Improvement of xylose fermentation in *Saccharomyces cerevisiae*

ACKNOWLEDGEMENTS

Tiivistelmä:

Alati kasvava öljyn tarve sekä keskustelu fossiilisten polttoaineiden negatiivisista vaikutuksista ilmastonmuutokseen ovat pääsyitä ympäristöystävällisten polttoaineiden sekä kemikaalien kehittämiselle. Biopolttoaineiden, kuten bioetanolin, tuotanto on lisääntynyt laajalti maailmalla viime vuosikymmenten aikana. Tuotannossa pääsääntöisesti käytettävät raaka-aineet kilpailevat elintarviketeollisuuden kanssa, jonka vuoksi mm. lignoselluloosapohjaisten raaka-aineiden käyttöä kehitetään intensiivisesti. Jotta etanolin tuotto lignoselluloosapohjaisesta raaka-aineesta olisi kyllin kannattavaa, tulisi sekä heksoosi- että pentoosisokerit saada fermentoitua. Leivinhiiva (*Saccharomyces cerevisiae*) ei kuitenkaan kykene luontaisesti fermentoimaan pentooseja. Hiivasta on rakennettu ksyloosia fermentoivia kantoja, mutta niiden etanolituottokyky ei ole riittävä taloudellisesti kannattavaan etanolituottoon. Eräitä pullonkauloja ovat mm. ksyloosin kuljetus soluun, riittämätön adenosiinitrifosfaatin (ATP) tuotto sekä ksyloosireduktaasi (XR) ja ksylitolidehydrogenaasi (XDH) entsyymien käyttämien eri kofaktoreiden synnyttämä hapetus- ja pelkistysepätasapaino.

Tässä työssä leivinhiivan ksyloosimetaboliaan syvennyttiin kolmen erillisen tutkimuksen kautta. Työn ensimmäisessä osiossa yritettiin rakentaa seulontamenetelmää tehokkaiden ksyloosipermeaasien löytämiseksi. Työn kokeellisessa osiossa *Tricoderma reesei*n ksylaania pilkkovaa β-ksylosidaasia (BXL1) yritettiin tuottaa *S. cerevisiae*:ssa. β-ksylosidaasi pilkkoo ksyloosianalogi p-nitrofenyyli-β-d-ksylopyranosidia, joka hajotessaan saa aikaan kromogeenisen värireaktion, jonka avulla solun sisäänottaman substraatin määrä voidaan laskea. Tutkielman toisessa osiossa leivinhiivaan siirrettiin *S. cerevisialle* kodonioptimoitu *Trypanosoma brucei*n NADH-riippuvainen fumaraattireduktaasi. Tavoitteena oli lisätä NAD$^+$ saatavuutta solun sisäisesti samalla korjaten XR ja XDH entsyymien synnyttämää hapetus- ja pelkistysepätasapainoa. Rakennetuilla hiivakannoilla suoritettiin entsyymiaktiivisuusmittauksia ja niiden kykyä kasvaa ksyloosilla seurattiin pullokasvatuksissa aerobisesti ja anaerobisesti. Ksyloosin kulutusta, metaboliitteja sekä etanolin tuottotasoa mitattiin nestekromatografisesti. Myös työn kolmas osuus liittyy aihepiiriltään *S. cerevisiaen* solunsisäisen pelkistystasapainoon. Tutkimuksessa mitattiin VTT:n kantakokoelmaan kuuluvista ksyloosilla anaerobisesti/mikroaerobisesti kasvavista hiivoista XR ja XDH entsyymien aktiivisuuksia. Seulonnan tavoitteena oli löytää hiivoja, joiden entsyymejä voitaisiin hyödyntää paremmin ksyloosia fermentoivien *S. cerevisiaen* kantojen rakentamisessa.

Tutkimusten tulokset osoittivat, ettei BXL1 entsyymin avulla kyetty rakentamaan toimivaa ksyloosipermeaasien seulontamenetelmää. Kyseinen geeni ilmentyi hiivassa, mutta varsinaisen proteiinituotteen ilmentymistä aktiivisena ei pystytty osoittamaan. Myöskään fumaraattireduktaasin aktiivisuutta hiivassa ei pystytty mittaamaan kokeessa käytetyllä menetelmällä. Geenin siirrolla havaittiin kuitenkin olevan pieni tehostava vaikutus ksyloosia hyödyntävän *S. cerevisiae* kannan etanolin tuottokykyyn anaerobisissa olosuhteissa. Myös XR ja XDH aktiivisuusmittauksissa löydettiin muutama mielenkiintoinen hiivakanta, joiden entsyymit tarjoavat potentiaalisen vaihtoehdon *Scheffersomyces stipitiksen (aiemmin tunnettu Pichia stipitiksenä)* entsyymeille mm. etanolin tai ksylitolin tuotantoon liittyvissä tutkimuksissa.

