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Biomass to Biofuel: The engineering of *Saccharomyces* species for the co-fermentation of cellulose and xylose

Thesis submitted for the Degree of Doctor of Philosophy

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Declaration

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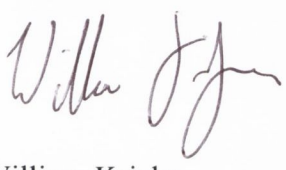

William Kricka

Summary

Lignocellulose represents one of the most abundant biomass sources in the world. Its renewable and abundant nature makes it a prime target for use in bioethanol production. The efficient conversion of lignocellulosic biomass into ethanol requires the utilisation of both the hemicellulosic and cellulosic fractions of biomass. Yeasts commonly used for ethanol production are unable to naturally utilise cellulose or the main component of hemicellulose, the pentose sugar xylose. Previous research has focussed on developing recombinant yeast strains capable of utilising either cellulose or xylose, however the co-utilisation of both cellulose and xylose has yet to be demonstrated.

The aim of this research was to engineer *Saccharomyces sp.* to co-utilise both cellulose and xylose for the fermentation of lignocellulosic biomass. Genes encoding for cellulases (*bgl1, cbh2, egl1, egl2*) and xylose utilising enzymes (*xyl1, xdh1*) were principally mined from the mesophilic fungus *Trichoderma reesei* and initially expressed individually in several *Saccharomyces sp.* Significantly more activity for both the cellulases and xylose utilising enzymes was observed in *Saccharomyces pastorianus* when compared to other *Saccharomyces sp.*, revealing this species to be a superior host of recombinant enzyme production. Recombinant protein production was optimised by examining expression of the genes from different promoters.

Fermentations with *S. pastorianus* strains expressing the genes for cellulase production, and using purified cellulose as a sole carbohydrate source, yielded low ethanol levels due to the reduced activity of cellulases at fermentation temperatures and by the lack of available sugars at the start of the fermentation. The introduction of a pre-fermentation hydrolysis step of cellulose by enzymes produced *in situ* improved ethanol yields.

The xylose reductase (XR)/xylitol dehydrogenase (XDH) and the xylose isomerase (XI) pathways have been previously introduced into *S. cerevisiae* to facilitate xylose utilisation. To compare these two pathways the *xi* gene from *Piromyces sp.* was also expressed in *S. pastorianus*. Ethanol production from xylose by engineered *S. pastorianus* strains via the XR/XDH pathway was significantly greater (7-fold) than that achieved by the XI pathway. Ethanol production using *xyl1* and *xdh1* co-expressing strains was enhanced by over-expression of a copy of the *S. cerevisiae*

XKS1 gene and by increasing the starting substrate concentration of xylose in the medium.

A strategy for co-utilisation of cellulose and xylose was developed whereby yeast strains co-expressing the recombinant cellulases and xylose utilising enzymes, in different combinations, were co-cultured to reconstitute the enzymatic pathways *in situ*. The co-production of cellulases and the xylose utilising enzymes, substantially increased cellulose hydrolysis *in situ* and resulted in an 82% conversion of available sugars to ethanol.

Different types of biomass were examined as a sugar source for ethanol production using the engineered strains. Due to the complex nature of lignocellulose, a physico-chemical pre-treatment is required to release sugars for fermentation. Xylose metabolism, cell growth and cellulase production were severely reduced in fermentations carried out with a liquor produced from acid-treated spent grain, a readily available waste biomass source. Due to the observed reduction in cellulase activity and xylose utilisation, alternative strategies are required if the engineered strains are to be developed for the fermentation of sugars extracted from biomass.

Taken together research presented in this thesis demonstrates that cellulose and xylose can be efficiently co-utilised using engineered *S. pastorianus*, however for the fermentation of actual biomass, an adapted fermentation strategy will be required. Strategies based on current brewing practises are proposed to facilitate the fermentation of biomass to bioethanol.

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Publications and Conferences

Publications:

James Fitzpatrick, William Kricka, Tharappel James and Ursula Bond. (2014). Expression of three *Trichoderma reesei* cellulase genes in *Saccharomyces pastorianus* for the development of a two- step process of hydrolysis and fermentation of cellulose. *Journal of Applied Microbiology*.

William Kricka, James Fitzpatrick and Ursula Bond. (2014). Metabolic engineering of yeasts by heterologous enzyme production for degradation of cellulose and hemicellulose from biomass: a perspective. *Frontiers in Microbiology*.

Conferences:

William Kricka and Ursula Bond. (2012). Biomass to Biofuel: engineering of *Saccharomyces sp* for the degradation and utilisation of cellulose. Poster presentation at the Irish Fungal Society meeting at Queens University, Belfast.

William Kricka, James Fitzpatrick and Ursula Bond. (2011). Biomass to Biofuel: engineering of *Saccharomyces sp* for the degradation and utilisation of cellulose. Oral presentation at the Irish Fungal Society meeting at Trinity College, Dublin.

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Chapter 1

Introduction

1.1 Biofuel

In the current environmental and economic climate there is a need to relinquish our dependence upon finite fuel sources such as petroleum and oil, and to look for an alternative renewable and environmentally friendly fuel. With the worlds energy consumption predicted to rise by 41% over the next 20 years (BP, 2014) along with imposition of reductions on CO_2 emissions (Cancun agreement, 2010) there is a need for alternative fuel sources. Biomass has been identified as a possible replacement for these fossil fuels due to its vast abundance and renewable nature. Biomass is classified based on its supply sector, these being agricultural residues, dedicated energy crops, industry, forestry and waste. Biomass can be used to generate many different biofuels from biodiesel to biohydrogen. The most successfully utilised commercial biofuel is bioethanol. In the 19th century bioethanol was widely used as a fuel source, however during the 20th century ethanol was replaced with oil derived products such as petroleum. Only recently due to environmental pressures and the requirement for energy security has bioethanol begun its resurgence. Today bioethanol is widely used in America and Brazil, although its use is in a blend with petroleum. Currently commercial bioethanol production is classified as 1st generation and produced from dedicated crops such as corn or sugar cane as a biomass source. Sugarcane offers the easiest model for 1st generation biofuels. Sugars such as sucrose are easily extracted directly from the crop then used as a carbohydrate source for fermentation and ethanol production. When using corn as a biomass source, an initial enzymatic hydrolysis step is required for the breakdown of starch. Although commercially successful, the use of edible raw material results in the cost of bioethanol being dictated by the international market. There is also the debate as to whether land should be used to feed or fuel a nation. This has led to a switch from edible to non-edible biomass, namely lignocellulosic biomass. Biofuel generated from this non-edible biomass was termed 2nd generation bioethanol. Lignocellulosic biomass represents one of the most abundant resources on the planet. Second generation biofuel from lignocellulose has the benefit of reduced biomass cost and better environmental performance (Farrell et al., 2006; Granda et al., 2007), however generally the starting material is much more complex than that used in 1st generation, requiring a thermochemical or biological pre-treatment. Second generation bioethanol can be produced by two mechanisms, the 'thermo' pathway or the 'bio' pathway. The 'thermo' pathway utilises heat and an oxidising agent to generate three

main products, bio char, bio oil and syngas. Biochar can be utilised as a solid fuel source for burning, while bio oil and syngas, through further processing, can be converted into transportation fuels (Lee and Lavoie, 2013). The 'bio' pathway requires the extraction of sugars from biomass using enzymes combined with a chemical pre-treatment step, which allows the enzymes access to polysaccharides within biomass. The released sugar is then fermented to produce ethanol. There is also a 3rd generation of biofuel, which utilises algal biomass, which offers several benefits over standard plant lignocellulosic biomass, such as the ability to produce substantial quantities of biomass in a short harvesting cycle. Chemical pre-treatment of algae has already shown great promise in the release of fermentable sugars and production of ethanol from various ethanologenic organisms (Ho et al., 2013).

1.2 Lignocellulosic Biomass

Lignocellulosic biomass is comprised of three main components, namely cellulose, hemicellulose and lignin. The percentage of each varies between different types of lignocellulosic biomass, although generally cellulose is found to be the most abundant (Saha, 2003). Cellulose is the major structural fibre found in plants. It is composed of repeating cellobiose units linked by a β 1-4 glucosidic linkage, that form linear polysaccharide chains of up to 15,000 units, with reducing and non-reducing ends. Intermolecular hydrogen bonding and Van der Waals forces allow cellulose chains to stack in parallel to form microfibrils. Microfibrils contain both highly ordered (crystalline regions) and disordered regions (amorphous regions) (Fernandes et al., 2011). Crystalline cellulose is classified into 4 allomorphs (I, II, III, IV) with cellulose type I being the predominant cellulose found in trees and plants. Within crystalline cellulose type I there are two structural polymorphs Iα and Iβ. The abundance of these two polymorphs varies depending on the cellulose source. Microfibrils are grouped and bound together by hemicellulose and lignin to form larger structures called macrofibrils (Figure 1.1).

The other major fraction of lignocellulosic biomass is hemicellulose. Hemicellulose is a heteropolymer composed of pentose (xylose and arabinose) and hexose sugars (glucose, mannose and galactose).

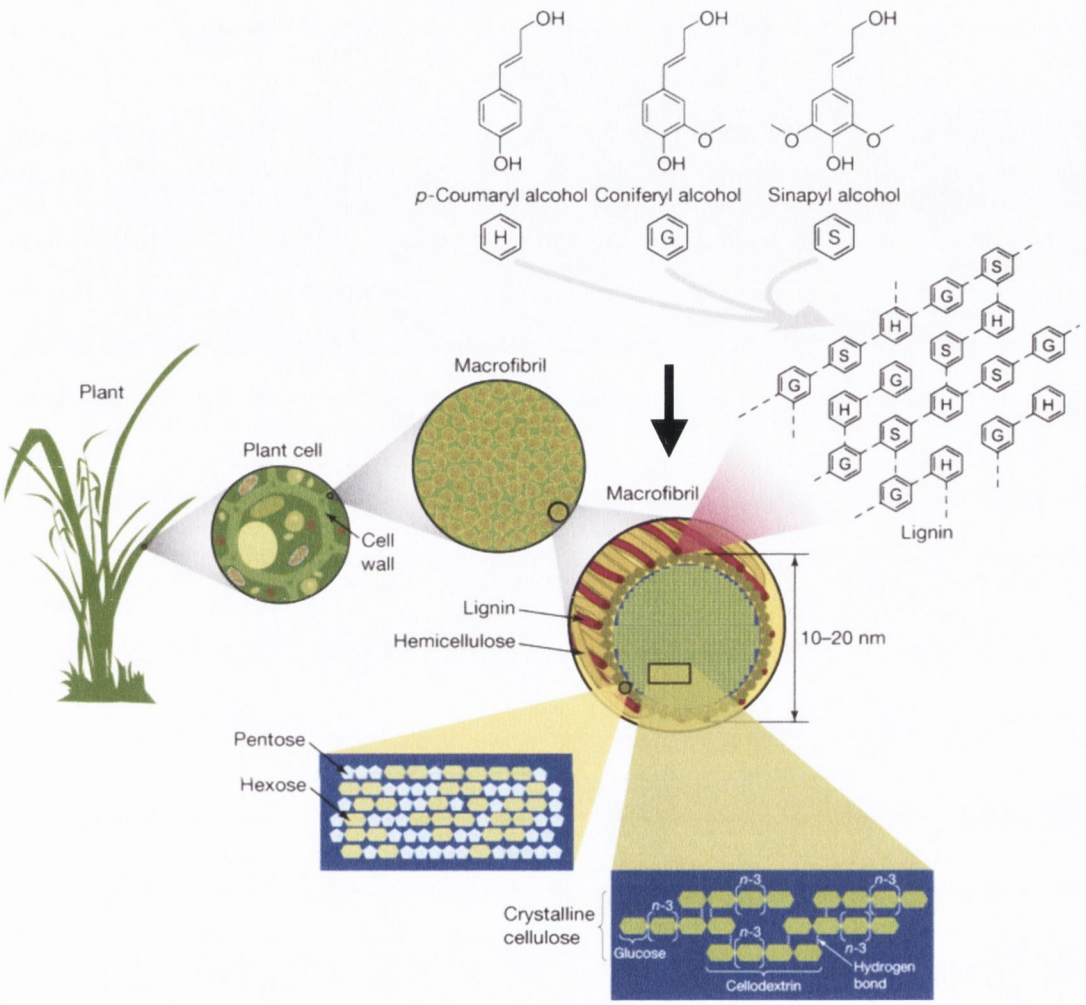


Figure 1.1 The structure and organisation of lignocellulose. The cellulose, hemicellulose and lignin components of the macrofibril are indicated in the arrowed window. Within the macrofibril cellulose microfibrils are represented by a green lattice. Hemicellulose is represented by yellow cylinders. Lignin is represented by red cylinders. The structural units of lignin are p-coumaryl alcohol (H), coniferyl alcohol (G) and sinpyl alcohol (S). Figure taken from Rubin (2008).

Generally hemicellulose is classified into four groups (xylan, xyloglucan, mannan and glucomannan) based on the β 1-4 linked backbone composition. Xylan is the major component of hard wood hemicellulose and has an important role in strengthening secondary cell wall structures. It is composed of β 1-4 linked xylose monomers, which can be modified through substitutions or branching. A usual modification is the addition of arabinose to the xylose backbone, these xylans are named glucuronoarabinoxylans (GAXs). Xyloglucan is a polymer composed of repetitive units of glucose and xylose. As with xylan, the xyloglucan backbone can be modified with substitutions and chain branching. Xyloglucans have an important role in cross linking adjacent cellulose microfibrils, via hydrogen bonding between xyloglucans and cellulose. The final groups in hemicellulose are the mannans and glucomannans, these are composed of mannose or mannose and glucose respectively.

Lignin is the third major component of lignocellulosic biomass, it is a heteropolymer comprised of p-hydroxyphenyl, guaiacyl, and syringyl monolignol units that form a complex branched network around cellulose microfibrils (Fig 1.1) (Weng et al., 2008). It is because of this complex network that the presence of lignin limits the accessibility to cellulose.

1.3 Biomass pre-treatment

The pre-treatment step is designed to effectively increase cellulose accessibility, generally through the alteration of the physical structure of cellulose and the solubilisation of the hemicellulose and lignin fractions of biomass. Pre-treatments are categorised as physical, chemical, biological and physico-chemical. All pre-treatment categories alter the cellulose structure and render it more accessible (Agbor et al., 2011), however the most commonly used are physico-chemical pre-treatments, which combine chemical with physical conditions (raised temperatures, increased pressure) to enhance pre-treatment efficacy.

The ideal pre-treatment allows for efficient cellulose disruption along with minimal inhibitor production, although the process must be cost effective to be applicable in industry. The most environmentally friendly pre-treatment methods involve steam explosion and hot water extraction. Steam explosion significantly solubilises the xylan fraction, allowing for increased cellulose accessibility, however a high percentage of lignin cannot be extracted (Liu et al., 2013). Hemicellulose is extracted by steam

explosion through the action of naturally occurring acetic and other acids released from biomass during treatment (Mosier et al., 2005). Extraction of hemicellulose from biomass can be enhanced by the addition of a dilute acid (DA) catalyst. The extracted hemicellulose is hydrolysed into xylose, glucose arabinose and galactose monomers. The release of sugars from biomass using dilute acid pre-treatment is dependent on factors such as acid concentration, pre-treatment time, solid loading and temperature (Ahmed et al., 2013; Kim et al., 2013b; Lim and Lee, 2013; Sindhu et al., 2011). As with steam explosion, dilute acid treatment does not significantly affect the lignin fraction of biomass. Pre-treatment of biomass using ammonia fibre expansion (AFEX) significantly alters the lignin structure within lignocellulose. The anhydrous ammonia cleaves the lignin-carbohydrate linkage, altering lignin structure and increasing enzyme accessibility to cellulose (Mosier et al., 2005). Ionic liquid (IL) pre-treatments interact with the hydrogen bonding network in cellulose, disrupting the 3D structure leading to amorphous cellulose generation (Moulthrop et al., 2005). Comparison of all three pre-treatments (DA, IL and AFEX) has shown significant levels of lignin (89.9%) and xylan (23.4%) extraction from biomass by IL pre-treatment, higher levels of xylan (87%) are extracted by dilute acid, however significantly lower levels of lignin (2.8%) and xylan (0.8%) are extracted by AFEX (Gao et al., 2014). Glucose release following biomass hydrolysis using commercial enzymes was highest from IL pre-treated biomass (Gao et al., 2014).

During pre-treatment, various by-products are released along with fermentable sugars. The hydrolysis of hemicellulose releases weak acids, such as acetic acid. These weak acids are thought to affect cellular growth through diffusion across the plasma membrane and altering cytosolic pH (Palmqvist and Hahn-Hagerdal, 2000). Other inhibitors such as furfurals and 5-hydroxymethyl furfural (HMF) are formed from the degradation of pentose and hexose sugars at high temperatures and pressure. Both these inhibitory compounds are naturally metabolised by yeast such as *Saccharomyces cerevisiae* into less toxic alcohols. Furfurals have been shown to affect cellular growth, although interestingly glycolytic activity is maintained (Horvath et al., 2001; Sarvari Horvath et al., 2003; Taherzadeh et al., 2000).

Pre-treatment of biomass using liquid based treatments such as dilute acid generate two distinct phases, a liquid phase generally containing solubilised hemicellulose (liquor) along with a insoluble phase generally containing cellulose and lignin.

1.4 Cellulase enzymes

The major component of lignocellulosic biomass is cellulose. Enzymes referred to as cellulases, which are part of the glycosyl-hydrolase group, hydrolyse this complex polysaccharide into glucose. Enzymes within this group hydrolyse glycosidic linkages and are classified into families based on similarities in amino acid sequence (Henrissat and Bairoch, 1993), generally resulting in families containing similar secondary structures such as active site architecture (Henrissat et al., 1996). To date there are 133 different families within the glycosyl-hydrolase group (http://www.cazy.org/Glycoside-Hydrolases.html). Cellulase enzymes differ from other glycosyl-hydrolase enzymes by their ability to hydrolyse β-1-4-glycosidic bonds and are found across families in the glycosyl-hydrolase group. There are three main classes of cellulase enzyme; endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidase (BGL) all of which have varying substrate specificities. Endoglucanase enzymes hydrolyse amorphous regions within the interior of the cellulose chain, allowing for the release of shorter chains along with new reducing and non-reducing chain ends. Cellobiohydrolase enzymes act in a highly processive manner moving along the reducing or non-reducing cellulose chain ends to release cellobiose units. β-Glucosidase enzymes hydrolyse the β-1-4 linkage between cellobiose to release glucose monomers.

Cellulase enzymes have been identified in a diverse range of fungal and bacteria species and are either defined as complexed or non-complexed.

Complexed cellulase enzymes, often referred to as cellulosomes are tethered to the cell wall. In cellulose utilising organisms such as *Clostridium thermocellum,* the cell wall attachment is mediated through the use of the scaffoldin protein CipA (Fujino et al., 1993), which binds to cellulase enzymes via highly conserved domains known as type 1 cohesins, allowing for multiple enzymes to be attached within close proximity. The cohesin domains bind to non-catalytic regions on cellulase enzymes known as type 1 dockerins forming the initial cellulosome structure. This complex is then attached to the cell wall through type 2 dockerin domain found at the C-terminus of CipA, which interacts with type 2 cohesion domains found on cell wall tethered proteins, allowing for cell wall attachment (Adams et al., 2006). The CipA protein also interacts with cellulose via a carbohydrate binding module (CBM), bringing the substrate into close proximity with the cellulosome structure (Poole et al., 1992). The ability to concentrate a high number of different enzymes close together is thought to be a major benefit of

using the cellulosome system compared to non-complexed free enzyme systems (Fierobe et al., 2002). The non-complexed cellulase system produces enzymes that are secreted from the cell into the surrounding environment and is the system of choice for industry, particularly the non-complexed cellulase system of the fungi *Trichoderma reesei* (Peterson and Nevalainen, 2012). Secreted cellulase enzymes are generally composed of three domains, a CBM, a catalytic domain and a linker region joining the CBM to catalytic domain.

1.5 Cellulose fermentation

Many different organisms are capable of naturally hydrolysing and utilising cellulose, however ethanol yields from these organisms is limited due to low ethanol tolerance. Two approaches have been taken to generate a cellulose utilising ethanologenic organism. One approach aims to increase ethanol production and tolerance in natural cellulose utilising organisms. The second utilises recombinant DNA technology to express cellulase enzymes in natural ethanologenic organisms.

1.5.1 Natural

Initial screening of various cellulose utilising organisms such as *Aspergilli sp.*, *Rhizopus sp.* and *Trichoderma sp.* showed extremely poor ethanol yields (gram of ethanol per gram of cellulose) from cellulose, with levels peaking at 0.016g/g, 0.028g/g and 0.04g/g respectively (Skory et al., 1997; Stevenson and Weimer, 2002). More recent studies identified cellulase producing organisms (*Fusarium verticillioides, Phlebia sp., Flammulina velutipes, Trametes hirsute*) with increased ethanol yields from cellulosic substrates with levels ranging from 0.05-0.42g/g (de Almeida et al., 2013; Kamei et al., 2012; Maehara et al., 2013; Okamoto et al., 2011). While ethanol yields in these organisms was reasonably high, the actual ethanol concentrations produced were low, with yields of 0.42g/g corresponding to an ethanol level of 8.4g/L (Kamei et al., 2012). This was likely to be due to low tolerance of cellulosic organisms to high ethanol concentrations.

1.5.2 Recombinant

Several different ethanologenic microorganisms can be used as a host for recombinant cellulase expression. Brewery contaminating bacteria, such as *Zymomonas mobilis* and lactic acid bacteria display good ethanol production from glucose (Liu and Qureshi,

2009), however the most common microorganism used for ethanol production is the yeast *S. cerevisiae*, which shows exceptional ethanol tolerance and production, reaching levels up to 162g/L (Casey et al., 1984). *Saccharomyces sp.* strains are unable to naturally utilise cellulose therefore heterologous expression of cellulase genes is required.

Cellulose hydrolysis and subsequent fermentation of released sugars by recombinant yeast requires high cellulase activity. If cellulose is used as a sole carbohydrate source there is a need to first produce sufficient levels of cellulase enzymes. However to produce recombinant cellulase enzymes, cell growth and metabolism is required, which is limited by the lack of fermentable sugars at the start of cellulose fermentation. This creates a 'chicken and egg' scenario between cellulase production and cellulose hydrolysis (Fig 1.2). The initial low cellulase expression due to limited growth and metabolism has been overcome through the tethering of cellulase enzymes to the cell wall (Baek et al., 2012; Fujita et al., 2004; Nakatani et al., 2013; Yamada et al., 2011) or by the addition of glucose or cellulase enzymes at the start of cellulose fermentation (Fitzpatrick et al., 2014).

The fermentation of purified cellulosic substrates such as phosphoric acid swollen cellulose (PASC) by engineered cellulase expressing *Saccharomyces sp.* strains (*S. cerevisiae* and *Saccharomyces pastorianus*) has been shown in previous studies (Table 1.1). Genes from 6 species, including *Aspergillus niger, C. thermocellulum, Aspergillus aculeatus, Saccharomycopsis fibuligera, Thermoascus aurantiacus* and *T. reesei* have been used for heterologous expression. Generally, rather than using genes from a single organism, the cellulase machinery has been reconstructed in yeast by combining cellulase genes from different species. The most commonly used host for cellulase expression is *S. cerevisiae*. Both complexed and non-complexed (tethered and cellulosome) systems (Figure 1.3) have been reconstituted within *S. cerevisiae*. A direct comparison of tethered and secreted systems suggested that the cellulosome approach was more efficient at PASC hydrolysis (Wen et al., 2010) with ethanol production being 3-fold greater than the secreted enzyme system (Goyal et al., 2011), however a comparison of all studies indicate similar levels of ethanol in both secreted and complexed systems (Table 1.1).

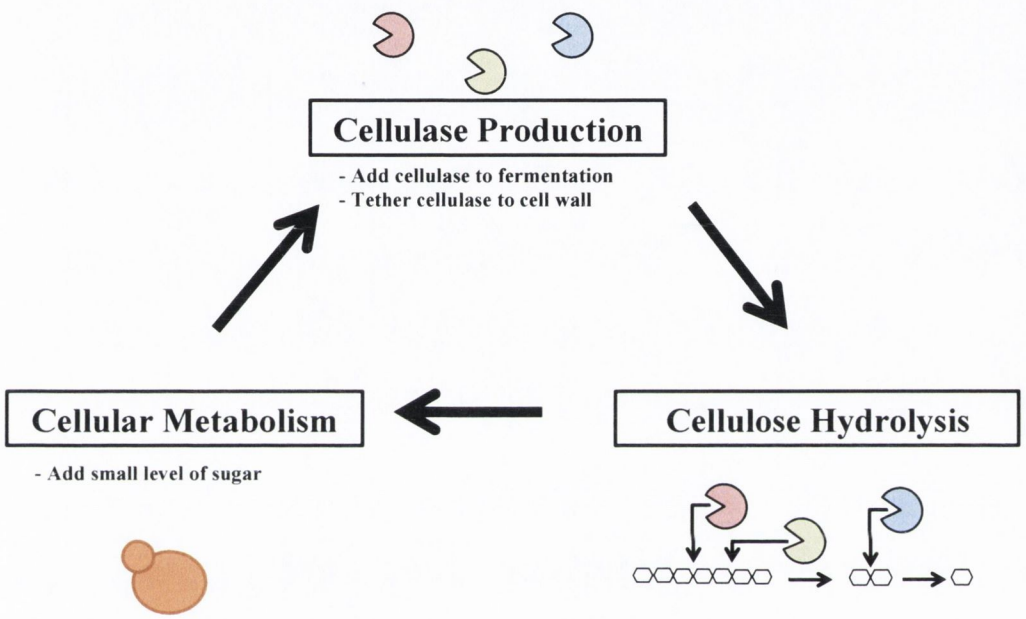


Figure 1.2 The chicken and egg conundrum when using cellulose as a sole carbohydrate source. Cellular metabolism is required for cellulase production, however this is dependent upon fermentable sugars released by cellulose hydrolysis. To avoid this scenario fermentable sugars can be added to increase cellular metabolism and kick start cellulase production, or high level of cellulase enzymes at the start of fermentations can be achieved through cell wall tethering or by the addition of cellulase enzymes.

Table 1.1 Ethanol production from simultaneous cellulose hydrolysis and fermentation using recombinant *Saccharomyces sp.*

Host	Cellulase enzyme	Tethered or Secreted	PASC (g/L)	Ethanol (g/L)	Yield (g/g)	Reference
S. cerevisiae EBY100	*T. aurantiacus* EGI	Tethered	10	2.12	0.21	(Baek et al., 2012)
	T. reesei CBHII	Tethered				
	A. aculeatus BGLI	Tethered				
S. cerevisiae EBY100	*C. thermocellum* CelA (EG)	Cellulosome	10	1.80	0.18	(Kim et al., 2013a)
	T. reesei CBHII	Cellulosome				
	A. aculeatus BGLI	Tethered				
S. cerevisiae MT8-1/cocδBEC3	*T. reesei* EGII	Tethered	20	7.60	0.38	(Yamada et al., 2011)
	T. reesei CBHII	Tethered				
	A. aculeatus BGLI	Tethered				
S. cerevisiae MT8-1	*T. reesei* EGII	Tethered	10	2.10	0.21	(Yanase et al., 2010)
	T. reesei CBHII	Tethered				
	A. aculeatus BGLI	Tethered				
S. pastorianus CM-51	*T. reesei* EGI	Secreted	25	5.00	0.20	(Fitzpatrick et al., 2014)
	T. reesei EGII	Secreted				
	T. ressei CBHII	Secreted				
	T. reesei BGLI	Secreted				
S. cerevisiae Y294	*T. reesei* EGI	Secreted	10	1.00	0.10	(Den Haan et al., 2007b)
	S. fibuligera BGLI	Secreted				
S. cerevisiae BY4742	*C. thermocellum* CelA (EG)	Cellulosome	10	1.25	0.12	(Goyal et al., 2011)
	T. reesei CBHII	Cellulosome				
	T.aurantiacus BGLI	Cellulosome				
S. cerevisiae BY4742	*C. thermocellum* CelA (EG)	Secreted	10	0.43	0.04	(Goyal et al., 2011)
	T. reesei CBHII	Secreted				
	T. aurantiacus BGLI	Secreted				
S. cerevisiae MT8-1	*T. reesei* EGII	Tethered	10	2.90	0.29	(Fujita et al., 2004)
	T. reesei CBHII	Tethered				
	A. aculeatus BGLI	Tethered				
S. cerevisiae EBY100	*T. reesei* EGII	Cellulosome	10	1.80	0.18	(Wen et al., 2010)
	T. reesei CBHII	Cellulosome				
	A. aculeatus BGLI	Cellulosome				

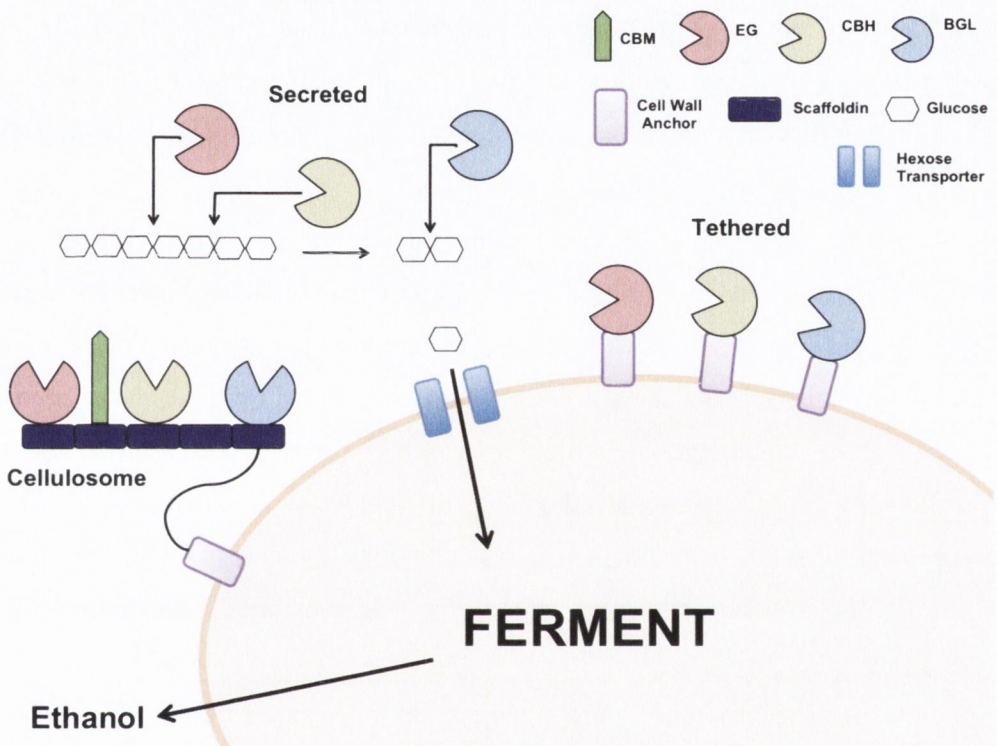


Figure 1.3 Model for heterologous cellulase expression by *Saccharomyces sp.* Cellulase enzymes can either be tethered individually or through a cellulosome to the cell wall or they can be fully secreted into the surrounding environment. CBM: Carbohydrate binding domain, EG: Endoglucanase, CBH: Cellobiohydrolase, BGL:β-glucosidase.

Initially cellulase gene expression by *S. cerevisiae* was accomplished using episomal plasmid vectors (YEp) (Baek et al., 2012; Den Haan et al., 2007b; Fitzpatrick et al., 2014; Fujita et al., 2004; Goyal et al., 2011; Kim et al., 2013a; Wen et al., 2010; Yanase et al., 2010). These high copy number plasmids offer high levels of cellulase expression, with 30-50 copies per cell, however continuous auxotrophic or antibiotic selection is required to maintain plasmid stability (Futcher and Cox, 1984). Thus the use of YEp vectors is not ideal for industrial applications. Integrative plasmids (YIp) offer an alternative allowing for stably maintained chromosomal integration of cellulase genes, however to generate high cellulase activity, multiple stable chromosomal integrations are required. YIp vectors typically target auxotrophic markers (*LEU*, *URA*, *HIS* etc), only allowing for 1-2 integrations per cell.

An alternative approach is to target gene integration to various repetitive DNA sequences thus allowing for multiple integrations. The long terminal repeats (δ regions) of the Ty retrotransposon have been used to allow for dispersed multiple stable chromosomal integrations (Lee and Da Silva, 1997). Cellulase genes have been stably integrated using this delta integration approach, with cellulase activity marginally improved compared to that achieved using YEp vectors (Yamada et al., 2010a). Using this approach for stable heterologous expression, the simultaneous PASC hydrolysis and fermentation by engineered *S. cerevisiae* has been demonstrated (Nakatani et al., 2013; Yamada et al., 2011).

Despite the approaches taken, a comparison of all reconstituted cellulase systems revealed that the conversion rate of cellulose to ethanol (0.043-0.38g/g) and ethanol production (1.8-7.6g/L) obtained are in fact similar to levels from natural cellulosic organisms and are yet to mirror those from traditional substrates such as oats or barley. This suggests that ethanol yields from recombinant yeasts are influenced by the complex and recalcitrant nature of the substrate, the limitation of heterologous cellulase expression and the conditions for optimal enzyme activity.

1.6 Optimisation of recombinant cellulase activity

Hydrolysis and fermentation of cellulose by recombinant yeast is limited by the incompatibility between the optimum temperature for cellulase activity and the optimum temperature for growth of mesophilic yeasts. Maximum cellulase activity is achieved at 50°C-70°C (Johnson et al., 1982; Kupski et al., 2014) while optimum growth for *Saccharomyces sp.* is between 20°C-30°C. Thus, for efficient cellulose hydrolysis at fermentation conditions cellulase enzyme activity needs to be optimised to compensate for the reduced temperature used.

The activities of recombinant cellulase enzymes from different organisms have been compared in an effort to identify cellulase enzymes with maximum activity. A comparison between studies is difficult due to the variation in units used, however a direct comparison within a single study removes this variability. A range of cellulase enzymes have been cloned and expressed in *S. cerevisiae*. Generally there appeared to be no consensus species that out performs others in all three enzyme classes. Recombinant BGL1 activity was highest from *S. fibuligera*, interestingly no activity was detected from *T. reesei* BGL1 (Tang et al., 2013; van Rooyen et al., 2005). A comparison of cellobiohydrolase enzyme activity identified that generally CBHII was more active than CBHI, with the highest activity from *Chrysosporium lucknowense* CBHII (Den Haan et al., 2007a; Ilmen et al., 2011). The comparison of recombinant endoglucanase activity has mainly been limited solely to genes from *T. reesei* (du Plessis et al., 2010), although endoglucanase enzymes from various protists isolated from termites have been expressed in *S. cerevisiae* (Todaka et al., 2011).

The ratio of individual enzymes also affects cellulose hydrolysis. The total cellulase secreted by *T. reesei* is represented by around 80% cellobiohydrolase and less than 5% of endoglucanase and β-glucosidase (Herpoel-Gimbert et al., 2008). Hence using equal amounts of cellulase enzymes may not yield the optimal cellulase synergy shown in nature. Using the delta integration approach cellulose hydrolysis was increased by altering cellulase ratios. The highest glucose yield was obtained in a strain containing 16, 2 and 6 copies of the *egl1, bgl1* and *cbh2* genes respectively. Hydrolysis decreased 1.4 fold when *egl1*, *cbh2* and *bgl1* copy number was varied to 5:9:6. The lowest activity was observed when *egl1*, *cbh2* and *bgl1* genes were present in single copies in a 1:1:1 ratio (Yamada et al., 2010b), showing that cellulase enzyme ratio and also copy number significantly affected cellulose hydrolysis.

Many different promoters have been used to increase recombinant gene expression (Nacken et al., 1996; Sun et al., 2012). This offers a simple method for optimising cellulase enzyme expression. Often constitutive promoters such as the PGK (Yamada et al., 2011), TEF1 (Fitzpatrick et al., 2014), SED (Inokuma et al., 2014) and ENO (Den Haan et al., 2007b) have been utilised for continuous expression of cellulase genes. Inducible promoters such as GAL1/10 have also been used (Jeon et al., 2009), however while these inducible promoters drive vastly higher expression, they are repressed by glucose the end product of cellulose hydrolysis.

Efficient secretion is required for high cellulase enzyme activity. In several studies the native secretory signals of cellulases have been exchanged for either *S. cerevisiae* secretory signals (mating factor-α) (Zhu et al., 2010) or the xyn2 secretory signal from *T. reesei* (Den Haan et al., 2007b; van Rooyen et al., 2005). Comparison in the activity of BGL1 (*S. fibuligera*) using various secretory signals, including the native secretory signal, revealed there was little difference between all secretory signals used (Tang et al., 2013). Modifications of disulphide bond formation, glycosylation, protein folding and trafficking have been shown to increase protein secretion and activity of cellulase enzymes, however modifications only resulted in a 1.3-fold (Xu et al., 2014) and 6-fold (Wang et al., 2013b) increase.

1.7 Xylose utilisation

The pentose sugar xylose is a major component found in hemicellulose. Its abundance and relative ease of extraction from biomass via pre-treatment (Weis et al., 2008, Lim & Lee., 2013, Ahmed et al., 2013) make it an attractive source of fermentable sugar. As with cellulosic organisms, ethanol production from xylose utilising organisms is often limited. This has led to the adaption and isolation of novel xylose utilising organisms, which have the potential for high ethanol production and tolerance.

1.7.1 Isolation and adaption of natural xylose utilising organisms

In nature xylose can be utilised by various yeast species and bacteria. These organisms are often associated with lignocellulose directly, or indirectly via association with another organism. The yeast species *Spathaspora passalidarum* isolated from the wood boring beetle *Odontotaenius disjunctus* displays a high capacity to ferment xylose, with ethanol levels exceeding 90% of theoretical yield (Hou et al., 2012). Xylose utilising species have also been isolated from other lignocellulosic associated beetles, such as the wood roach *Cryptocercus sp.* (Urbina et al., 2013). Examination of buffalo faeces led to

the identification of 28 isolates, comprising of 6 species (*Candida tropicalis, Candida parasilosis, Geotrichum sp., Candida mengyuniae, Sporopachydermis lactativora* and *Trichosporon asahii*), which were capable of xylose fermentation, generating ethanol levels ranging from 0.006-0.602g/L (Lorliam et al., 2013). The yeast *Pichia stipitis* has been well characterised and naturally utilises xylose, however low ethanol production and tolerance has limited its use in bioethanol production (Meyrial et al., 1997). Ethanol production by *P. stipitis* was increased by genome shuffling (Bajwa et al., 2010; Shi et al., 2014) and mutagenesis (Grabek-Lejko et al., 2006; Watanabe et al., 2011) with levels reaching 43g/L. The use of natural xylose fermenting organisms removes the need for metabolic engineering, with xylose metabolism also likely to be more efficient than that of non-xylose fermenting organisms (Fiaux et al., 2003).

1.7.2 Recombinant

The yeast *S. cerevisiae* would normally be an ideal candidate for bioethanol production, due to its high stress-tolerance and ethanol yields. While various xylose utilising *Saccharomyces sp.* have been identified (Wenger et al., 2010, Schwartz et al., 2012), poor growth and alcohol production have led to the use of recombinant DNA technology to engineer *S. cerevisiae* to increase xylose utilisation efficiency.

In nature there are two pathways that are used for xylose metabolism (Fig 1.4). Pathway 1 requires the use of two enzymes (Xylose reductase (XR), followed by a xylitol dehydrogenase (XDH)) for sequential xylose metabolism whereas pathway 2 requires only a single enzyme, xylose isomerase (XI). The product of these two pathways, xylulose, is then metabolised via the pentose phosphate (PPP), glycolytic and fermentation pathways by the natural enzyme machinery present in *S. cerevisiae* (Fig 1.5). *S. cerevisiae* strains have been engineered to express both the XI and XR/XDH pathways to utilise xylose (Table 1.2/1.3).

