *S. cerevisiae* (La Grange et al. 2000; La Grange et al. 2001).

The additional production of recombinant β-xylanases in *P. stipitis*, a yeast known to produce low xylanase activity when cultured on xylan as sole carbon source (Özcan et al. 1991), enhanced its growth on xylan as sole carbon source (Den Haan and Van Zyl, 2001). In our study the recombinant strains over-expressing fungal xylanolytic enzymes show a dramatic increase in biomass formation on xylan as sole carbon source (Fig. 6,

Table 3). The expression of the *XLO2* gene alone also resulted in a marked increase of 6.9 ± 0.72% in biomass formation over the control strain. This could be attributed to very weak native *P. stipitis* β-xylosidase activity, as is the case with the native β-xylanase activity (Basaran et al. 2001). The production of either of the fungal β-xylanases resulted in higher biomass yields, with the expression of *A. kawachii xynC* yielding about 16% more and of *T. reesei xyn2* about 10% more biomass than the control strain. During co-expression of the β-xylanases and the β-xylosidase, the simultaneous action of the two heterologous xylanolytic enzymes gave further improvements in biomass yields. The *P. stipitis* [*xynC XLO2*] strain showed the maximum biomass yield improvement of about 27% and the *P. stipitis* [*xyn2 XLO2*] strain showed about 20% improvement over the *P. stipitis* [VECT] control strain. It is likely that the *P. stipitis* [*xynC*] and the *P. stipitis* [*xynC XLO2*] strains produce higher biomass yields from xylan as sole carbon source than the *T. reesei xyn2* expressing strains because of the higher β-xylanase activity observed in these strains (Fig. 4). Furthermore, the pH of the cultures at inoculation was pH 6 but dropped to pH 3 during the course of the experiment. It was previously shown that the *A. kawachii xynC* gene product has a higher activity at pH 3 than the *T. reesei xyn2* gene product (Crous et al. 1995; La Grange et al. 1996). Thus the fungal β-xylanase produced by the *P. stipitis* [*xynC*] and the *P. stipitis* [*xynC XLO2*] strains is more active at pH 3, probably leading to better xylan degradation and higher biomass production. It is interesting to note that the percentage of improvements in biomass yields of the strains co-expressing the xylanolytic genes is more than the sum of the percentages of improvement of the strains expressing the genes individually. This showed the synergistic action of the heterologous xylanolytic enzymes on xylan. The production of heterologous enzymes by the recombinant *P stipitis* strains might account for lower than expected biomass yields as energy and therefore carbon is required for heterologous protein production. Furthermore, as the birchwood glucoronoxylan used contained a certain amount of glucoronic acid side chains, the degradation of the polymer by the xylanases expressed was therefore limited because of the limitations of family 11 xylanases (Biely et al. 1997). The biomass yield could therefore be further increased with the expression of an α-glucuronidase. According to the invention, recombinant *P. stipitis* strains expressing fungal xylanolytic enzymes that are more efficient in degrading

xylan than native *P. stipitis* strains were created. These strains represent a step towards the efficient degradation and utilisation of hemicellulosic materials by ethanol-producing yeasts.

Table 3 Maximum levels of biomass achieved by recombinant *P. stipitis* strains

Recombinant strain	**Maximum cell count achieved on 2% xylan (10^6 cells per ml)**	**Percentage of theoretical maximum cell count***	**Percentage of improvement over control strain**
P. stipitis [VECT]	18.9 ± 0.87	6.1 ± 0.28	(control strain)
P. stipitis [*XLO2*]	40.3 ± 2.22	13.0 ± 0.72	6.9 ± 0.72
P. stipitis [*xyn2*]	49.1 ± 1.94	15.8 ± 0.63	9.7 ± 0.63
P. stipitis [*xynC*]	67.6 ± 3.21	21.8 ± 1.04	15.7 ± 1.04
P. stipitis [*xyn2 XLO2*]	79.1 ± 2.09	25.5 ± 0.67	19.4 ± 0.67
P. stipitis [*xynC XLO2*]	102 ± 2.66	32.8 ± 0.86	26.7 ± 0.86

*Maximum theoretical cell count was taken as the maximum cell count achieved when *P. stipitis* was grown on 2% xylose as sole carbon source (3.10E+08 cells/ml).