Avainsanat: bioetanoli, lignoselluloosa, ksyloosi, *Saccharomyces cerevisiae*, ksyloosireduktaasi, ksylitolidehydrogenaasi, fumaraattireduktaasi

University of Jyväskylä   **Abstract of Master´s Thesis**
Faculty of Mathematics and Science

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Title of thesis	Improvement of xylose fermentation in *Saccharomyces cerevisiae*
Finnish title:	*Saccharomyces cerevisiae* hiivan ksyloosifermentaation tehostaminen
Date:	2.5.2012 **Pages:** 71

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Abstract:

The increasing need of oil and the negative impacts of fossil fuels on climate change have motivated the research towards environmental friendly alternatives to diminish the use of fossil resources. Until today, bioethanol production and use has increased worldwide. The raw materials used today compete with food industry and conquently, the ethanol production from lignocellulose has been developed intensively. However, to make the processes economically competitive, also pentoses in lignocellulose should be fermented at high rate. *Saccharomyces cerevisiae*, alias baker's yeast has an excellent ethanol production capacity but it can not naturally utilize xylose. Several xylose-utilizing *S. cerevisiae* strains have been constructed; however the fermentation rate is still lower than that of hexose sugars. The major obstacles are thought to be the xylose transport, ATP depletion during the fermentation and the redox imbalance due to the different cofactor specifities of xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes in the xylose utilization pathway.

This thesis includes three separate studies related to xylose metabolism of recombinant *S. cerevisiae*. In the first part the aim was to set up a high throughput xylose uptake assay by overexpressing a gene encoding β-xylosidase enzyme from *Tricoderma reesei* in *S. cerevisiae*. The experimental part included the enzyme activity measurements by using commercial xylose analogue p-nitrophenyl-β-d-xylopyranoside, which creates yellow colour when hydrolyzed with β-xylosidase. In the second study *Trypanosoma brucei*'s NADH dependent fumarate reductase (FRDg) encoding gene was overexpressed in xylose utilizing *S. cerevisiae* in order to improve ethanol fermentation by providing intracellular NAD^+ for the XDH reaction. Aerobic and anaerobic shake flask cultivations were carried out with the constructed strains. The xylose utilization and metabolite and ethanol productivities were measured with high pressure liquid chromatography (HPLC). In the third part of the study, XR and XDH activities were measured spectrophotometrically from the xylose-utilizing yeast strains from the VTT Culture collection. The aim of the study was to find new enzymes to be exploited in the engineering of xylose metabolism of *S. cerevisiea*.

The xylose transporter assay was not functional. The BXL1 gene was expressed in yeast, but no β-xylosidase activity was detected. The expression of FRDg slightly improved the xylose fermentation in xylose-utizing *S. cerevisiae* but the activity measurements did not show FRDg activity. In the XR and XDH activity measurements of strains from the VTT Culture collection a couple of strains with potentially interesting XR and XDH activities were found. These enzymes offer alternatives for *S. stipitis* XR and XDH enzymes in engineering of baker's yeast strains for ethanol or xylitol production.

Keywords: bioethanol, lignocellulose, xylose, *Saccharomyces cerevisiae*, xylose reductase, xylitol dehydrogenase, fumarate reductase

LIST OF ABBREVIATIONS

ATP	Adenoside triphosphate
BXL	β-xylosidase
bxl1	*T. reesei* gene encoding β-xylosidase
DDIW	Double distilled ion changed water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FADH₂	Flavin adenine dinucleotide in a hydroquinone form
FRD	Fumarate reductase
FRDg	*T. brucei* gene coding for fumarate reductase
GRAS	An organism classified as Generally Regarded As Safe
HPLC	High Perfomance Liquid Chromatography
HXT	Hexose transporter protein
LB	Luria-Bertani growth medium
Leu	Leucine
LEU2	*S. cerevisiae* gene coding for β-isopropylmalate dehydrogenase
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
K_m	Characterizes enzyme's affinity for a substrate
PAN	Peroxideacetylnitrates
PCR	Polymerase Chain Reaction
***PGK1* promoter/terminator**	Promoter region of *S. cerevisiae* gene encoding 3-phosphoglycerate kinase
PPP	Pentose Phosphate Pathway
RNA	Ribonucleic acid
rpm	Rounds Per Minute
SC	Synthetic Complete
SCD	Synthetic Complete medium with D-glucose
SCX	Synthetic Complete medium with D-xylose
SDS	Sodium Dodecyl Sulphate
SOC	Recovery broth for *E. coli* used after electroporation
TCA	Tricarboxylic Acid Cycle
Tris	Tris(hydroxymethyl)methylamine
ura	Uracil
URA3	*S. cerevisiae* gene encoding orotidine-5'-phosphate decarboxylase
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulokinase
XR	Xylose reductase
XYL1	Xylose reductase gene of *S. stipitis*
XYL2	Xylitol dehydrogenase gene of *S. stipitis*
XKS	Gene encoding xylulokinase enzyme