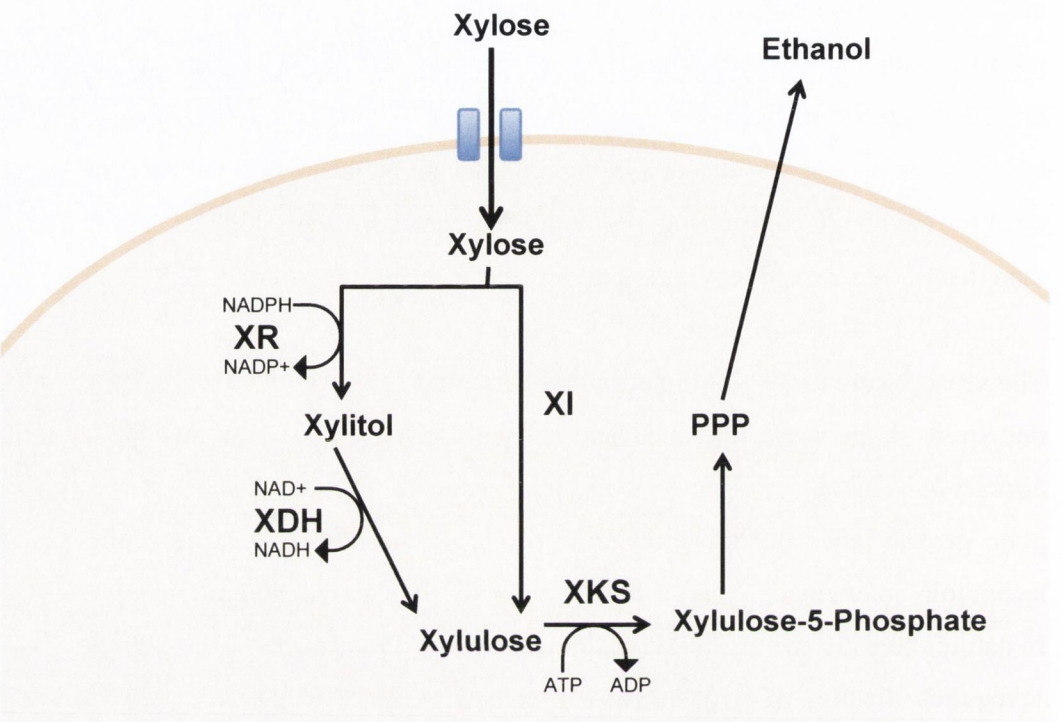
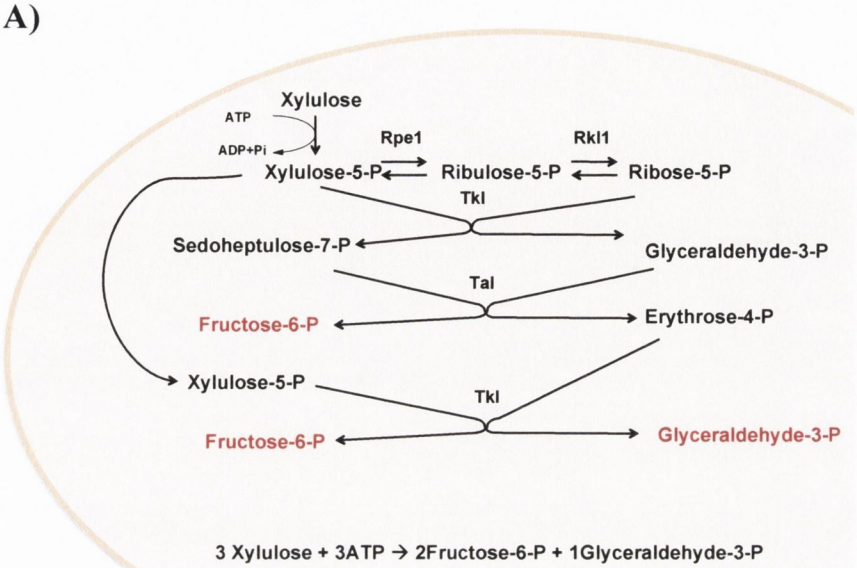


Figure 1.4 Model for the heterologous expression of xylose utilizing pathways by *Saccharomyces sp*. Xylose can be metabolized to xylulose sequentially by xylose reductase (XR) and xylose dehydrogenase (XDH) or in a single step by the xylose isomerase (XI) enzyme. Xylulose is then metabolized by the native xylulose kinase (Xks) enzyme and by the pentose phosphate pathway (PPP).

A)



B)

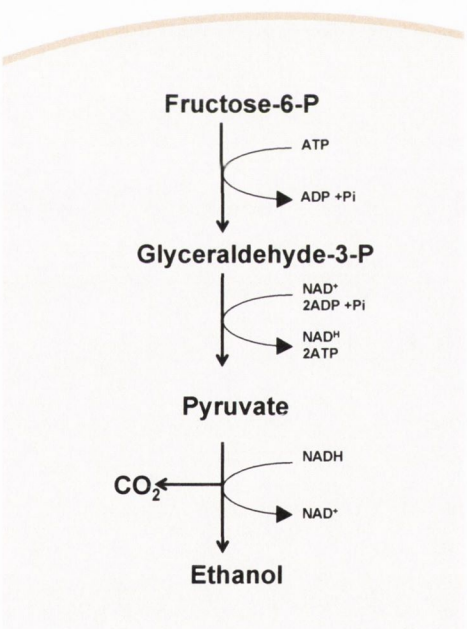


Figure 1.5 Metabolic pathway for xylulose utilization by *Saccharomyces sp.* A) The metabolism of xylulose into fructose-6-phosphate and glyceraldehyde-3-phosphate by the PPP. Products are highlighted in red. P: phosphate, Rpe: D-ribulose-5-phosphate 3-epimerase, Rkl: ribose-5-phosphate ketol-isomerase, Tal: transaldolase, Tkl: transketolase. B) The conversion of the products from the PPP into ethanol via the glycolytic and fermentation pathway.

Table 1.2 Ethanol production from xylose using recombinant *Saccharomyces sp.* strains expressing the XR/XDH pathway. XR: Xylose reductase, XDH: Xylitol dehydrogenase, XK: Xylulose kinase, PPP: Yes indicates the overexpression of pentose phosphate pathway genes.

Host	Enzyme				Ethanol	Theoretical Yield	Reference
	XR	XDH	XK	PPP	(g/L)	(g/g)	
S. cerevisiae 424A	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	-	27.50	0.40	(Bera et al., 2011)
S. cerevisiae 424A	*Neurospora crassa*	*Pichia stipitis*	*S. cerevisiae*	-	27.50	0.40	
S. cerevisiae 424A	*Candida parapsilosis*	*Pichia stipitis*	*S. cerevisiae*	-	7.50	0.23	
S. cerevisiae F106X	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	Yes	12.30	0.24	(Xiong et al., 2011)
S. cerevisiae YY5A	*Candida guilliermondii*	*Pichia stipitis*	*Pichia stipitis*	Yes	6.10	0.33	(Ma et al., 2012)
S. cerevisiae MA-N4	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	-	12.60	0.35	(Matsushika et al., 2009b)
S. cerevisiae YPH499XU	*Scheffersomyces stipitis*	*Scheffersomyces stipitis*	*S. cerevisiae*	-	-	0.24	(Kato et al., 2013)
S. cerevisiae 1-δX-70	*Scheffersomyces stipitis*	*Scheffersomyces stipitis*	*S. cerevisiae*	-	-	0.36	
S. cerevisiae TMB 3057	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	Yes	13.30	0.33	(Karhumaa et al., 2007b)
S. cerevisiae TMB 3400	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	-	12.10	0.34	
S. cerevisiae BY4741X	*Scheffersomyces stipitis*	*Scheffersomyces stipitis*	*S. cerevisiae*	-	25.40	0.28	(Fujitomi et al., 2012)
S. cerevisiae W303-1ATe	*Scheffersomyces stipitis*	*Scheffersomyces stipitis*	*S. cerevisiae*	-	12.20	0.27	(Ismail et al., 2013)
S. cerevisiae sun048T	*Scheffersomyces stipitis*	*Scheffersomyces stipitis*	*S. cerevisaie*	-	16.60	0.34	
S. cerevisiae YRH388	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	-	5.60	0.23	(Hector et al., 2011)
S. cerevisiae YRH396	*Pichia stipitis*	*Pichia stipitis*	*S. cerevisiae*	-	7.80	0.27	

Table 1.3 Ethanol production from xylose using recombinant *Saccharomyces sp.* strains expressing the XI pathway. XI: Xylose isomerase, XK: Xylulose kinase, PPP: Yes indicates the overexpression of pentose phosphate pathway genes.

Host	Enzyme			Ethanol	Theoretical Yield	Reference
	XI	XK	PPP	(g/L)	(g/g)	
S. cerevisiae TMB 3066	*Piromyces sp. E2*	*S. cerevisiae*	Yes	7.30	0.43	(Karhumaa et al., 2007b)
S. cerevisiae BY4741	*Burkholderia cenocepacia*	-	-	-	0.23	(de Figueiredo Vilela et al., 2013)
S. cerevisiae YRH631	*Prevotella ruminicola*	*S. cerevisiae*	-	4.10	0.35	(Hector et al., 2013)
S. cerevisiae YRH1114	*Prevotella ruminicola*	*S. cerevisiae*	-	13.60	0.42	
S. cerevisiae Y7092	*Piromyces sp E2*	*S. cerevisiae*	-	5.30	0.35	(Usher et al., 2011)
S. cerevisiae HDY.GUF5	*Clostridium phytofermentans*	*S. cerevisiae*	-	-	0.23	(Demeke et al., 2013)
S. cerevisiae GS1.11-26	*Clostridium phytofermentans*	*S. cerevisiae*	-	-	0.46	
S. cerevisiae MT8-1	*Orpinomyces sp.*	*S. cerevisiae*	-	6.93	0.32	(Tanino et al., 2010)
S. cerevisiae CIBTS0735	*Piromyces sp. E2*	*S. cerevisiae*	Yes	17.50	0.45	(Diao et al., 2013)
S. cerevisiae BY4741-S2A3K	*Piromyces sp. E2*	*S. cerevisiae*	Yes	7.00	0.42	(Lee et al., 2012)
S. cerevisiae H158	*Thermus thermophilus*	-	-	1.30	0.13	(Walfridsson et al., 1996)
S. cerevisiae BarraGrande	*Clostridium phytofermentans*	-	-	8.00	0.43	(Brat et al., 2009)
S. cerevisiae RWB202	*Piromyces sp. E2*	-	-	8.05	0.42	(Kuyper et al., 2004)
S. cerevisiae INVSc1	*Orpinomyces sp.*	*S. cerevisiae*	-	4.06	0.39	(Madhavan et al., 2009)

Genes encoding XR and XDH enzymes are most commonly sourced from *P. stipitis*, while a more diverse range of XI genes from different organisms have been examined (Table 1.2/1.3). The direct comparison of the two pathways suggested that the XR/XDH pathway although less efficient, produces higher ethanol levels from xylose (Karhumaa et al., 2007b). Once xylose is metabolised into xylulose it is then phosphorylated by the natural xylulokinase (Xks1) enzyme present in *S. cerevisiae*. The overexpression of the native *S. cerevisiae XKS1* gene in a XR/XDH background increased ethanol production and reduced by-product formation (Johansson et al., 2001; Toivari et al., 2001). This increase in conversion of xylulose to xylulose-5 phosphate is thought to facilitate an increase in flux of xylose to ethanol through the alteration of intercellular metabolites (Toivari et al., 2001).

The cofactor requirement of the XR and XDH system (Fig 1.4) is thought to result in redox imbalances affecting ethanol production (Jeffries and Jin, 2004). Altering co-factor specificity to enable co-factor recycling between the enzymes is one possible approach to reduce the redox imbalance and increase ethanol yields (Ghosh et al., 2011). The alteration of XDH co-factor specificity from NAD^+ to $NADP^+$ has been reported to increase ethanol yields and decrease xylitol excretion, although ethanol levels were not increased greatly (Matsushika et al., 2008; Watanabe et al., 2007b) and no distinct correlation between XDH ($NADP^+$) activity and alcohol production was observed (Watanabe et al., 2007b). The alteration of XR co-factor specificity from NADPH to NADH allowing for the natural recycling of co-factors, resulted in increased ethanol production and reduced xylitol formation (Bengtsson et al., 2009; Krahulec et al., 2012; Petschacher and Nidetzky, 2008; Watanabe et al., 2007a; Xiong et al., 2011).

To improve metabolic fluxes toward ethanol production in *S. cerevisiae* manipulation of specific enzymes within the PPP as well as other metabolic pathways have been extensively examined. Deletions and overexpression of PPP enzymes was shown to have a mixed effect on xylose metabolism (Matsushika et al., 2009a). Two of the key enzymes involved in the PPP are transaldolase (Tal1) and transketolase (Tkl1). Overexpression of both these enzymes along with Rkl1 and Rpe1 (PPP enzymes, Fig 1.5A) increased growth rates on xylose, although ethanol levels were only increased fractionally (Bera et al., 2011; Karhumaa et al., 2007a). Deletion of either *TAL1* or *TKL1* led to decreased growth and ethanol production from xylose, demonstrating the essential nature of these enzymes in xylose fermentation (Matsushika et al., 2012). Interestingly when the secondary transaldolase (*NQM1*) and transketolase (*TKL2*) genes

were deleted, growth and ethanol production increased compared to the control (Matsushika et al., 2012). The use of inverse metabolic engineering has become a very useful tool for the identification of not only PPP gene targets, but also other genes for optimising xylose utilisation. Transcriptome analysis identified that mRNA levels of genes such as *TAL1*, *TKL1*, *SOL3* and *GND1* are increased when grown in xylose. Interestingly non PPP genes such as those involved within galactose metabolism were also up-regulated (*GAL1/7/10*) (Bengtsson et al., 2008; Wahlbom et al., 2003). The validation of these results has only shown a benefit of *TAL1* and *SOL3* overexpression, with growth rates increasing 24% and 19% respectively compared to the control strain (Bengtsson et al., 2008). The notable down regulation of genes was also used as a guide to possible targets. Knockout mutants were used for expression validation. Strains carrying the deletions YLR042C, *MNI1* and *RPA49*, showed a vast improvement in growth rates compared to the control strain (173%, 62% and 90% increase respectively) (Bengtsson et al., 2008). The deletion of YLR042C along with the overexpression of the PPP genes (*TAL1, TKL1,RKL1* and *RPE1*) was shown to increase ethanol yield 2-fold (Parachin et al., 2010). The deletions of other genes such as *PHO13* (Van Vleet et al., 2008), *ALP1*, *ISC1*, *RPL20B* and *BUD21* (Usher et al., 2011) have shown to increase ethanol yields from xylose. The mechanism of how these deletions increase xylose fermentation however is unknown.

It is clear that an improved pentose metabolism phenotype is controlled by the expression of a variety of genes and involves a complex metabolic pathway. To mimic this phenotype through overexpression of key genes may prove complex and unpredictable. The identification of key genes required for optimum PPP metabolism has been invaluable, however an alternative strategy may be to develop an evolutionary approach to identify natural mutations that lead to increased xylose utilisation in *S. cerevisiae*.

1.8 Hexose and pentose sugar co-utilisation

Optimal bioethanol production requires the utilisation of both the hexose sugars from cellulose and pentose sugars from hemicellulose. In order to reduce costs the ideal strain should utilise both cellulose and xylose in a single fermentation vessel. The ability of recombinant yeast to co-utilise cellulose and xylose without the addition of commercial enzymes has yet to be demonstrated.

The co-utilisation of xylose and glucose has identified various issues with pentose and hexose co-fermentation. The first stage in both xylose and glucose metabolism is the transport of the sugar into the cell. *S. cerevisiae* can naturally transport both hexose and pentose sugars across the membrane by various hexose specific sugar transporters (Hxt7/Hxt5/Hxt4/Hxt2 and Gal2) (Hamacher et al., 2002). Although xylose can enter the cell using the same transporters, the affinity of the transporters for xylose is much lower than glucose. This difference in affinity between hexose and pentose sugars results in the inhibition of xylose uptake in the presence of glucose. Glucose catabolism was also shown to affect xylose utilisation through variation in metabolic fluxes within xylose metabolism (Pitkanen et al., 2003), although the inhibition of xylose transport by the presence of glucose was shown to be the main road block for the co-utilisation of glucose and xylose (Subtil and Boles, 2012). The overexpression of various native hexose transporters led to increased xylose transport (Hxt1, Hxt7, Hxt13 and Gal1) (Tanino et al., 2012; Wang et al., 2013a; Young et al., 2011), although ethanol production was not significantly increased.

The co-utilisation of xylose with more complex sugars such as cellobiose has been accomplished. Two different approaches for cellobiose and xylose co-utilisation have been tested. Firstly the xylose utilising enzymes (XR/XDH/Xks1) and an intracellular β-glucosidase were co-expressed along with a cellodextran transporter to allow for intracellular cellobiose hydrolysis (Chomvong et al., 2014; Ha et al., 2011; Ha et al., 2013). This approach removes glucose inhibition of xylose transport. The second approach expressed xylose utilising enzymes and a secreted form of β-glucosidase to allow for extracellular cellobiose hydrolysis followed by glucose and xylose transport into the cell (Saitoh et al., 2010). Both strategies were able to co-utilise xylose and cellobiose with similar ethanol yields (g of ethanol/g of sugar) observed (0.29-0.40g/g).

1.10 Objectives of this study

The aim of this research was to generate engineered *Saccharomyces sp.* strains capable of co-utilising xylose and cellulose for the direct application of ethanol production from pre-treated biomass. Within the literature the host yeast used for recombinant expression of cellulase and xylose utilising enzymes has almost exclusively been *S. cerevisiae*, often utilising cellulase genes from different microorganisms. This study

aims to address the ability to utilise cellulose and xylose using the machinery from a single species (*T. reesei*) and examine the use of alternative *Saccharomyces sp.* as expression hosts.

The chicken and egg conundrum and reduced cellulase activity at fermentation temperatures limit ethanol production by engineered yeast. Several strategies were examined to increase cellulase activity. First the inclusion of a pre-hydrolysis step prior to fermentations. Secondly a strategy in which the co-utilisation of xylose and cellulose was examined. Substrates such as purified cellulose and xylose were used to test the various strategies for ethanol production. Finally the approaches were applied to fermentations using real biomass as a starting substrate.

In an approach to optimise recombinant enzyme activity initially cellulase and xylose utilising genes from the mesophilic fungi *T. reesei* were individually cloned into several *Saccharomyces spp.* strains. Cellulase activity was further optimised through promoter swaps and co-expression. Xylose fermentation was optimised through comparison of the XR/XDH (*T. reesei*) and the XI (*Piromyces sp. E2*) pathways. Both the cellulose and xylose utilising systems were combined to develop a strategy for optimising the co-utilisation of purified cellulose and xylose.

Finally the extraction of sugars from biomass using both chemical and biological methods was examined. Fermentation strategies developed from purified substrates were applied to actual biomass for the production of ethanol from natural biomass sources.

Chapter 2

Materials and Methods

2.1 Strains, growth media and growth conditions

2.1.1 Yeast

The strains CM-51, C10 and C10-51 are stress tolerant derivatives (James et al., 2008) of the *S. pastorianus* yeast strain CMBS-33 obtained from the Centre for Malting and Brewing Science, Leuven, Belgium. The *S. cerevisiae* strain L6440 (4n) is a lab generated tetraploid *S. cerevisiae* yeast strain (Galitski et al., 1999), the haploid *S. cerevisiae* strain BY4741 (MAT**a**, his3Δ, leu2Δ, met15Δ, ura3Δ) was obtained from Euroscarf (Johann Wolfgang Goethe University Frankfurt, Germnay). Industrial *S. cerevisiae* strains Y2699, K103 and R130 were obtained from Diageo Ireland, St James Gate, Dublin. The yeast strain *Saccharomyces eubayanus* (PYCC6148) was obtained from the Portugeuse Yeast Culture Collection (PYCC) (Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (FCT/UNL, Caparica) (Libkind et al., 2011). The *T. reesei* strain QM9123 was obtained from Commonwealth Agricultural Bureaux International, CABI, (Surrey, UK).

All standard media in this study was autoclaved at 120°C prior to use, unless otherwise stated. Yeast strains were routinely cultured in rich media composed of Yeast Extract Peptone (YEP) (Formedium) (30g/L) or synthetic complete media (SC) (1.7g/L yeast nitrogen base (Melford), 0.5g/L ammonium sulfate (Melford), 0.59g/L synthetic drop out (Formedium), 0.02g/L adenine (Fluka), 0.012g/L lysine (Fluka), 0.008g/L trptophan (Fluka), 0.008g/L leusine (Sigma-Aldrich), 0.002g/L histidine (Fluka), 0.003g/L uracil (Sigma-Aldrich)) supplemented with glucose (D) (Fisher Science), xylose (X) (Carbosynth), cellobiose (C) (Carbosynth) or sucrose (S) (Fluka) at concentrations ranging from 20g/L-50g/L. Media was supplemented with hygromycin (Invitrogen) (300μg/mL) and G418 (Melford) (200μg/mL) where applicable. Yeast strains were grown in 250mL closed top conical flasks at 30°C and 150rpm or in micro-cultures (200μL) at 30°C in a 96 flat bottom well plate (Sarstedt) with no agitation.

The growth of cultures was monitored using a haemocytometer. Samples were taken and diluted in dH_2O and cells were counted using a light microscope (Nikon).

2.1.2 Bacteria

The *Escherichia coli* strain XL1-Blue (*recA1*, *endA1m*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [*F'proAB*, *lacIZ_M15*, *Tn10*]) (Stratagene, La Jolla, CA, USA) was used for plasmid propagation. For plasmid propagation *E. coli* strains were cultured overnight in

5mL Luria broth (LB) (10g/L tryptone, 5g/L Yeast Extract (YE), 10g/L NaCl) supplemented with carbenicillin (Melford) (100μg/mL) at 37°C and 200rpm.

Bacterial cell growth was monitored using optical density measurements at 600nm (OD_{600}) using Biophotometer spectrophotometer (Eppendorf).

2.1.3 Phosphoric acid swollen cellulose (PASC)

PASC was generated as previously described (Fitzpatrick et al., 2014) with minor modifications. Avicel-PH-101 (0.8g) (Fluka) was mixed with 3mL of dH_2O and vortexed for 5 minutes. To this slurry, 2x20mL of phosphoric acid (85% wt. in H_2O, Fluka) was slowly added. After each 20mL addition the mixture was vortexed for 5 minutes. The mixture was left at 4°C over night. Ice-cold water was then added in 40mL aliquots, followed by vigorous mixing. This was repeated three more times, until a total of 160mL of water had been added. The solution was centrifuged at 4000rpm for 10 minutes at 4°C, and the supernatant discarded. The ice cold water wash step was repeated two more times. After the third centrifugation the supernatant was discarded and 4x40ml of 50mM sodium carbonate was added and vigorously mixed and the pH of the solution adjusted to pH6.0 (using 2M sodium carbonate). The solution was centrifuged at 4000rpm for 20 minutes at 4°C and the supernatant discarded, and the ice cold wash step was repeated a further three times. After the final centrifugation the supernatant was discarded and the remaining solid (PASC) was autoclaved.

The acid treatment of Avicel-PH-101 causes the microcrystalline cellulose to swell resulting in 40-fold increase in weight (Fitzpatrick et al., 2014). PASC concentration is stated throughout as theoretical glucose concentration, this is calculated by dividing the PASC concentration by a factor of 40 to account for the increase in weight caused by acid treatment (i.e 40g PASC = 1g glucose). PASC fermentation media was composed of YEP (30g/L) supplemented with 416-1000g/L PASC (10.4-25g/L theoretical glucose).

2.1.4 Biomass

Lignocellulosic biomass from industrial and agricultural sources are listed in Table 2.1.

Table 2.1 Lignocellulosic biomass. Forms of biomass used in this study

Biomass	Source
Spent Grain Batch 1[A]	Diageo, Pilot brewery, St James's Gate, Co Dublin
Spent Grain Batch 2[A]	Diageo, Commercial brewery, St James's Gate, Co Dublin
Spent Grain Batch 3[A]	Seamus O'Hara, 5 Lamps Brewery, Dublin
Miscanthus giganteus Batch 1	Dr. Trevor Hodkinson (Botany Department, Trinity College Dublin)
Miscanthus sinensis Batch 1-3	Dr. Trevor Hodkinson (Botany Department, Trinity College Dublin)
Eucalyptus nitens	Dr. Denis Lobby, DL Biotechnology, Co. Carlow

[A]- Spent grain is the residue remaining in the mash kettle after the mashing and lautering processes.

Biomass samples were first dried at 60°C. Spent grain and *E. nitens* samples were supplied pre-milled. The stem and the leaf of *Miscanthus* grasses were separated and milled individually using a blender (Phillips) for 5 minutes at full speed. H_2SO_4 (4% w/v) was added to the dried biomass to yield a final biomass concentration of 50-125g/L. Biomass solutions were heated on a low pressure (100°C, 5Psi) setting for 40 minutes in a pressure cooker (Moulinex, Minut' cook), and subsequently centrifuged to separate out the soluble and insoluble material. The soluble fraction (liquor) from the pre-treatment was neutralised by the addition of NaOH (6.66M) until pH5.5 was reached. The neutralised liquor was passed through Whatman No 1 filter paper to remove insoluble precipitate arising from the neutralisation and any remaining biomass. Biomass liquor was supplemented with YEP (30g/L) and filter sterilised through 0.20μm polyethersulfone (PES) syringe filter before use as a fermentation medium.
The insoluble material from the spent grain pre-treatment (referred here as spent spent grain (SSG)), was washed three times in water, then neutralised using a 2.5mM Na_2CO_3 solution, followed by 2 further washes with water. The SSG was then autoclaved. For SSG and spent grain liquor combined fermentations (SG-SSG) spent grain liquor was supplemented with insoluble SSG (500g/L) and YEP (30g/L).

2.1.5 Fermentation conditions

Yeast strains were individually cultured in YEP-D (20g/L) at 30°C, 150rpm for 24Hrs. Cells were harvested washed and inoculated into fermentation media at a starting cell

density of $1x10^8$ cells/mL. In co-culture fermentations, cells were inoculated in a 1:1:1 ratio to a final cell density of $1x10^8$cells/mL. Fermentations were carried out in sealed 250mL conical flasks or 50mL Falcon tubes at 30°C and 150rpm for up to 14 days.

2.2 Molecular biology techniques

2.2.1 DNA extraction

DNA extraction from yeast strains was carried out using the phenol:chloroform extraction method (Cassago et al., 2002) with slight modifications. Strains were cultured overnight in 5mL YEP-D (20g/L) media. The cells were harvested, then re-suspended in 400µL of DNA re-suspension buffer (1% (w/v), sodium dodecyl sulphate (SDS), 10 mM EDTA and 0.1 M Tris- HCl (pH 8)). Tris HCl (50mM, pH 8) buffered phenol was mixed in a 1:1 ratio with chloroform and added in a 1:1 ratio to re-suspended cells along with a ½ volume of glass beads (Sigma-Aldrich). The mixture was then vortexed for 5 minutes followed by centrifugation at 14,000 rpm for 5 minutes at 4°C. The aqueous top layer was removed and used for another phenol chloroform extraction as described above but without glass beads. The aqueous top layer was removed and mixed in a 1:1 ratio with chloroform and vortexed for 10 minutes, followed by centrifugation for 10 minutes at 4°C. The top aqueous layer was removed and stored on ice.

Sodium acetate and ethanol were added to extracted DNA to a final concentration of 0.3M and 80% respectively, and the mixture incubated at -70°C for 45 minutes. Precipitated DNA was pelleted by centrifugation (14,000rpm, 4°C), the pellet was then washed in 70% ethanol and then dried and re-suspended in dH_2O.

2.2.2 Polymerase chain reaction (PCR)

PCR reactions contained 1x reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.3), dNTP (2.5mM) oligonucleotides (0.5µM) and 5ng-500ng DNA template, in a final reaction volume of 25µL. Template concentration varied depending on whether template was genomic DNA (500ng) or purified plasmid (5ng). Two different PCR cycle times were used (Table 2.2) dependent on the polymerase enzyme used. For the generation of large quantities of specific DNA fragments 10U of the DNA polymerase enzyme *Verizyme* (*Pyrococcus furiosus*) (BioYork) was used. For confirmation of transformants, 10U of the polymerase enzyme *Taq* (*Thermus aquaticus*) (Bioyork) was used.

Table 2.2 PCR reaction programs used for DNA amplification using either *Taq* or *Verizyme* polymerase

Step	*Taq*	*Verizyme*
1/ Denature	4min, 95°C	4min, 95°C
2/ Denature	30sec, 95°C	45 sec, 95°C
3/ Annealing	45sec[A]	60 sec[A]
4/ Extension	1min, 72°C	120sec, 72°C
5/ Extension	12min, 72°C	12min, 72°C
Cycle 2-4	25	35

[A]- Annealing temperatures of primers were based on melting temperatures determined using ApE-plasmid editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/).

2.3 Oligonucleotides

A full list of the oligonucleotides used in this study for cloning are shown below in Table 2.3, all oligonucleotides were ordered from Sigma-Aldrich.

Table 2.3 Oligonucleotides used for cloning

Name	Sequence (5'-3')
bgl1_MidR	GCCAACATCCTGCCGCTCAAGAAGCC
bgl1_MidF	GGCTTCTTGAGCGGCAGGATGTTGGC
cbh2_Rec1_F	gaattcgatatcaagcttatcgataccgtcgacaATGATTGTCGGCATTCTCACCAC GC
cbh2_MidR	GCATAGTTACCGCCATTCTTGTTGGCGGTGCG
cbh2_MidF	CGCACCGCCAACAAGAATGGCGGTAACTATGC
cbh2_Rec2_R	gcgtgacataactaattacatgactcgaggtcgac**CTAGTGATGGTGATGGTGAT G**CAGGAACGATGGGTTTGCGTTTGTG
egl1_Rec1_F	gaattcgatatcaagcttatcataccgtcgacATGGCGCCCTCAGTTACACTGCCG T
egl1_new_Rec1_F	gaattcgatatcaagcttatcgataccgtcgacaATGGCGCCCTCAGTTACACTGCC GT
egl1_Rec2_R	gcgtgacataactaattacatgactcgaggtcgac**CTAGTGATGGTGATGGTGAT G**AAGGCATTGCGAGTAGTAGTCGTTG
egl2_Rec1_F	gaattcgatatcaagcttatcgataccgtcgacaATGAACAAGTCCGTGGCTCCAT TGC
egl2_MidR	GGATAAACCTTCGAGGTAACGCAAGTGCCATCTGTGGTACAGC CAAAGTCAAAAC
egl2_MidF	GCGGGTTTTGACTTTGGCTGTACCACAGATGGCACTTGCGTTA CCTCGAAGGTTTATCCT
egl2_Rec2_R	gcgtgacataactaattacatgactcgaggtcgacCTACTTTCTTGCGAGACACGA GCTG
Psi_PGK_F	tttaaccaataggccgaaatcggcaaaatcccttaCCAAGAATTACTCGTGAGTAA GGAAAGA
Psi_Cyc_R	cactcaaccctatctcggtctattcttttgatttaGGCCGCAAATTAAAGCCTTCGAG CG
Psi_TEF_F	ccaataggccgaaatcggcaaaatcccttaAATGTTTCTACTCCTTTTTTACTCT
TEF_R	ggtgatggtgatgcgatcctctcatactagtgcggccgctctagaTTGTAATTAAAACTTA GATTAGATTGC
Psi_HXT7_F	ccaataggccgaaatcggcaaaatcccttaGGGGTTGTTAAGCATGCCCTGCTA AAC
HXT7_R	ggtgatggtgatgcgatcctctcatactagtgcggccgctctagaTTTTGATTAAAATTAA AAAAACTTTTTG

Table 2.3 continued

Name	Sequence (5'-3')
xdh_Rec1_F	gaattcgatatcaagcttatcgataccgtcgacaATGGCGACTCAAACGATCAACA AGG
xdh_IntF	CGATCAACAAGGATGCGATCAGCAACCTCTCC TTCGTCCTCAACAAGCCC
xdh_IntR	GGGCTTGTTGAGGACGAAGGAGAGGTTGCTGATCGCATCCTT GTTGATCG
xdh_Rec2_R	gcgtgacataactaattacatgactcgaggtcgacTTACACCTTCTCGTTGGGCCCG GCA
xr_Rec1_F	gaattcgatatcaagcttatcgataccgtcgacaATGGCGTCTCCCACGCTCAAGC TCA
xr_IntR1	CCGCACTCCTTTTCGTTGCCATAGTCACAGGCACCGTCAAACA GGCGG
xr_IntF1	CCGCCTGTTTGACGGTGCCTGTGACTATGGCAACGAAAAGGA GTGCGG
xr_IntR2	ctaattacatgactcgaggtcgacTTAGCCAAACAGGTAGAGCTTGTTGGCG GAGAAGTAGTTTGTAGGCTTGTTGAACC
xr_Rec2_R	gcgtgacataactaattacatgactcgaggtcgacTTAGCCAAACAGGTAGAGCTT GTTG
xr_MidF	GCGCCATGGAGCGCCTCGTCGACAAGGGCC
xr_MidR	GGCCCTTGTCGACGAGGCGCTCCATGGCGC
xks_Rec1_F	aagttttaattacaatctagagcggccgcactagtATGTTGTGTTCAGTAATTCAGA GAC
xks_MidR	CATATAGTGGCTAAATTATCCCCAGTCATG
xks_MidF	CATGACTGGGGATAATTTAGCCACTATATG
xks_Rec2_R	gcgtgacataactaattacatgactcgaggtcgacTTAGATGAGAGTCTTTTCCAGT TCG
xi_Rec1_F	gaattcgatatcaagcttatcgataccgtcgacaATGGCTAAGGAATACTTCCCAC AAA
xi_MidF	TCGAACCAAAGCCAATGGAACCAACCAAGCACCAA
xi_MidR	TTGGTGCTTGGTTGGTTCCATTGGCTTTGGTTCGA
xi_Rec2_R	gcgtgacataactaattacatgactcgaggtcgacTTATTGGTACATAGCAACGAT AGCT

Table 2.3 continued

Name	Sequence (5'-3')
xdh_R	ACCAGTGAATAATTCTTCACCTTTAGACATCACCTTCTCGTTG GGCCCGGCAATC
GFP_F	ATTCTGATTGCCGGGCCCAACGAGAAGGTGATGTCTAAAGGT GAAGAATTATTCA
GFP_R	ACATAACTAATTACATGACTCGAGGTCGACTTATTTGTACAAT TCATCCATACCA
Acc_TEF_F	atacgactcactatagggcgaattgggtacAATGTTTCTACTCCTTTTTTACTCT
Acc_R	GCGATAGTTCCTCACTCTTTCCTTACTCACGAGTAATTCTTGGG GCCGCAAATTAAAGCCTTCGAGCG
Acc_PGK_F	gcgtaatacgactcactatagggcgaattgggtaccCCAAGAATTACTCGTGAGTA AGGAAAGA
Kpn_F	atacgactcactatagggcgaattgggtacCCAAGAATTACTCGTGAGTAAGGA A
Spe_R	ccaccgcggtggcggccgctctagaactagGGCCGCAAATTAAAGCCTTCGAG CG

lowercase sequence- plasmid specific sequence

UPPERCASE sequence – gene or promoter specific sequence

UNDERLINED UPPERCASE sequence – GFP specific sequence

UPPERCASE BOLD sequence – 6x His tag sequence

2.4 Cloning strategy

The co-ordinates and origin of genes used in this study are shown in Table 2.4. Using a cDNA approach cellulase genes (*bgl1*, *cbh2*, *egl1*) had previously been inserted individually via homologous recombination into the low copy number plasmid pGREG586 (Fitzpatrick et al., 2014). These plasmids contain a modular cloning system allowing for easy replacement of the gene or promoter within the cassette (Jansen et al., 2005). The original pGREG586 modular cloning cassette consisted of a GAL1 promoter with Sac1 and Spe1 restriction digest sites upstream and downstream of the promoter respectively for promoter replacement. Downstream of the GAL1 promoter there is a 6xHis tag and the *HIS3* gene. The *HIS3* gene was flanked either side by two recombination sites (Rec1 and Rec2) (34bp), which contain Sal1 restriction enzyme sites for gene replacement (Fig 2.1A).

Table 2.4 Gene co-ordinates. The source organism and co-ordinates of target genes used in this study. *T. reesei* gene co-ordinates were obtained from the Fungal Genomic Resource (Grigoriev et al., 2012), compiled from original *T. reesei* sequencing data (Martinez et al., 2008), *Piromyces sp* E2 gene co-ordinates were obtained from the Joint Genome Institute (JGI) genome portal (Nordberg et al., 2014), *S. cerevisiae* gene co-ordinates were obtained from *Saccharomyces* Genome Database (SGD).

Organism	Gene	Scaffold/Chromosome	Gene co-ordinates
T. reesei	*bgl1*	Scaffold 6	104478-107104
T. reesei	*cbh2*	Scaffold 3	12521-14440
T. reesei	*egl1*	Scaffold 10	665665-667273
T. reesei	*egl2*	Scaffold 3	33633-35550
T. reesei	*xyl1*	Scaffold 10	509692-511253
T. reesei	*xdh1*	Scaffold 23	402101-404144
Piromyces sp. E2	*xi*	Scaffold 20	199178-200795
S. cerevisiae	*XKS1*	Chromosome VII	886073-887875

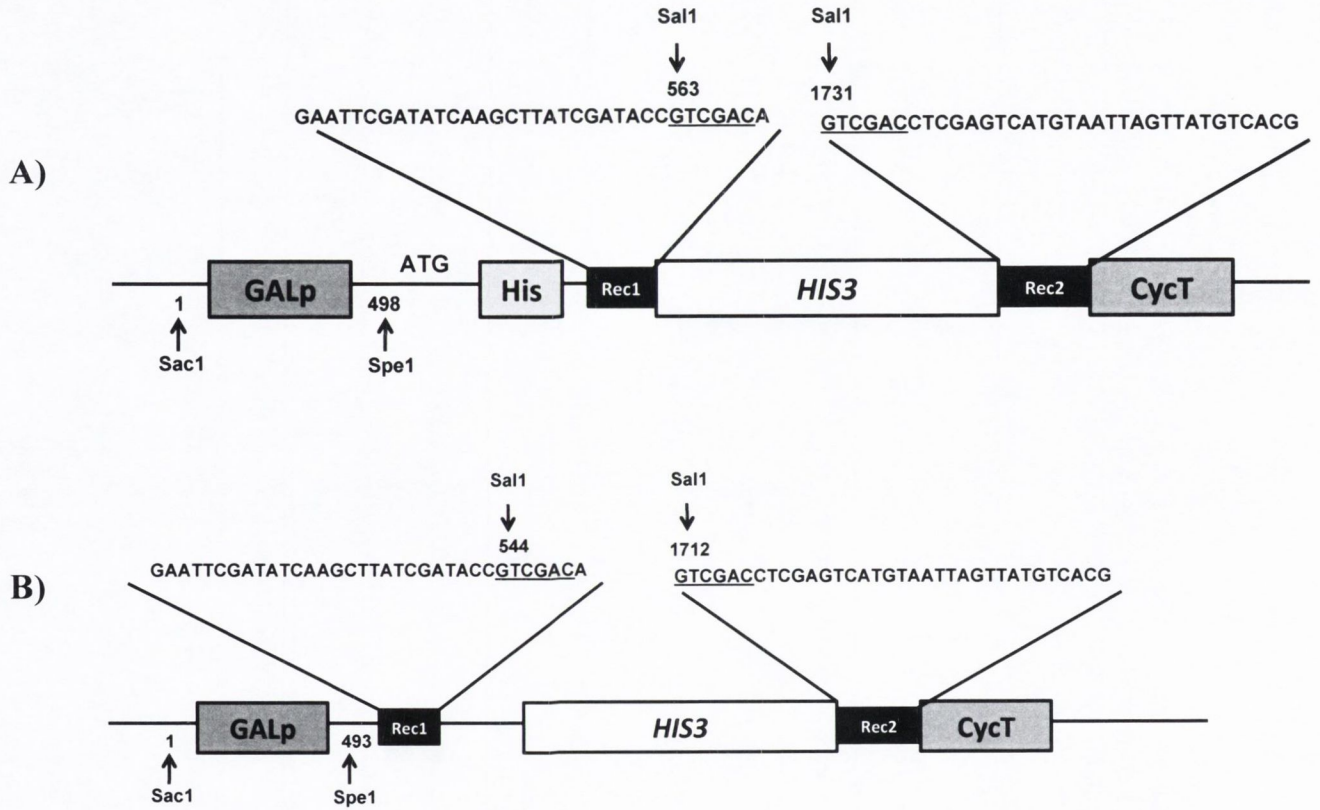


Figure 2.1 Drag and Drop modular cloning cassette (Jansen et al., 2005). A) pGREG586 B) pGREG505. Rec site sequences shown, along with integrated SalI sites (shown as underlined nucleotides) for *HIS3* excision. The restriction enzyme sites SacI and SpeI are used for promoter excision. GALp:GAL promoter. His:6xHistidine N-terminal tag. Rec1:Recombination anchor site 1. *HIS3*: Imidazoleglycerol-phosphate dehydratase. Rec2:Recombination anchor site 2. CycT: Cyc terminator.