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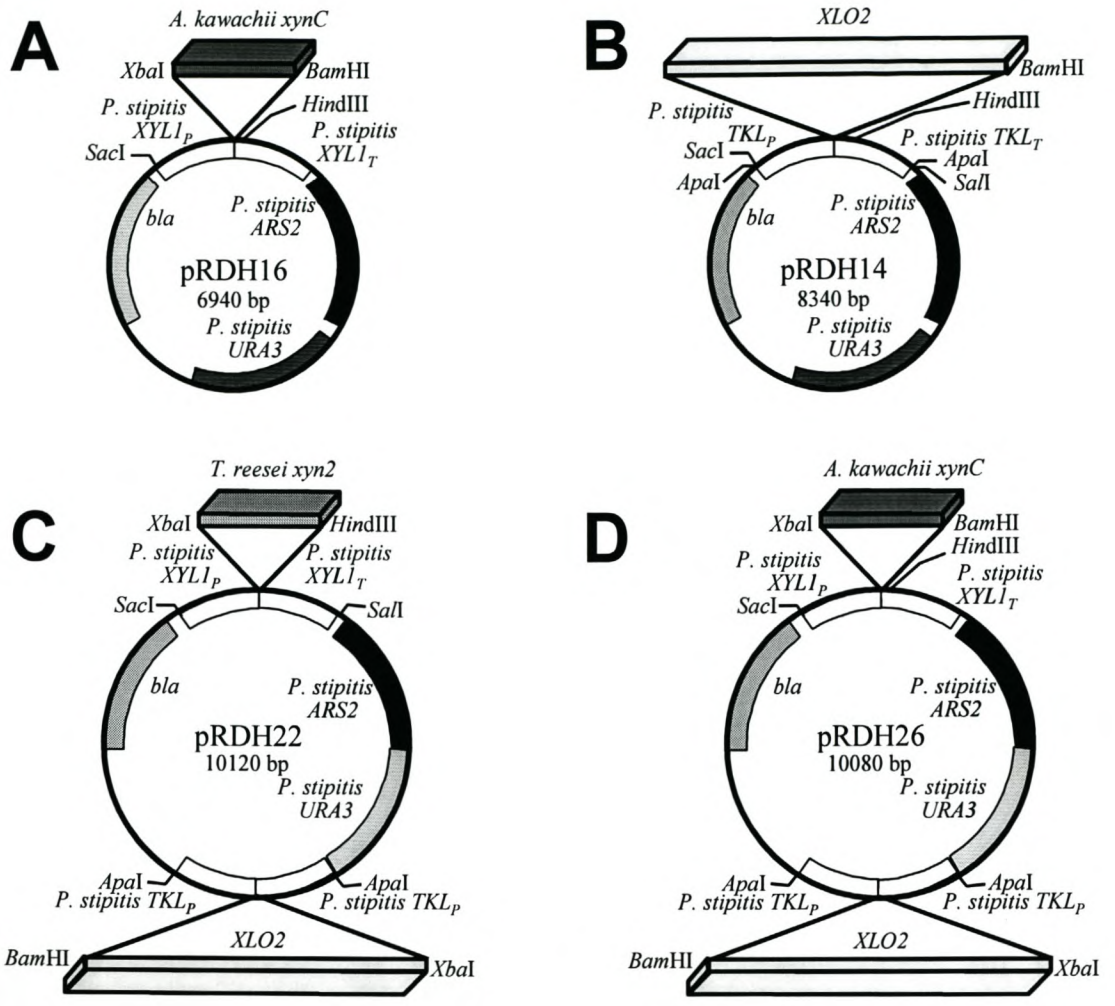