YPD   Yeast extract peptone with D-glucose
YPX   Yeast extract peptone with D-xylose

1   INTRODUCTION

1.1   Ethanol as a transportation fuel

The world wide energy crisis in the early 1970 initiated determined research and development aiming at sustainable production of fuels, *e.g.* bioethanol, and chemicals for biomaterials. Today the motivation towards production of environmentally friendly alternatives arises from the will to reduce the dependency on oil imported from the unstable regions of the world, depletion of fossil resources and wish to reduce the climate impact of fossil fuel combustion (Delucchi, 2010). By far the most common renewable fuel produced microbially, particularly by yeast, is ethanol (Gray et al., 2006). Plant biomass is a good option as raw material for the renewable bioethanol production since in theory additional CO_2 is not released to the atmosphere as the raw material binds the same amount of carbon when growing that is liberated during combustion of ethanol (Hill et al., 2006).

Bioethanol as a transportation fuel is an environmentally friendly alternative since its combustion produces mostly carbon dioxide, water and only minor amounts of carbon monoxide and nitrogen oxides. Furthermore, ethanol has high octane number and heat of evaporation, but is not as toxic as petrol. Ethanol, however, does not contain as much energy as petrol and its incomplete combustion liberates acetaldehyde to the atmosphere which can react with nitrogen and smog producing peroxideacetylnitrates (PAN). Both acetaldehyde and PAN are greenhouse gasses. Yet, the biggest drawback is its high price, which is the reason why ethanol is generally used as an additive in petrol. Together with gasoline, the oxygen contained in ethanol reduces the carbon monoxide, nitrogen oxide and hydrocarbon discharges. (Delucchi, 2010; Gray et al., 2006; Hill et al., 2006)

1.2   Oxidative phosphorylation and fermentation

Adenonine triphosphate (ATP) is the main energy source needed to drive the biochemical processes in the cells. Under aerobic conditions ATP is produced in glycolysis and in

oxidative phophorylation, which involves the electron transfer chain and ATP synthesis. When oxygen is not available ATP is only generated by glycolysis. In this section the principals of energy metabolism and ATP synthesis are explained under both conditions mentioned. The central carbon pathways and some of the closely connected reactions are presented in Figure 1.
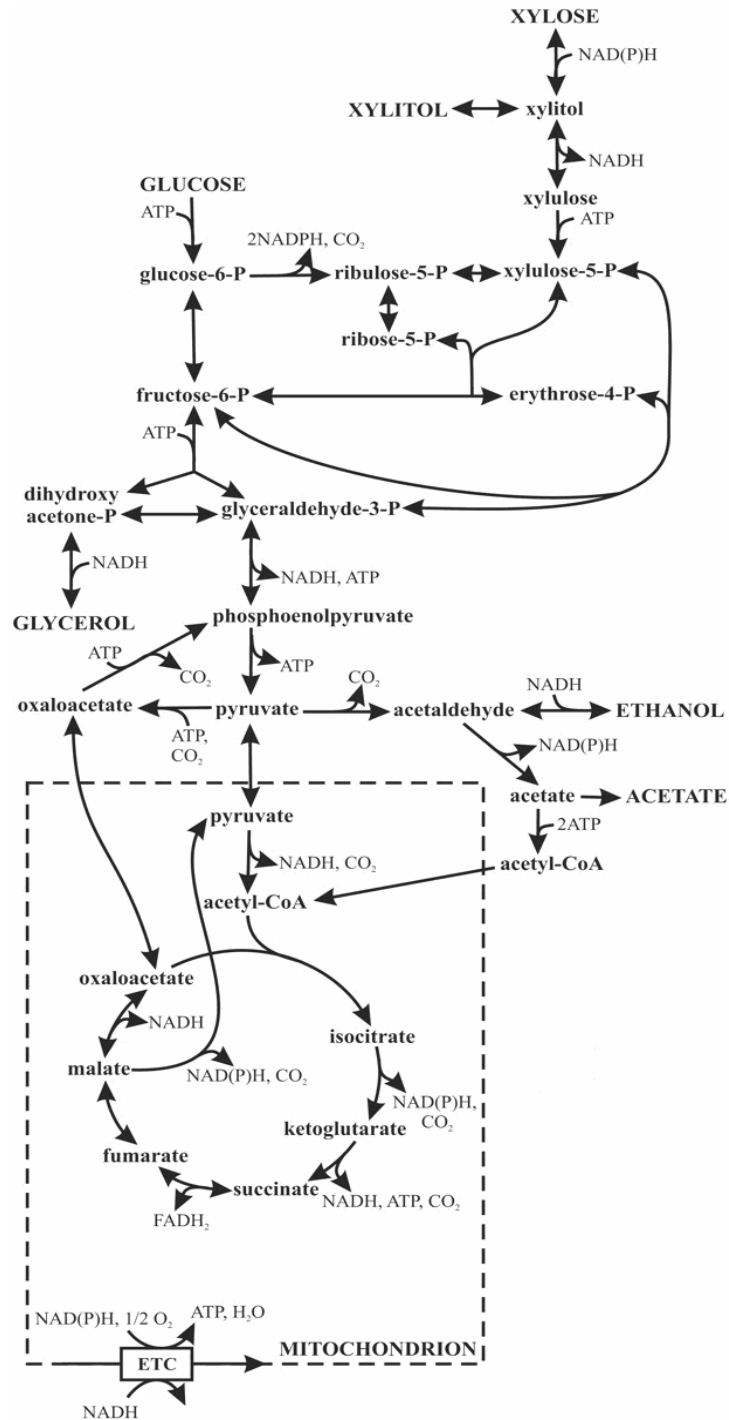