The *bgl1*, *cbh2* and *egl1* cDNAs were cloned into pGREG586 downstream of the GAL1 promoter via *in vivo* homologous recombination. Subsequently the GAL1 promoter was replaced by the PGK promoter (Fitzpatrick et al., 2014). The xylose utilising genes *xyl1* and *xdh1* were individually inserted into the pGREG505 plasmid (Fig 2.1B) using the same approach, however the genes were amplified in fragments from *T. reesei* genomic DNA to remove introns (T C James, unpublished). The *xdh1* gene was amplified in 2 fragments from *T. reesei* genomic DNA. Fragment 1 was amplified using primers xdh_Rec1_F and xdh_IntR. The amplified DNA contained a 5'end extension (34bp) homologous to the Rec1 site of pGREG505 and a 3'end extension (27bp) homologous to the 5'end of fragment 2. Fragment 2 was amplified using primers xdh_IntF and xdh_Rec2_R. The amplified DNA contained a 5'end extension (22bp) homologous to the 3'end of fragment 1 and a 3'end extension (34bp) homologous to the Rec2 site.

The *xyl1* gene was amplified in 2 fragments from *T. reesei* genomic DNA. Fragment 1 was amplified using primers xr_Rec1_F and xr_IntR1. The amplified DNA contained a 5'end extension (34bp) homologous to the Rec1 site of pGREG505 and a 3'end extension (25bp) homologous to the 5'end of fragment 2. Fragment 2 was amplified using primers xr_IntF and xr_IntR2. The amplified DNA contained a 5'end extension (23bp) homologous to the 3'end of fragment 1 and a 3'end extension (34bp) homologous to the Rec2 site. The fragments were inserted into Sal1 (New England BioLabs, UK) digested pGREG505 plasmid via *in vivo* homologous recombination, generating plasmids pGREG505 PGKxdh and pGREG505 PGKxr (T.C James, personal communication).

The cloned genes were subsequently transferred from the low copy number plasmid pGREG series to the high copy number plasmid pRS42H (Taxis and Knop, 2006). The PGKbgl1 gene cassette, consisting of the PGK promoter, N-terminal 6xHis tag, *bgl1* gene, and the Cyc terminator, was amplified in two fragments from pGREG586 PGKbgl1 plasmid using primers Kpn_F and bgl1_midR for fragment 1 and bgl1_midF and Spe_R for fragment 2. DNA fragments were inserted into pRS42H digested with Kpn1 (New England BioLabs, UK) and Spe1 (New England BioLabs, UK) via *in vivo* homologous recombination. The new plasmid was named pRS42H PGKbgl1 (Fig 2.2) and was used as the skeleton for all cloning strategies used in this study (Fitzpatrick et al., 2014).

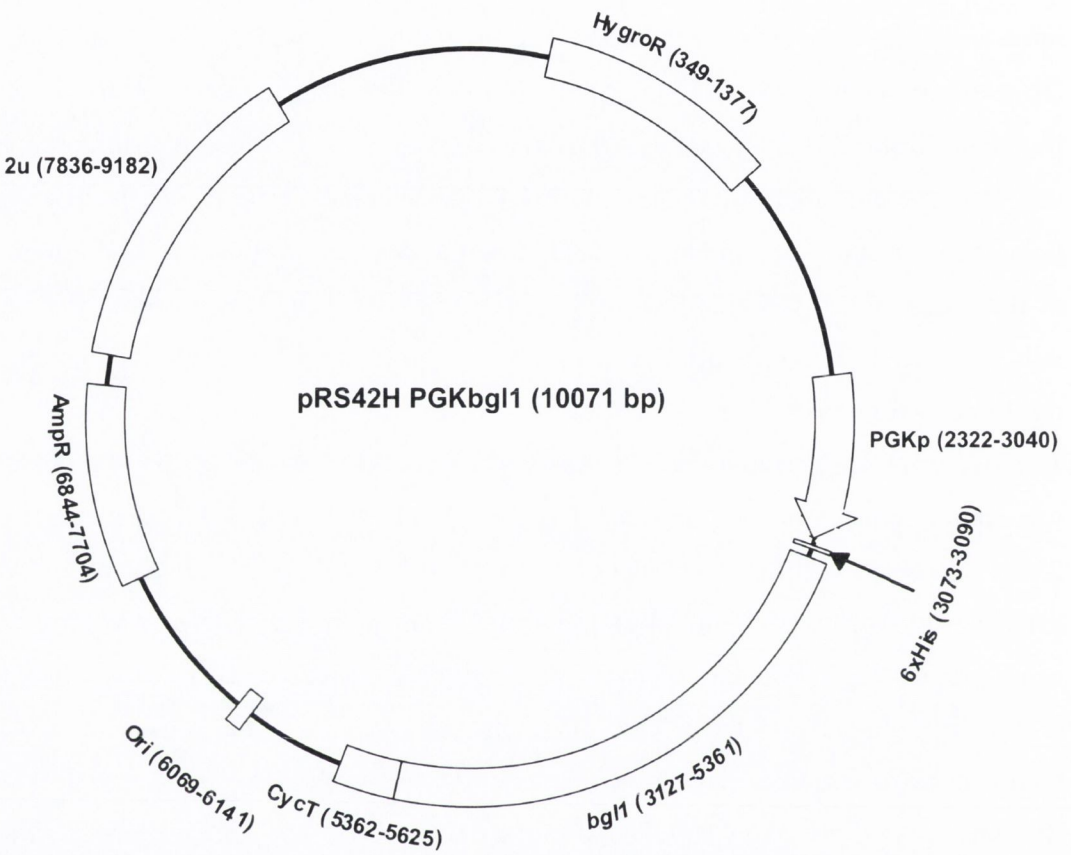


Figure 2.2 Schematic of the pRS42H PGKbgl1 high copy number plasmid. HygroR: Hygromycin resistance cassette. PGKp: PGK promoter from *S. cerevisiae*. 6xHis: Inbuilt 6xHistidine N-terminal tag. *bgl1*: β-glucosidase gene from *T. reesei*. CycT: Cyc terminator from *S. cerevisiae*. Ori: Origin of replication for *E.coli*. AmpR: *bla* Ampicilin resistance cassette. 2u: 2 micron origin of replication for yeast.

2.4.1 Single gene insertion strategy

Single gene expression cassettes in the high copy number plasmid pRS42H were generated using the single gene insertion strategy. The plasmid pRS42H PGKbgl1 was either digested with Sal1 (excising the *bgl1* gene) or Sal1 and Spe1 (excising the *bgl1* gene and the inbuilt 6xHis tag) (Fig 2.1). Replacement genes were amplified using gene specific primers and template shown in Table 2.5. Genes were amplified in 1 or 2 fragments depending on the size of the gene or to facilitate the removal of introns. The PCR products from the amplified genes contained 5' and 3' end extensions of 34bp, homologous to Rec sites and to the upstream or downstream contiguous fragments of the gene insert. The PCR products were inserted into digested pRS42H PGKbgl1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51 (Fig 2.3/2.4).

The plasmid pRS42K (containing Geneticin® resistance marker, *kanMX4*) (Taxis and Knop, 2006) did not contain the pGREG modular cloning cassette, necessitating the insertion of the whole cassette (promoter, gene and terminator) into pRS42K for single gene expression in a Geneticin® resistant background. Gene cassettes were amplified in 2 fragments using primers and template shown in table 2.5. Fragment 1 contained a 5' end extension homologous to a 35bp region directly upstream of the Psi1 cut site on pRS42K, and a 3' end extension of 30bp homologous to the 5'end of fragment 2. Fragment 2 contained a 5'end extension of 30bp homologous to the 3'end of fragment 1, along with a 3'end extension homologous to a 35bp region directly downstream of the Psi1 (New England BioLabs, UK) cut site on pRS42K. Fragments were inserted into Psi1 digested pRS42K plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51.

2.4.2 Second gene insertion strategy

Plasmids containing single gene cassettes were digested and linearised using the restriction enzyme Psi1. Gene cassettes (promoter, 6xHis tag, gene and terminator) were amplified in two fragments using specific primers (Table 2.5). Fragment 1 incorporated a 5' end extension homologous to a 35bp region directly upstream of the Psi1 cut site and a 3' end extension of 30bp homologous to the 5'end of fragment 2. Fragment 2 contained a 5'end extension of 30bp homologous to the 3'end of fragment 1, and a 3'end extension homologous to a 35bp region directly downstream of the Psi1 cut site on the plasmid. Fragments were inserted into digested plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51 (Fig 2.5). The two gene cassettes were inserted in a tandem orientation with a space of 562bp between cassettes.

Table 2.5 Cloning strategies for single, double, triple gene expression and promoter swaps.

Plasmid	Cloning Method	Template DNA	Fragment	Primers		Plasmid used for insertion	Restriction enzyme (feature excised)
				F	R		
PGKcbh2	Single gene insertion	pGREG586 PGKcbh2	1	cbh2_Rec1_F	cbh2_Rec2_R	pRS42H PGKbgl1	Sal1 (*bgl1*)
PGKegl1 (1.1)	Single gene insertion	pGREG586 PGKegl1	1	egl1_Rec1_F	egl1_Rec2_R	pRS42H PGKbgl1	Sal1 (*bgl1*)
PGKegl1 (1.2)	Single gene insertion	pGREG586 PGKegl1	1	egl1_new_Rec1_F	egl1_Rec2_R	pRS42H PGKbgl1	Sal1 (*bgl1*)
PGKegl2	Single gene insertion	*T. reesei* Genomic DNA (QM9123)	1	egl2_Rec1_F	egl2_MidR	pRS42H PGKbgl1	Sal1 (*bgl1*)
			2	egl2_MidF	egl2_Rec2_R		
PGKxr	Single gene insertion	pGREG505 PGKxr	1	xr_Rec1_F	xr_Rec2_R	pRS42H PGKbgl1	Sal1 (*bgl1*)
PGKxdh	Single gene insertion	pGREG505 PGKxdh	1	xdh_Rec1_F	xdh_Rec2_R	pRS42H PGKbgl1	Sal1 (*bgl1*)
TEFxks	Single gene insertion	*S. cerevisiae* genomic DNA (288C)	1	xks_Rec1_F	xks_MidR	pRS42H TEFbgl1	Sal1 and Spe1 (N-His Tag, *bgl1*)
			2	xks_MidF	xks_Rec2_R		
PGKxdhGFP	Single gene insertion	pGREG505 PGKxdh	1	xdh_Rec1_F	xdh_R	pRS42H PGKbgl1	Sal1 (*bgl1*)
		pGREG599	2	GFP_F	GFP_ R		
TEFxks^{Km}	Single gene insertion	pRS42H TEFxks	1	Psi_TEF_F	xks_MidR	pRS42K	Psi1
			2	xks_MidF	Psi_Cyc_R		
PGKxi	Single gene insertion	pUC57 xi	1	xi_Rec1_F	xi_MidR	pRS42H PGKbgl1	Sal1 (*bgl1*)
			2	xi_MidF	xi_Rec2_R		
PGKegl2-PGKegl1	Second gene insertion	pRS42H PGKegl2	1	Psi_PGK_F	egl2_MidR	pRS42H PGKegl1	Psi1
			2	egl2_MidF	Psi_Cyc_R		

Table 2.5 continued

Plasmid	Cloning Method	Template DNA	Fragment	Primers		Plasmid used for insertion	Restriction enzyme (feature excised)
				F	R		
PGKxr-PGKxdh	Second gene insertion	pRS42H PGKxr	1	Psi_PGK_F	xr_MidR	pRS42H PGKxdh	Psi1
			2	xr_MidF	Psi_Cyc_R		
PGKxr-TEFxks-PGKxdh	Third gene insertion	pRS42H TEFxks	1	Acc_TEF_F	xks_MidR	pRS42H PGKxr-PGKxdh	Acc65I
			2	xks_MidF	Acc_R		
PGKxr-TEFbgl1-PGKxdh	Third gene insertion	pRS42H TEFbgl1	1	Acc_TEF_F	bgl1_MidR	pRS42H PGKxr-PGKxdh	Acc65I
			2	bgl1_MidF	Acc_R		
PGKxr-TEFcbh2-PGKxdh	Third gene insertion	pRS42H TEFcbh2	1	Acc_TEF_F	cbh2_MidR	pRS42H PGKxr-PGKxdh	Acc65I
			2	cbh2_MidF	Acc_R		
PGKxr-PGKegl2-PGKxdh	Third gene insertion	pRS42H PGKegl2	1	Acc_PGK_F	egl2_MidR	pRS42H PGKxr-PGKxdh	Acc65I
			2	egl2_MidF	Acc_R		
TEFbgl1	Promoter Swap	pGREG505 TEF1	1	Psi_TEF_F	TEF_R	pRS42H PGKbgl1	Spe1 and Psi1 (PGK promoter)
HXT7bgl1	Promoter Swap	*S. cerevisiae* genomic DNA (288C)	1	Psi_HXT7_F	HXT7_R	pRS42H PGKbgl1	Spe1 and Psi1 (PGK promoter)
TEFcbh2	Promoter Swap	pGREG505 TEF1	1	Psi_TEF_F	TEF_R	pRS42H PGKcbh2	Spe1 and Psi1 (PGK promoter)

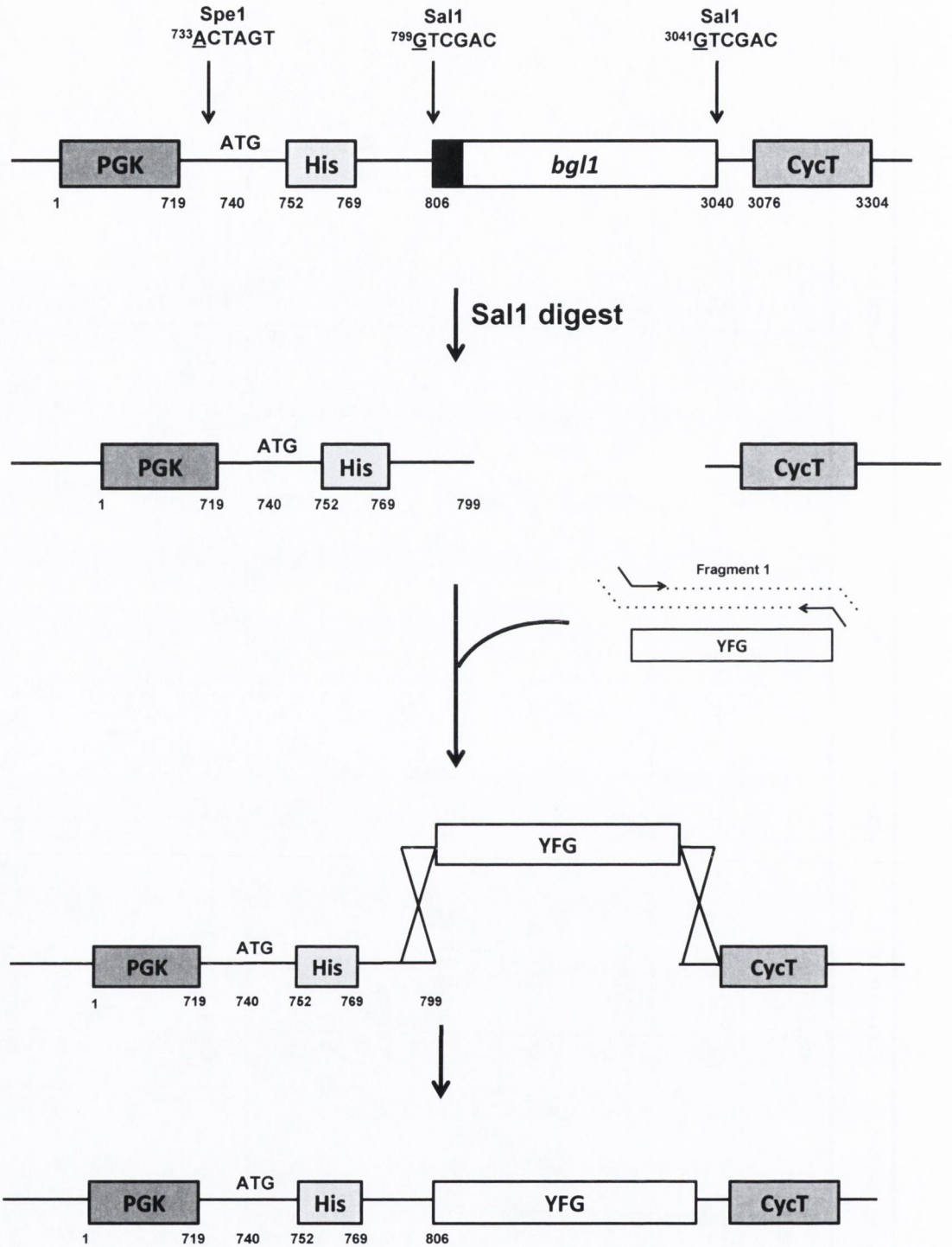


Figure 2.3 Single gene insertion strategy. Host plasmid is digested using Sal1 and amplified new DNA is inserted via *in vivo* homologous recombination into host plasmid. PGK:PGK promoter. His:6xHistidine N-terminal tag. YFG: Your Favourite Gene. CycT:Cyc terminator. Black box (■) represents native *bgl1* secretory signal.

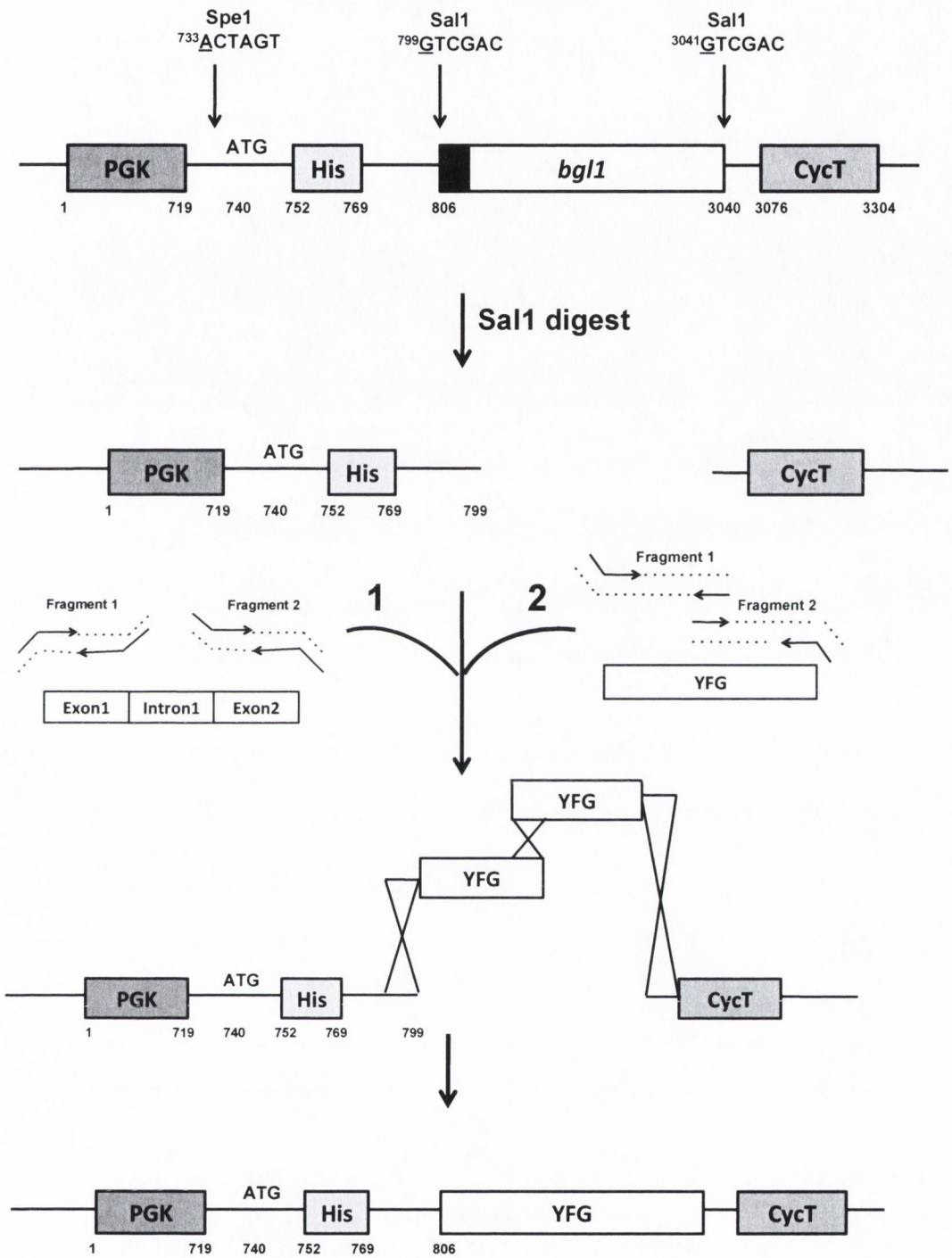


Figure 2.4 Single gene insertion strategy using 2 fragments. Host plasmid is digested using Sal1 and new DNA is then amplified in 2 fragments ((1) to remove introns or (2) due to size of the gene) and inserted via *in vivo* homologous recombination into digested host plasmid. PGK:PGK promoter. His:6xHistidine N-terminal tag. YFG: Your Favourite Gene. CycT:Cyc terminator. Black box (■) represents native *bgl1* secretory signal.

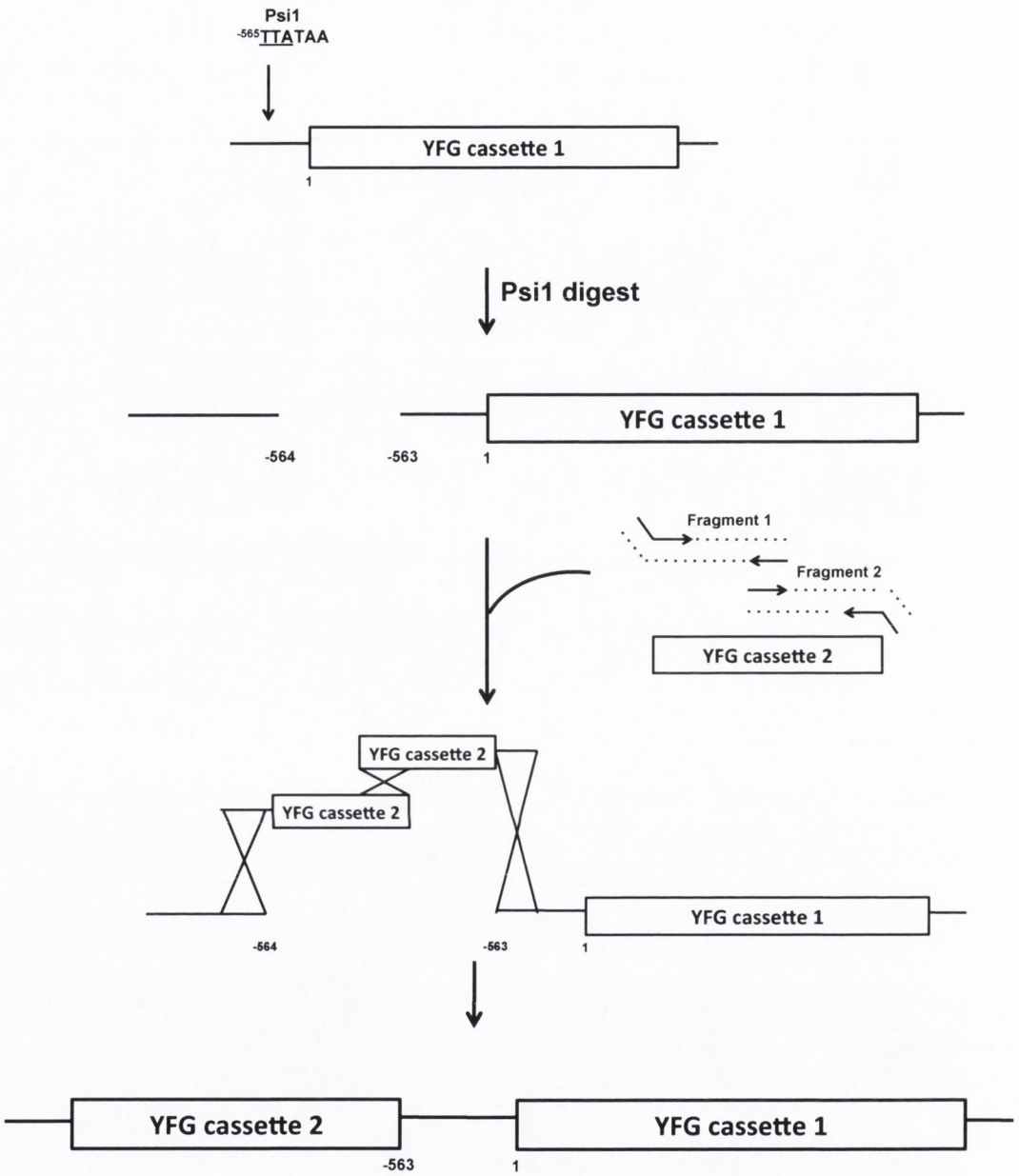


Figure 2.5 Second gene insertion strategy. Host plasmid is first digested using Psi1, new DNA is amplified in 2 fragments and then inserted via *in vivo* homologous recombination into digested plasmid. YFG cassette 1: Your Favourite Gene pre-existing cassette within plasmid. YFG cassette 2: Your Favourite Gene cassette to be inserted into digested plasmid.

2.4.3 Third gene insertion strategy

Plasmids containing two gene cassettes were digested and linearised using the restriction enzyme Acc65I (New England BioLabs, UK). Gene cassettes (promoter, 6xHis tag, gene and terminator) were amplified in two fragments using specific primers (Table 2.5). Fragment 1 incorporated a 5' end extension homologous to a 35bp region directly upstream of the Acc65I site along with a 3' end extension of 30bp homologous to the 5' end of fragment 2. Fragment 2 contained a 5' end extension of 30bp homologous to the 3'end of fragment 1, and a 3' end extension homologous to a 35bp region directly downstream of the Acc65I cut site on the plasmid. The fragments were inserted into Acc65I digested plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51 (Fig 2.6). The three gene cassettes were all in a tandem orientation, with cassette 2 being 557bp upstream of the newly inserted cassette, which was directly followed by gene cassette 1.

2.4.4 Promoter swap strategy

Plasmids were digested with the restriction enzymes Spe1 and Psi1, excising the existing promoter in the plasmid along with 562bp upstream of the promoter (Fig 2.7). Replacement promoters, *S. cerevisiae* HXT7 (1162308-1163278 Chromosome IV) and *S. cerevisiae* TEF1 (700191-700591 Chromosome XVI) were amplified as single fragments from template DNA (pGREG505 TEF1 was gifted by Karreman RJ & Morrissey JP, UCC, Cork) using the primers outlined in Table 2.5. The resultant PCR products contained 5' and 3' end extensions (30bp), the 5' end extension homologous to the region directly upstream of the Psi1 cut site and the 3' end extension homologous to the region directly downstream of the Spe1 cut site on the digested plasmid. Fragments were inserted into digested plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51 (Fig 2.7). The 5'UTR length differed between promoters (PGK, TEF and HXT7) (Fig 2.8).

An outline of all gene cassettes generated in this study is shown in Fig 2.9-2.11.

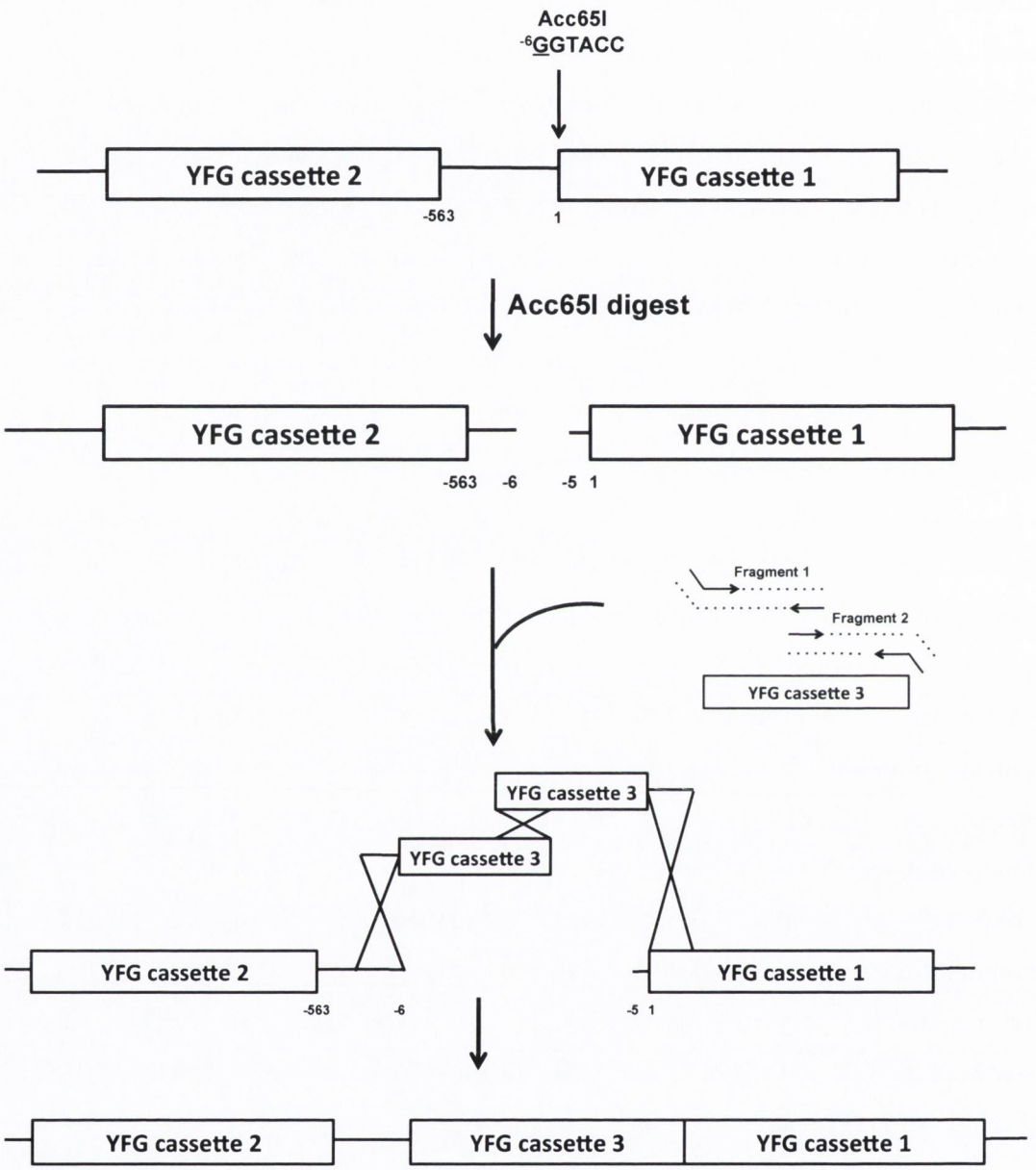


Figure 2.6. Third gene insertion strategy. Host plasmid is first digested using Acc65I and new gene cassette for insertion is amplified in 2 fragments and then inserted via *in vivo* homologous recombination into digested host plasmid. YFG cassette 1: Your Favourite Gene pre-existing cassette within plasmid. YFG cassette 2: Your Favourite Gene pre-existing cassette within plasmid. YFG cassette 3: Your Favourite Gene cassette to be inserted into digested plasmid.

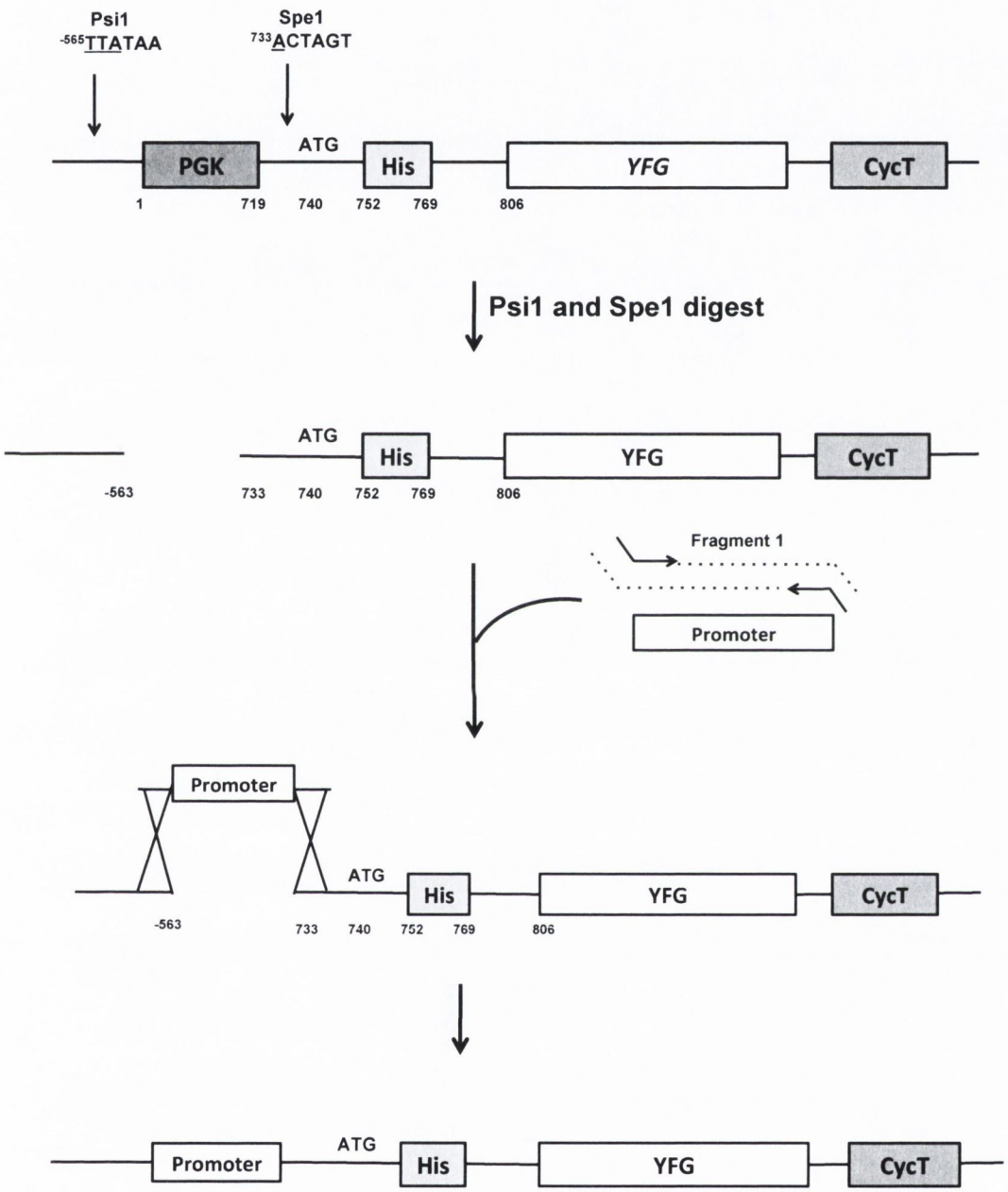


Figure 2.7 Promoter swap strategy. Host plasmid is first digested using SpeI and PsiI, this removing existing promoter. New DNA is amplified in a single fragment then inserted via *in vivo* homologous recombination into digested host plasmid. PGK: PGK promoter. His: 6xHistidine N-terminal tag. YFG: Your Favourite Gene. CycT: Cyc terminator. Promoter: New promoter (TEF1/HXT7).

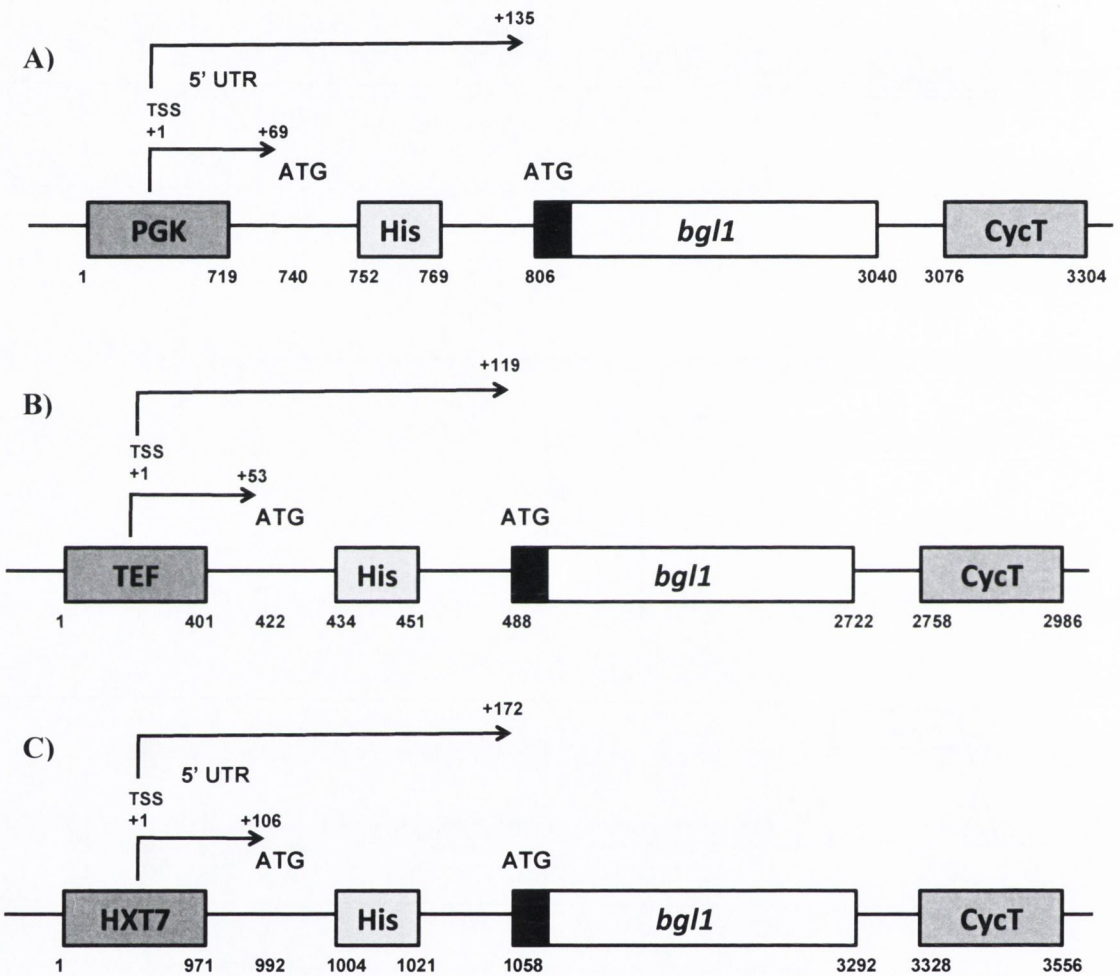


Figure 2.8 Comparison of the 5' untranslated regions in the promoter swap cassettes for *bgl1*. (A) PGKbgl1 (B) TEFbgl1 (C) HXT7bgl1. Transcriptional Start Sites (TSS) for TEF and PGK were obtained from the Yeast Promoter Alas (http://ypa.ee.ncku.edu.tw/index.html). TSS for HXT7 was obtained from Zhang and Dietrich (2005). Arrows represent the 5'UTR from TSS to ATG present within the plasmid and the start codon of the gene. Black box (■) represents native *bgl1* secretory signal. His: 6xHistidine N-terminal tag. CycT: Cyc terminator. The 5'UTR for the other promoter swap cassette, TEFcbh2, is identical to TEFbgl1 5'UTR.