Figure 1

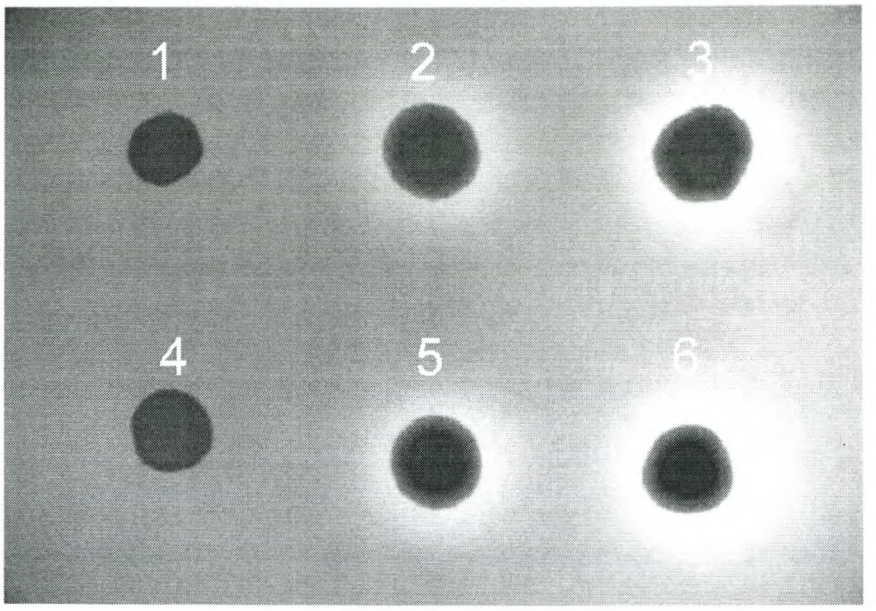
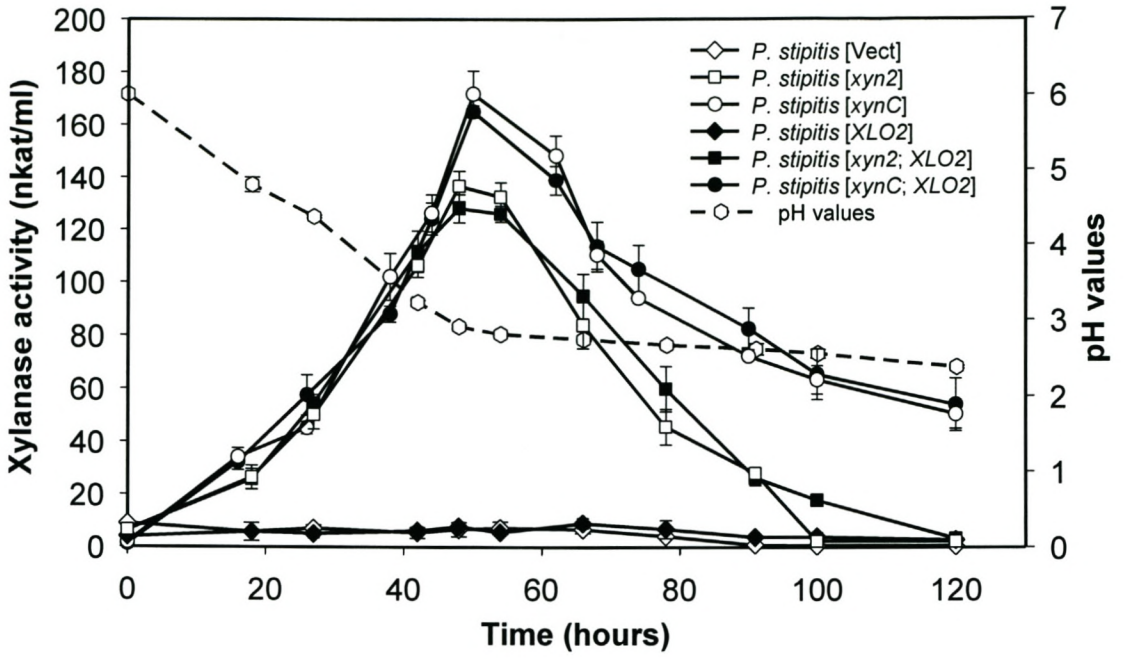