Figure 1. The central carbon catabolite pathways of xylose utilising Saccharomyces cerevisiae (The figure modified from Sonderegger et al. 2004).

In the cytosol, glycolysis produces ATP (ADP and P_i), pyruvate and NADH from glucose. When oxygen is present pyruvate is coverted to acetyl-CoA which is completely oxidized in the citric acid cycle (TCA) generating CO_2, H_2O and highly energetic electrons which are stored within the coenzymes NADH and $FADH_2$. The ATP generating process in the inner mitochondrial membrane is called oxidative phosphorylation and it involves the reduction of O_2 to H_2O through the electron transfer chain with the electrons donated by coenzymes NADH and $FADH_2$. The electron transfer begins when an H^- ion is removed from NADH and converted to an H^+ proton. The electrons travel through the chain until they reach the Complex IV and reduce O_2 simultaneously producing H_2O. The electronegative oxygen acts as a terminal electron acceptor of the chain. During the electron transfer the enzymes in the electron transfer chain and their prosthetic groups transfer H^- -ions across the inner mitochondrional membrane from the matrix into the intermembrane space, creating a proton gradient. H^- -ions can not return into the matrix without special transporters, ATP synthases. Besides ATP synthase, oxidative phosphorylation needs two other transfer proteins, one for ADP and ATP transport, the other to carry phosphates into the matrix. The oxidative phosphorylation produces 34 ATPs from one glucose molecule (at the most), water, NAD^+ and FAD^+. As glycolysis produces four ATP molecules and consumes two, the total ATP yield from one glucose molecule is 36 ATPs (Jouhten et al., 2008).

In oxygen-depleted conditions NADH generated by glycolysis can not be oxidized by O_2, leading to a situation where the cells do not have an electron acceptor for the oxidation of glyseraldehyde-3-phosphate, thus the energy yielding reactions of glycolysis would stop. Therefore, the cells must have an alternative to oxidize NADH to overcome the depletion of NAD^+. Some micro-organisms have the ability to continually generate NAD^+ during anaerobic glycolysis by transferring electrons from NADH to form reduced end products such as lactate and ethanol; this is called fermentation. Many micro-organisms such as yeast *Saccharomyces cerevisiae* prefer fermenting glucose to ethanol and CO_2 over lactate. The conversion of pyruvate to ethanol takes place in two steps (Fig. 1); first pyruvate is decarboxylated to acetaldehyde in a reaction catalysed by pyruvate decarboxylase (CO_2 released) and in the second reaction alcohol dehydrogenase reduces acetaldehyde to ethanol by using hydride ions from NADH (Jouhten et al., 2008; Bakker et al., 2000). The

fermentation generates only two molecules of ATP (in glycolysis) per one molecule of glucose since in the absence of oxygen the electron transfer chain is not functional.

1.3   Lignocellulosic hydrolysates as raw material for fermentation

To date the ethanol industry uses, for the most part, sugar cane, barley, corn, grain, wheat, straw and cotton as raw material for fermentation. The so called second generation bioethanol production aims at use of raw materials that do not compete with the food industry. The biofuel industry should also be able to exploit all sugars present in the raw material in order to make the production economically feasible. The raw material could be *i.e.* municipal solid waste or wood and agricultural residues. (Gray et al., 2006) Lignocellulose containing plant biomass is composed of cellulose (40-50%), hemicellulose (25-35%) and lignin (15-20%). Cellulose is an insoluble homopolymer of glucose monomers linked via β-1,4 glycosidic linkages making it a highly crystalline and compact structure that is very resistant to microbial degradation, while hemicellulose is a heteropolymer containing a xylan backbone with a random and amorphous structure with little strength (Horn et al., 2006). Hemicellulose contains different D-pentose sugars, mainly xylose, but also arabinose, and rhamnose and additional hexose sugars such as galactose and mannose (Gray et al., 2006; Binder and Raines, 2010). Lignin is covalently attached to hemicellulose by ferulic acid ester linkages. It is a heterologous polymer consisting of aromatic compounds that can not be fermented (van Maris et al., 2006). The relative proportion of the individual sugars depends on the raw material; hardwood and agricultural raw materials are rich in pentose sugars, while softwood contains only minor fractions of xylose and arabinose (Aristidou and Penttilä, 2000).