Cassette Overview

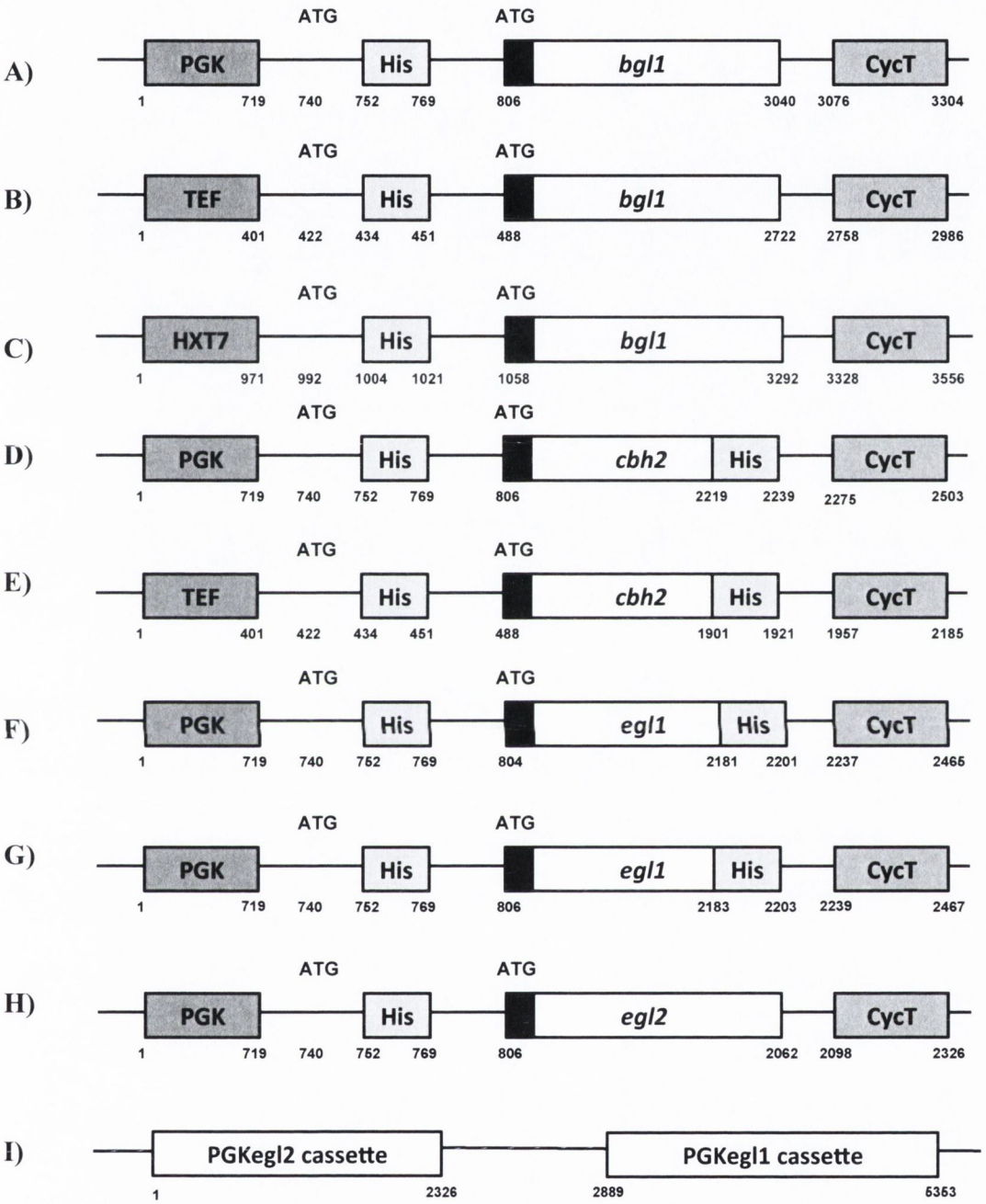


Figure 2.9 Cellulase plasmid cassettes. Images showing features of individual cassettes and their co-ordinates. PGK:PGK promoter. TEF: TEF promoter. HXT7: HXT7 promoter. HIS: 6xHistidine tag. CycT: Cyc terminator. Black box (■) native secretory signal. A: PGKbgl1, B: TEFbgl1, C:HXT7bgl1, D: PGKcbh2, E: TEFcbh2, F:PGKegl1 1.1, G: PGKegl1 1.2, H: PGKegl2, I: PGKegl2-PGKegl1.

Cassette Overview

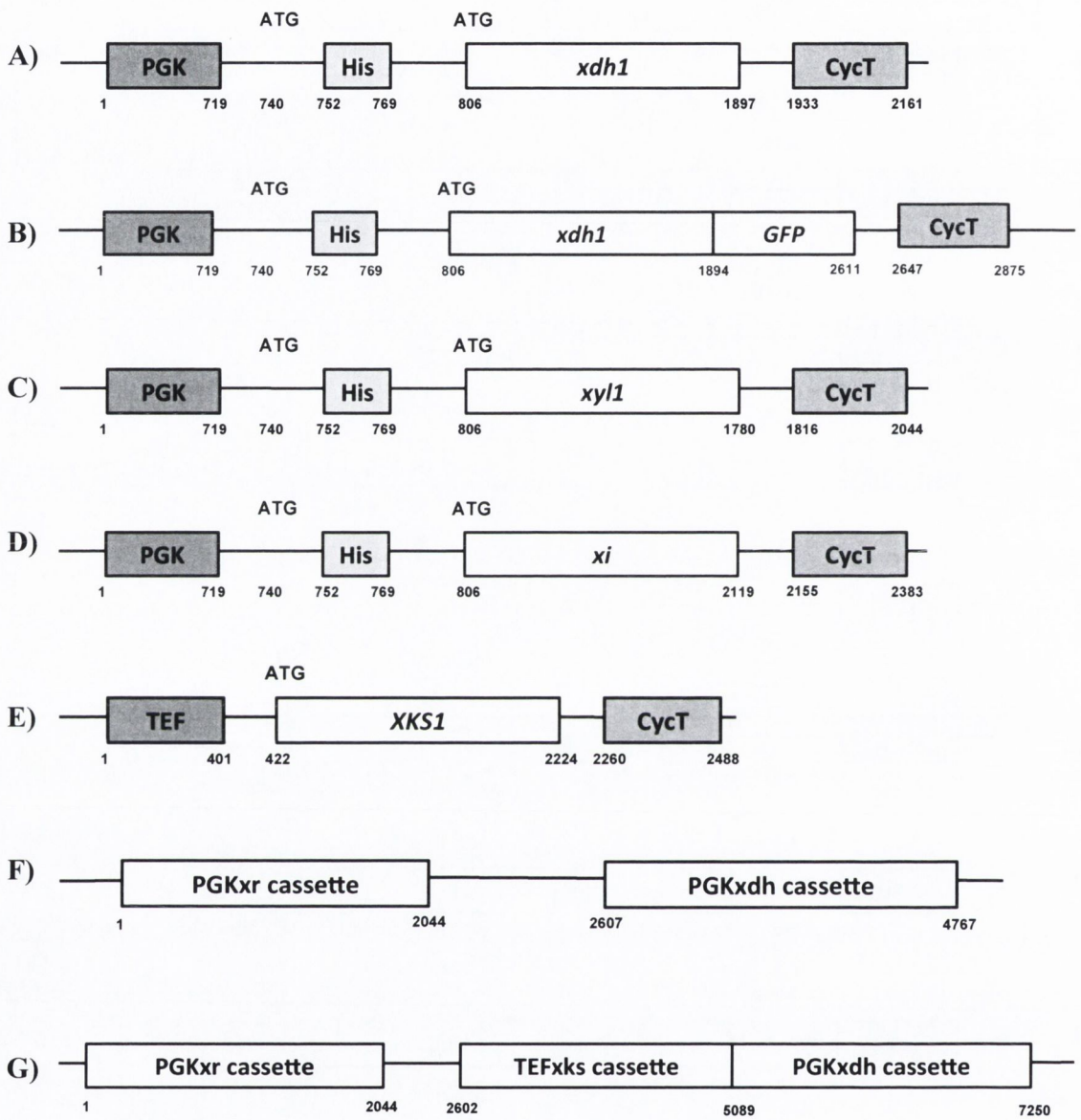


Figure 2.10 Xylose utilising plasmid cassettes. Images showing features of individual cassettes and their co-ordinates. PGK:PGK promoter. HIS: 6xHistidine tag. CycT: Cyc terminator. GFP: Green fluorescent protein. A: PGKxdh, B: PGKxdhGFP, C: PGKxr, D: PGKxi, E: TEFxks, F: PGKxr-PGKxdh, G: PGKxr-TEFxks-PGKxdh.

Cassette Overview

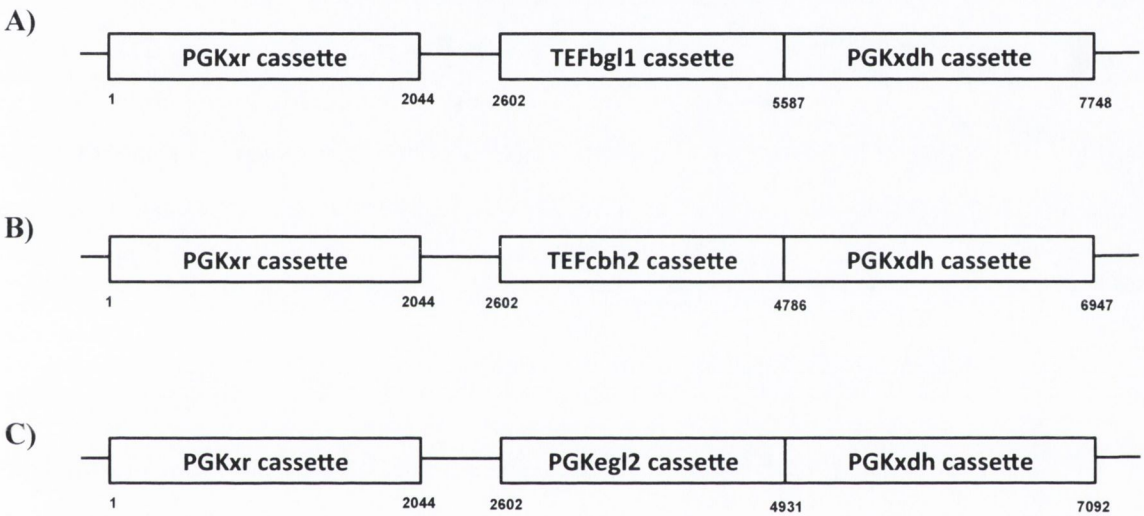


Figure 2.11 Plasmid cassettes for xylose and cellulose co-utilisation. The orientation of the gene cassettes and coordinates are shown. A: PGKxr-TEFbgl1-PGKxdh, B: PGKxr-TEFcbh2-PGKxdh, C: PGKxr-PGKegl2-PGKxdh.

2.5 DNA Transformation

2.5.1 Yeast Transformation

Yeast transformations were carried out using the lithium acetate method. Yeast cells were grown overnight on YEP-D (20g/L) agar plates, cells were harvested and washed in 0.1M lithium acetate. Transformation mixtures contained 50% (w/v) PEG3500, 0.1M Lithium acetate and 100μg carrier DNA (salmon sperm) with either 1μg of plasmid for general plasmid transformations or cut plasmid (0.1μg) along with DNA fragment for new plasmid generation. Cells were incubated at 42°C for 1 hour and recovered overnight in YEP-D (20g/L) at 30°C. The cells were then harvested and plated on YEP-D (20g/L) agar containing the antibiotic required for positive selection.

Colony PCR reactions were carried out using the *Taq* protocol (Section 2.3.2) to identify positive transformants. Template DNA was extracted by lysing individual colonies in 25μL, 25mM NaOH and heating to 95°C for 5 minutes. For the PCR reaction 1μL of cell lysate was used as template DNA. Alternatively functional assays (X-glu activity, CMC activity and fluorescence microscopy) were used to identify positive yeast transformants (see below).

2.5.2 Bacterial Transformation

The *E. coli* strain XL1-Blue was transformed with either plasmid or total DNA extract from yeast. Competent *E. coli* XL1-Blue cells were mixed with plasmid (50ng) or total DNA extract (5μg) and incubated on ice. After 1 hr incubation, cells were subject to heat shock at 42°C (90 seconds) followed by further incubation on ice for 60 seconds. Cells were then allowed to recover for 3Hrs in pre-warmed LB at 37°C and then plated onto LB agar, containing carbenicillin (100μg/mL).

2.5.3 Isolation of plasmid from bacteria

Single colonies were picked and inoculated into a 5mL LB culture (carbenicillin 100μg/mL) and incubated overnight at 37°C and 200rpm. The cells were then harvested and the supernatant discarded. The cell pellet was then resuspended in 200μL of resuspension buffer (25mM Tris-HCl pH8, 10mM EDTA pH8) and vortexed for 1 minute. To the cell suspension, 200μL of lysis buffer (0.2M NaOH, 1% SDS) was added and the mixture slowly inverted continuously for 5 minutes, followed by the addition of 350μL of neutralisation buffer (3M Pottasium acetate, 5M Glacial acetic acid). The mixture was then inverted a further 4 times and the insoluble and soluble material separated by centrifugation at 14,000rpm for 10 minutes at 4°C. The soluble

fraction was added to a DNA binding column (Econospin) and incubated at room temperature for 5 minutes. The column was then centrifuged at 13,000 rpm for 1 minute at 4°C. The flow through was discarded and the column was then washed with 200µL of wash buffer (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol) and centrifuged twice at 13,000rpm for 1 minute. After the second spin, 50µL of dH_2O was added to the column and incubated at room temperature for 5 minutes. The plasmid was eluted from the column in a fresh eppendorf by a final centrifugation step at 13,000rpm for 1 minute at 4°C.

2.6 DNA sequencing

Gene cassettes generated in this study were sequenced by GATC (GATC Biotech AG, Konstanz). The generated sequence was compared to wild type sequence using the ApE sequence alignment program. Several point mutations were discovered in the sequences of the cloned genes. The mutations did not affect the open reading frame of the gene. The plasmid sequences for all gene cassettes generated in this study and a summary of the point mutations in the genes are contained in the supplemental CD.

2.7 Enzymatic assays

2.7.1 XDH and XR activity

XDH and XR activities were quantified by measuring nicotinamide adenine dinucleotide (NAD^+) reduction to NADH and oxidation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$ respectively. Yeast strains expressing XR and XDH were harvested after 24Hrs of growth in YEP-D (20g/L) at 30°C, and were washed twice and re-suspended in 1/10 of the original culture volume in lysis buffer (250mM potassium phosphate (Fisher Scientific) pH7.0). The cells were lysed using Zirconia beads with agitation at 4°C. Following removal of cell debris by centrifugation (14,000rpm for 20 minutes at 4°C), XDH and XR activity in the cell free extract was measured. The XDH assay buffer contained 0.15M xylitol (Fluka), 0.4mM NAD^+ (Melford) or $NADP^+$ (Sigma-Aldrich) and 25mM potassium phosphate pH 7.0. The XR reaction buffer contained 0.34mM NADPH or NADH (Sigma-Aldrich), 50mM Xylose and 25mM potassium phosphate pH 7.0. Buffers were equilibrated to room temperature before use. For each reaction cell-free extract was mixed in 1:10 ratio with reaction buffer and absorbance at 340nm was measured at 10

second intervals for 2 minutes at 30°C. XDH activity is defined as NAD^+ or $NADP^+$ (μM) reduced per minute. XR activity was defined as NADH or NADPH (μM) oxidised per minute. NADH and NADPH concentrations were calculated using a standard curve (0-800μM). Enzyme activity was standardised against protein levels (Section 2.11) with the crude specific activity being defined as $\mu M.min^{-1}.\mu g^{-1}$ (U^{XDH} and U^{XR}). Enzyme assays and standards were measured using an Ascent Multiscan spectrophotometer.

2.7.2 β-Glucosidase enzyme activity

The activity of BGL1 in yeast strains was qualitatively examined using the chromogenic substrate X-Glucoside (5-bromo-4-chloro-3-indolyl-B-D-glucopyranoside) (Melford), an analogue of cellobiose the natural substrate of BGL1. The screening of BGL1 expressing strains was carried out in 96 flat bottom well plates. Single colonies of strains expressing BGL1 were inoculated into YEP-D (20g/L) containing 5mg/mL X-glucoside. Cultures were incubated at 30°C with no shaking for 3 days. Cultures producing a green colour were deemed to be BGL1 expressing positive. The natural colour produced from the hydrolysis of X-glucoside is blue, however when strains are grown in YEP-D the colour of the media results in a green colour.

For quantitative analysis of BGL1 activity, yeast strains were grown in SC-S (20g/L) media in a 96-well plate without shaking at 30°C. After 48Hrs cells were counted using a haemocytometer and then separated from spent media via centrifugation at 14,000rpm for 5 minutes at 4°C. The culture supernatant (25μL) was added to 500μl of reaction buffer (47.5mM Sodium Acetate pH5.0 (Sigma-Aldrich), 1mM p-Nitrophenyl β-D-Glucopyranoside (Sigma-Aldrich) (p-NPG)) and incubated at 50°C. The reaction was stopped after 30 minutes by the addition of 500μL 50mM sodium carbonate (Fluka). The absorbance of the reaction mix was measured in a 96 flat bottom well plate at 405nm using an Ascent Multiscan spectrophotometer. A standard curve generated using various concentrations (0-300mU) of commercial *T.reesei* cellulase (*T.reesei* ATCC-26921, 6mU/μg, Sigma-Aldrich) using the same protocol as that described above allowed for the calculation of activity in terms commercial cellulase units (mU). The activity was either expressed solely as mU or units were standardised against the final cell number and expressed in terms of mU per $1x10^8$ cells (U^{BGL1}).

2.7.3 Endoglucanase activity

Endoglucanase activities in recombinant yeast strains were qualitatively examined using the soluble cellulosic substrate carboxymethyl cellulose sodium salt (CMC) (Fluka). Individual colonies of strains expressing endoglucanase were inoculated into YEP-D (20g/L) (200µL) and incubated at 30°C for 2 days. The cells were then pelleted by centrifugation at 14,000 rpm for 5 minutes at 4°C and the supernatant spotted (10µL) onto CMC agar plates (1g/L CMC, 10g/L agar). After 24Hrs incubation at 30°C the plates were stained using 1% (w/v) Congo Red (Fluka) solution (20mL per plate) and incubated at room temperature for 1Hr followed by 2 rounds of de-staining using 2M NaCl (20mL per de-stain). Yellow zones of hydrolysis indicated endoglucanase activity. Endoglucanase activity was quantified using the 2,4-dinitrosalicyclic acid (DNS) method (Miller et al., 1960). Yeast strains were grown in SC-S (20g/L) media in a 96-well plate at 30°C. After 48Hrs cells were counted using a haemocytometer and then separated from spent media via centrifugation at 14,000rpm for 5 minutes at 4°C. The spent media was mixed in a 1:1 ratio with a 1% CMC 50mM Sodium Acetate (pH5.0) solution and incubated at 50°C for 1Hr. The reaction was stopped by the addition of 3 volumes of DNS reagent (0.7M Potassium sodium tartrate Tetrahydrate (Fluka), 45mM DNS (Sigma-Aldrich), 4mM Sodium Sulfite (Sigma-Aldrich), 1% NaOH (Fluka), 80µL Tris-HCl (50mM pH8.0) buffered Phenol) and heated to 100°C for 5 minutes. The reaction was allowed to cool to room temperature and the absorbance was measured at 540nm using an Ascent Multiscan spectrophotometer. Endoglucanase activity was converted into mU of commercial cellulase using a standard curve (0-24mU) of commercial *T.reesei* cellulase (*T.reesei* ATCC-26921). The enzyme activity units were standardised against the final cell number and expressed in terms of mU per $1x10^8$ cells (U^{EG}).

2.7.4 Cellobiohydrolase activity

Cellobiohydrolase activity was quantified using an indirect assay of glucose release from PASC as described previously (Fitzpatrick et al., 2014). Briefly cellobiose released by the activity of CBHII is further hydrolysed to glucose by the action of added BGL1. Yeast strains CM-51 pRS42H, CM-51 PGKcbh2, CM-51 TEFcbh2 and CM-51 TEFbgl1 were individually cultured in SC-S (20g/L) at 30°C and 200rpm. After 48Hrs spent media was harvested and were mixed in different combinations in a 1:1 ratio and then added to PASC (100g/L) (2.5g/L theoretical glucose, see Section 2.1.3) sodium acetate (pH5.0) solution, and incubated at 50°C for 24Hrs. Glucose release from

reactions was measured at time 0Hr and 24Hr using the glucose assay kit (Sigma-Aldrich). Enzyme activity was defined as glucose released from PASC in terms of g/L.

2.7.5 PASC hydrolysis

Yeast strains were cultured in SC-S (20g/L) or YEP-D (20g/L) media at 30°C, 200rpm. After 48Hrs cells were separated from the media via centrifugation. Supernatants were mixed with PASC (100-1000g/L PASC, 2.5-25g/L theoretical glucose, see Section 2.1.3) and incubated at 50°C for 0-4 days. Glucose concentrations from reactions were determined using the glucose assay kit (Sigma-Aldrich). Enzyme activity was defined as glucose released from PASC in terms of g/L.

2.8 Ethanol quantification

Ethanol produced from fermentations was determined using the alcohol dehydrogenase (ADH) assay. Fermentation samples (10µL) were mixed with 200µL of ADH buffer (0.5M Tris (Fluka), 65mM succinic acid (Sigma-Aldrich), 5mM EDTA (Fluka), 25mM NAD, 16.5U/mL ADH (Fluka)) and incubated at 30°C for 8 minutes. The reduction of NAD+ by ADH was measured at 340nm in a 96 flat bottom well UV transparent plate using an Ascent Multiscan spectrophotometer. Samples were diluted in dH_2O to ensure that the values were within the absorbance range generated using ethanol standards (0-0.4g/L).

2.9 Glucose quantification

Glucose concentrations were determined using a glucose assay kit (Sigma-Aldrich). Samples (20µL) were mixed with 100µL of reaction buffer (1.5mM NAD+, 0.1mM ATP, 1U/mL hexokinase, 1U/mL glucose-6-phosphate dehydrogenase) and incubated at 30°C for 15 minutes. The absorbance at 340nm was then read using a Multiscan Ascent spectrophotometer. A standard curve was generated using the glucose standard (1mg/mL) supplied in the kit at concentrations ranging from 0-0.2g/L.

2.10 Xylose quantification

Xylose concentrations were determined using a xylose mutarotase assay kit (Megazyme, Dublin, Ireland). Samples (5µL) were mixed with 146µL of reaction buffer 1 (supplied by meazyme) and incubated at 30°C for 5 minutes and absorbance read at 340nm to determine background. To the reaction mix 2.5µL of reaction buffer 2 (xylose

mutoratase (XMR)) was then added and incubated at 30°C for 6 minutes followed by absorbance reading at 340nm. Values were compared to a standard curve generated using a xylose standard (0.25mg/mL) with concentrations ranging from 0-0.25g/L using the same protocol described above.

2.11 Protein quantification

The protein concentration of samples was determined using Bradford reagent (Sigma-Aldrich). The assay was carried in a standard 96-well plate, 250µL of Bradford reagent (Sigma-Aldrich) was added to 5µL of diluted samples. The plate was shaken for 5 seconds (900rpm) and the absorbance measured at 610nm using Multiscan Ascent spectrophotometer. A standard curve was generated using Bovine Serum Albumin (BSA) (Sigma-Aldrich) (0-2mg/mL).

2.12 Green fluorescent protein (GFP) analysis

2.12.1 GFP Microscopy

Yeast strains expressing GFP fusion genes were cultured in YEP-D (20g/L) at 30°C for 24Hrs. Samples were taken and imaged using Nikon eclipse E400 and Nikon digital camera DXM1200, using light microscopy and an fluorescein isothiocyanate (FITC) filter (excitation 490nm and emission 525nm). Images were taken using a 40x objective magnification and the program ACT-1.

2.12.3 Quantification of GFP florescence

Strains expressing GFP fusion genes were grown in YEP-D (20g/L) at 30°C for 24Hrs at 150rpm. Cells were then counted using a haemocytometer and a total of $7x10^8$ cells were harvested by centrifugation. Due to the high level of auto fluorescence background generated from YEP-D medium cells were washed 3 times using dH_2O. After the final wash the cell pellet was re-suspended in dH_2O (10mL). Washed cells (100µL) were added to a 96 flat bottom black well plate and fluorescence was measured using a Synergy H1 Hybrid reader (Biotek). The excitation and emission wavelengths were set at 480nm and 510nm respectively.

2.13 Theoretical yield calculations

Theoretical ethanol yields were expressed as a percentage of the maximum possible ethanol production from xylose and glucose levels at time zero. The calculation of

maximum theoretical ethanol from glucose and xylose was calculated using these equations:

Glucose

$$\text{Maximum ethanol (g/L)} = \left(\left(\frac{\text{Glucose day 0 (g/L)}}{\text{MW (Glucose)}} \right) \times 2 \right) \times \text{MW (ethanol)}$$

Xylose

$$\text{Maximum ethanol (g/L)} = \left(\left(\frac{\text{Xylose day 0 (g/L)}}{\text{MW (Xylose)}} \right) \times 1.6 \right) \times \text{MW (ethanol)}$$

MW ethanol - 46

MW glucose - 180

MW xylose - 150

The percent of theoretical yield was then calculated by dividing the actual ethanol level produced by the combined maximum ethanol levels from glucose and xylose and multiplied by 100.

The number of moles of ethanol produced from each sugar was calculated by using these equations for glucose and xylose fermentation.

Glucose

$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$

Xylose

$3C_5H_{10}O_5 \rightarrow 5CH_3CH_2OH + 5CO_2$

Chapter 3

Optimisation of cellulase expression for simultaneous cellulose hydrolysis and fermentation

3.1 Introduction

Current 2nd generation bioethanol production is dependent on the addition of commercial cellulase enzymes sourced from various cellulytic organisms. One such organism is the mesophilic fungi *T. reesei*. Analysis of the *T. reesei* genome identified at least 9 putative cellulase encoding genes, consisting of two cellobiohydrolase (CBHI, CBHII), five endoglucanase (EGI, EGII, EGIII, EGIV, EGV) and two β-glucosidase enzymes (BGLI, BGLII) (Martinez et al., 2008). Cellulase enzymes represent around 31% of total secreted protein by *T. reesei* (Adav et al., 2012). The cellobiohydrolase enzymes are by far the most abundant, representing around 70-80% of cellulase enzymes secreted (Adav et al., 2013), with CBHI being the most abundant (Herpoel-Gimbert et al., 2008).

A strategy of heterologous expression of cellulase enzymes in natural ethanologenic *Saccharomyces sp.* has been adopted to allow for simultaneous cellulose hydrolysis and fermentation avoiding the expense of buying commercial enzymes (Section 1.5.2). Previously, using a cDNA approach cellulase genes (*cbh2, egl1* and *bgl1*) were amplified and inserted individually via homologous recombination into the low-copy number plasmid pGREG586. The expression and production of the enzymes was examined in two different host strains, *S. cerevisiae* S150 and the industrial lager strain *S. pastorianus* C10-51 (James et al., 2008). Low levels of ethanol (mg/L) were produced from purified cellulose fermentations, likely as a result of the low expression by pGREG586 plasmids (J Fitzpatrick, PhD thesis, 2011). Ethanol levels from cellulose fermentations using the low copy number plasmid were significantly less than levels reported in the literature by engineered *S. cerevisiae* (Table 1.1), although a distinct advantage of expression in *S. pastorianus* and the limitation of cellulase activity at 30°C were identified.

To achieve efficient cellulose hydrolysis and fermentation a high level of cellulase activity is required, however at optimal temperatures for fermentation (15°C-30°C) cellulase activity is greatly reduced. This together with the chicken and egg conundrum (Section 1.5.2) limits current ethanol production from recombinant ethanologenic organisms.

The aim of this chapter was to improve cellulase expression for the optimisation of ethanol production using cellulose as a sole carbohydrate source. To improve cellulase activity expression of the cellulase genes from high copy number plasmid was

compared to that from low copy number plasmid in various *Saccharomyces sp.* hosts. Promoter swaps and the co-expression of enzymes were examined for optimisation of cellulase activity. Through enzyme quantification, optimised strains with increased activity can be identified and then subsequently used for cellulose hydrolysis and fermentation.

3.2 Quantification of high and low copy number plasmid activity in different *Saccharomyces sp.*

To date, the choice of host for heterologous expression of cellulases has been *S. cerevisiae*. Experiments had demonstrated that significantly higher levels of cellulase activity were observed in *S. pastorianus* compared to *S. cerevisiae* (Fitzpatrick et al., 2014). To confirm *S. pastorianus* as a superior host, BGL1 activity from various *Saccharomyces sp.* strains was examined using both high and low copy number plasmids. The environmental stress associated with fermentations requires the use of stress tolerant strains. The strains CM-51, C10 and C10-51 are stress tolerant derivatives of the lager strain *S. pastorianus* CMBS-33 (James et al., 2008), while the strains K103 and R130 are industrial *S. cerevisiae* strains obtained from Diageo, St James Gate, Dublin.

The *bgl1* gene from *T. reesei* was initially cloned into the low copy number plasmid pGREG586 using a cDNA approach (Fitzpatrick et al., 2014). The PGKbgl1 cassette in pGREG586 was then inserted into the high copy number plasmid pRS42H as described in section 2.4. *Saccharomyces sp.* strains (Table 3.1) were transformed individually with pRS42H PGKbgl1 and pGREG586 PGKbgl1. Strains were grown in SC-S media, after 48Hrs of growth the supernatants were harvested and BGL1 activity quantified as described in section 2.7.2.

Comparison of high and low copy number plasmids indicated enzyme expression was dependent on gene copy number, as higher activity was produced by high copy number plasmids. However no difference was seen between the high and low copy number plasmid in *S. cerevisiae* s150. The highest BGL1 activity was observed using the high copy number plasmid in *S. pastorianus* strains, which displayed approximately 8-fold greater activity than that of any of the *S. cerevisiae* strains (Table 3.1). Based on this information the strain that was selected as the ideal host for the expression of cellulases in this study was the strain *S. pastorianus* CM-51.

Table 3.1 BGL1 activity from culture supernatants of various industrial and lab *Saccharomyces sp* strains. Strains were grown in SC-S (20g/L) media at 30°C. After 48Hrs supernatant was harvested and BGL1 activity quantified. Activity (mU) was standardised against final cell number and expressed in terms mU per $1x10^8$ cells (U^{BGL1}). Values are the average of two independent experiments, with triplicate readings in both experiments, ± represents the standard deviation between the average of two independent experiments. Background activity from empty vector (pRS42H) containing control strains was subtracted from activity from PGKbgl1 containing strains.

Strain	Low copy number (U^{BGL1})	High copy number (U^{BGL1})
S. cerevisiae s150	5.16±0.62	3.96±0.06
S. cerevisiae R130	-	7.17±3.02
S. cerevisiae K103	-	8.32±1.53
S. pastorianus CMBS-33	3.52±1.23	38.40±1.22
S. pastorianus CM-51	3.68±0.40	50.99±0.73
S. pastorianus C10	5.23±2.32	71.89±11.91
S. pastorianus C10-51	4.76±2.59	53.91±16.75

3.3 Optimisation of BGL1 activity through promoter swaps

To optimise BGL1 activity, the promoter used to drive expression was varied. The standard promoter used throughout this study was the *S. cerevisiae* promoter PGK. This was substituted by the *S. cerevisiae* TEF1 or the *S. cerevisiae* HXT7 promoter using the promoter swap cloning strategy (Section 2.4.3). Both PGK and TEF1 are constitutive promoters, allowing for continual transcription of their associated genes, while HXT7 is a glucose-regulated promoter, allowing for increased transcription during glucose starvation conditions. The TEF1 promoter was amplified in a single fragment from pGREG505 TEF1 (gifted by Karreman RJ & Morrissey JP, UCC, Cork) using primers Psi_TEF_F and TEF_R. The HXT7 promoter was amplified in a single fragment from *S.cerevisiae* 288c genomic DNA using primers Psi_HXT7_F and HXT7_R (Table 2.5). The DNA fragments were individually inserted into pRS42H PGKbgl1 digested with PsiI and Spe1 to remove the PGK promoter, via *in vivo* homologous recombination in *S. pastorianus* CM-51 (Fig 2.7).

Yeast strains CM-51 PGKbgl1, CM-51 TEFbgl1, CM-51 HXT7bgl1 and CM-51 pRS42H were grown in SC-S media for 48 Hrs. The supernatants were collected and BGL1 activity quantified as described in Material and Methods (Section 2.7.2). The highest activity was obtained using the TEF1 promoter, which showed a 1.7-fold increase in activity compared to the PGK promoter cassette (Fig.3.1). The HXT7 promoter produced similar levels of BGL1 activity as the PGK promoter. Although beneficial, the limited increase of BGL1 activity via promoter swaps is unlikely to completely overcome the road blocks associated with reduced enzyme activity at fermentation temperatures.

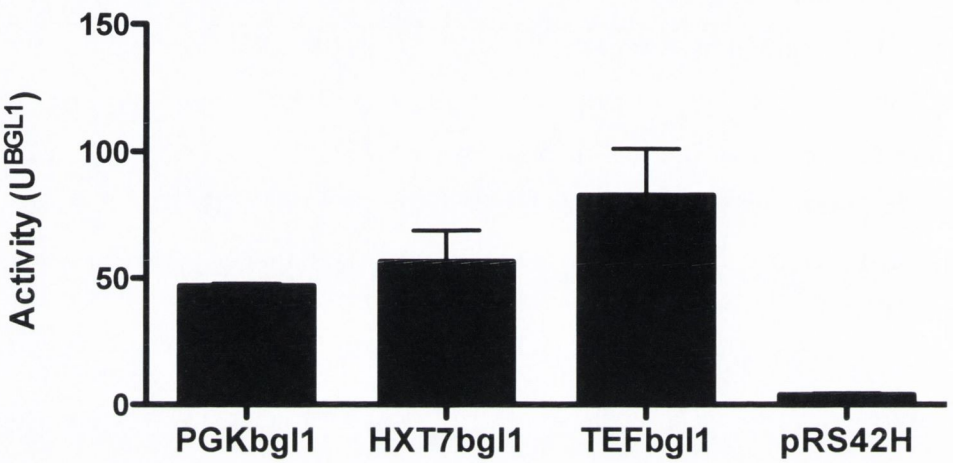


Figure 3.1 BGL1 activity from *S. pastorianus* CM-51 expressing *bgl1* under the control of PGK, HXT7 or TEF1 promoter. Activity was quantified from culture supernatant after 48Hrs growth at 30°C in SC-S (20g/L). Activity (mU) was standardised against final cell number and expressed in terms mU per $1x10^8$ cells (U^{BGL1}). PGKbgl1: CM-51 PGKbgl1, HXT7: CM-51 HXT7bgl1, TEFbgl1: CM-51 TEFbgl1, pRS42H: CM-51 pRS42H. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the average of two independent experiments.

3.4 Quantification of recombinant CBHII activity

CBHII activity was quantified using an indirect assay (glucose assay) as outlined in section 2.7.3 (Fitzpatrick et al., 2014). The *cbh2* gene was inserted into the high copy number plasmid using the single gene insertion strategy (section 2.4.1). Briefly, the *cbh2* gene was amplified in a single fragment from the previously generated plasmid pGREG586 PGKcbh2 using the primers cbh2_Rec1_F and cbh2_Rec2_R. The single fragment was inserted into SalI digested pRS42H PGKbgl1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51 (Fig 2.3). The standard PGK promoter was also swapped for the TEF1 promoter using the promoter swap strategy (Section 2.4.3). The TEF1 promoter was amplified in a single fragment from pGREG505 TEF1 using the primers Psi_TEF_F and TEF_R. The single fragment was inserted into SpeI and PsiI digested pRS42H PGKcbh2 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. Individual strains (CM-51 PGKcbh2, CM-51 TEFcbh2, CM-51 TEFbgl1 and CM-51 pRS42H) were grown in SC-S media for 48Hrs. The culture supernatants were harvested and then mixed in different combinations in a 1:1 ratio. These cellulase mixes were then added to PASC and incubated at 50°C for 24Hrs. Glucose release was quantified using the glucose assay kit. Approximately 1.7 times more glucose was released from PASC using TEFcbh2 construct compared to PGKcbh2 (Fig 3.2), mirroring results shown for BGL1 activity. Although beneficial, this again demonstrates the limitations of promoter exchange to generate significant increases in enzyme activity. A small amount of glucose was released using BGL1 enzyme alone suggesting that either cellobiose was released during the acid treatment of cellulose or BGL1 is able to act on amorphous cellulose to release glucose.

3.5 Quantification of recombinant endoglucanase activity in single and co-expressing cassettes

The two most studied endoglucanase enzymes from *T. reesei* are EGI and EGII. The *egl1* and *egl2* genes from *T. reesei* were cloned into the high copy number vector pRS42H using the single gene insertion strategy (section 2.4.1). The *egl1* gene was amplified in a single fragment from the previously generated plasmid pGREG586 PGKegl1 using primers egl1_Rec1_F and egl1_Rec2_R.

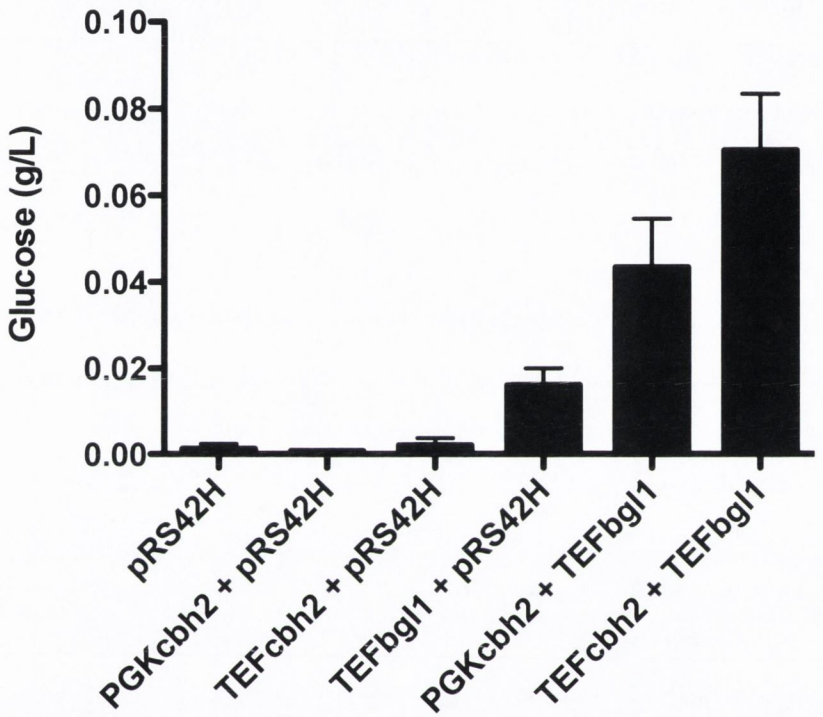


Figure 3.2 Cellobiohydrolase activity from *S. pastorianus* CM-51 strains expressing *cbh2* under the control of the PGK or TEF1 promoter. Enzyme activity was quantified from culture supernatant after 48Hrs growth at 30°C in SC-S (20g/L) media. Activity was quantified indirectly through measuring glucose release using mixtures (1:1 ratio) of CM-51 TEFcbh2 or CM-51 PGKcbh2 with either CM-51 TEFbgl1 or CM-51 pRS42H supernatant. Cellulase cocktails were mixed with PASC (100g/L) (2.5g/L theoretical glucose, see Section 2.1.3) and incubated at 50°C for 24Hrs. Activity is defined as glucose release in g/L. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the average of two independent experiments.

The DNA fragment was inserted into SalI digested pRS42H PGKbgl1 via *in vivo* homologous recombination. Sequencing of this plasmid identified a two nucleotide deletion within the RecI site (Fig 3.3). The original error was found within the forward primer egl1_RecI_F, which was shown to be missing 2 nucleotides of the RecI site. The gene was re-inserted using the same strategy, however an alternative forward primer, egl1_new_RecI_F was used for DNA amplification. The two plasmids generated were given the names PGKegl1 1.1 (2 nucleotide deletion in RecI site) and PGKegl1 1.2.

To compare the endoglucanase activity of the two constructs, PGKegl1 1.1 and PGKegl1 1.2 strains were individually grown in SC-S media for 48Hrs. The supernatants were harvested and endoglucanase activity quantified using the soluble cellulose substrate CMC (section 2.7.4). The activity of CM-51 PGKegl1 1.1 was 2-fold greater than that of the CM-51 PGKegl1 1.2 construct (Fig 3.4), showing the 2 base pair deletion is beneficial towards enzyme activity. It was decided that CM-51 PGKegl1 1.1 would be taken forward due to the increased activity shown.

To optimise endoglucanase activity a second endoglucanase enzyme EGII was individually cloned and co-expressed along with the PGKegl 1.1 cassette. The *egl2* gene was individually cloned using the single gene insertion strategy (Section 2.4.1). The gene was amplified in 2 fragments from *T. reesei* genomic DNA using primers egl2_RecI_F and egl2_MidR for fragment 1 and egl2_MidF and egl2_Rec2_R for fragment 2. The two fragments were inserted into SalI digested pRS42H PGKbgl1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. For co-expression (Section 2.4.2) the PGKegl2 cassette (promoter, gene and terminator) was amplified in two fragments from pRS42H PGKegl2 plasmid using primers Psi_PGK_F and egl2_MidR for fragment 1 and egl2_MidF and Psi_Cyc_R for fragment 2. The two fragments were inserted into PsiI digested pRS42H PGKegl1 1.1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51.