Figure 2



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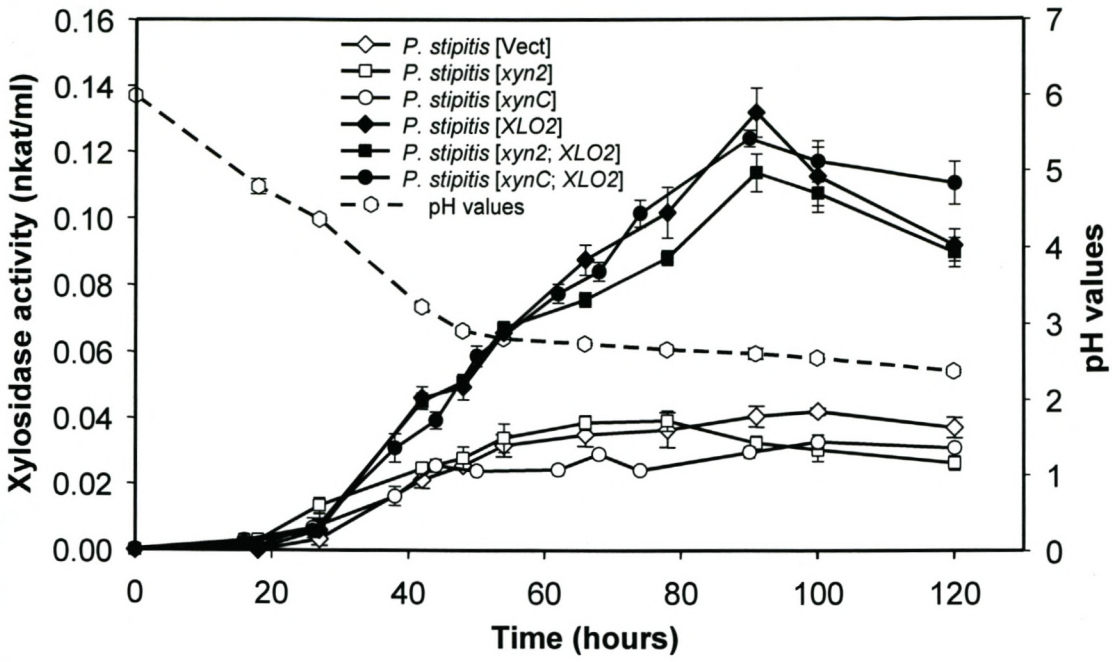