Release of sugars from the compact and heterogenous structure of lignocellulose requires enzymatic, physical and/or chemical disruption methods. Enzymatic saccharification methods are the most common ones; however, the physical and chemical pre-treatment processes are usually used together with cellulases to produce soluble sugar monomers (Binder and Raines, 2010). The enzymatic hydrolysis generally uses different thermophilic and hyperthermophilic cellulases including endo-acting (endoglucanases) and exo-acting (cellobiohydrolases) enzymes, mainly from filamentous fungi (*Tricoderma reesei* and

Aspergillus niger) and anaerobic bacteria (*Clostridium thermocellum* and *Clostridium cellulovorans*) (Fujita et al., 2004). However, the expense of the enzymes can be as much as one-third of the total cost of ethanol production from cellulose which is propably the biggest drawback of enzymatic hydrolysis (Kim and Ishikawa, 2010). One relatively novel approach to reduce the enzyme costs and to optimally utilize all sugars generated from lignocellulose would be the recycling of process water, however; this can lead to increased concentration of compounds that inhibit *S. cerevisiae*. These include low molecular weight organic acids, furans and aromatics that slow down the growth of *S. cerevisiae* and therefore reduce ethanol yield and productivity. However, utilizing *A. niger*, an organism that can utilize these inhibiting compounds as nutrients, would be convenient and thereby make the process water recycling more feasible. (Alriksson et al., 2009; Yang et al., 2010)

Engineering yeast strains that are able to both break down and ferment cellulose simultaneously is one approach to reduce the fermentation costs. For example Fujita *et al.* (2004) were among the first ones to demonstrate a successful saccharification and fermentation of *S. cerevisiae* codisplaying β-glucosidase 1, endoglucanase II, and cellobiohydrolase II from *T. reesei*, with a yield of 0.45 g g^{-1}. In order to further improve cellulose degradation for cost-effective ethanol production, Yamada *et al.* (2010) developed a simple method, named cocktail δ-integration, to optimize cellulase expression levels of recombinant *S. cerevisiae* under the desired conditions. With cocktail δ-integration several genes are introduced simultaneously with only a single recombination operation, and the strain with the highest activity can be improved by repeated cocktail δ-integration. Recently, instead using the conventional yeast *S. cerevisiae*, Yanase *et al.* (2010) engineered a *Kluyveromyces marxianus* strain to harbour *T. reesei* endoglucanase II and *Aspergillus aculeatus* β-glucosidase. The strain was able to ferment β-glucan to ethanol at 48 °C with a yield of 0.47 g g^{-1}, corresponding 92.2% of the theoretical yield. However, the effectiveness is, at least partly, due to the higher temperature used in the fermentation; the activity of cellulotic enzymes is usually highest at ~50 °C and decreases significantly while the temperature drops (Yanase et al., 2010).

Besides enzymatic pre-treatment processes, chemical methods such as dilute acid pre-treatment *e.g.* with HCl and H_2SO_4 and ammonia fiber expansion (AFEX) are regarded as

promising methods for the hydrolysis of lignocellulose in large-scale cellulosic biofuel production (Bosch et al., 2010; Lau et al., 2009). Dilute acid pre-treatment has been extensively investigated and developed both in the laboratory and at pilot scale to disrupt lignocellulosic biomass for fuel production. Dilute acid method is a dry-to-slurry process which effectively hydrolyzes hemicellulose to soluble sugars. In contrast, AFEX is a dry-to-dry process at alkaline pH using anhydrous ammonia as the reaction catalyst to reduce the degree of polymerization of cellulose and hemicellulose to increase enzyme accessibility for hydrolysis (Lau et al., 2009; Lau and Dale, 2009). AFEX technology can alleviate fermentation costs through the reduction of inoculum size and by practically eliminating nutrient costs during bioconversion. However, AFEX requires additional xylanases as well as cellulase activity to gain a complete hydrolysis. (Brown et al., 2010)

None of these pre-treatment methods alone are effective enough and therefore the proper combination of methods, along with optimized pH and temperature for a given feedstock is required for high yields of sugars from both cellulose and hemicellulose (Lloyd and Wyman, 2005). Yet, even more importantly, efficient bioethanol production from non-starch hydrolysates requires a fermenting organism that converts all types of sugars (hexoses and pentoses) to ethanol in high yield and high rate (Matsushika et al., 2008).