Strains (CM-51 PGKegl1 1.1, CM-51 PGKegl2, CM-51 PGKegl2- PGKegl1 1.1 and CM-51 pRS42H) were grown in SC-S media for 48Hrs and the supernatants assayed for endoglucanase activity using the CMC/DNS assay (section 2.7.4) (Fig 3.5). Activity of EGII was almost two fold greater than EGI 1.1. The co-expression of both *egl1*1.1 and *egl2* genes further increased endoglucanase activity.

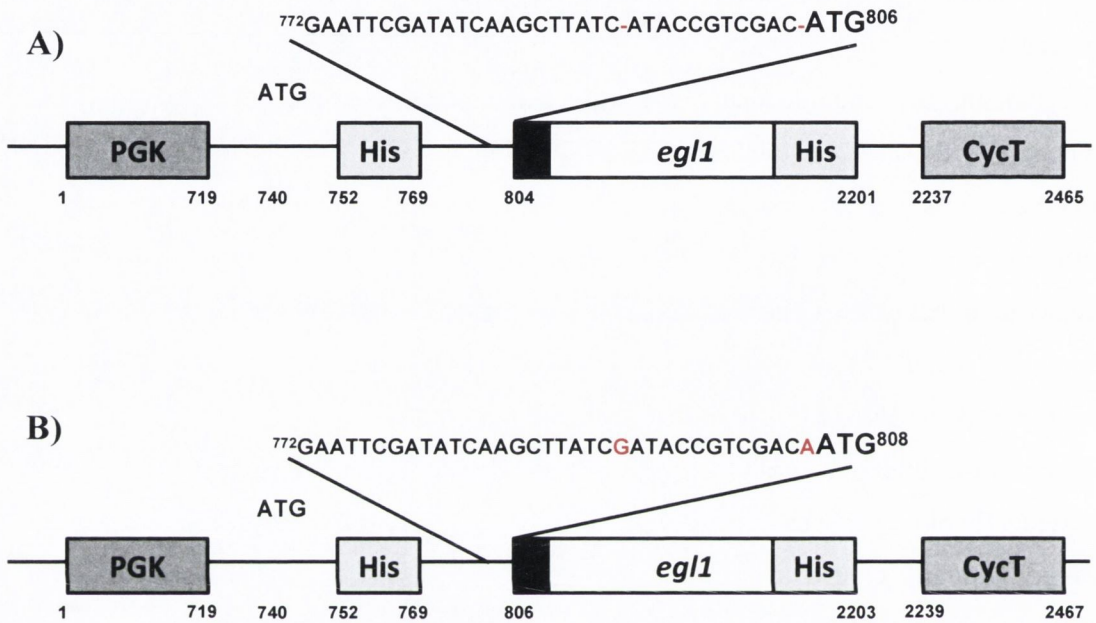


Figure 3.3 Diagram of PGKegl1 1.1 and PGKegl1 1.2 expression cassettes. (A) Schematic of the PGKegl1 1.1 cassette with the location of the two deleted nucleotides (-) highlighted within the Rec1 sequence. (B) PGKegl1 1.2 cassette showing the correct Rec1 sequence, with the two nucleotides added (red). Black box (■): native *egl1* secretory signal. PGK: PGK promoter. His: 6xHistidine tag. CycT: Cyc terminator.

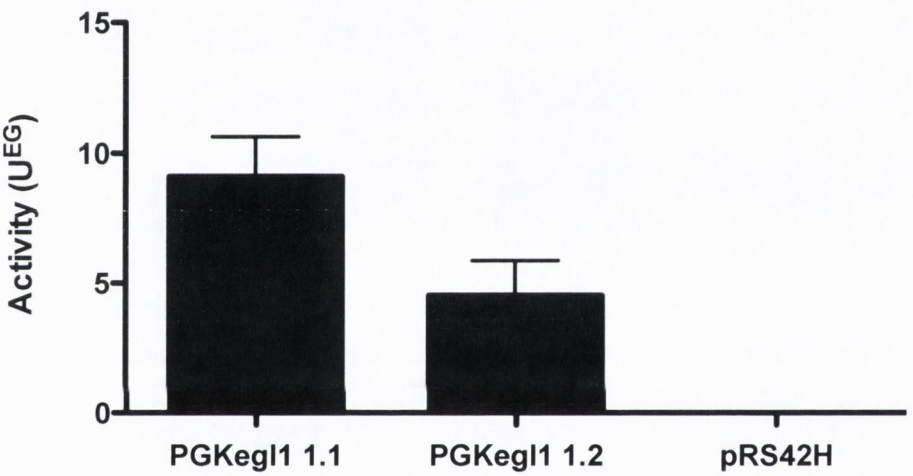


Figure 3.4 Enzyme activity of EGI constructs. Enzyme activity was quantified from culture supernatants after 48Hrs growth at 30°C in SC-S (20g/L) media using the CMC/DNS assay. Activity (mU) was standardised against final cell number and expressed in terms mU per $1x10^8$ cells (U^{EG}). PGKegl1 1.1 contains a 2 nucleotide deletion in the Rec1 sequence, while PGKegl 1.2 represents the wildtype sequence cassette. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the average of two independent experiments.

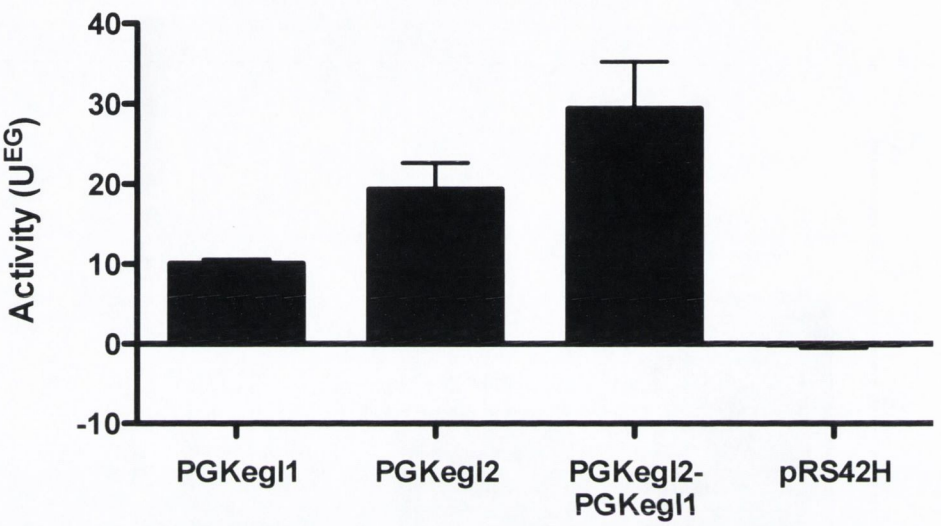


Figure 3.5 Endoglucanase activity in single EGI and EGII and co-expression constructs. Enzyme activity was quantified from culture supernatants after 48Hrs growth at 30°C in SC-S (20g/L) media using the CMC/DNS assay. Activity (mU) was standardised against final cell number and expressed in terms mU per $1x10^8$ cells (U^{EG}). Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the average of two independent experiments.

The co-expression produced an additive effect (EGII activity + EGI activity = EGII-EGI activity) while no clear synergistic benefit was seen from the co-expression.

3.6 Reconstitution of *T. reesei* cellulase system in *S. pastorianus*

Having expressed and quantified individual cellulase enzymes in the high copy number plasmid, the combined effect of all three cellulases on degrading cellulose was next examined. A co-culture approach was pursued, in which yeasts expressing each cellulase gene individually were cultured together to reconstitute the full cellulase machinery. The use of individual expressed genes allows for easy manipulation of enzyme combinations and ratios. Yeast strains (CM-51 PGKbgl1, CM-51 PGKcbh2, CM-51 PGKegl1 1.1 and CM-51 pRS42H) were grown individually in SC-S media for 48 Hrs. The cultures were harvested and the supernatants were tested individually, in pair wise combinations or combined all together in a 1:1:1 ratio for PASC hydrolysis. The cellulase cocktails were mixed with PASC and incubated at 50°C. The level of glucose released was measured using the glucose assay kit after 48 Hrs (Fig 3.6). Glucose release from individual cellulase enzymes was very low, however CM-51 PGKbgl1 and CM-51 PGKegl1 1.1 individual supernatants released some glucose, while no glucose release was detected using CM-51 PGKcbh2 supernatant. Low levels of glucose were released from CM-51 PGKcbh2 + CM-51 PGKegl1 1.1 supernatants. This was expected due to the lack of a β-glucosidase enzyme. Levels of glucose were increased using CM-51 PGKcbh2 + CM-51 PGKbgl1 supernatant, however a 2-fold increase was obtained using CM-51 PGKegl1 1.1 + CM-51 PGKbgl1 supernatants. When all three enzymes were combined glucose release peaked at 0.18g/L, representing a 6-fold increase compared to the sum of individual enzymes (0.0306g/L). The importance of expressing all three classes was also shown, as cellulose hydrolysis from the maximum double cellulase mix (CM-51 PGKbgl1 + CM-51 PGKegl1 1.1) was half of that achieved when all three enzymes were present (CM-51 PGKbgl1 + CM-51 PGKcbh2 + CM-51 PGKegl1 1.1).

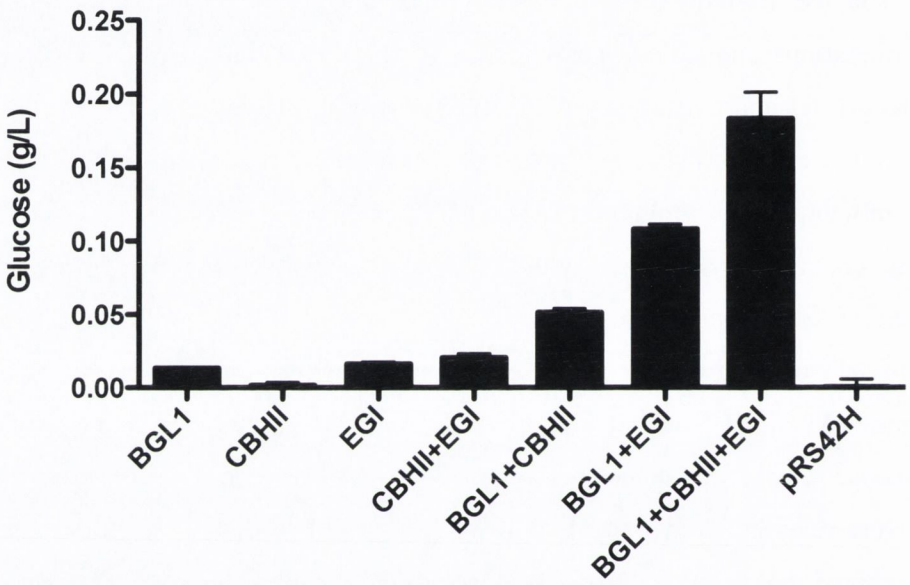


Figure 3.6 Synergistic recombinant enzyme hydrolysis of PASC. Strains CM-51 PGKbgl1, CM-51 PGKcbh2, CM-51 PGKegl1 1.1 and CM-51 pRS42H were cultured in SC-S (20g/L) media at 30°C for 48Hrs. Supernatants were incubated with PASC (100g/L) (2.5g/L theoretical glucose, see Section 2.1.3) either individually, pairwise or in triplicate, individual enzyme concentrations within single, double or triple cellulase mix were maintained equal throughout by the addition of CM-51 pRS42H supernatant. Enzyme activity was quantified after 48Hrs at 50°C and activity is expressed in terms of glucose release in g/L. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the average of two independent experiments.

3.7 Quantification of Cel1.0 and Cel2.0 activity

Utilising the data from promoter swap and co-expression studies two distinct groups of cellulase expressing strains were generated. The first generation cellulase system in which genes were expressed from the PGK promoter was designated Cel1.0 (CM-51 PGKbgl1, CM-51 PGKcbh2 and CM-51 PGKegl1 1.1), while the optimised system was designated Cel2.0 (CM-51 TEFbgl1, CM-51 TEFcbh2 and CM-51 PGKegl2-PGKegl1). The overall effect of enzyme optimisation on cellulose degradation was examined. Yeast strains Cel1.0 and Cel2.0 and CM-51 pRS42H were grown individually in YEP-D media for 48 Hrs. Cel1.0 and Cel2.0 individual supernatants were harvested and pooled in a 1:1:1 ratio to generate Cel1.0 and Cel2.0 cellulase cocktails. The cellulase cocktail mixes were mixed with PASC and incubated at 50°C, and samples were taken at intervals for glucose quantification (Fig 3.7). The final concentration of glucose release from Cel2.0 strains (6.29g/L) was twice the level released using Cel1.0 strains (2.82g/L), showing that the optimisation through promoter swaps and co-expression was beneficial towards cellulose breakdown. Increased hydrolysis was achieved by increasing substrate concentration and extending the length of incubation time (compared to Fig 3.6). Although glucose release was increased, the yield of glucose from cellulose using Cel2.0 only reached 25.16% of the theoretical maximum yield, with the rate of glucose release being $0.98 g.L^{-1}.day^{-1}$.

3.8 Fermentation of PASC using Cel2.0

The data presented thus far, indicates that optimum cellulose hydrolysis was achieved using the Cel2.0 strains. The ability of the strains to degrade cellulose and convert the released sugars into ethanol was next examined. To overcome the 'chicken and egg' conundrum associated with cellulose hydrolysis and fermentation, a pre-hydrolysis stage using previously generated recombinant cellulase enzymes was required. Cel 2.0 strains were individually grown in YEP-D media, cells were harvested and combined (1:1:1 ratio) and inoculated into fresh YEP-D media to a final cell density of $1x10^8$cells/mL. As a control CM-51 pRS42H strain was inoculated into YEP-D media to a final cell density of $1x10^8$cells/mL.

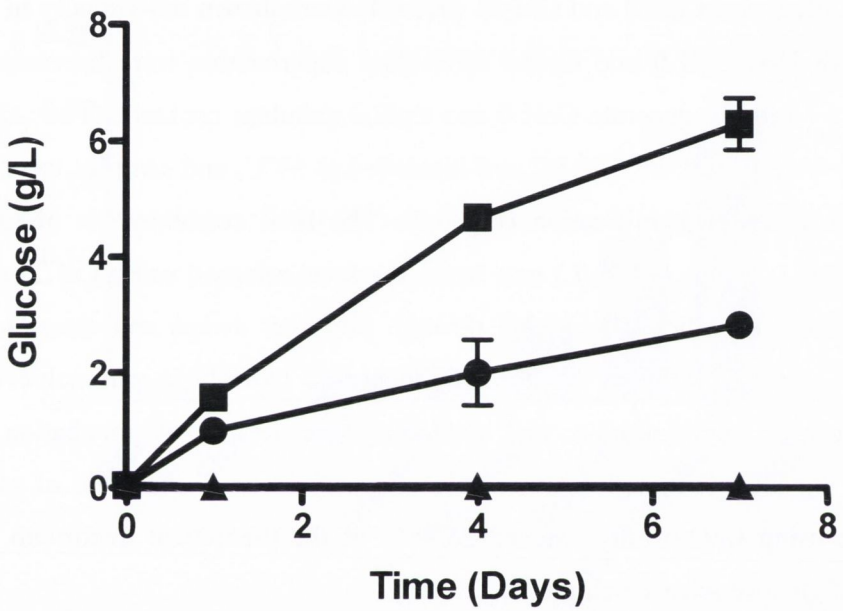


Figure 3.7 Comparison of optimised cellulase expression cassettes on PASC hydrolysis. PASC hydrolysis by recombinant enzymes Cel1.0 (●), Cel2.0 (■) and empty vector containing control pRS42H (▲). Cel 1.0 strains (CM-51 PGKbgl1, CM-51 PGKcbh2, CM-51 PGKegl1 1.1) and Cel 2.0 strains (CM-51 TEFbgl1, CM-51 TEFcbh2, CM-51 PGKegl1-PGKegl2) were grown individually in YEP-D (20g/L) media at 30°C for 48Hrs, supernatants were harvested and combined in a 1:1:1 ratio then incubated with PASC (1000g/L) (25g/L theoretical glucose, see Section 2.1.3) at 50°C. The empty vector control CM-51 pRS42H was grown in YEP-D (20g/L) media, after 48Hrs the supernatant was harvested and mixed with PASC (25g/L) and incubated at 50°C. Enzyme activity is defined as glucose release from PASC in g/L. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the average of two independent experiments.

Cultures were grown for 48Hrs to generate recombinant cellulase enzymes used for the pre-hydrolysis step. Supernatants were harvested and mixed with PASC and incubated at 50°C for 24Hrs. After the pre-hydrolysis step PASC-supernatant mixtures were supplemented with fresh YEP and corresponding PASC-supernatant mixtures were inoculated with fresh Cel2.0 cells or CM-51 pRS42H to a final cell density of $1x10^8$cells/mL. Fermentations were incubated at 30°C for up to 14 days and samples were taken at intervals for ethanol quantification. Background ethanol levels from CM-51 pRS42H fermentations were subtracted from Cel2.0 fermentations (Fig 3.8). Cel2.0 co-culture produced peak ethanol levels of 2.16g/L at day 14. Although the ability of Cel2.0 co-culture to hydrolysis and ferment cellulose was shown, it is clear there is room for improvement as ethanol levels produced only represented 17.11% of the theoretical ethanol yield from PASC.

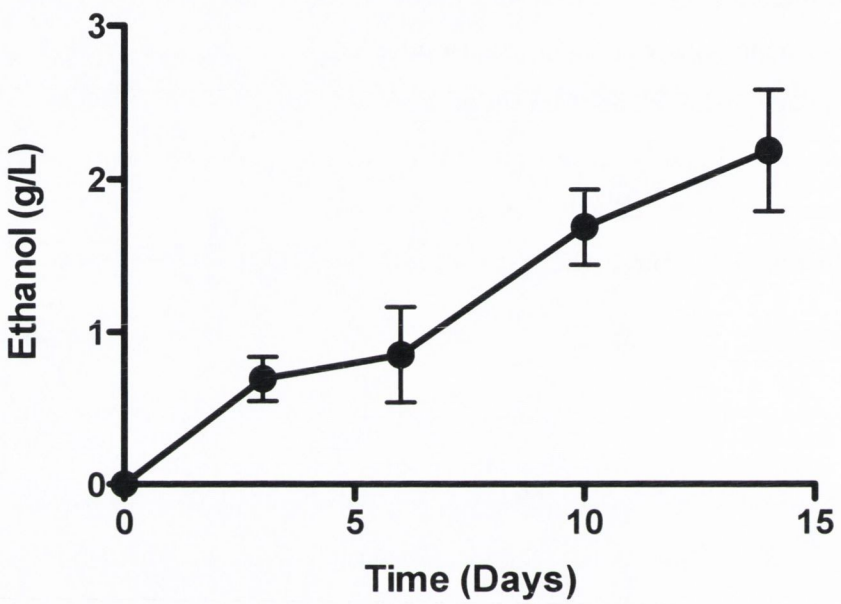


Figure 3.8 Fermentation of PASC using CM-51 Cel2.0 strains. Cel 2.0 strains (CM-51 TEFbgl1, CM-51 TEFcbh2 and CM-51 PGKegl1-PGKegl2) were grown in a high cell density ($1x10^8$cells/mL) co-culture in a 1:1:1 ratio in YEP-D (20g/L) media for 48Hrs. Empty vector strain CM-51 pRS42H was grown in a high cell density ($1x10^8$cells/mL) culture in YEP-D (20g/L) media for 48Hrs. The supernatants were harvested and then incubated with PASC (1000g/L) (25g/L theoretical glucose, see Section 2.1.3) at 50°C for 24Hrs. PASC and supernatant mixtures were then supplemented with fresh YEP (30g/L) and fresh Cel2.0 (1:1:1 ratio of each strain) or CM-51 pRS42H cells were inoculated to a final cell density of $1x10^8$cells/mL. Ethanol levels were determined using the ADH assay, and values are expressed as ethanol produced in g/L. Ethanol levels from CM-51 pRS42H were subtracted from Cel2.0 ethanol levels to remove background levels. Values are the average of four independent experiments, with duplicate readings in all four experiments, error bars represent the standard error between the average of four independent experiments.

Chapter 4

Engineering of *Saccharomyces sp.* for xylose fermentation

4.1 Introduction

The pentose sugar xylose is a major component of hemicellulose. Although various xylose utilising *Saccharomyces sp.* have been identified (Schwartz et al., 2012; Wenger et al., 2010), poor growth and alcohol production have led to the use of recombinant DNA technology to increase xylose utilisation efficiency.

In this study xylose utilising genes (XR-XDH pathway) were mined form the mesophilic fungi *T. reesei*. Both xylose reductase (*xyl1*) and xylitol dehydrogenase genes (*xdh1*) are found in single copies within the *T.reesei* genome. The *xyl1* gene is essential for xylose metabolism (Seiboth et al., 2007), however the presence of a second xylitol dehydrogenase like enzyme, known as L-arabinatol-4-dehydrogenase (LAD1) demonstrates some redundancy in the metabolism of xylitol to xylulose in *T. reesei* (Seiboth et al., 2003).

The redox imbalances caused by the co-factor requirements of the XR-XDH pathway remain a roadblock in efficient xylose metabolism in *Saccharomyces sp.* (Jeffries and Jin, 2004). It is for this reason several groups have examined the use of the XI pathway. This pathway directly converts xylose to xylulose in a single step without any co-factor requirements.

Both XR/XDH and XI pathways convert xylose into xylulose, which is then phosphorylated by xylulose kinase (Xks) to Xylulose-5-phosphate. While the *S. cerevisiae* genome encodes a single *XKS1* gene the activity of the enzyme is insufficient, limiting xylose fermentation efficiency. The reduced efficiency has led to the overexpression of native *Saccharomyces sp.* genes such as *XKS1* and others genes involved in the PPP to improve ethanol production.

In this chapter the *T. reesei xyl1* and *xdh1* genes were expressed in various *Saccharomyces sp.* Enzyme activities were quantified with the aim of identifying an ideal host for expression. A comparison of the two xylose utilising pathways had previously identified that higher ethanol levels were produced using the XR/XDH pathway (Karhumaa et al., 2007b). To confirm this the XR/XDH and XI pathways (*Piromyces sp. E2*) were compared in *S. pastorianus*, along with the effect of the overexpression of the native *XKS1* gene for the optimisation of xylose fermentation.

4.2 Quantification of XR and XDH co-factor specificity

The *xyl1 and xdh1* genes were individually inserted into the high copy number plasmid (pRS42H) using the single gene insertion strategy (Section 2.4.1). Briefly, the *xyl1* and *xdh1* genes were amplified from pGREG505 PGKxr and pGREG505 PGKxdh in single fragments using the primers xr_Rec1_F, xr_Rec2_R and xdh_Rec1_F, xdh_Rec2_R respectively. The single fragments were inserted into Sal1 digested pRS42H PGKbgl1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51.

To determine if the *T. reesei* XR and XDH enzymes display the same specific co-factor requirements as previously cloned genes from other species, the XR and XDH activity was quantified using different co-factors as outlined in section 2.7.1

Strains (CM-51 PGKxdh, CM-51 PGKxr and CM-51 pRS42H) were grown in YEP-D media for 24Hrs. Cells were harvested and enzyme activities were quantified from cell lysate as described in Materials and Methods (Section 2.7.1). The cofactor specificity of both enzymes was as previously identified with XR and XDH demonstrating high specificity for NADPH and NAD+ respectively (Fig 4.1A/B).

4.3 Quantification of XR and XDH activity in various *Saccharomyces sp.*

To identify an ideal host for expression, the *xyl1* and *xdh1* genes were co-expressed in different *Saccharomyces sp.* and activities quantified. The co-expression of the PGKxr and PGKxdh cassettes was carried out using the second gene insertion strategy (Section 2.4.2). Briefly, the PGKxr cassette was amplified from pRS42H PGKxr in 2 fragments. Fragment 1 was amplified using primers Psi_PGK_F and xr_MidR and fragment 2 was amplified using primers xr_MidF and Psi_Cyc_R. The two fragments were inserted into Psi1 digested pRS42H PGKxdh plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51.

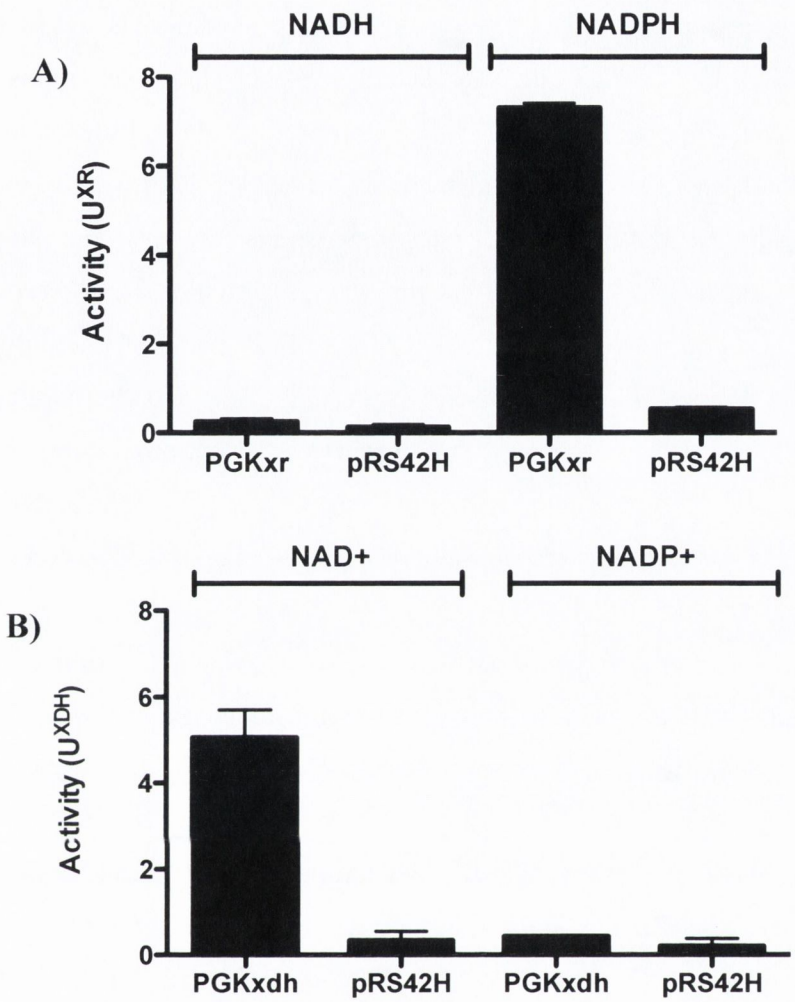


Figure 4.1 Enzyme activities and co-factor preference of XR and XDH. XR (A) and XDH (B) activity using NADPH/NADH and NAD+/NADP+ as co-factors respectively. Strains (CM-51 PGKxr, CM-51 PGKxdh and CM-51 pRS42H) were cultured in YEP-D (20g/L) media at 30°C for 24Hrs, cells were harvested, washed and then enzyme activity was quantified using cell lysate. XR activity is defined as NADPH/NADH (μM) oxidised per minute, XDH enzyme activity is defined as NAD^+/$NADP^+$ (μM) reduced per minute, both were standardised against protein level (μg) to give crude specific activity (U^{XR}, U^{XDH}). Values are the average of two independent experiments with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments.

The plasmid pRS42H PGKxr-PGKxdh was transformed into various *Saccharomyces sp.* strains as outlined in Table 4.1. Strains were cultured for 24Hrs in YEP-D media. Enzyme activity was quantified from cell lysate as described in Materials and Methods (Section 2.7.1) (Table 4.1). Both XR and XDH activity was detected in all strains apart from the *S. cerevisiae* lab strain BY4741, which appeared to show little or no XR activity. *S. pastorianus* CM-51 displayed substantially higher levels of activity of both XDH and XR compared to all other *Saccharomyces sp.* strains (Fig 1A). The ploidy of *S. cerevisiae* strains appears to have an affect on enzyme activity. The *S. cerevisiae* tetraploid strain, *S. cerevisiae* 4n produced higher levels of XR and XDH activity than that observed in the diploid industrial strain *S. cerevisiae* R130 and the haploid lab strain *S. cerevisiae* BY4741. *S. pastorianus* is a hybrid yeast containing both genomes from *S. cerevisiae* and *S. eubayanus* (Bond, 2009; Libkind et al., 2011). The activities in the parental strain *S. eubayanus* were substantially lower than that observed in *S. pastorianus*.

To determine why *S. pastorianus* produces much higher activity a C-terminal Green Fluorescence Protein (GFP) tagged XDH protein was generated and transformed into *S. pastorianus* strain CM-51 and *S. cerevisiae* strains BY4741 and R130. The *xdh1*-GFP fusion construct was generated from 2 fragments using the single gene insertion strategy (Section 2.4.1). The *xdh1* gene (fragment 1) was amplified from pRS42H PGKxdh plasmid using primers, xdh_Rec1_F and a modified reverse primer, xdh_R, to remove the native stop codon of the *xdh1* gene. The GFP gene was amplified from pGREG599 (Jansen et al., 2005) using the primers GFP_F and GFP_ R. The two fragments were inserted into Sal1 digested pRS42H PGKbgl1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. GFP and enzyme activity was examined using fluorescence microscopy and XDH activity assay respectively as outlined in sections 2.12 and 2.7.1 in Materials and Methods.

Table 4.1 Enzyme activities of XR and XDH in different *Saccharomyces sp.* Strains containing PGKxr-PGKxdh or pRS42H plasmid were cultured in YEP-D (20g/L) media at 30°C for 24Hrs, cells were harvested, washed and then enzyme activity was quantified using cell lysate. XR enzyme activity is defined as NADPH (μM) oxidised per minute, this was then standardised against protein level (μg) to give crude specific activity (U^{XR}). XDH enzyme activity is defined as NAD+ (μM) reduced per minute, this was then standardised against protein level (μg) to give crude specific activity (U^{XDH}). The activity of empty vector containing (pRS42H) strain was subtracted from PGKxr-PGKxdh to remove background activity. Values are the average of two independent experiments with duplicate readings in both experiments, ± represent the standard deviation between the averages of two independent experiments

Strain	Activity	
	U^{XR}	U^{XDH}
S. pastorianus CM-51	10.92±0.21	3.36±0.26
S. cerevisiae 4n	3.96±1.15	1.98±0.29
S. cerevisiae R130	0.95±0.57	0.51±0.23
S. cerevisiae BY4741	0.04±0.02	0.44±0.01
S. eubayanus PYCC6148	2.34±0.95	1.45±0.43

Fluorescence was clearly evident in strains containing the XDH-GFP fusion protein (Fig 4.2) while none was observed in CM-51 PGKxdh and CM-51 pRS42H. Microscopy images indicated that fluorescence was localised in the cytoplasm of the cell.

The comparison of the strains showed a 4-fold increase in fluorescence from *S. pastorianus* CM-51 PGKxdhGFP compared to *S. cerevisiae* strain R130 PGKxdhGFP and a 9-fold increase compared to *S. cerevisiae* strain BY4741 PGKxdhGFP (Fig 4.3A). This trend was mirrored in XDH activity from PGKxdhGFP constructs (Fig 4.3B), with *S. pastorianus* CM-51 showing the highest XDH crude specific activity with a 2.6-fold increase in activity compared to *S. cerevisiae* strain R130 and a 5.5-fold increase compared to *S. cerevisiae* strain BY4741, indicating the reduced activity displayed by *S. cerevisiae* compared to *S. pastorianus* is due to reduced recombinant protein level.

The XDH activity was reduced by almost half in the GFP tagged construct, CM-51 PGKxdhGFP compared to CM-51 PGKxdh (Fig 4.4), demonstrating that the presence of the large GFP tag at the C-terminal has a detrimental effect on XDH activity.

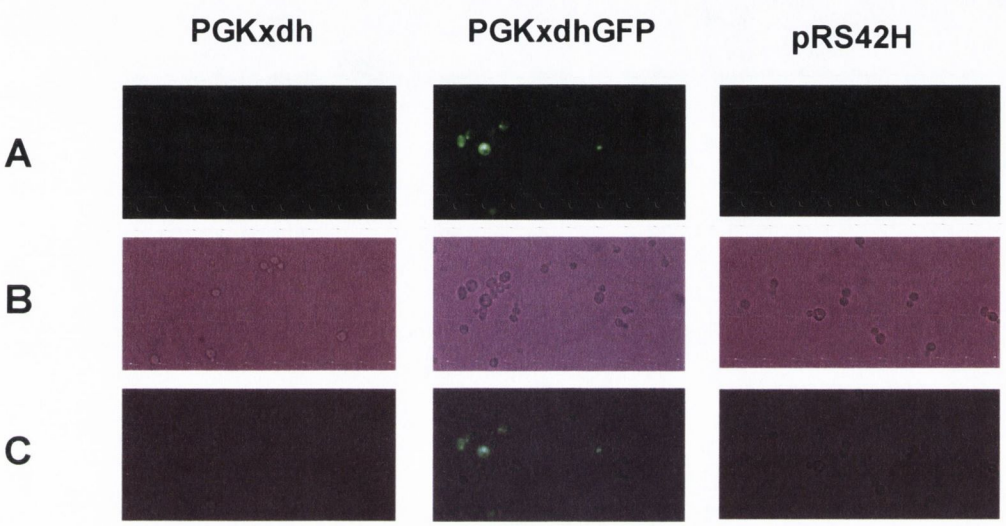


Figure 4.2 Fluorescence microscopy images of cells producing GFP tagged XDH. Microscopy images of *S. pastorianus* CM-51 cells expressing XDH (PGKxdh), C-terminal GFP fusion XDH (PGKxdhGFP) or the empty vector control (pRS42H). Strains were grown in YEP-D (20g/L) for 24Hrs. Cells were visualised using a Nikon eclipse E400 and Nikon digital camera by light microscopy using a fluorescien isothiocyanate (FITC) filter (A) (excitation 490nm and emission 525nm) or by simple light microscopy (B). The two corresponding images were over laid (C) using Microsoft powerpoint software.

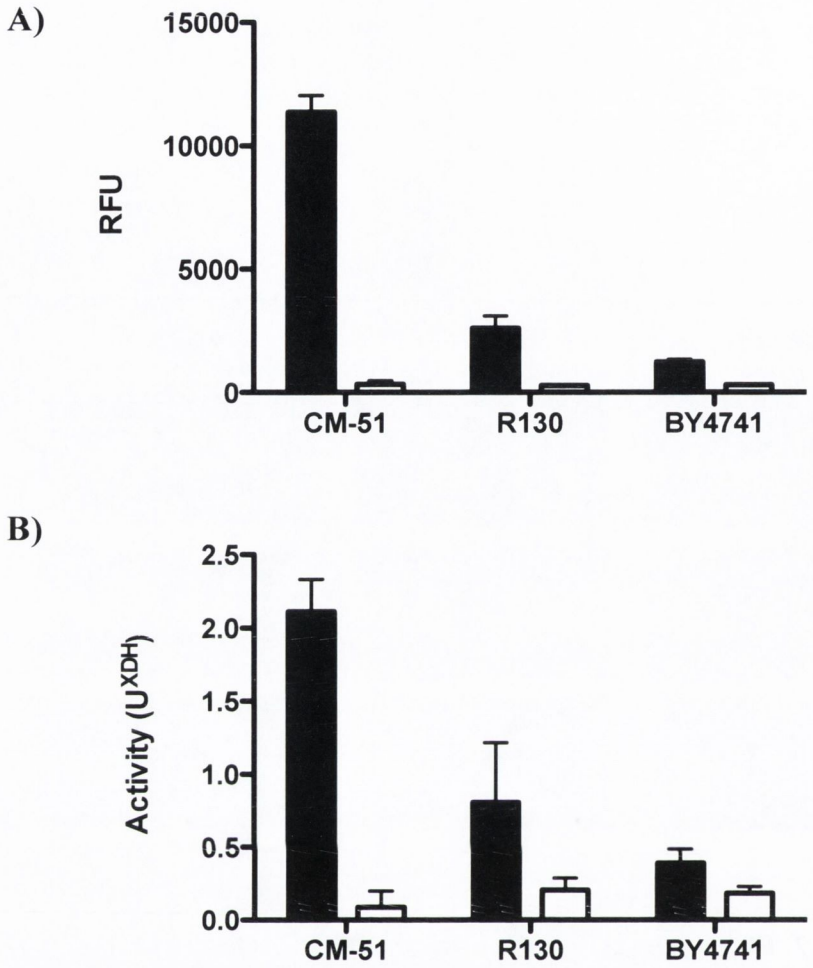


Figure 4.3 Characterisation of XDH-GFP fusion protein in various *Saccharomyces sp.* strains. Fluorescence levels (A) and XDH activity (B) produced by various *Saccharomyces sp.* strains containing the PGKxdhGFP (■) or the pRS42H (□) plasmid. For fluorescence quantification strains were cultured in YEP-D (20g/L) at 30°C for 24Hrs, cells were then harvested, washed and approximately $7x10^6$ cells were used for fluorescence quantification. Measurements were taken using excitation at 480nm and emission at 510nm and fluorescence was expressed in relative fluorescence units (RFU). For XDH enzyme activity quantification strains were cultured in YEP-D (20g/L) at 30°C for 24Hrs, cells were harvested, washed and then enzyme activity was quantified using cell lysate. XDH Enzyme activity is defined as NAD+ (μM) reduced per minute, this was then standardised against protein level (μg) to give crude specific activity (U^{XDH}). Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments. CM-51- *S. pastorianus* CM-51, R130- *S cerevisiae* R130, BY4741- *S. cerevisiae* BY4741.

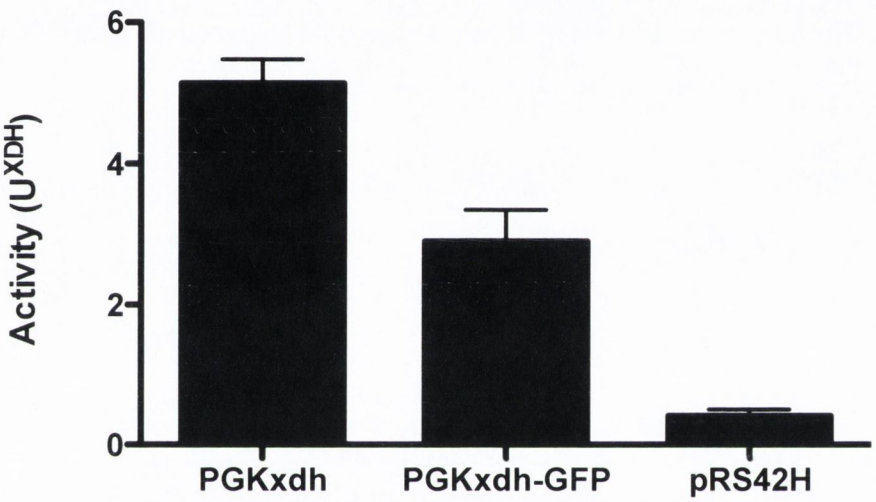


Figure 4.4 Enzyme activities of XDH and XDH-GFP fusion. Strains CM-51 PGKxdh, CM-51 PGKxdhGFP and CM-51 pRS42H were cultured in YEP-D (20g/L) media at 30°C for 24Hrs, cells were harvested, washed and enzyme activity was quantified using cell lysate. XDH enzyme activity is defined as NAD+ (μM) reduced per minute, this was then standardised against protein level (μg) to give a crude specific activity (U^{XDH}). Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments.

4.4 Xylose utilisation

In addition to the XR-XDH utilising pathway the XI pathway was individually cloned into the high copy number plasmid pRS42H using the single gene insertion strategy (section 2.4.1). The protein sequence of *xi* gene from *Piromyces sp.* was obtained from www.ebi.ac.uk (database ID TR:Q9P8C9_PIRSE) and was reverse translated (yeast codon optimized) using GeneDesign and this DNA sequence was synthesized and cloned into pUC57 by GenScript. Inc. (Piscataway, NJ) (T.C James, unpublished). The *xi* gene was amplified from pUC57 in 2 fragments. Fragment 1 was amplified using primers xi_Rec1_F and xi_MidR, fragment 2 was amplified using primers xi_MidF and xi_Rec2_R. The two fragments were inserted into SalI digested pRS42H PGKbglI plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51.

Yeast strains (CM-51 PGKxdh, CM-51 PGKxr, CM-51 PGKxr-PGKxdh, CM-51 PGKxi, and CM-51 pRS42H) were grown overnight in YEP-D media, serially diluted and then spotted onto SC-D or SC-X agar plates and incubated at 30°C for up to 5 days (YEP agar was not used due to background growth observed resulting in false positive results). All strains grew on SC-D (Fig 4.5). Growth on xylose was only observed in strains expressing both *xyl1* and *xdh1* (PGKxr-PGKxdh). The individual expression of *xyl1* (PGKxr) or *xdh1* (PGKxdh) was not sufficient to support growth on xylose, indicating that both enzymes are key for xylose utilisation. While the *xyl1* and *xdh1* (PGKxr-PGKxdh) co-expressing strains showed growth on xylose, CM-51 PGKxi strain, did not sustain growth on xylose.