Figure 4

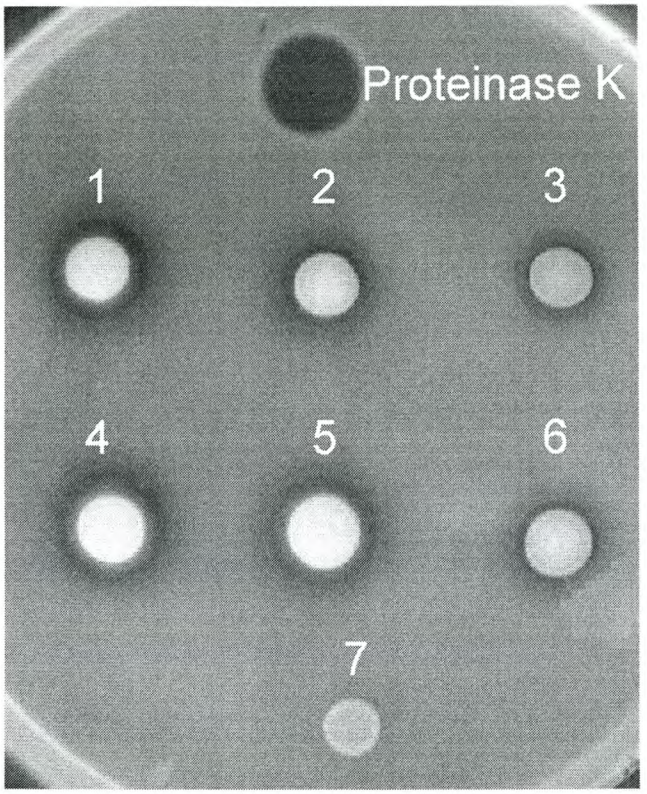

Figure 5

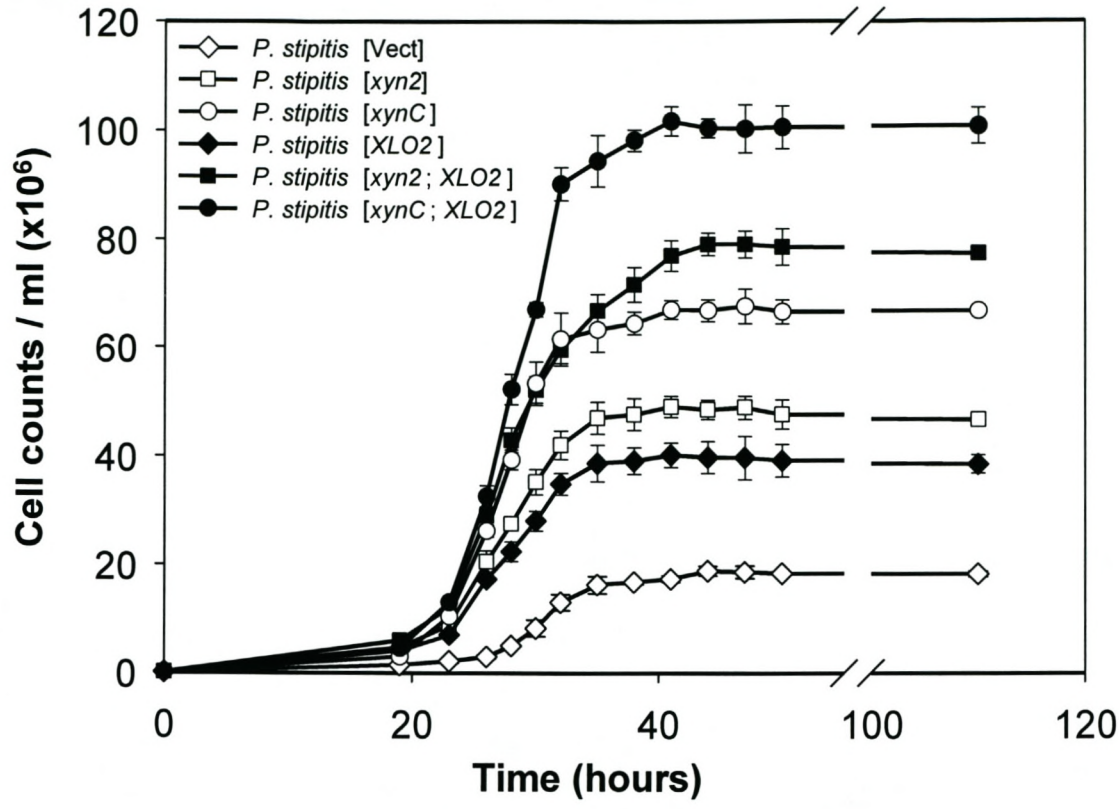

Figure 6