1.4 Pentose metabolism – bacteria, yeasts and filamentous fungi

In bacteria, xylose isomerase (XI, E.C 5.3.1.5) converts D-xylose to D-xylulose. The same enzyme catalyses also glucose isomerisation to fructose and therefore it is also called glucose isomerase (GI, XylA) (Lonn et al., 2002). Also some fungi have been reported to harbour XI activity. However, their xylose metabolism most often relies on an oxidoreductive pathway, where xylose taken up into the cell is first reduced to xylitol by xylose reductase (XR, EC 1.1.1.21) (Verduyn et al., 1985) and then oxidized to xylulose by xylitol dehydrogenase (XDH, EC 1.1.1.9) (Rizzi et al., 1989). In the pentose phosphate pathway xylulokinase (XK, EC 2.7.1.17) phosphorylates xylulose to xylulose-5-phosphate which enters the central carbon metabolism. The native anaerobic pentose assimilation, alongside with ethanol production, is limited only to a small number of organisms, since

many can utilize pentoses for growth, but only under non-fermentative conditions when oxygen is available (Wang and Schneider, 1980; Wang et al., 1980). To make ethanol production from lignocellulosic hydrolysates fully economically competitive, complete substrate utilization and high ethanol and inhibitor tolerance in anaerobiosis is essential. Hence, extensive amount of metabolic engineering has been carried out over the last couple of decades to improve pentose metabolism in microbes. (Bärbel Hahn-Hägerdal et al., 2000; Salusjärvi et al., 2008)
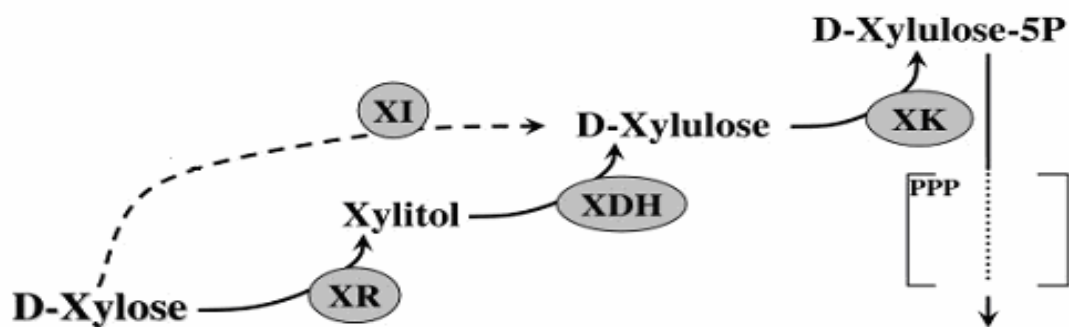
Figure 2. XR-XDH (yeast and fungi) and XI (bacteria) xylose utilization pathways (The figure modified from Bettiga et al., 2008).

The group of xylose utilizing bacteria comprises of native and genetically modified strains. The most extensively studied and engineered strains are gram-negative bacteria *Escherichia coli* and *Zymomonas mobilis*. With metabolic engineering both bacteria were able to ferment xylose and proved to be effective ethanol producing organisms, however, unfortunately most of these genetically modified bacterial strains are still extremely sensitive for high ethanol and inhibitor concentrations; in addition they produce high amounts of various side-products (Yang et al., 2010). Additionally, other bacteria, not so commonly known, have been studied. For instance, deletion of several genes of the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum* (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase involved in organic acid formation) resulted in a strain ALK2 able to produce ethanol from xylose as the only detectable organic product; 37 g/liter, which is the highest reported yield for a thermophilic anaerobe (Shaw et al., 2008).

The most studied native xylose-utilizing yeast are *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*) and different *Candida* species. *S. stipitis* can ferment xylose to ethanol at theoretical yields, but requires oxygen for complete xylose utilization, mainly because its XR prefers NADPH as a cofactor and XDH is strictly NAD^+-specific. This causes redox cofactor imbalance that under anaerobiosis results in xylitol and glycerol accumulation as oxidation of NADH in the electron transfer chain is not possible. (Salusjärvi et al., 2008; Karhumaa et al., 2005; Bruinenberg et al., 1983) XR of *S. stipitis* is, however, also able to use NADH as a cofactor which enables ethanol fermentation at low oxygen levels. In most yeast such as *Candida tropicalis* and *Candida guilliermondii* ethanol production is barely observed due to their strictly NADPH-specific XR, which on the other hand, results in large quantities of xylitol under oxygen-limited conditions. Therefore new species for pentose fermentation are looked for continuously, *e.g.* one of the new interesting candidates is *C. maltosa* that was shown to have an exclusively NADPH-dependent XR, but on the other hand, NADP-dependent XDH activity was measured, which lead to a significant accumulation of ethanol under oxygen-limited conditions. Furthermore, *C. maltosa* showed a strong ability to produce ethanol under aerobic conditions in high glucose concentrations, similar to that of *S. cerevisiae*. The alcohol dehydrogenases related to ethanol production of *C. maltosa* are still being investigated (Lin et al., 2010).