The ability of strains CM-51 PCKxr-PGKxdh and CM-51 PGKxi to ferment xylose was next examined. Yeast strains were cultured in YEP-D media for 24Hrs, cells were then harvested, washed and inoculated into YEP-X media at a high cell density ($1x10^8$ cells/mL). Cultures were incubated at 30°C for 12 days with samples being taken at intervals for ethanol and xylose quantification (Fig 4.6). While CM-51 PGKxi displayed no growth on minimal media containing xylose, low levels of xylose utilisation and ethanol production were observed in rich liquid medium. The strain CM-51 PGKxr-PGKxdh displayed much higher levels of xylose consumption and a 7-fold increase in ethanol production was observed. Ethanol levels peaked in CM-51 PGKxr-PGKxdh fermentations at 8.83g/L, representing a theoretical ethanol yield of 38.78%. No ethanol production or xylose utilisation was observed using the empty vector containing strain CM-51 pRS42H.

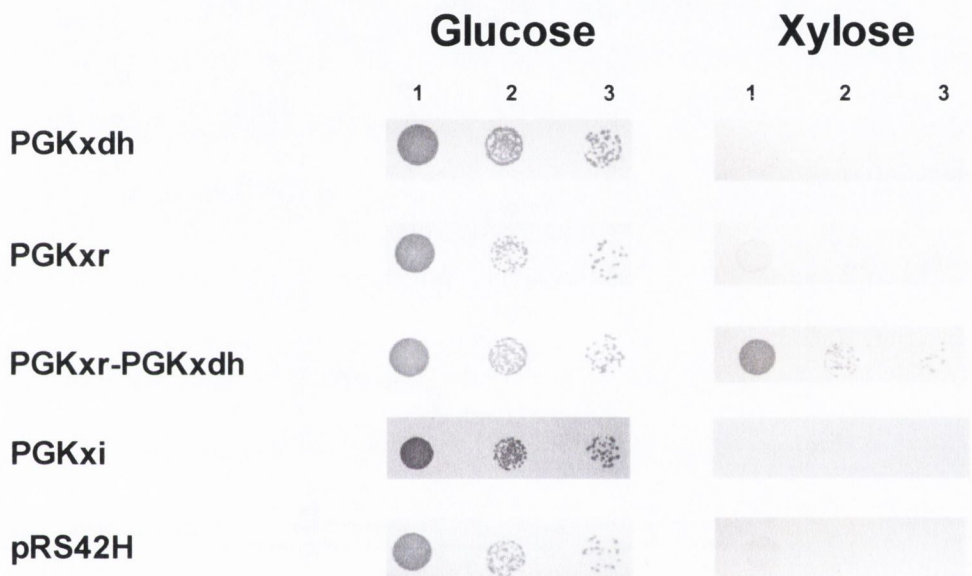


Figure 4.5 Growth of *S. pastorianus* CM-51 strains on glucose or xylose. Strains were grown overnight in YEP-D (20g/L) media at 30°C, and then serially diluted and samples were spotted onto SC-D (20g/L) and SC-X (20g/L) agar media and plates were incubated at 30°C for up to 5 days before images were taken. 1) 1 in 100 dilution. 2) 1 in 10,000 dilution. 3) 1 in 50,000 dilution.

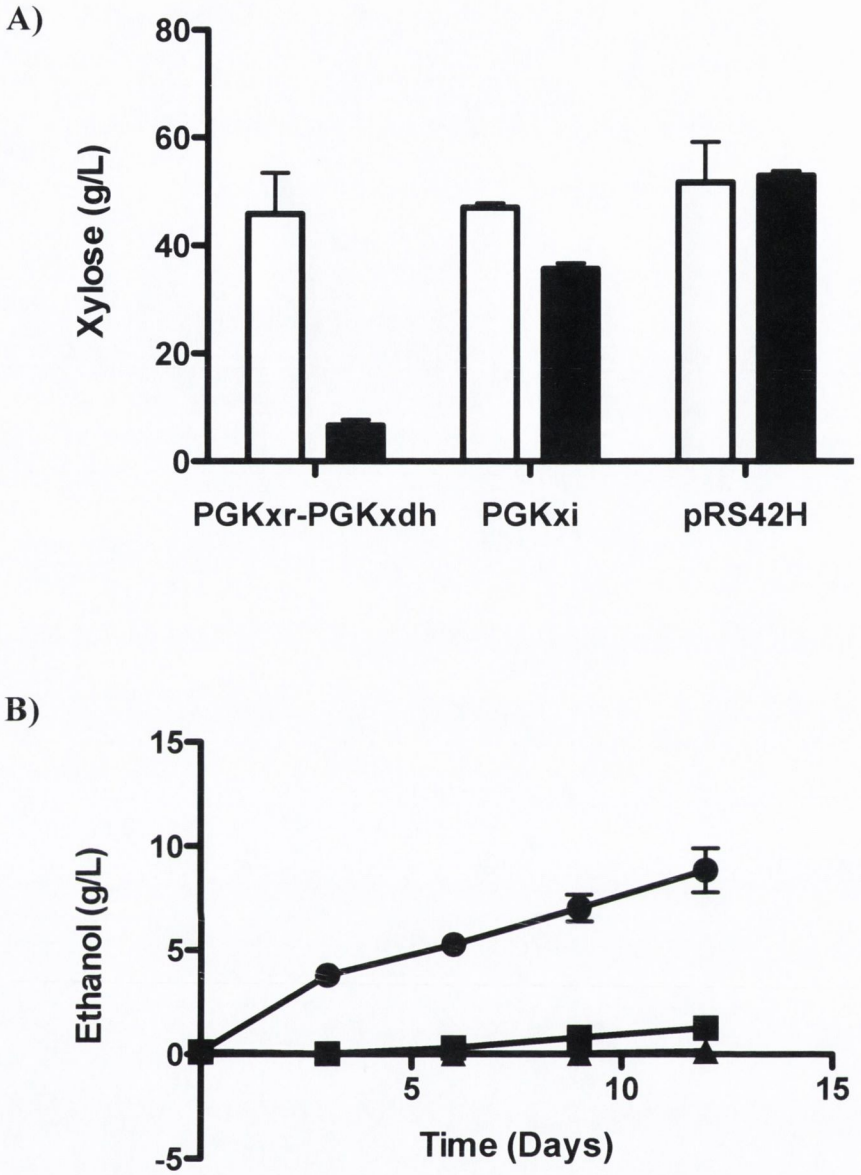


Figure 4.6 Xylose metabolism using the XR/XDH or XI pathway in *S. pastorianus* CM-51. Xylose levels (A) and ethanol production (B) at day 0 (□) and day 12 (■) and over time respectively in YEP-X (50g/L) fermentations using strains CM-51 PGKxr-PGKxdh (●), CM-51 PGKxi (■) and CM-51 pRS42H (▲) Strains (CM-51 PGKxr-PGKxdh, CM-51 PGKxi and CM-51 pRS42H) were intially grown in YEP-D (20g/L) for 24Hrs. Cells were harvested, washed and inoculated into YEP-X (50g/L) at a starting cell density of $1x10^8$cells/mL and incubated at 30°C for up to 12 days. All values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments.

Although CM-51 PGKxr-PGKxdh produced higher levels of ethanol than CM-51 PGKxi, the percentage of the theoretical ethanol yield from xylose was still very low. To increase ethanol yield the native *XKS1* gene from *S. cerevisiae* was inserted in tandem orientation to *xyl1* and *xdh1* in the plasmid PGKxr-PGKxdh.

The native *XKS1* gene from *S. cerevisiae* was first inserted into the high copy number plasmid using the single gene insertion strategy (section 2.4.1). The *XKS1* gene was amplified from *S. cerevisiae* 288c genomic DNA in 2 fragments. Fragment 1 was amplified using the primers xks_Rec1_F and xks_MidR and fragment 2 was amplified using the primers xks_MidF and xks_Rec2_R. The 2 fragments were inserted into Sal1 and Spe1 digested pRS42H TEFbgl1 plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. For co-expression with PGKxr and PGKxdh cassette, the third gene insertion strategy was used (Section 2.4.3). The TEFxks cassette was amplified in two fragments from pRS42H TEFxks. Fragment 1 was amplified using the primers Acc_TEF_F and xks_MidR and fragment 2 was amplified using the primers xks_MidF and Acc_R (Table 2.5). The 2 fragments were inserted into Acc65I digested PGKxr-PGKxdh plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. The PGKxks cassette was inserted 557 bp downstream of the PGKxr cassette and directly upstream of the PGKxdh cassette (Fig 2.6).

Ethanol production in xylose fermentations was increased up to 3-fold in the XR/XDH background through the overexpression of the native *S. cerevisiae* gene *XKS1,* with levels reaching 19.06g/L (Fig. 4.7A), representing 63.84% of the theoretical ethanol yield. Demonstrating the key requirement for the overexpression of *XKS1* for efficient ethanol production from xylose. Xylose utilisation however was similar between both overexpressing and non-overexpressing *XKS1* strains (Fig 4.7B).

Surprisingly, reducing the starting concentration of xylose in the medium from 50g/L to 20g/L abrogated the influence of *XKS1* overexpression on final ethanol levels (Fig 4.8), although the rate of ethanol production was still greater in *XKS1* overexpressing strain. This indicates that substrate concentration is as important as the overexpression of *XKS1*, as without high xylose loading the benefit of the overexpression of *XKS1* on final ethanol yield is nullified.

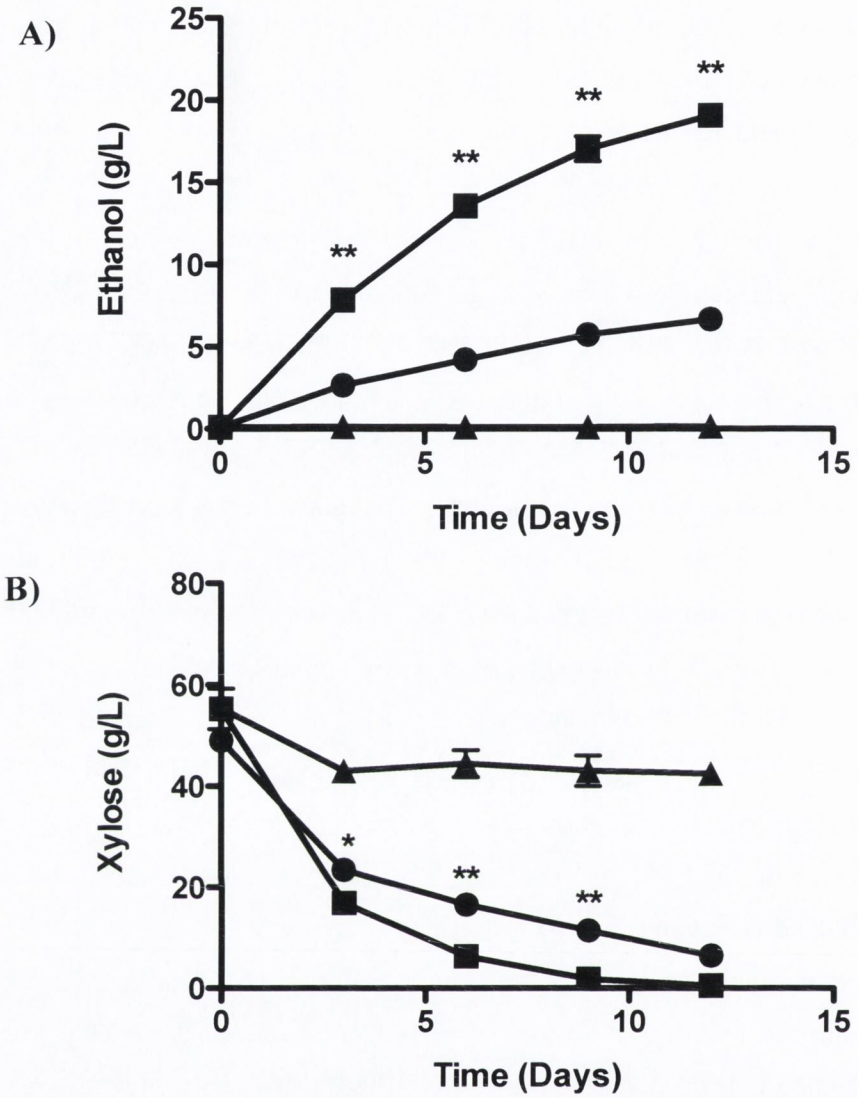


Figure 4.7 Effect of the overexpression of *XKS1* in high level xylose (50g/L) fermentations. Ethanol production (A) and xylose utilisation (B) in YEP-X (50g/L) fermentations using strains CM-51 PGKxr-PGKxdh (●), CM-51 PGKxr-TEFxks-PGKxdh (■) and CM-51 pRS42H (▲). Strains (CM-51 PGKxr-PGKxdh, CM-51 PGKxr-TEFxks-PGKxdh and CM-51 pRS42H) were cultured in YEP-D (20g/L) media at 30°C for 24Hrs, cells were harvested, washed and inoculated into YEP-X (50g/L) media at a starting cell density of $1x10^8$cells/mL and incubated at 30°C for up to 12 days. Values are the average of three independent experiments, with duplicate readings in both experiments, error bars represent the standard error between the averages of three independent experiments. Statistical significance of ethanol production (A) and xylose utilisation (B) between CM-51 PGKxr-PGKxdh (●), CM-51 PGKxr-TEFxks-PGKxdh (■) at individual time points was calculated using two-tailed students *t* test, *-$p<0.05$, **-$p<0.001$.

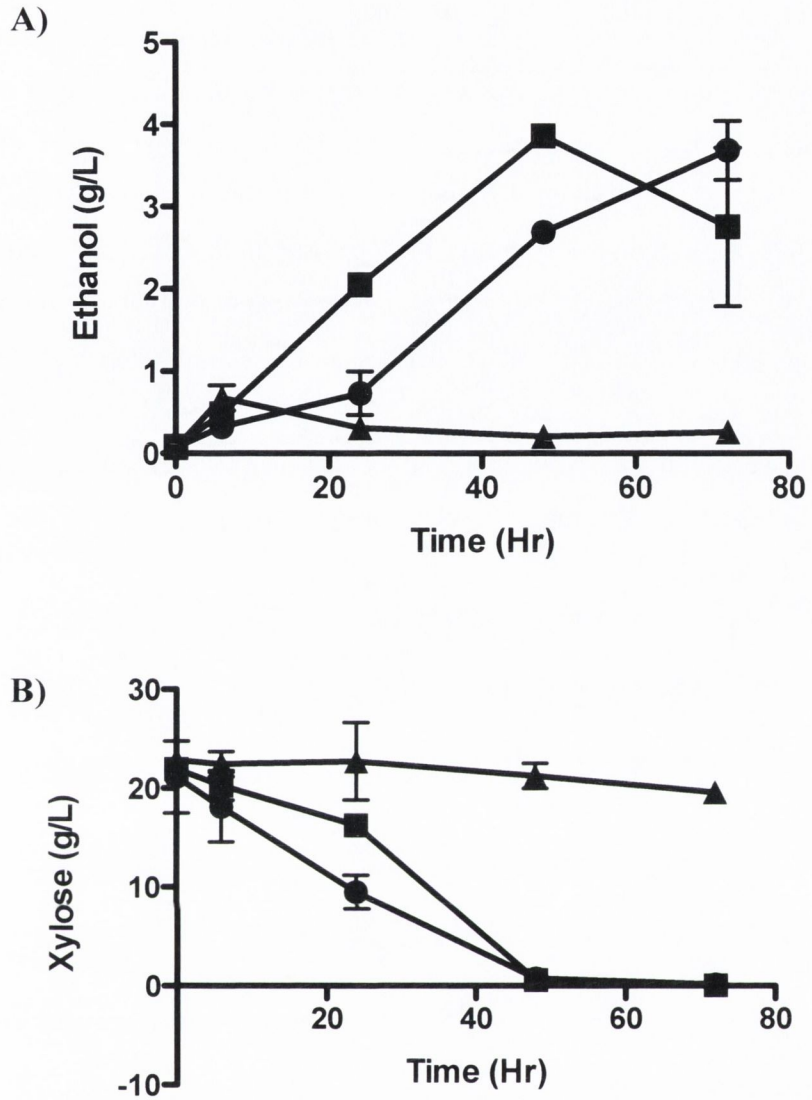


Figure 4.8 Effect of the overexpression of *XKS1* in low level xylose (20g/L) fermentations. Ethanol production (A) and xylose utilisation (B) in YEP-X (20g/L) fermentations using strains CM-51 PGKxr-PGKxdh (●), CM-51 PGKxr-TEFxks-PGKxdh (■) and CM-51 pRS42H (▲). Strains (CM-51 PGKxr-PGKxdh, CM-51 PGKxr-TEFxks-PGKxdh and CM-51 pRS42H) were cultured in YEP-D (20g/L) media at 30°C for 24Hrs, cells were harvested, washed and inoculated into YEP-X (20g/L) media at a starting cell density of $1x10^8$cells/mL and incubated at 30°C for up to 12 days. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments.

To enter the cell xylose must hijack various hexose transporters. At low xylose concentrations it is thought that xylose transport is inefficient. (Runquist et al., 2009). It was therefore rational to think that this could be the reason for the difference in ethanol yields seen between 20g/L and 50g/L xylose. To examine this, strains CM-51 PGKxr-TEFxks-PGKxdh and CM-51 pRS42H were cultured in YEP-D media for 24Hrs. Cells were then harvested, washed and inoculated into YEP-X (20g/L or 50g/L) media at a final cell density of $1x10^8$cells/mL, with samples being taken at intervals for xylose quantification (Fig 4.9). The xylose utilisation rates in low (20g/L) and high (50g/L) xylose fermentations were 0.40g/Hr and 0.50g/Hr respectively. From the rates it appears that the difference seen in ethanol production and yields previously cannot be explained through xylose transport, as the rate of xylose utilisation appears to be the same between 20g/L and 50g/L.

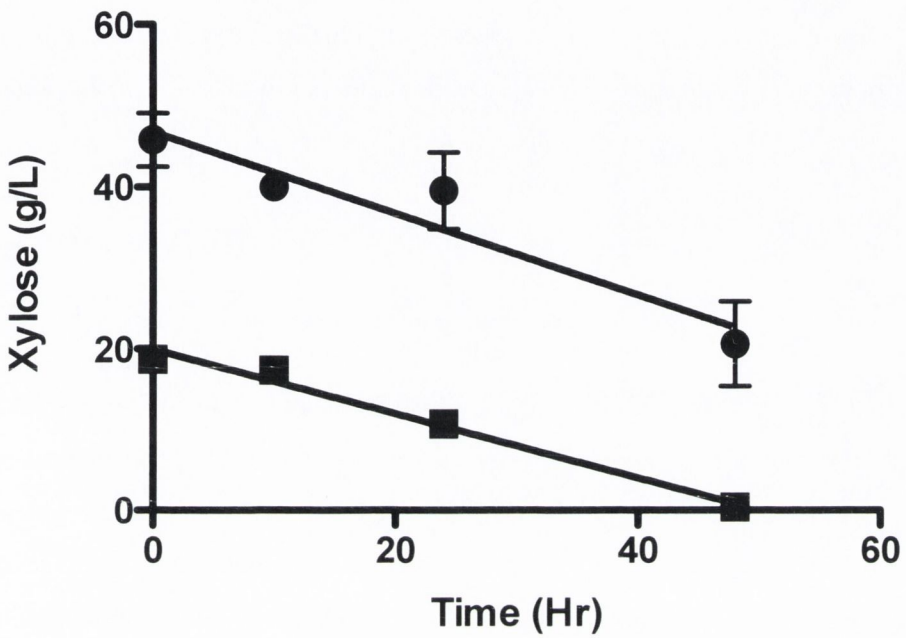


Figure 4.9 Xylose utilisation rate at high and low xylose concentrations. Xylose utilisation in 50g/L (●) and 20g/L (■) xylose fermentations using CM-51 PGKxr-TEFxks-PGKxdh. Strains were cultured in YEP-D (20g/L) media at 30°C for 24Hrs, cells were harvested, washed and inoculated into xylose fermentations at a high starting cell density ($1x10^8$cells/mL) and incubated at 30°C. Values are the average of two independent experiments with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments. Lines of best fit were calculated using linear regression, gradients were calculated to be -0.503±0.089 and -0.399±0.025 for 50g/L and 20g/L fermentations respectively. Strain CM-51 pRS42H showed no xylose consumption (data not shown).

4.5 Discussion

The utilisation of lignocellulosic biomass requires the efficient fermentation of both the cellulosic and the hemicellulosic fraction of biomass. In this chapter the metabolism of the pentose sugar xylose, a main component of hemicelluose was examined. The genes *xyl1* (XR) and *xdh1* (XDH) from *T. reesei* were individually expressed and enzyme activity quantified in *S. pastorianus* CM-51. Co-factor usage was found to be highly specific for both XR (NADPH) and XDH (NAD+). The use of different co-factors between XR (NADPH) and XDH (NAD+) results in an intracellular redox imbalance (Jeffries and Jin, 2004). The co-factor alteration of XR from NADPH to NADH is a strategy used to increase ethanol yields via co-factor recycling in the XR-XDH pathway (Bengtsson et al., 2009; Petschacher and Nidetzky, 2008; Xiong et al., 2011).

Strain comparison of XR and XDH activity identified that *S. pastorianus* CM-51 produced the highest activity. Enzyme activity was lower in other *Saccharomyces sp.* such as *S. cerevisiae*, mirroring results seen for BGL1 activity (Chapter 3). Further analysis using a XDH-GFP fusion construct suggested that recombinant protein levels were decreased in *S. cerevisiae* compared to *S. pastorianus*, this is likely the reason for the decreased activity seen within *S. cerevisiae* strains. Despite possible redox imbalances, the XR/XDH pathway produced significantly more ethanol than the XI pathway. In *S. pastorianus* CM-51 the XR-XDH pathway produced not only a higher level of ethanol but also higher ethanol yield from consumed xylose, confirming previous comparisons between the XR/XDH and XI pathways in a *S. cerevisiae* host (Bettiga et al., 2008; Karhumaa et al., 2007b). The poor xylose utilisation and ethanol production shown by CM-51 PGKxi would suggest that the enzyme activity may be limiting xylose metabolism. Increasing XI activity (2-fold) through adaptive and directed evolution produced significantly higher ethanol yields (Diao et al., 2013; Lee et al., 2012), identifying enzyme activity as the limiting factor in xylose utilisation via the XI pathway. Xylose metabolism was further optimised in *S. pastorianus* through the overexpression of the native *XKS1* gene from *S. cerevisiae*. Ethanol production increased approximately 3-fold through the overexpression of *XKS1* although this was only seen at high xylose loading concentrations (50g/L) and not at lower concentrations (20g/L). Ethanol yields were increased to 0.32g/g (ethanol g/xylose g) through the overexpression of *XKS1*, similar to yields shown in other studies of 0.34g/g (Matsushika

and Sawayama, 2011), 0.35 g/g (Kato et al., 2013), 0.34 g/g (Ismail et al., 2013) and 0.39g/g (Bera et al., 2011). The increase in ethanol yield seen through *XKS1* overexpression is thought to be due to a reduction in by-product formation, in particular a decrease in xylitol yield (Johansson et al., 2001; Matsushika and Sawayama, 2011; Toivari et al., 2001).

Data presented in this chapter has again demonstrated *S. pastorianus* to be a superior host for recombinant enzyme expression over *S. cerevisiae* as a result of reduced recombinant protein level within *S. cerevisiae*. Data confirmed previous studies identifying higher ethanol production from the XR-XDH over the XI pathway, along with the essential requirement of *XKS1* overexpression and high xylose concentrations for efficient pentose sugar fermentation.

Chapter 5

Engineering of *S. pastorianus* for xylose and cellulose co-fermentation

5.1 Introduction

The production of ethanol from cellulose and xylose individually has been studied in depth (Section 1.5.2, 1.7.2), however the co-fermentation of complex sugars using solely recombinant *Saccharomyces sp.* has yet to advance past xylose and cellobiose (Ha et al., 2011; Li et al., 2010; Saitoh et al., 2010). When switching from purified substrates to actual biomass it is unlikely that cellobiose will be readily available in fermentations without the addition of commercial enzymes, therefore it is important to develop a strategy that better represents the complex nature of lignocellulose and develop strains that are able to co-utilise cellulose and xylose to optimise ethanol production from lignocellulose.

To combine both the cellulose and xylose utilising machinery shown in previous chapters (Chapter 3 and Chapter 4) individual cellulase genes (*bgl1*, *cbh2*, *egl2*) were co-expressed with xylose utilising enzymes (XR/XDH). Using a co-culture system of strains a strategy was developed to allow for xylose and cellulose co-utilisation in a single fermentation, in order to optimise ethanol production from lignocellulose.

Problems associated with hexose and pentose co-utilisation such as inhibition of sugar transport and inefficient metabolic fluxes have been shown to effect glucose and xylose co-utilisation in *S. cerevisiae* (Subtil and Boles, 2012), however the effect of more complex sugars such as cellulose has yet to be investigated. This chapter describes the investigation of pentose and hexose sugar co-utilisation by engineered *S. pastorianus* using a co-culture approach, with the aim of optimising ethanol production from cellulose and xylose.

5.2 Quantification of cellulase and XR/XDH activity in triple expressing strains

All three classes of cellulase enzymes are required for the efficient degradation of cellulose (Section 3.6). Based on previous data the optimal individual cellulase cassettes were identified as TEFbgl1, TEFcbh2 and PGKegl2 (Chapter 3). These cassettes were individually cloned into the PGKxr-PGKxdh plasmid using the third gene insertion strategy (Section 2.4.3) using primers and templates outlined in Table 2.5. Cellulase cassettes were amplified in 2 fragments. The TEFbgl1 cassette was amplified from pRS42H TEFbgl1 plasmid using primers Acc_TEF_F and bgl1_MidR for fragment 1 and primers bgl1_MidF and Acc_R for fragment 2. The TEFcbh2 cassette was amplified from pRS42H TEFcbh2 plasmid using primers Acc_TEF_F and cbh2_MidR for fragment 1 and primers cbh2_MidF and Acc_R for fragment 2. The PGKegl2 cassette was amplified from pRS42H PGKegl2 plasmid using primers Acc_PGK_F and egl2_MidR for fragment 1 and primers egl2_MidF and Acc_R for fragment 2. Amplified fragments were inserted individually into the Acc65I digested PGKxr-PGKxdh plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. Each cellulase cassette was inserted in a tandem orientation, 557bp downstream of the PGKxr cassette and directly upstream of the PGKxdh cassette.

To ensure that the triple expression cassette design did not effect enzyme activity, cellulase activity was quantified in the triple cassette background and compared to corresponding individual cassettes. Xylose and cellulose co-utilising strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh) and corresponding cellulase expressing strains (CM-51 TEFbgl1, CM-51 TEFcbh2 and CM-51 PGKegl2) were grown individually in SC-S media. After 48Hrs, supernatants were harvested and used for PASC hydrolysis. Individual supernatants were combined in a 1:1:1 ratio. The resulting cellulase cocktails were then individually mixed with PASC and incubated at 50°C. The level of glucose released was measured using the glucose assay kit after 24Hrs (Fig 5.1). Glucose levels released from PASC using xylose and cellulose co-utilising strains supernatant showed no difference to that of single cellulase strains supernatant, indicating that the activity of cellulase enzymes is unaffected through the triple expression cassette.

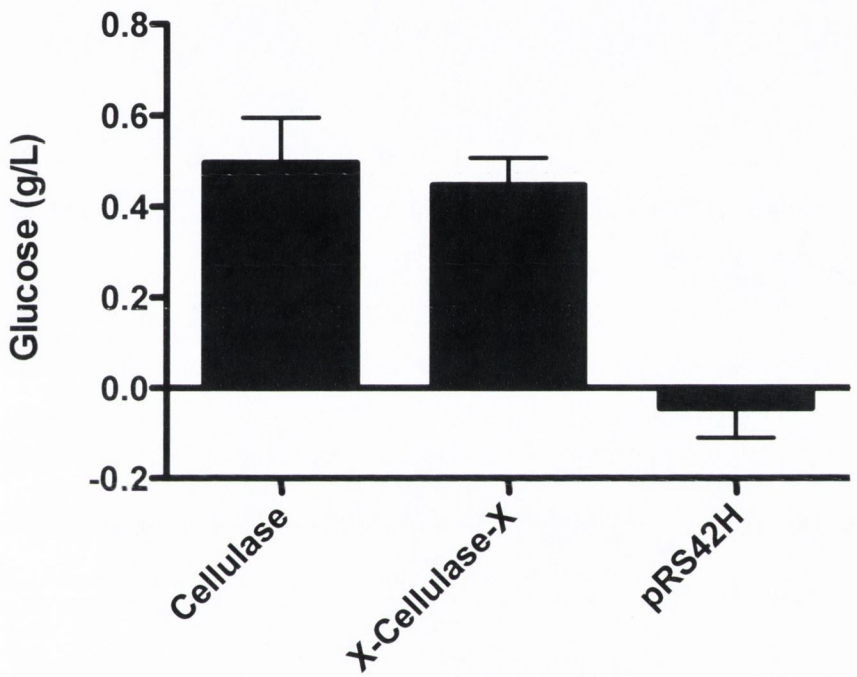


Figure 5.1 PASC hydrolysis by recombinant cellulase enzymes from individual and triple cassette expressing *S. pastorianus* CM-51. Individual cellulase expressing strains (CM-51 TEFbgl1, CM-51 TEFcbh2 and CM-51 PGKegl2) and triple expressing strains were (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh) were grown individually in SC-S (20g/L) media at 30°C for 48Hrs, supernatants were harvested and combined in a 1:1:1 ratio to generate single (Cellulase) and triple cassette (X-Cellulase-X) cellulase mixtures. Empty vector containing strain CM-51 pRS42H was cultured in SC-S (20g/L) media at 30°C for 48Hrs, supernatant was harvested and used in hydrolysis reaction (pRS42H). Cellulase cocktails and pRS42H were individually mixed with PASC (1000g/L) (25g/L theoretical glucose, see Section 2.1.3) and incubated at 50°C for 24Hrs. Activity is defined as glucose release and expressed as glucose in g/L. Values are the average of two independent experiments with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiments.

XR and XDH enzyme activity was quantified to ensure xylose utilisation was not affected in the triple expression cassette. Individual strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh, CM-51 PGKxr-PGKxdh and CM-51 pRS42H) were cultured in YEP-D media for 24Hrs. XDH and XR activities were quantified from cell lysate as described in materials and methods (Section 2.7.1). No difference was seen in activity between triple expressing XR and XDH strains and that produced by the strain CM-51 PGKxr-PGKxdh (Fig 5.2), again demonstrating that triple expression does not have any negative effect on enzyme activity.

5.3 Xylose and cellobiose co-fermentation

The ability to co-utilise and ferment xylose and complex sugars was next examined. Cellulose is composed of repeating cellobiose units, which is hydrolysed to glucose through the action of BGL1. The simplest model for xylose and cellulose utilisation is the co-fermentation of xylose and cellobiose. Therefore the ability of the strain CM-51 PGKxr-TEFbgl1-PGKxdh to co-utilise xylose and cellobiose was first tested. Strains (CM-51 PGKxr-TEFbgl1-PGKxdh and CM-51 pRS42H) were grown in YEP-D media for 24Hrs. Cells were harvested, washed and then inoculated into YEP-C, YEP-X or YEP-C+X at a starting cell density of $1x10^8$ cells/mL. Fermentations were carried out at 30°C and samples were taken over time for ethanol quantification. The results indicated that strain CM-51 PGKxr-TEFbgl1-PGKxdh utilised both cellobiose and xylose individually and also xylose and cellobiose in a co-fermentation, with ethanol levels at day 14 reaching 16.29g/L, 5.00g/L and 27.35g/L respectively (Fig 5.3A). No ethanol was detected from the empty vector containing strain CM-51 pRS42H in any of the fermentation conditions (data not shown). Previous results demonstrated that the overexpression of *XKS1* was required for efficient ethanol production from xylose. The overexpression of the *XKS1* gene was supplied in trans on a separate plasmid (pRS42K) (Table 2.3). The TEFxks cassette was amplified from pRS42H TEFxks plasmid in 2 fragments using primers Psi_TEF_F and xks_MidR for fragment 1 and xks_MidF and Psi_Cyc_R for fragment 2.

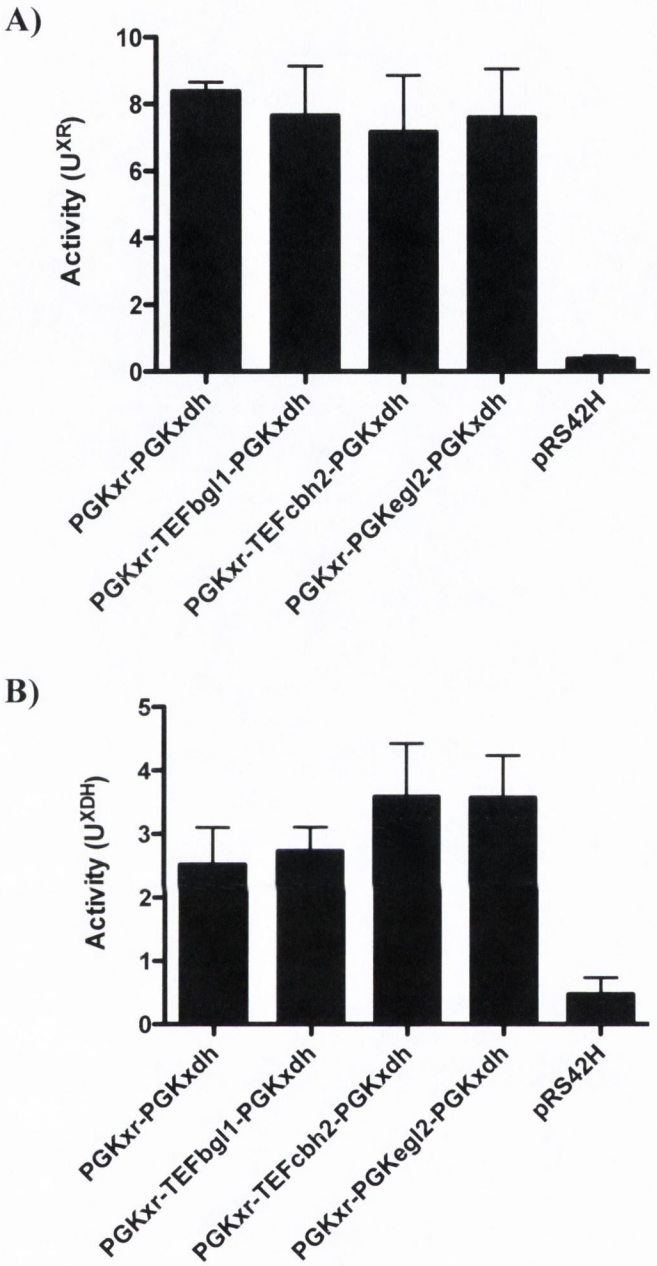


Figure 5.2 Activities of XR and XDH in double and triple cassette expressing *S. pastorianus* CM-51. XR (A) and XDH (B) enzyme activity of triple and double expressing strains. Strains (CM-51 PGKxr-TEFbgl-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh, CM-51 PGKxr- PGKxdh and CM-51 pRS42H) were cultured in YEP-D (20g/L) media for 24Hrs, cells were harvested, washed and enzyme activity was quantified using cell lysate. XR enzyme activity is defined as NADPH (μM) oxidised per minute, XDH enzyme activity is defined as NAD+ (μM) reduced per minute. Enzyme activities were standardised against protein level (μg) to give crude specific activity (U^{XR}, U^{XDH}). Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the averages of two independent experiment.

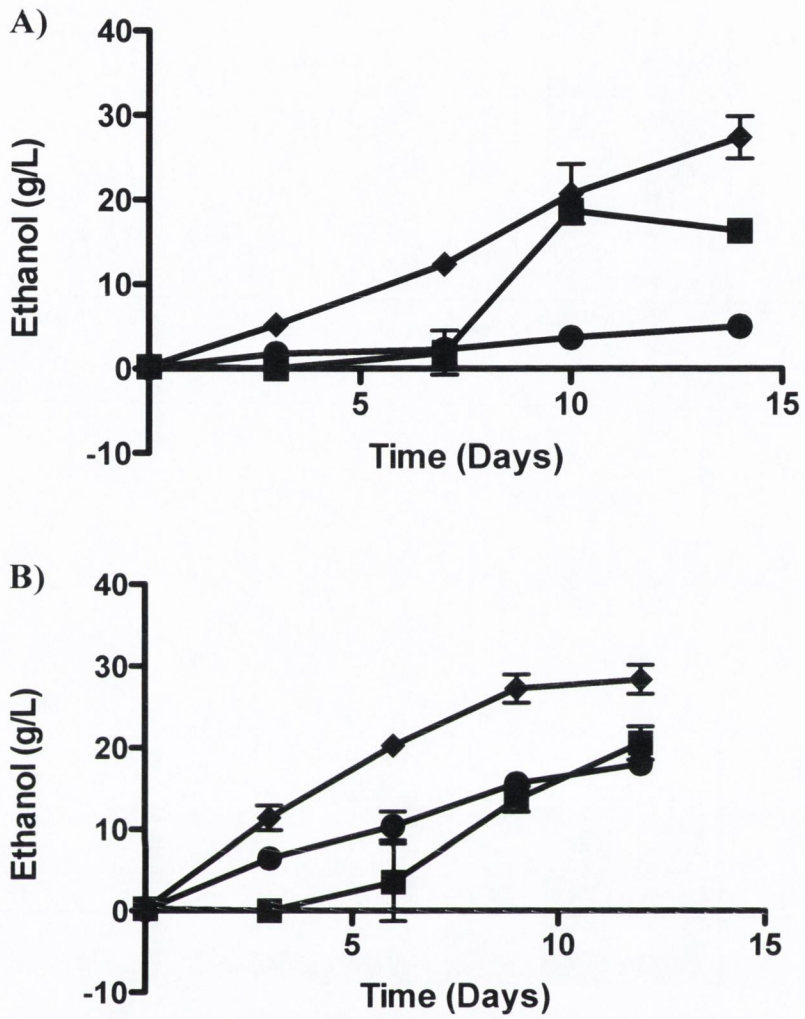


Figure 5.3 Co-fermentation of xylose and cellobiose. *S. pastorianus* CM-51 expressing the gene cassette PGKxr-TEFbgl1-PGKxdh (A) or additionally TEFxks^{Km} in trans (B) were cultured in YEP-D (20g/L) for 24Hrs at 30°C, cells were harvested and used to inoculate fermentations YEP-C (50g/L) (■), YEP-X (50g/L) (●) and YEP-C(50g/L)+X(50g/L) (♦) at a high cell density (1x10^{8}cells/mL). Fermentations were conducted at 30°C for up to 14 days and samples were taken at intervals and levels are expressed in terms of ethanol in g/L. Values are the average of two independent experiments, with duplicate readings in both experiments, error bars represent the standard deviation between the averages of independent experiments.

The 2 fragments were inserted into PsiI digested pRS42K plasmid via *in vivo* homologous recombination in *S. pastorianus* CM-51. The strain CM-51 TEFxks^{Km} was subsequently transformed with the PGKxr-TEFbgl1-PGKxdh hygromycin resistant plasmid, and the ability of this new strain to co-utilise cellobiose and xylose was next examined. Yeast strains (CM-51 PGKxr-TEFbgl1-PGKxdh+TEFxks^{Km} and CM-51 pRS42H+pRS42K) were grown in YEP-D media. Cells were harvested, washed and inoculated into YEP-C, YEP-X or YEP-C+X media at a starting cell density of 1x10^8 cells/mL and incubated at 30°C for up to 12 days. Ethanol levels reached 18.02g/L and 20.62g/L in xylose and cellobiose fermentations respectively (Fig 5.3B). When both xylose and cellobiose were combined in a single fermentation ethanol production increased to 28.36g/L. Although ethanol levels increased in xylose and cellobiose co-fermentations, the percentage of theoretical ethanol yield (55.61%) was reduced by 20-30% compared to individual xylose (73.70%) and cellobiose (80.69%) fermentations using *XKS1* overexpressing strains, indicating a possible problem in efficient ethanol production in mixed sugar fermentations.

5.4 Xylose and cellulose co-fermentation

The ability of recombinant strains to ferment cellulose along with xylose was next examined. Individual strains (PGKxr-TEFbgl1-PGKxdh, PGKxr-TEFcbh2-PGKxdh, PGKxr-PGKegl2-PGKxdh and CM-51 pRS42H as a control) were grown in YEP-D for 24Hrs. Cells were harvested, washed, and combined in a 1:1:1 ratio and inoculated into YEP-X, YEP-PASC or YEP-X+PASC media. All fermentations were inoculated at a high starting cell density (1x10^{8}cells/mL). Fermentations were incubated at 30°C, with samples being taken over time for ethanol quantification. The empty vector containing strain CM-51 pRS42H produced no significant levels of ethanol in any of the fermentations (data not shown). The co-cultured strains were unable to utilise PASC media alone, producing little to no ethanol (Fig 5.4A). Ethanol production from xylose alone peaked at 5.43g/L at day 14 and increased in xylose and PASC co-fermentations to 9.61g/L, indicating that xylose utilisation can support cellulose fermentation when both xylose-utilising enzymes and cellulases are co-produced during fermentation.

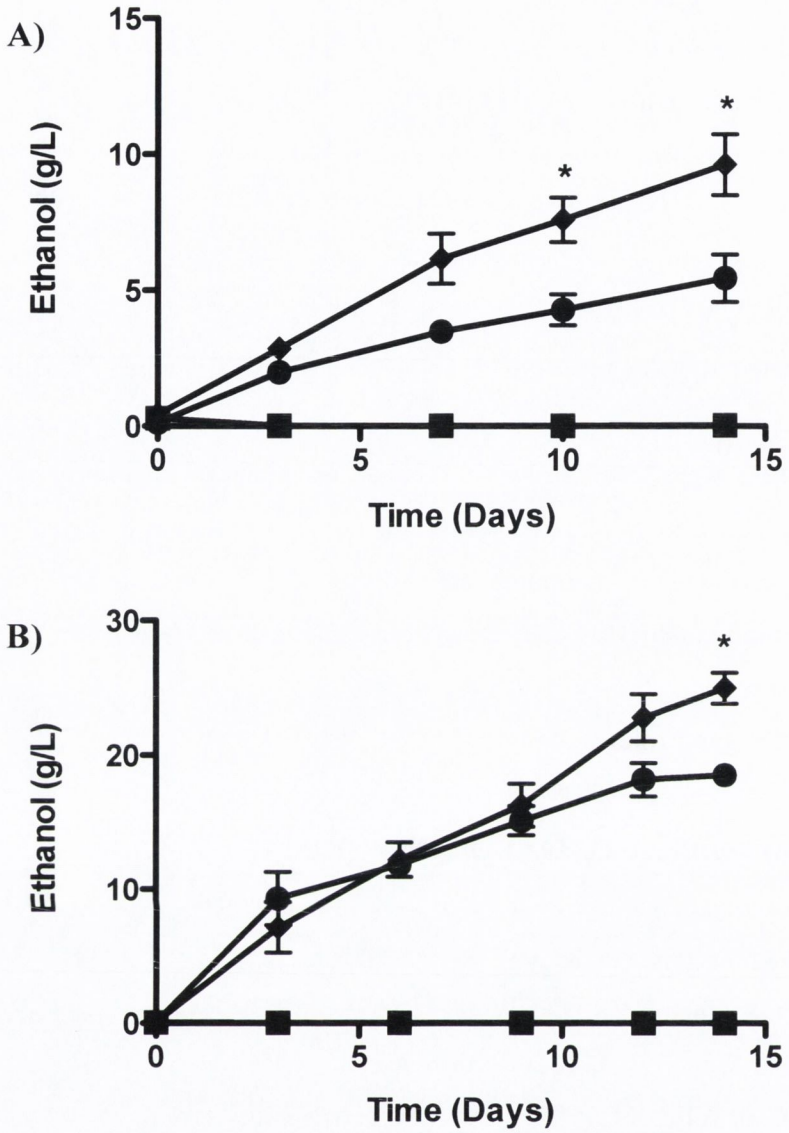


Figure 5.4 Co-fermentation of xylose and cellulose (PASC). *S. pastorianus* CM-51 expressing the gene cassettes PGKxdh-TEFbgl1-PGKxr, PGKxdh-TEFcbh2-PGKxr and PGKxdh-PGKegl2-PGKxr (A) or additionally TEFxks^{Km} in trans (B) were individually cultured in YEP-D (20g/L) at 30°C for 24Hrs. Cells were harvested, combined in a 1:1:1 ratio and used to inoculate fermentations containing PASC (■), xylose (●), or xylose+PASC (♦). Substrate concentrations were 416g/L PASC (10.4g/L theoretical glucose, see Section 2.1.3) and 41g/L xylose (A) and 416g/L PASC (10.4g/L theoretical glucose, see Section 2.1.3) and 50g/L xylose (B). Fermentations were conducted at 30°C for 14 days and samples were taken at the intervals shown and levels are expressed in terms of ethanol in g/L. Values are the average of three (A) and four (B) independent experiments, with duplicate readings in all experiments, error bars represent the standard error between the averages of independent experiments. Statistical significance of ethanol production between Xylose (●) and Xylose+PASC media (♦) at individual time points was calculated using two-tailed students *t* test, *- $p<0.05$.

To improve ethanol yields from xylose and PASC co-fermentations, triple-expressing strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh and CM-51 PGKxr-PGKegl2-PGKxdh) were transformed with the plasmid pRS42K TEFxks. Yeast strains (CM-51 PGKxr-TEFbgl1-PGKxdh+TEFxks^{Km}, CM-51 PGKxr-TEFcbh2-PGKxdh+TEFxks^{Km}, CM-51 PGKxr-PGKegl2-PGKxdh+TEFxks^{Km} and CM-51 pRS42H+pRS42K) were cultured individually in YEP-D media for 24Hrs Fermentations were set up as described previously with cellulose and xylose utilising strains being combined in a 1:1:1 ratio. Cells were inoculated at a high starting cell density (1x10^{8}cells/mL) into YP-X, YP-PASC and YP-X+PASC media (Fig 5.4B). Strain CM-51 pRS42H+pRS42K containing the empty vectors produced no ethanol in any fermentation conditions (data not shown). Levels of ethanol produced from xylose fermentations were similar to levels shown in this study by *XKS1* overexpressing strains (CM-51 PGKxr-TEFxks-PGKxdh) (Chapter 4) with levels peaking at 18.51g/L, while no ethanol production was seen in PASC alone fermentations. When xylose and PASC were combined in a single fermentation vessel ethanol production increased to 24.99g/L.

The percentage of theoretical ethanol yield was significantly higher at day 14 in xylose and PASC co-fermentations compared to xylose alone for both overexpressing and non-overexpressing *XKS1* strains, with the percentage ethanol yields peaking at 81.86% in xylose and PASC fermentations using *XKS1* overexpressing strains (Fig 5.4B).

5.5 Discussion

The co-utilisation of xylose and cellulose is seen as an efficient method of maximising the conversion of lignocellulosic biomass to ethanol. The fermentation of xylose and cellulose individually by recombinant *Saccharomyces sp.* strains has been demonstrated previously, however little work has focused on combining the two within a single reaction vessel.

To enable the co-utilisation of cellulose and xylose the cellulase genes *bgl1*, *cbh2* and *egl2* were individually co-expressed with *xyl1* and *xdh1*. As with previous double expression plasmids there were no negative effects on enzyme activity of multiple gene expression on a single plasmid.

Strains expressing *bgl1* along with the xylose utilising enzymes *xyl1* and *xdh1*, were capable of co-fermenting both cellobiose and xylose. The overexpression of *XKS1* supplied in trans however interestingly offered little benefit in xylose and cellobiose co-utilisation, suggesting that xylose fermentation efficiency, which is increased through the overexpression of *XKS1,* is affected in xylose and cellobiose co-fermentation. Xylose transport along with metabolic fluxes within the cell are effected at high glucose concentrations, with an increased flux towards the citric acid cycle shown at increasing glucose:xylose ratios (Kim et al., 2010; Pitkanen et al., 2003). The preferential utilisation of glucose over xylose may explain the reduced yield shown in cellobiose and xylose co-fermentations compared to individual fermentations using *XKS1* overexpressing strains. The percentage of ethanol yield from cellobiose and xylose co-fermentations (55.61%) was lower in this study compared to previous studies, with yields reaching 73.52% (Ha et al., 2011), 75.46% (Ha et al., 2013) and 78.38% (Saitoh et al., 2010), suggesting the strategy of pentose and hexose sugar co-utilisation developed in this study may not be the most efficient strategy.

The fermentation of cellulose as a sole carbohydrate source is impeded by the chicken and egg conundrum. The co-expression of xylose utilising enzymes along with cellulase genes removes this limitation. The initial metabolism of the simple sugar xylose facilitates cellulase production resulting in sequential pentose and hexose sugar utilisation. Ethanol yields (% of theoretical ethanol) reached 81.86% in xylose and PASC fermentations using *XKS1* overexpressing strains. The reduction in yield using *XKS1* overexpressing strains in cellobiose and xylose co-fermentations (55.61%) was not observed in xylose and PASC co-fermentations, most likely because of the

sequential nature of xylose and cellulose utilisation or the slow glucose release over time from PASC enhancing co-utilisation. It must be noted that the reduced theoretical glucose concentration of PASC (25g/L theoretical glucose, see Section 2.1.3) compared to cellobiose (50g/l) in fermentations may have an effect on ethanol production efficiency.

Cellulose fermentation even in co-utilising strains is still severely limited by low cellulase activity at mesophilic fermentation temperatures, highlighted in *XKS1* overexpressing strains (Fig 5.4B) where no difference between xylose alone and xylose + PASC fermentations was seen until day 10.

The slow and minimal release of glucose from PASC due to low level cellulase activity at 30°C, however appears to be beneficial towards the co-utilisation of xylose and cellulose. The controlled low-level of glucose release from complex sugars minimises inhibitory effects of glucose on xylose utilisation and fluxes within the cell. Low level cellulase activity as seen within this study might allow for a more efficient co-utilisation of xylose and cellulose within a single reaction, although the increased complex nature of lignocellulosic biomass compared to PASC may actually cause severely extended fermentation times as a result of limited cellulase activity.

The new data presented within this chapter demonstrates the sequential utilisation of pentose and hexose sugars allowing for efficient ethanol production from xylose and cellulose. The utilisation of cellulose is dependent upon the presence of the pentose sugar xylose as no ethanol was produced in any PASC alone fermentations. Initial pentose sugar metabolism facilitates the production of cellulase enzymes resulting in gradual cellulose hydrolysis and subsequent hexose sugar fermentation, without the need for an elevated temperature pre-hydrolysis step or the addition of commercial cellulase enzymes.

Chapter 6

Extraction and fermentation of sugars from lignocellulosic biomass by engineered *S. pastorianus*

6.1 Introduction

The ability of strains to co-utilise xylose and cellulose has been shown in the previous chapter. As a proof of principle it is important to show that purified cellulose and xylose can be co-utilised efficiently without the addition of commercial enzymes, however for application to industry, production of ethanol from actual biomass must be demonstrated.

Many different sources of biomass have been identified for bioethanol production, these include waste products from industry or dedicated fuel crops.

In previous chapters recombinant yeast strains expressing genes encoding xylose utilising enzymes and cellulase enzymes were used to demonstrate the co-fermentation of purified xylose and cellulose. Lignocellulosic biomass is far more complex and recalcitrant than purified substrates and chemical or physical pre-treatments are required. A commonly used pre-treatment involves dilute acid under high temperature and pressure. Acid treatment exposes amorphous cellulose, and increases the effective concentration of cellulose through the solubilisation of the hemicellulosic fraction. Dilute acids can hydrolyse hemicellulose very efficiently, however during the pre-treatment process various compounds are released that limit the efficiency of biomass fermentation. The three main inhibitors released are acetic acid, furfurals and phenols. Acid hydrolysis of hemicellulose releases acetic acid along with other components such as xylose, mannose, galactose and glucose. The high temperatures and pressure used during pre-treatment can cause xylose to be further degraded into furfurals. Phenols can be formed by the continued carbohydrate degradation and also through the partial breakdown of lignin. The presence of these inhibitory compounds along with the increased complexity of biomass limit current ethanol production from lignocellulose.

The aim of this chapter is to compare sugar extraction from various biomass sources using a dilute acid pre-treatment, and the subsequent fermentation of the extracted sugar in the liquid phase. To fully utilise biomass both the solid and liquid phase post acid pre-treatment must be utilised. The hydrolysis of the insoluble phase using commercial and recombinant cellulase enzymes for further sugar extraction was examined. The overall aim of this chapter is to combine both liquid and insoluble phases into a single fermentation to allow co-utilisation within a single vessel.

6.2 Dilute acid pre-treatment of biomass and hemicellulosic hydrosylate (liquor) fermentation

To produce ethanol from lignocellulosic biomass sugars must first be extracted from biomass by physical, biological or chemical means. Initially hexose and pentose sugars were extracted from biomass by dilute acid pre-treatment (Section 2.1.4). Various types of biomass were obtained from industrial and agricultural sources. The milling prior to pre-treatment enabled easier handling of biomass. Spent grain, obtained from various breweries and *E. nitens* samples were milled prior to acquisition. The stem and leaf components of *M. sinensis* and *M. giganteus* were separated and milled individually prior to pre-treatment to determine which part was more beneficial for sugar extraction. The milling of the stem from *M. giganteus* was found to be extremely difficult, the size of stem fragments were still large even after milling (Fig 6.1), due to its robust nature compared to other grass stems. Three different batches of spent grain and *M. sinensis* were used, while only one batch of both *E. nitens* and *M. giganteus* were examined.

For sugar extraction, biomass sources were treated with 4% H_2SO_4 (w/v) under heat and pressure conditions. After pre-treatment the remaining solid biomass was separated from the liquid phase (referred to here as liquor) by centrifugation and neutralised as described in Materials and Methods (Section 2.1.4). The glucose and xylose content of the liquor was then quantified (Table 6.1). The xylose content of the liquor was relatively consistent between different biomass types, however a reduced level was seen from *M. giganteus* stem and leaf fractions. In comparison the glucose content varied greatly between biomass types. The highest glucose yield was achieved using spent grains (16.97g/L), however glucose concentrations showed large variation between batches (5.43-31.22g/L). The glucose content of both *M. giganteus* and *M. sinensis* stem liquor was 2-fold greater than that of the corresponding leaf liquor. The lowest glucose content was from *E. nitens* liquor, which contained little to no glucose.

Figure 6.1 Lignocellulosic biomass. Different biomass sources (Table 2.1) after drying at 60°C and milling. Spent grain and *E. nitens* were pre-milled before they were obtained, however *M. sinensis* and *M. giganteus* stem/leaf fraction were milled using a blender at full speed for 5 minute.

Table 6.1 Sugar extraction by dilute acid pre-treatment of biomass. Glucose (g/L) and xylose (g/L) concentration in liquor after 4% (w/v) H_2SO_4 treatment of biomass (100g/L) at high temperature (100°C) and pressure (5Psi) for 40 minutes. After pre-treatment liquor was neutralised and filtered (Section 2.1.4) before glucose and xylose quantfication. Spent grain and *M. sinensis* values are the averages of duplicate treatments from three different batches. ± represents the standard deviation between the averages of the three different batches. *M. giganteus* and *E. nitens* values are the averages of duplicate treatments from a single batch, with duplicate readings in all experiments. ± represents the standard deviation between the averages of the two individual pre-treatments.

Biomass	Xylose (g/L)	Glucose (g/L)
Spent grain	11.63±2.13	16.97±13.11
M. sinensis stem	12.17±1.09	9.56±2.55
M. sinensis leaf	11.82±0.64	3.70±0.46
M. giganteus stem	4.98±1.85	11.18±0.28
M. giganteus leaf	8.33±1.06	6.25±2.23
E. nitens	15.71±0.64	0.98±0.088

Liquor generated from the three batches of spent grain and *M. sinensis* stem/leaf were used as a sole carbohydrate source for fermentations. Strains CM-51 PGKxr-PGKxdh and CM-51 pRS42H were grown in YEP-D media for 24Hrs, cells were harvested, washed and then inoculated into biomass liquor (plus 30g/L YEP) at a final cell density of $1x10^8$ cells/mL. Fermentations were incubated at 30°C for up to 5 days and samples taken at intervals for ethanol quantification. Peak ethanol levels were seen after 2 days (Fig 6.2). For all biomass sources the xylose utilising strain CM-51 PGKxr-PGKxdh produced higher levels of ethanol than the empty vector containing strain CM-51 pRS42H. Ethanol levels (Day 2) were similar between *M. sinensis* stem (5.72g/L) and spent grain (6.65g/L) liquor fermentations, however the variation between spent grain batches was far greater, mirroring the large variation in glucose sugar concentrations between spent grain batches. The biomass source that produced the lowest ethanol level was *M. sinensis* leaf, with levels being two fold less than *M. sinensis* stem and spent grain liquor fermentations.

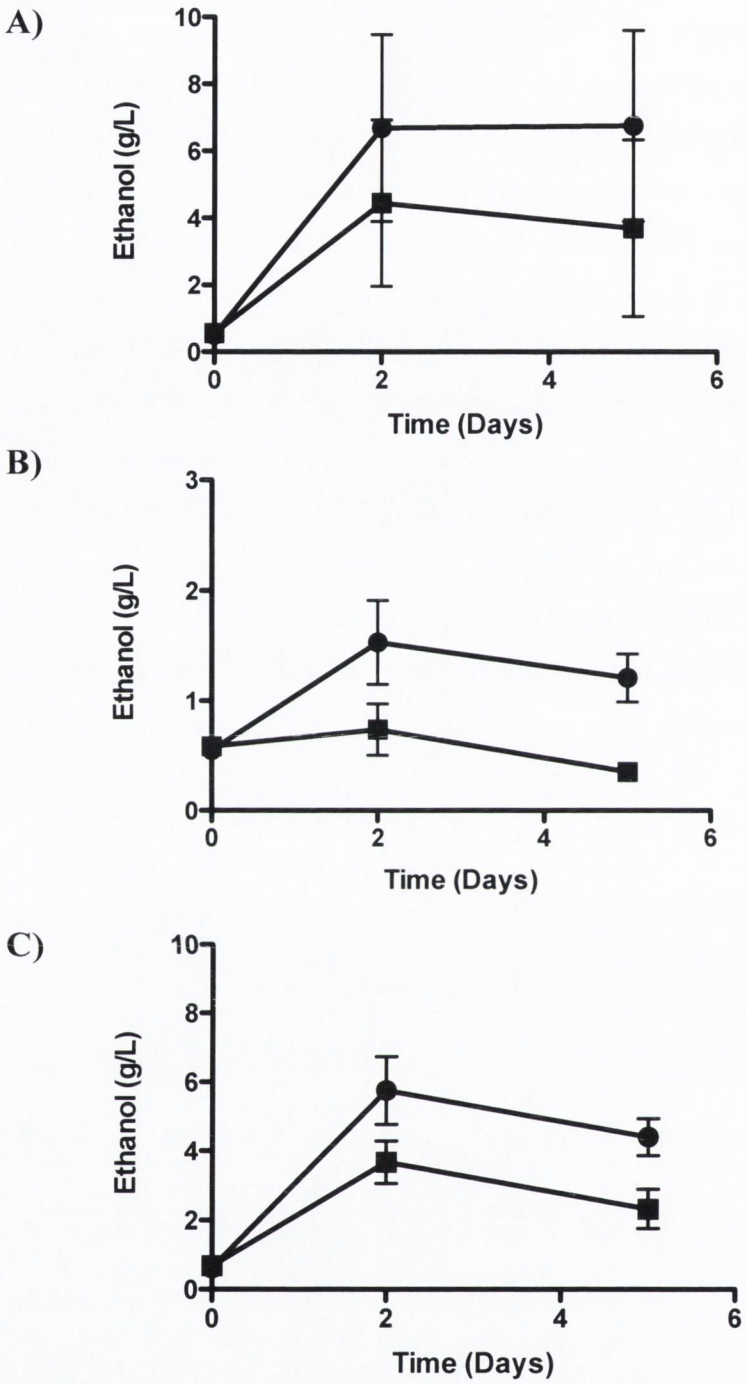


Figure 6.2 Biomass liquor fermentations. Ethanol production (g/L) from spent grain liquor (A), *M. sinensis* leaf liquor (B) and *M. sinensis* stem liquor (C) fermentations using strains CM-51 PGKxr-PGKxdh (●) and the empty vector containing strain CM-51 pRS42H (■). Strains were grown in YEP-D (20g/L) at 30°C. After 24Hrs cells were harvested, washed and inoculated at a high cell density ($1x10^8$ cells/mL) into fermentation media and incubated at 30°C for up to 5 days. All biomass liquor was supplemented with YEP (30g/L). Liquor was generated using dilute acid (4% H_2SO_4) pre-treatment of biomass at a final concentration of 100g/L. Values are the average of three independent experiments, with duplicate readings in all experiments, error bars represent the standard error between triplicate experiments.

6.2.1 Comparison of spent grain liquor and purified xylose and glucose

Ethanol production from biomass liquor was compared to a purified sugar mixture to identify if inhibitors produced in the pre-treatment of biomass limit fermentation efficiency. Spent grain biomass was chosen to be the focus of the remaining work in this study due to its relatively high glucose and xylose extraction and its classification as a by product. From the three different batches of spent grain it was decided that batch 2 would better mimic the industrial application of bioethanol production.

To increase sugar extraction from spent grain, the amount of biomass that was pre-treated was increased from 100g/L to 250g/L. The increase in biomass loading led to an increase in xylose and glucose release, with levels in spent grain liquor reaching 29.95g/L and 12.20g/L respectively.

The overexpression of *XKS1* along with *xyl1* and *xdh1* has previously shown to improve xylose fermentation at high xylose concentrations. For this reason the strain CM-51 PGKxr-TEFxks-PGKxdh was used along with the empty vector containing strain CM-51 pRS42H. Strains were grown in YEP-D media for 24Hrs. Cells were harvested, washed and inoculated into either spent grain liquor or a purified sugar mixture (15g/L glucose, 30g/L xylose) to a final cell density of $1x10^8$cells/mL (it must be noted that glucose concentration varied slightly between purified sugar media and spent grain liquor media). Fermentations were incubated at 30°C for up to 5 days, with samples being taken at intervals for ethanol, glucose and xylose quantification. Ethanol levels peaked after 24Hrs in spent grain liquor, with CM-51 PGKxr-TEFxks-PGKxdh reaching 10.16g/L and CM-51 pRS42H reaching 7.86g/L (Fig 6.3A). Levels of ethanol produced by CM-51 PGKxr-TEFxks-PGKxdh in purified sugar fermentations peaked at day 5 with levels reaching 15.10g/L (Fig 6.3B). The strain CM-51 pRS42H reached peak ethanol levels in purified sugar fermentations faster than in spent grain liquor fermentations with levels peaking at 7.47g/L after 3 hours. The strain CM-51 pRS42H showed ethanol consumption in both fermentation conditions (spent grain liquor and purified sugar mix). This was not observed in xylose utilising strain CM-51 PGKxr-TEFxks-PGKxdh fermentations.

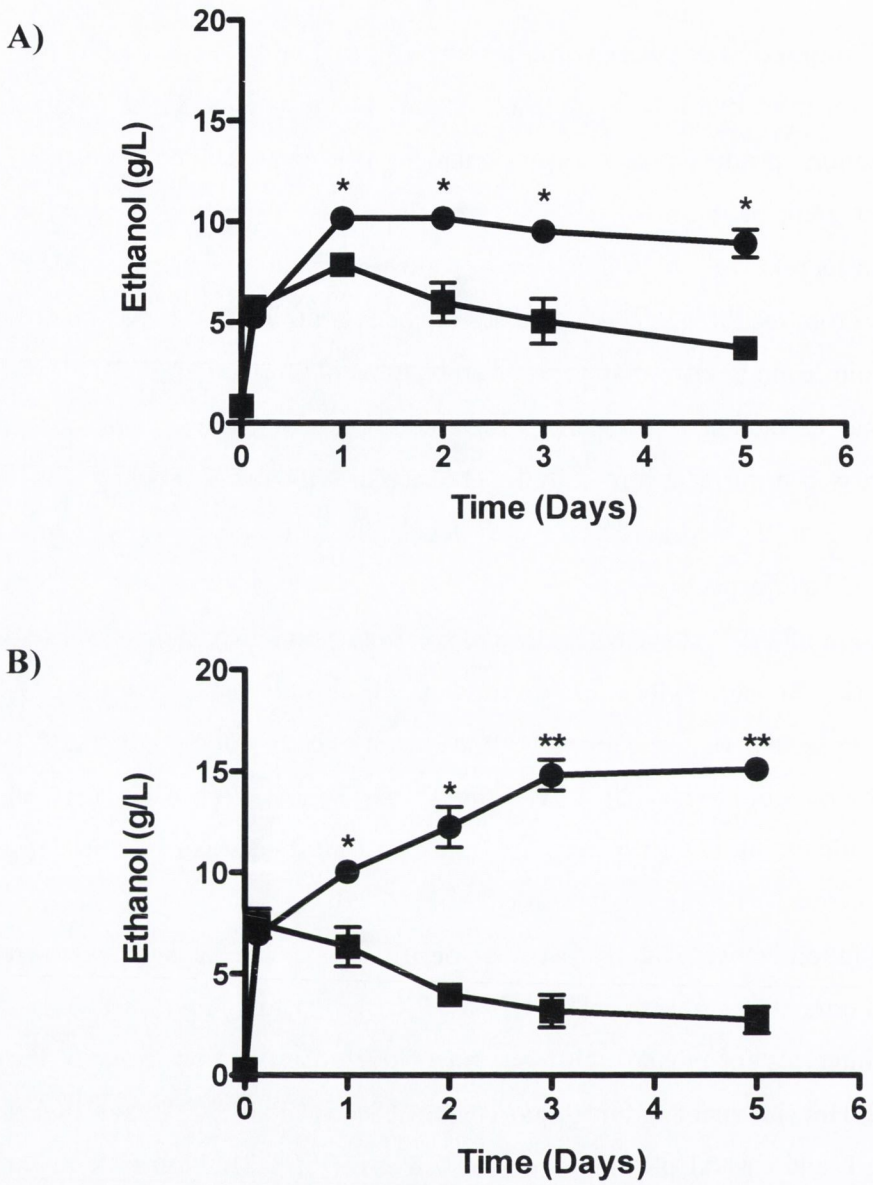


Figure 6.3 Comparison of ethanol production from spent grain liquor and purified sugar fermentations. Ethanol production (g/L) from spent grain liquor (A) and purified xylose (30g/L) + glucose (15g/L) (B) fermentations using CM-51 PGKxr-TEFxks-PGKxdh (●) and the empty vector containing control strain CM-51 pRS42H (■). Strains were grown in YEP-D (20g/L) at 30°C. After 24Hrs cells were harvested, washed and inoculated at a high cell density ($1x10^8$ cells/mL) into fermentation media and incubated at 30°C for up to 5 days. Liquor was generated using dilute acid (4% H_2SO_4) pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of three independent experiments, with duplicate readings in all experiments, error bars represent the standard error between triplicate experiments. Statistical significance of ethanol production between CM-51 PGKxr-TEFxks-PGKxdh (●) and CM-51 pRS42H (■) at individual time points was calculated using two-tailed students *t* test, *- $p<0.05$, **-$p<0.001$.

Comparison of glucose utilisation between spent grain liquor and purified sugar fermentations shows that glucose was more rapidly utilised in purified fermentations, however in all fermentations glucose was depleted by day 1 (Fig 6.4 A/B). Interestingly xylose utilisation was severely inhibited in spent grain liquor fermentations, with only 8.54g/L of xylose utilised by CM-51 PGKxr-TEFxks-PGKxdh by day 5 compared to 28.57g/L in purified sugar fermentations (Fig 6.5 A/B), showing that while hexose fermentation seems to be un-affected in spent grain liquor the utilisation of pentose sugars is greatly affected. This is supported by the similar ethanol levels in spent grain liquor and purified sugar fermentations by CM-51 pRS42H, as strains are only able to utilise hexose sugars.

To ensure that the reduced xylose utilisation observed in spent grain liquor fermentations was not due to the presence of non-fermentable xylose, purified xylose (20g/L) was added to spent grain liquor. Fermentations were set up as described previously using strains CM-51 PGKxr-TEFxks-PGKxdh and CM-51 pRS42H (Fig 6.6). Xylose utilisation was similar in spent grain liquor with (9.56g/L utilised) or without (11.21g/L utilised) added xylose, with both being severely reduced compared to the purified xylose and glucose mixture (25.97g/L utilised). This indicates that it is unlikely the xylose within the liquor is non-fermentable, and reduced xylose utilisation is associated with cellular xylose metabolism.

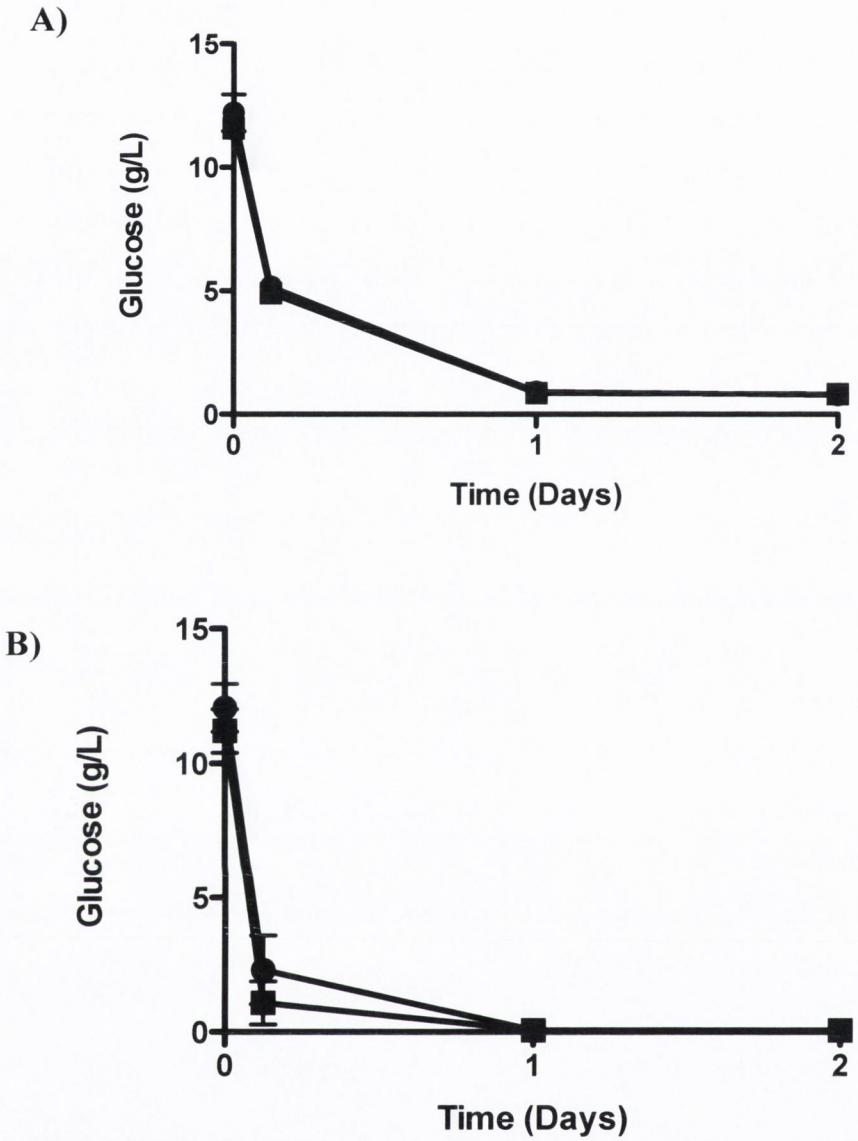


Figure 6.4 Comparison of glucose utilisation in spent grain liquor and purified sugar fermentations. Glucose utilisation (g/L) in spent grain liquor (A) and purified xylose (30g/L) + glucose (15g/L) (B) fermentations using CM-51 PGKxr-TEFxks-PGKxdh (●) and the empty vector containing control strain CM-51 pRS42H (■). Strains were grown in YEP-D (20g/L) at 30°C. After 24Hrs cells were harvested, washed and inoculated at a high cell density ($1x10^8$ cells/mL) into fermentation media and incubated at 30°C for up to 5 days (data only shown up till day 3). Liquor was generated using dilute acid (4% H_2SO_4) pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of three independent experiments, with duplicate readings in all experiments, error bars represent the standard error between triplicate experiments.

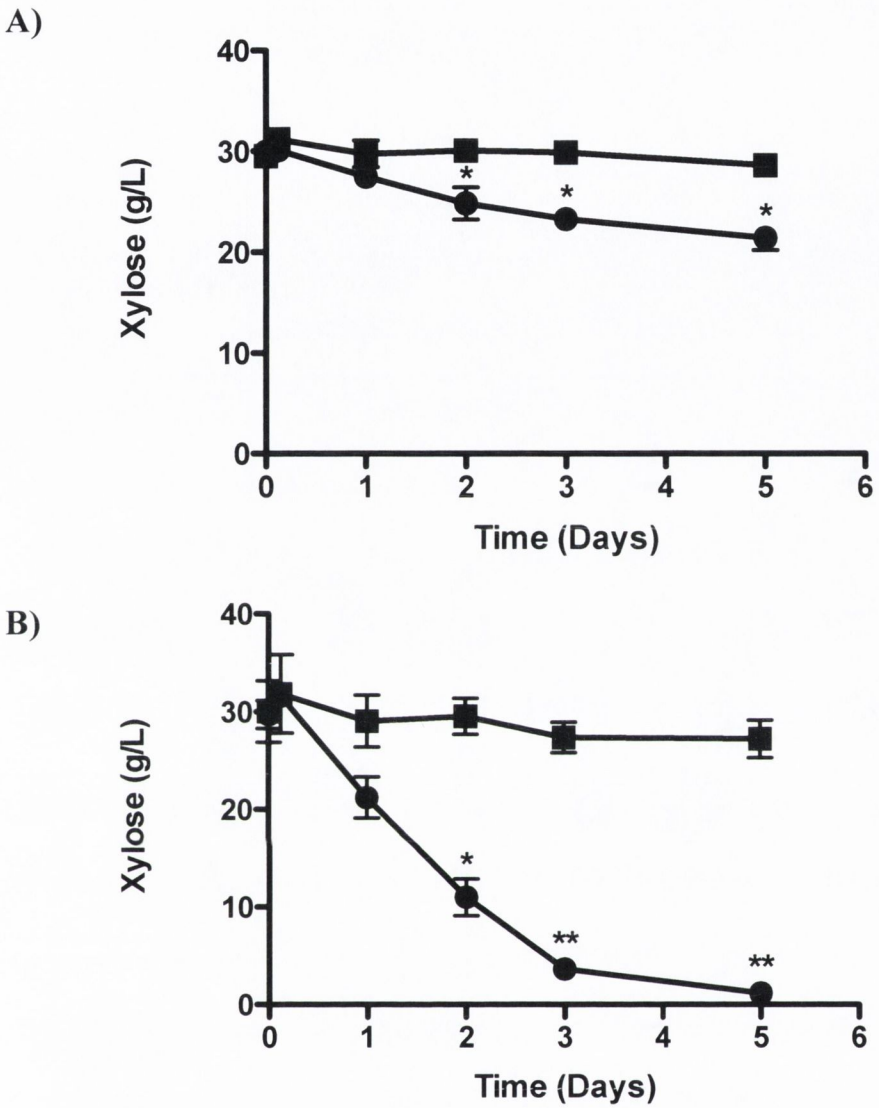


Figure 6.5 Comparison of xylose utilisation in spent grain liquor and purified sugar fermentations. Xylose utilisation (g/L) in spent grain liquor (A) and purified xylose (30g/L) + glucose (15g/L) (B) fermentations using CM-51 PGKxr-TEFxks-PGKxdh (●) and the empty vector containing control strain CM-51 pRS42H (■). Strains were grown in YEP-D (20g/L) at 30°C. After 24Hrs cells were harvested, washed and inoculated at a high cell density ($1x10^8$ cells/mL) into fermentation media and incubated at 30°C for up to 5 days. Liquor was generated using dilute acid (4% H_2SO_4) pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of three independent experiments, with duplicate readings in individual experiments, error bars represent the standard error between triplicate experiments. Statistical significance of ethanol production between CM-51 PGKxr-TEFxks-PGKxdh (●) and CM-51 pRS42H (■) at individual time points was calculated using two-tailed students *t* test, *- $p<0.05$, **- $p<0.001$.

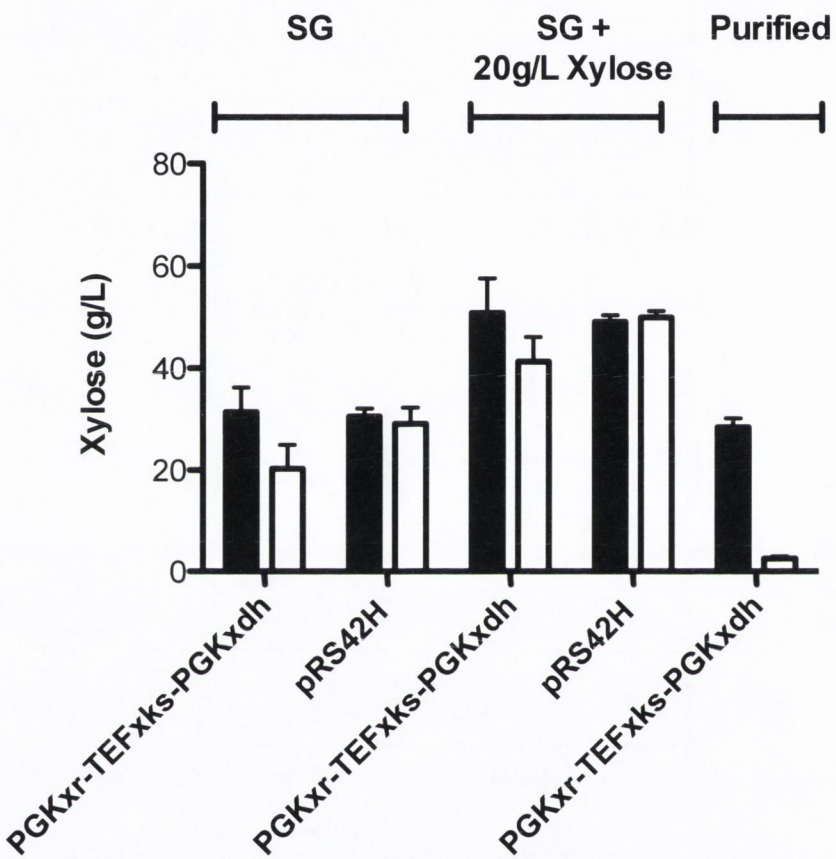


Figure 6.6 Inhibition of xylose utilisation in spent grain liquor fermentations. Xylose concentration (g/L) in spent grain liquor (SG), spent grain liquor + purified xylose (20g/L) (SG+20g/L Xylose) and purified sugar (30g/L xylose, 15g/L glucose) (Purified) fermentations at day 0 (□) and day 3 (■) using strains CM-51 PGKxr-TEFxks-PGKxdh (PGKxr-TEFxks-PGKxdh) and CM-51 pRS42H (pRS42H). Strains were grown in YEP-D (20g/L) at 30°C. After 24Hrs cells were harvested, washed and inoculated at a high cell density ($1x10^8$ cells/mL) into fermentation media and incubated at 30°C for up to 3 days. Liquor was generated using dilute acid pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of two independent experiments, with duplicate readings in individual experiments, error bars represent the standard deviation between the averages of two independent experiments.

To rule out the possibility that reduced xylose utilisation in spent grain liquor results from direct inhibition of the recombinant enzymes, XR and XDH activity was quantified at day 0 and day 3 of fermentations. Spent grain and purified sugar fermentations were set up as described previously, and samples were taken at day 0 and day 3 for XR and XDH enzyme quantification (Section 2.7.1). XDH activity was similar on day 0 and day 3 in both spent grain liquor and purified sugar mixture media (Fig 6.7), however XR activity was slightly reduced (1.4 fold) at day 3 compared to day 0 in spent grain liquor, while in purified sugar mixture XR activity showed no difference. This indicates that both enzymes are active in spent grain liquor fermentations at least until day 3. It is unlikely that the observed 1.4-fold reduction in XR activity observed on day 3 can account for the significant reduction in xylose utilisation in spent grain liquor fermentations.