1.5   *Saccharomyces cerevisiae*

Yeast are eukaryotic fungal micro-organisms (Smith, 1989). The best known yeast *S. cerevisiae,* alias baker's yeast, is a facultative anaerobe *i.e.* it can grow under both aerobic and anaerobic conditions. It was the first organism that had its whole genome sequenced and published in 1996 (Goffeau et al., 1996). *S. cerevisiae* has 16 chromosomes and about 70% of its genome codes for proteins. Most of the strains include 50-100 copies of so called 2 μm plasmid, which has been a preminent tool of its molecular biology. As a GRAS (Generally Regarded As Safe) organism it is widely used in food industry *e.g.* in bakery, manufacturing of milk products and alcoholic beverages. Besides this, *S. cerevisiae* is used as a eukaryotic model/experimental system for molecular biology thus

offering new applications and commercial interests to biotechnology, agriculture and medicine (Goffeau et al., 1996; Ostergaard et al., 2000; Nevoigt, 2008).

Furthermore, compared to other organisms, the exceptional properties of *S. cerevisiae*, such as its high ethanol tolerance and efficient ethanol production from hexoses (90-95% of the theoretical yield) even at relatively low pH, makes it the prime choice for the industrial bioethanol production (Wenger et al., 2010). *S. cerevisiae* has also a high tolerance towards inhibitors generated as side products in the hydrolysis processes.

S. cerevisiae is able to ferment glucose to ethanol even under aerobic conditions. This is due to the so called Crabtree effect; *S. cerevisiae* has a completely respiratory metabolism at low glycolytic fluxes, but when the specific glucose uptake rate (or the glycolytic flux) exceeds a threshold rate, ethanol and glycerol are formed (Wiebe et al., 2008; Vemuri et al., 2007; Van Hoek et al., 1998). This is unfavourable in aerobic bioprocesses, but in fermentative or low oxygen conditions this may become an advantage. Still it is not completely solved whether the Crabtree effect is triggered by an overflow in the capacity of metabolism at the pyruvate branchpoint when the generation of glycolytic NADH leads to reduced conditions, or is it due to a limited capacity of the respiratory system to oxidize NADH (Vemuri et al., 2007; Krahulec et al., 2010). In addition, because the fermentative pathway leading to ethanol generates less ATP than the respiratory pathway, the cells respond by increasing the glycolytic flux to meet the ATP demand, which might further induce the overflow of metabolism (Gancedo, 1998).

In spite of *S. cerevisiae*'s capacity for efficient aerobic and anaerobic hexose utilization, it exhibits only insignificant metabolism of D-xylose and L-arabinose (Karhumaa et al., 2005; Bengtsson, 2009; Matsushika and Sawayama, 2008). A long time it was thought that natural xylose utilising strains of *S. cerevisiae* did not exist at all, however, natural selection and fermentation have been detected in few winery strains (Garcia Sanchez et al., 2010; Sonderegger and Sauer, 2003; Wisselink et al., 2009).

1.6 Fundamentals of xylose metabolism in genetically modified *S. cerevisiae* – The strategies and bottlenecks

1.6.1 Xylose uptake

The first obligatory and essential step in the utilization of carbohydrates is the transport of sugars into the cells across the plasma membrane. Different mechanisms, alongside with tranporter affinity, mediating the glucose/pentose transport have evolved in various organisms, *i.e* proton symport systems, phosphotranferase systems and facilitated diffusion systems (Hamacher et al., 2002). However, only recently two transporters specific for D-xylose were identified from *S. stipitis* and *Neurospora crassa* (Du et al., 2010). The hexose transporter family of *S. cerevisiae* comprises 18 transporter proteins (Hxt1-17, Gal2) which deliver sugars into the cell (Wieczorke et al., 1999). *S. cerevisiae* does not have specific xylose transporters but at least Hxt4, Hxt5, Hxt7 and Gal2 are known to transport xylose by facilitated diffusion and are expressed when xylose is the only sugar present (Hamacher et al., 2002; Olofsson et al., 2010; Rossi et al., 2010; Saloheimo et al., 2007).

The affinity and velocity of transport by HXTs is regulated by the presence and concentration of sugars available. The affinities (K_m) and substate specifities of different Hxt proteins for different hexoses (glucose, fructose and mannose) vary considerably; *e.g.* Hxt6 and Hxt7 have been classified as high (K_m 1 to 2 mM), Hxt2 and Hxt4 as moderately low and Hxt1 and Hxt3 as low affinity (K_m 15 to 20 mM) glucose transporters (Ozcan and Johnston, 1999; Rintala et al., 2008; Reifenberger et al., 1995). Distinctly lower affinity for xylose and competitive inhibition by glucose result in condiderably lower xylose uptake rate by HXTs compared with glucose (Hamacher et al., 2002; Runquist et al., 2010). Interestingly, the specific xylose uptake rate of *S. cerevisiae* could be enhanced with 0.1 g g^{-1} of glucose (Pitkänen et al., 2003). Also Krahulec *et al.* (2010) showed that in the precense of both glucose and xylose, the uptake of xylose was raised to a detectable level only at glucose concentrations lower than 4 g/L, but the uptake dropped when the glucose concentration was below 2 g/L. This could be explained by the induction of xylose transporting *HXT*s, improved cofactor generation and by the induction of glycolytic enzymes (Krahulec et al., 2010; Olofsson et al., 2010). However, the most relevant issue in

improvement of xylose uptake of *S. cerevisiae* is to find xylose-specific transporters from other organisms with high capacity and ability to transport specifically xylose.