Analysis of yeast cell counts in spent grain liquor and purified sugar mixture fermentations using CM-51 PGKxr-TEFxks-PGKxdh uncovered a two fold increase in the final cell density in purified sugar mixture compared to spent grain liquor fermentations (data not shown). The use of high cell inoculums in fermentations may actually mask the true affects of spent grain liquor on growth. To examine the affects of spent grain liquor on growth strains CM-51 PGKxr-TEFxks-PGKxdh and CM-51 pRS42H were inoculated at a low starting cell density, cultures were incubated at 30°C and growth monitored over time (Fig 6.8). Growth rates and final cell density were reduced dramatically in spent grain liquor compared to purified sugar mixture.

6.3 Biological extraction of sugars from spent spent grain (SSG)

The pre-treatment of spent grain biomass generated a liquid and solid phase. The liquid (liquor) phase was examined as a sugar source for fermentations as described above. The remaining solid phase was referred to as spent spent grain (SSG). To optimise the fermentation capacity of spent grain as a whole, the ability to further release glucose from SSG through cellulose hydrolysis was examined. After pre-treatment, the insoluble and soluble fraction were separated, via centrifugation. The remaining solid fraction (SSG) was washed and neutralised prior to use in hydrolysis reactions (as described in Materials and Methods).

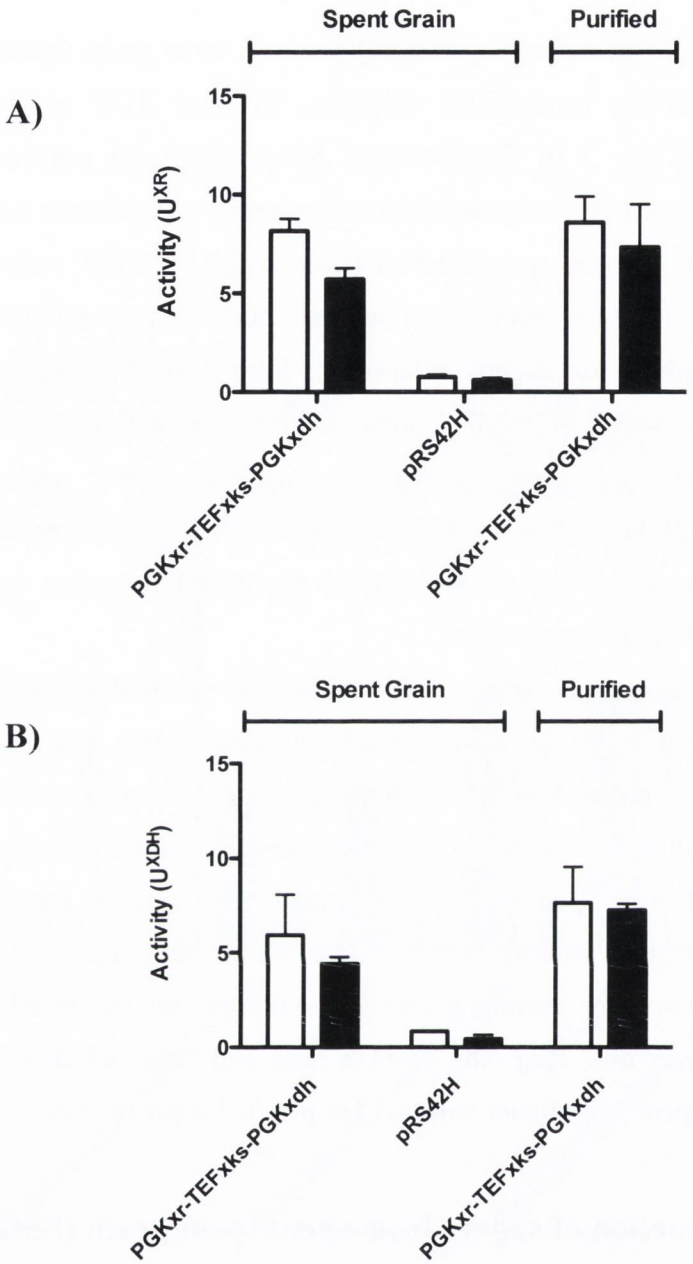


Figure 6.7 Enzyme activity of XR and XDH in spent grain liquor and purified sugar fermentations. XR (A) and XDH (B) activity at day 0 (□) and day 3 (■) in spent grain liquor (spent grain) and purified sugar (30g/L xylose,15g/L glucose) (purified) fermentations. Strains (CM-51 PGKxr-TEFxks-PGKxdh and CM-51 pRS42H) were grown in YEP-D (20g/L) at 30°C. After 24Hrs cells were harvested, washed and inoculated ($1x10^8$cells/mL) into fermentation media and incubated at 30°C for up to 3 days. Enzyme activity was determined from cell lysate. XR activity is defined as NADPH (μM) oxidised per minute, XDH activity is defined as NAD+ (μM) reduced per minute, both were standardised against protein level (μg) to give crude specific activity (U^{XR}, U^{XDH}). Liquor was generated using dilute acid pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of three independent experiments with duplicate readings in individual experiments, error bars represent the standard error between averages of three independent experiments.

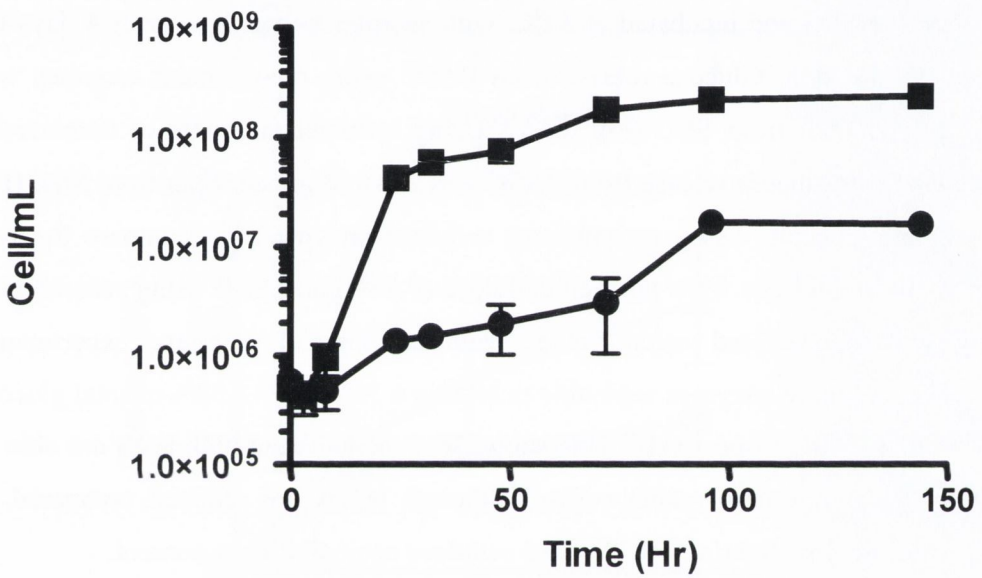


Figure 6.8 Cell growth in spent grain liquor and purified sugar media. Growth of xylose utilising strain CM-51 PGKxr-TEFxks-PGKxdh in spent grain liquor (●) and purified xylose (30g/L) + glucose (15g/L) media (■). Liquor was generated using dilute acid (4% H_2SO_4) pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of two independent experiments with duplicate readings in individual experiments, error bars represent the standard deviation between the averages of two independent experiments.

The ability of recombinant enzymes to hydrolyse SSG was examined. Strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh and CM-51 pRS42H) were grown individually in YEP-D media for 24Hrs. Cells were then harvested, washed, and cellulase expressing strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh) were combined in a 1:1:1 ratio and inoculated into fresh YEP-D media at a starting cell density of $1x10^8$cells/mL. The empty vector containing strain CM-51 pRS42H was also inoculated into YEP-D media to a final cell density of $1x10^8$cells/mL. After 48Hrs. supernatants from cultures were harvested and mixed with either PASC or SSG and incubated at 50°C, with samples being taken over 4 days for glucose determination. Glucose release from PASC using recombinant enzymes was 3.5-fold greater than from SSG (Fig 6.9A). Using saturated amounts of commercial cellulase enzymes glucose release from PASC was 2.5-fold greater than from SSG (Fig 6.9B), mirroring results using recombinant cellulase enzymes. To compare the two substrates glucose release from purified cellulose (PASC) and SSG using recombinant cellulase was standardised using values from commercial cellulase experiments. Recombinant cellulase enzymes were able to release 6.55% and 13.58% of total glucose from SSG and PASC respectively. This shows that recombinant cellulases are able to breakdown SSG into fermentable sugars, although levels are reduced compared to purified cellulose, highlighting SSG limited cellulose accessibility or content.

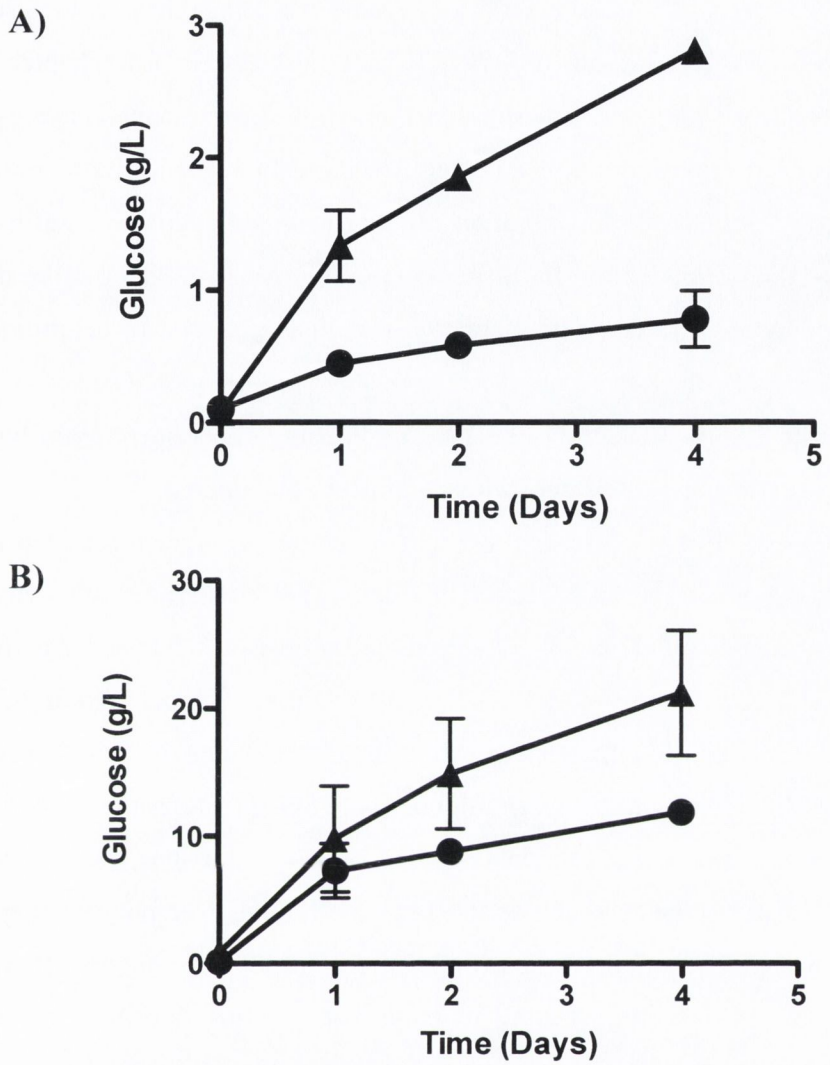


Figure 6.9 Hydrolysis of purified and natural cellulose using recombinant and commercial cellulase enzymes. Glucose release (g/L) over time from PASC (▲) or SSG (batch 2) (●) using recombinant (A) or commercial cellulase enzymes (A). To generate recombinant enzymes strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh) were grown in a co-culture (1:1:1 ratio) in YEP-D (20g/L) at 30°C. After 48Hrs supernatant was harvested. Recombinant or commercial cellulase enzymes (3U/mL) were incubated with either PASC (1000g/L) (25g/L theoretical glucose, see Section 2.1.3) or SSG (1000g/L) at 50°C for up to 4 days. Activity is defined in terms of glucose in g/L. Values are the average of two independent experiments with duplicate readings in individual experiments, with error bars representing the standard deviation between the averages of two independent experiments. Supernatant from CM-51 pRS42H was used as a control and no glucose release was observed (data not shown).

6.4 SSG and SG liquor co-utilisation

Xylose and cellulose co-utilisation has been demonstrated previously through the use of a co-culture system of three individual strains (Chapter 5) and in this chapter the fermentation of spent grain liquor, as well the ability of recombinant enzymes to hydrolyse SSG has been demonstrated. It was therefore logical to try and combine these two sugar sources within a single fermentation using xylose and cellulose co-utilising strains to optimise ethanol production from spent grains. If spent grains are to be used as a sole source of carbohydrates for fermentation then it is essential to determine if recombinant cellulase enzymes can be produced from spent grain liquor. The activity of BGL1 produced from CM-51 PGKxr-TEFbgl1-PGKxdh using either spent grain liquor or a purified sugar mixture as a sole carbohydrate source was compared.

Strains (CM-51 PGKxr-TEFbgl1-PGKxdh and CM-51 pRS42H) were inoculated into either spent grain liquor or a purified sugar mixture at a low starting cell density. Cultures were incubated at 30°C and samples were taken on day 3 for BGL1 activity quantification. BGL1 raw activity (mU) was greatly reduced in spent grain liquor media, with activities being 5-fold higher in purified sugar mixture media (Fig 6.10A).

The reduction in BGL1 activity in spent grain liquor could either be a result of the direct inhibition of BGL1 by spent grain, or the inhibition of BGL1 production. When enzyme activity was standardised to the final cell count (Fig 6.10B), BGL1 activity from spent grain liquor was similar to that found in purified sugar mixture. This suggests that the inhibition of growth observed in fermentations using spent grain liquor as a medium (Fig 6.8) results in low levels of BGL1 activity, and it is unlikely that the BGL1 enzyme is directly affected by spent grain liquor.

Although enzyme activity was severely affected when strains were grown in spent grain liquor, the co-utilisation of both SSG and spent grain liquor was examined. Strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh, CM-51 PGKxr-PGKxdh and CM-51 pRS42H) were grown individually in YEP-D. Cells were then harvested, washed and cellulase expressing strains (CM-51 PGKxr-TEFbgl1-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh, CM-51 PGKxr-PGKegl2-PGKxdh) were combined in a 1:1:1 ratio and inoculated into spent grain liquor and SSG (Section 2.1.4) to a starting cell density of $1x10^8$cells/mL.

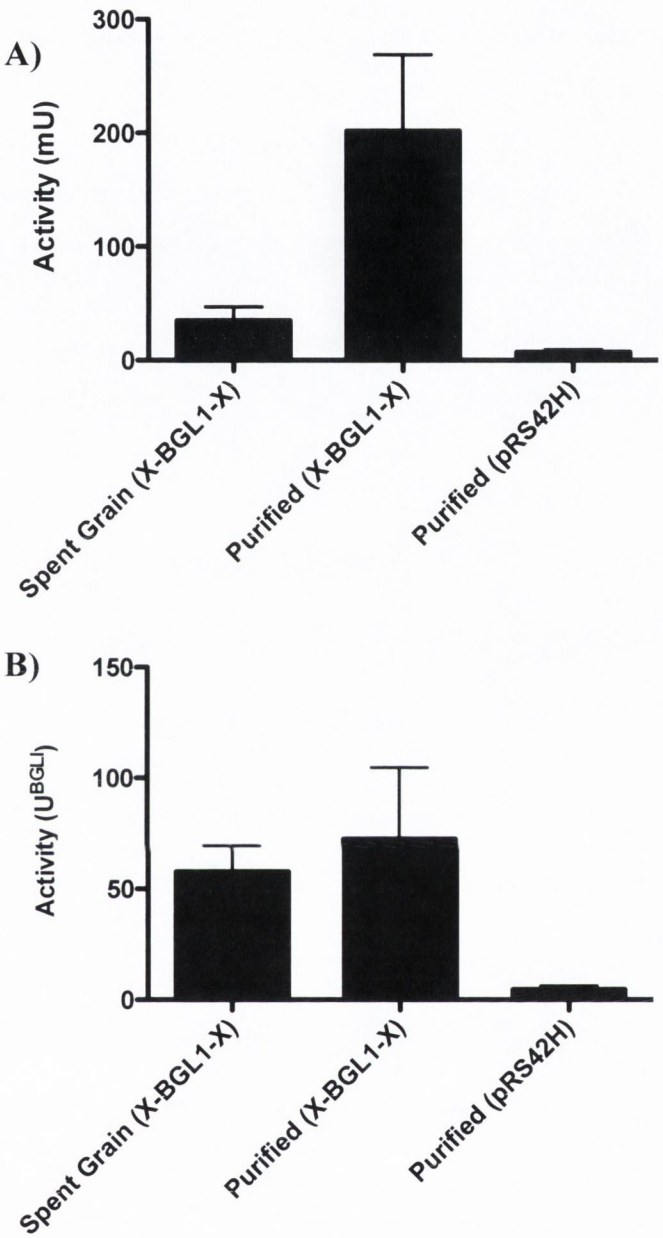


Figure 6.10 BGL1 activity in spent grain liquor and purified sugar media cultures. BGl1 activity in terms of raw activity in mU (A) or activity standardised against final cell number expressed in terms mU per $1x10^8$ cells (U^{BGL1}) (B) from spent grain liquor and purified sugar (30g/L xylose, 15g/L glucose) (Purified) fermentations using strains CM-51 PGKxr-TEFbgl1-PGKxdh (X-BGL1-X) and CM-51 pRS42H (pRS42H). Strains were inoculated into media at a low cell density ($1x10^5$cells/mL), cultures were incubated at 30°C. After 3 days BGL1 activity was quantified. Liquor was generated using dilute acid (4% H_2SO_4) pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of two independent experiments with duplicate readings in individual experiments, error bars represent the standard deviation between the averages of two independent experiments.

The empty vector containing control strain (CM-51 pRS42H) and the xylose utilising strain (CM-51 PGKxr-PGKxdh) were used as controls. Fermentations were incubated at 30°C, and samples were taken over a 10-day period for ethanol quantification. Ethanol production was similar in cellulase expressing and non-expressing strains and in strains containing the empty vector (Fig 6.11), indicating that no additional ethanol was produced as a result of the hydrolysis of SSG by recombinant cellulase enzymes. Therefore while the co-utilisation of purified xylose and cellulose was shown to be efficient, when using actual biomass it would appear that the effects on xylose utilisation, cellular growth and the increased complexity of the substrate limit the use of the co-utilisation strategy developed in chapter 5.

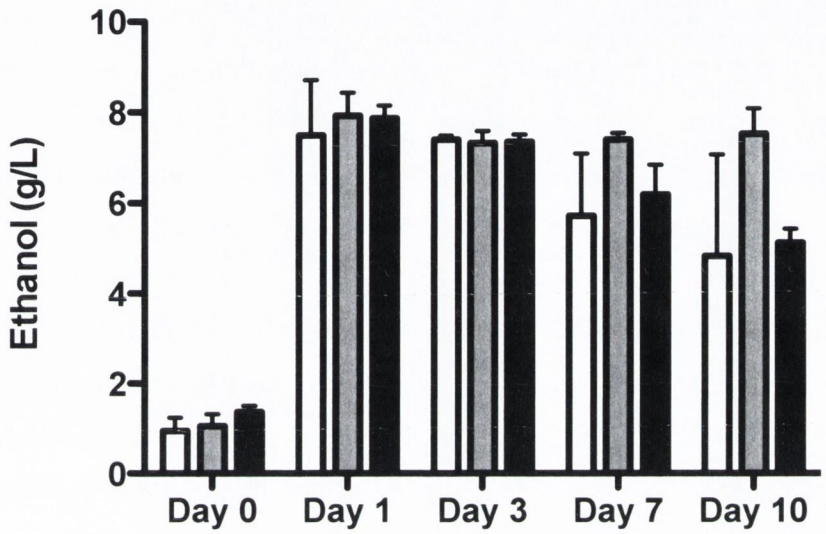


Figure 6.11 Spent grain liquor and SSG co-utilisation. Ethanol production (g/L) from spent grain liquor and SSG (500g/L) co-fermentations using either a co-culture of CM-51 PGKxr-PGKegl2-PGKxdh, CM-51 PGKxr-TEFcbh2-PGKxdh and CM-51 PGKxr-TEFbgl1-PGKxdh (X-Cel-X) (□) or CM-51 PGKxr-PGKxdh individually (▩) and CM-51 pRS42H individually (■). Strains were grown for 24Hrs in YEP-D (20g/L), harvested, washed and inoculated into fermentation media at a high cell density ($1x10^8$cells/mL) and incubated at 30°C for up to 10 days. Liquor and SSG were generated using dilute acid pre-treatment of spent grain batch 2 at a final concentration of 250g/L. Values are the average of two independent experiments with duplicate readings in individual experiments, error bars represent the standard deviation between the averages of the two experiments.

6.5 Discussion

The ability to produce ethanol from actual biomass is crucial if bioethanol is to be commercially viable in the future. Although in previous chapters ethanol from purified media (xylose and PASC) was shown, it is important to be able to transfer this into a biomass context.

The release of fermentable sugars from biomass is the first stage in bioethanol production from lignocellulosic biomass. Different strategies for initial pre-treatment of biomass have been examined (outlined in chapter 1). Within this study dilute sulphuric acid was used to fractionate the hemicellulosic from the cellulosic component. The use of dilute acid pre-treatment efficiently released xylose and glucose into the liquid phase from various biomass sources. Levels of xylose release were highest in *E.nitens*, although glucose release was almost not detectable. This trend has been shown previously in the acid pre-treatment of other *Eucalyptus sp.* (Gutsch et al., 2012; Pereira et al., 2014; Romani et al., 2011; Wei et al., 2012). Comparison of three different batches of spent grain showed great variation in glucose release. The reason for the difference is most likely due to either different variants of grain used or to the efficiency in initial sugar extraction from grains (resulting in higher levels of starch remaining within spent grain). Although this is hard to prove in this study as chemical compositions of spent grain batches have not been quantified, a significant difference between starch composition of spent grains produced from two barley variants was shown previously, while xylose composition between the two was similar (Vietor et al., 1993). Fermentation data showed ethanol levels from spent grain (6.65g/L) and *M.sinensis* stem liquor (5.72g/L) were similar to values shown previously for the fermentation of hemicellulosic hydrolysate (1.4-30g/L) from various biomass sources (Chandel et al., 2011; Gao and Xia, 2012; Katahira et al., 2006; Martin et al., 2007; Sasaki et al., 2013). Interestingly the ethanol production from *M.sinensis* leaf liquor was greatly reduced compared to that of *M.sinensis* stem liquor. The presence of various inhibitory molecules released by pre-treatment has been shown to reduce ethanol production and severely affect growth (Bajwa et al., 2013; Wang et al., 2014). The comparison of soluble phenols found in *M.sinensis* leaf and stem showed there to be an almost 3-fold increase in *M.sinensis* leaf (Parveen et al., 2013). Phenol compounds have been shown to greatly affect ethanol production and yield (Klinke et al., 2003), although

other compounds from pre-treatment such as weak acids also affect fermentation efficiency (Huang et al., 2011).

Ethanol production from spent grain (batch 2) liquor was reduced by 30% compared to purified sugar fermentations. Interestingly xylose metabolism was also severely reduced, while glucose utilisation seemed to be unaffected. The main inhibitory by-products of pre-treatment (weak acids, furfurals and phenols) have been shown to severely affect xylose utilisation (Wang et al., 2014). The addition of increasing concentrations of acetic acid to xylose and glucose co-fermentations showed a similar decrease in xylose utilisation, while glucose utilisation was unaffected (Hasunuma et al., 2011). In this study, the activity of both XR and XDH was not decreased significantly when strains were grown in spent grain liquor compared to purified glucose and xylose, suggesting that it is unlikely XR and XDH activity is the cause of the decreased xylose utilisation. Metabolome analysis identified that xylose metabolism in the presence of inhibitors is hindered due to the accumulation of various PPP intermediates (ribulose-5-phosphate, ribose-5-phosphate, eryhtrose-4-phosphate and sedoheptuose-7-phosphate) (Hasunuma et al., 2011), fitting with results indicating that XR/XDH activities are unlikely to be a cause for this inefficient xylose utilisation, rather that metabolism further down stream may be the problem. The overexpression of various PPP enzymes has little effect on ethanol yields from purified xylose (Bera et al., 2011), however an increase in ethanol levels in the presence of inhibitors has been shown in particular with the over-expression of the PPP gene *TAL1* (Fig 1.5)(Hasunuma et al., 2014; Hasunuma et al., 2011). Inhibitors clearly have global effects on the cells metabolism as studies have shown that the transcription of various genes involved with transcriptional and translational control, ribosomal and carbohydrate metabolism associated proteins are reduced in the presence of inhibitors (Bajwa et al., 2013; Li and Yuan, 2010).

The severely reduced cellulase enzyme activity produced from spent grain liquor is another road block in the co-utilisation of the spent grain liquor and SSG. Similar to other studies, the addition of commercial cellulase enzymes to insoluble acid pre-treated biomass was effective at releasing glucose from acid treated biomass (Lim and Lee, 2013; Wei et al., 2012), however the addition of commercial cellulase enzymes is a major cost. If cellulase enzyme production could be improved in spent grain liquor it would remove the cost of commercial cellulase, or at least reduce the required level used.

To improve fermentation efficiency from biomass it is clear that the presence of inhibitors must be addressed. One method has been to remove inhibitors chemically or by filtration (Chandel et al., 2007; Grzenia et al., 2012; Sasaki et al., 2014; Zhuang et al., 2009). This however increases the processing and hence the cost associated with biomass fermentation. The use of evolutionary engineering and mutagenesis to try and adapt yeast to better tolerate inhibitors has allowed for the identification of increased tolerant strains (Demeke et al., 2013; Koppram et al., 2012). Increased tolerance has been linked to an increase in fitness and cell viability in the presence of inhibitors (Almario et al., 2013; Heer and Sauer, 2008; Koppram et al., 2012; Wallace-Salinas and Gorwa-Grauslund, 2013), however significant increases in ethanol production from tolerant strains has been limited (Wallace-Salinas and Gorwa-Grauslund, 2013). Through the subsequent use of transcriptome analysis of tolerant strains possible key target genes leading to inhibitor tolerance have been identified, such as *ATP5* (ATP synthase) for acetic acid inhibition and various genes associated with furfural detoxification (*GRE3*) (Almario et al., 2013). A novel approach is to engineer strains to utilise the inhibitors for added ethanol production. Furfurals are already naturally metabolised by *S.cerevisiae* into corresponding alcohols (Taherzadeh et al., 1999), however acetic acid is not metabolised naturally by *S.cerevisiae*. Through the expression of acetylating acetaldehyde dehydrogenase (AADH) from *E.coli* (Wei et al., 2013), demonstrated that acetate was fully metabolized, improving growth and ethanol production.

Within this study inhibitor tolerance has not been addressed, however the approach of adaptive evolution through repetitive stress and selection for a required phenotype was used to previously generate the host strain in this study, *S.pastorianus* CM-51 (James et al., 2008). The high degree of genome plasticity observed within lager yeast could be utilised through repetitive culturing in stress conditions and subsequent selection, with the aim to generate a *S.pastorianus* strain able to tolerate the stresses associated with biomass hydrolysate fermentation. The generation of stress tolerant strains with improved xylose utilisation would allow for the possibility for the co-utilisation of spent grain liquor and SSG, although an elevated temperature hydrolysis step is likely to be required due to the complex nature of SSG.

Chapter 7

General Discussion

As the world enters the post fossil fuel era the need to identify renewable and more environmentally friendly fuel sources has grown. The development of 2nd generation bioethanol production sought to utilise lignocellulosic biomass over edible food crops as a substrate for fermentation. Due to increased complexity of lignocellulosic biomass, a pre-treatment step followed by enzymatic hydrolysis is required to release fermentable sugars.

To optimise ethanol production from lignocellulosic biomass both the cellulosic and hemicellulosic fractions must be utilised, however natural ethanologenic *Saccharomyces sp.* are unable to utilise cellulose or xylose. The requirement for the addition of commercial enzymes for hydrolysis prior to fermentations represents a significant cost in 2nd generation bioethanol production. If natural ethanologenic yeast could be engineered to express the required enzymes for simultaneous cellulose and xylose fermentation the cost of production would be decreased.

Individual cellulose and xylose fermentation by engineered *Saccharomyces sp.* has been demonstrated, however ethanol production from cellulose is limited by cellulase activity at fermentation temperatures and the complex nature of the substrate, while insufficient metabolic fluxes within the cell affect xylose fermentations.

The aim of this study was to demonstrate the co-fermentation of both C5 and complex C6 sugars, with the aim of optimising ethanol production from lignocellulosic biomass.

Cellulase activity at fermentation conditions limits cellulose fermentation

The cellulase genes selected for heterologous expression in this study were, *egl1*, *egl2*, *cbh2* and *bgl1*, all of which were sourced from the mesophilic fungal species *T. reesei*. Cellulase activity was increased through varying the host *Saccharomyces sp.*, promoter swaps and co-expression, however low activity at fermentation temperatures limited ethanol production from purified cellulose. The chicken and egg conundrum of cellulose fermentation was overcome in this study by the use of a pre-hydrolysis step using recombinant cellulase enzymes or by the co-utilization of xylose with cellulose. Ethanol levels reported using cellulose as a sole carbohydrate source (Chapter 3) (2.16g/L) were similar to values from the literature (Table 1.1), although fermentation times were far greater (14 days) and required an elevated pre-hydrolysis step. Fermentations yields were increased to 81% through the co-utilisation of xylose and cellulose, however fermentations still took up to 14 days. The length of

fermentation highlights the limitation of cellulase activity from recombinant yeast in fermentation conditions. Recombinant cellulase activity is clearly linked to gene copy number, although a 1:1:1 ratio of enzymes may limit the synergistic effect of enzymes. Cellobiohydrolase enzymes dominate expression in *T. reesei*, representing around 80% of secreted cellulase enzymes (Adav et al., 2013). Mimicking the natural cellulase enzyme ratio may enable the reconstruction of the natural synergy and high cellulose degradation shown by *T. reesei*. The heterologous expression of cellobiohydrolase enzymes is thought to be the limiting step in efficient cellulose hydrolysis and fermentation, due to low recombinant enzyme production by yeast (Den Haan et al., 2007a). The calculation of required total cellulase production by *S.cerevisiae* for efficient cellulose degradation was calculated to be around 1.5% of the total cellular protein (Lynd et al., 2005). Recent work has achieved this level of cellobiohydrolase expression in *S. cerevisiae*, with levels reaching 4% of total protein (Ilmen et al., 2011), although efficient cellulose fermentation has yet to be demonstrated using this specific cellobiohydrolase enzyme.

The identification of cold-adapted cellulase enzymes for heterologous expression could be key to improving activity at fermentation temperatures. The current practise of heterologously expressing cellulase enzymes with optimal activities between 50°C-70°C such as those from *T. reesei* (Kupski et al., 2014) and *C. thermocellum* (Johnson et al., 1982) could be modified by using cellulase enzymes from psychrophilic organisms with optimal cellulase activity temperatures similar to those found in fermentations (Ueda et al., 2014; Yang and Dang, 2011).

If the approach of using thermophilic cellulase enzymes from organisms such as *T. reesei* and *C. thermocellum* continues, an alternative approach is to tailor the fermentation process for optimal cellulase activity. The secretion of enzymes by the cell allows for the inclusion of an elevated temperature hydrolysis step prior to fermentations. This 2-stage fermentation process is already used in industrial brewing for the generation of wort and may offer the simplest solution to increasing ethanol production from cellulose.

Optimisation of xylose fermentation via XR/XDH pathway

The co-factor requirements of the XR/XDH pathway are thought to limit ethanol yield from xylose due to redox imbalances. Analysis of enzyme activity of both XR and XDH from *T. reesei* identified that enzymes were highly specific for the cofactors

NADPH and NAD+ respectively, however ethanol production still far exceeded that of the non-cofactor requiring XI pathway, fitting with previous studies (Bettiga et al., 2008; Karhumaa et al., 2007b). The comparison of enzyme activity between various *Saccharomyces sp.* showed that *S. pastorianus* produced the highest activity when compared to *S. cerevisiae* and *S. eubayanus*, with some evidence suggesting that ploidy may affect activity in *S. cerevisiae*. This trend of higher activity within *S. pastorianus* was due to higher recombinant protein levels, as demonstrated through GFP fusion constructs.

As with previous studies (Johansson et al., 2001; Matsushika and Sawayama, 2011; Toivari et al., 2001) the overexpression of *XKS1* further increased ethanol production and yield via the XR/XDH pathway. Interestingly this was only the case at high xylose concentrations, indicating that xylose fermentation via the XR/XDH pathway is limited by xylose concentration, and by Xks1 activity. Ethanol yields in this study reached 0.32g/g after 12 days, this suggesting that there are still limitations within xylose fermentations by *Saccharomyces sp.*

Generally there are two approaches that have been used to increase the xylose fermentation efficiency of the XR/XDH pathway. Alteration of the co-factor specificity of XR and XDH increases ethanol yields by alleviating redox imbalances through co-factor recycling, however only a limited increase in ethanol yields has been shown using this strategy. The other strategy is the manipulation of the PPP to improve metabolic fluxes within the cell. The overexpression of PPP enzymes Tal1, Rke1, Tkl1 and Rpe1 have all been examined in *S. cerevisiae*, however the effect of overexpression of these enzymes within a *S. pastorianus* background has yet to be examined. The use of an allotetraploid strain such as *S. pastorianus* in itself may already allow for varied expression of genes associated with PPP through increased copy number and its hybrid genome nature. Analysis of the *S. pastorianus* group 2 genome sequence identified that *S. pastorianus* CM-51 is likely to contain multiple copies of genes associated with the PPP from both *S. cerevisiae* and *S. eubayanus* (2 copies *TAL1*, 4 copies *TKL1*, 4 copies *RKI1*, 4 copies *RPE1*) (Monerawela and Bond, unpublished). The fermentation of the pentose sugar xylulose would allow a direct comparison of the hosts natural ability for pentose metabolism. The comparison of various *Saccharomyces sp.* natural ability for pentose sugar metabolism may offer an easier approach to optimising xylose fermentation by minimising the requirement for further genetic engineering.

Biomass fermentation limited by inhibitors

In this study the pre-treatment of biomass using dilute acid generated two distinct phases, a liquid phase (liquor) and an insoluble phase. Analysis of the liquid phase revealed both xylose and glucose could be efficiently extracted from biomass. The fermentation of biomass liquor by xylose utilising strains showed high levels of ethanol could be obtained from both spent grain and *M. sinensis* stem.

At high biomass loading sugar extraction from spent grain was increased using dilute acid pre-treatment. Interestingly the utilisation of the pentose sugar xylose in spent grain liquor fermentations was severely inhibited, whereas hexose sugar utilisation was unaffected. The analysis of enzyme activity indicated only a slight decrease in XR activity, however cell growth was severely reduced in spent grain liquor cultures. The ability to fully utilise glucose and not xylose sugars would suggest that the inhibition is within the PPP and the metabolism of xylose to fructose-6-phosphate and glyceraldehyde-3-phosphate. The analysis of metabolites would give a better insight as to where metabolism is affected, although previous studies would suggest the effect to be more global rather than a specific enzyme.

Adaptive evolution has successfully been used to improve tolerance towards inhibitors produced during pre-treatment (Wallace-Salinas and Gorwa-Grauslund, 2013).The improvement of stress tolerance of *S. pastorianus* strains has been demonstrated before using a chemical and heat shock strategy (James et al., 2008). This strategy could be applied to the generation of *S. pastorianus* strains with increased tolerance to inhibitors. The model of xylose and cellulose co-fermentation was unable to be applied to actual biomass fermentations due to reduced cellulase production and xylose utilisation using spent grain liquor. The use of actual biomass as opposed to purified cellulose and xylose identified the significant role inhibitors play in limiting ethanol production from lignocellulose.

In conclusion the research presented within this thesis has shown that through the genetic engineering of *S. pastorianus* the co-utilisation purified cellulose and xylose in a single fermentation vessel is possible. The pre-treatment of biomass using dilute acid allowed for high sugar extraction into the liquid phase, however inhibitors limited the ability to mirror results shown using purified substrates. The data shows the clear road-blocks in lignocellulose fermentation, these being the reduced cellulase activity at fermentation temperatures and the effect of inhibitors on xylose utilisation, however

using a devised 2-step fermentation process both spent grain liquor and SSG could be utilised (Fig 7.1). Initially xylose and cellulose strains are grown in rich media. The cells generated are then used in spent grain liquor fermentation, while the supernatant containing recombinant cellulase enzymes is incubated with SSG (Mashing stage) at an optimal temperature for cellulase activity. The SSG cellulase slurry is then separated (Laureting stage) into soluble (containing glucose release from cellulose) and insoluble fractions, with the soluble fraction being combined with spent grain liquor for a secondary fermentation. This process could easily be integrated into present day commercial brewery practise due to the presence of an elevated heating step already within the process, however success is heavily dependable on improved tolerance towards inhibitors allowing for improved xylose utilisation and increased recombinant cellulase yields.

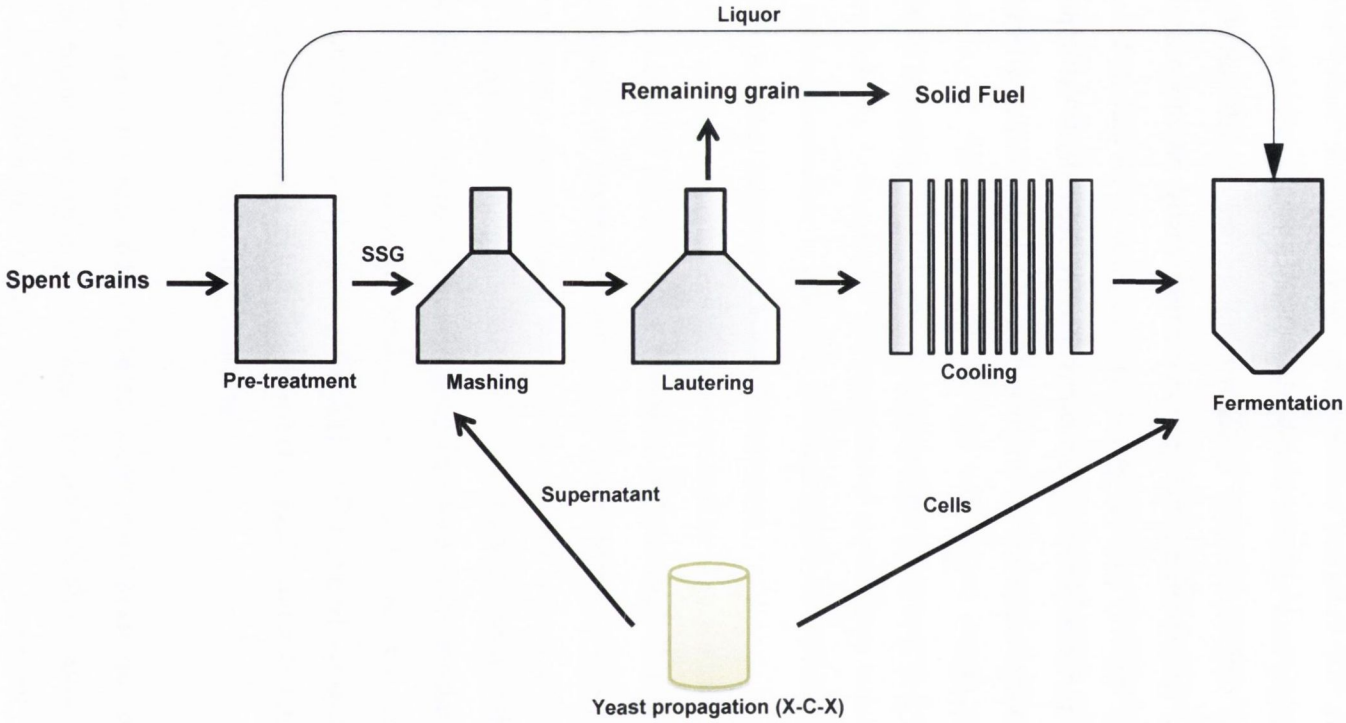


Figure 7.1 2-stage industrial fermentation of spent grain. Diagram illustrating a two stage process for spent grain liquor and SSG to be efficiently fermented to produce ethanol. The first stage involves the pre-treatment of spent grains to hydrolysis the hemicellulose and expose cellulose. The spent grain liquor and SSG are then separated and the liquor is combined with xylose and cellulose utilising strains (X-C-X) for 1st fermentation. The supernatant (cellulase containing) from yeast X-C-X propagation is incubated at high temperatures with SSG to hydrolyse cellulose in the mashing stage. The SSG and soluble sugars are then separated in the lautering stage. The soluble sugar mixture from SSG hydrolysis is added to liquor fermentation in a secondary fermentation. The remaining SSG can then be utilised as solid fuel

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