1.6.2   Xylose utization pathways

Even though wild-type *S. cerevisiae* is unable to grow and ferment xylose; it can easily use xylulose (Fig. 1), an isomer of xylose, since it has a xylulokinase encoding gene *XKS1* that converts xylulose to xylulose-5-phosphate. But, to get xylulose from xylose, either XR-XDH patway or XI has to be expressed in yeast.

Oxidoreductive Pathway. The *XYL1* and *XYL2* coding for XR and XDH of *S. stipitis* were the first genes chosen to be expressed in *S. cerevisiae.* Even though *S. stipitis* is a naturally xylose-utilizing yeast, it can not ferment xylose under anaerobic conditions (Verduyn et al., 1985; Toivola et al., 1984). Since *Ss*XR has a preference for NADPH and on the other hand *Ss*XDH is strictly NAD^+ -specific, the introduction of XR-XDH-pathway into *S. cerevisiae* does not alone allow sufficient xylose fermentation in industrial bioprocesses. On the contrary, the cofactor imbalance leads to unfavourable excretion of the by-product xylitol, resulting in ethanol yields far below of the theoretical 0.51 g g^{-1} (Bengtsson, 2009; Jeppsson et al., 2002; Saleh et al., 2006; Jin et al., 2005). Genes resembling *Ss*XR and *Ss*XDH have been identified from *S. cerevisiae* (Toivari et al., 2004). *GRE3* encodes a non-specific aldo reductase with 72% amino acid similarity to *Ss*XR. Gre3p converts xylose to xylitol, but uses only NADPH as a cofactor. *SOR1* encoding for a sorbitol dehydrogenase (SDH), on the other hand resemble the *XYL2* gene encoding for XDH. Besides sorbitol SDH can also use xylose as a substrate. However, the activity of the endogenous xylose pathway of *S. cerevisiae* is not high enough for efficient ethanol fermentation from xylose and therefore several genes for xylose metabolism have been cloned from other organisms in order to construct an efficient xylose fermenting *S. cerevisiae* strain (Toivari et al., 2004). Furthermore, modifying the coenzyme specifities of these oxidoreductive enzymes by protein engineering has been one of the approaches for achieving improved fermentation rates from xylose using recombinant *S. cerevisiae* as a production organism (Karhumaa et al., 2005; Matsushika and Sawayama, 2008; Saleh et

al., 2006; Verho et al., 2003). Next the most well known strategies and some of the latest achievements are discussed.

The first improvement in ethanol production achieved by engineering of XR specifity was obtained with the recombinant *S. cerevisiae* strain carrying K270R (Lys270Arg) *S. stipitis* XR mutant with increased K_m for NADPH and *wt* XDH expressed together (Kostrzynska et al., 1998). XR belongs to the superfamily of aldo-keto reductases (AKR) (Bengtsson, 2009; Jeppsson et al., 2006). Most members of this superfamily are strongly NADPH dependent, but a few members are both NADH and NADPH specific *e.g.* XR from *S. stipitis*, *Pachysolen tannophilus, Candida shehatae* and *Candida tenuis* (Verduyn et al., 1985; Lee et al., 2003; Ho et al., 1990). For example, Petschacher and Nidetzky (2008) and Bengtsson *et al.* (2009) improved the redox balance with mutated *C. tenuis* and *S. stipitis* XR (NADH preference increased); the modified strain was suggested to provide more NAD^+ for the XDH reaction, resulting in higher xylose consumption rate and lower xylitol production. Bengtsson *et al.* (2009) suggested that a conserved Ile-Pro-Lys-Ser motif among NADPH-dependent xylose reductases relates to the cofactor specificity of these enzymes (Bengtsson, 2009). Based on the information from these studies several NADH-preferring *Ss*XR mutants have been generated by site-directed mutagenesis (Petschacher et al., 2005; Watanabe et al., 2007a). For example when the bindig pocket was altered by introducing amino acids with shorter and less hydrophilic side chains it became less favourable for NAD(P)H increasing its K_m value. On the other hand, glycine and proline, freguently occuring in turn regions of proteins, caused turns in the eight α/β loops and led to a smaller coenzyme binding pocket favouring NADH (Liang et al., 2007).

In studies aiming at altering the coenzyme specifity of XDH enzymes, the amino acid residues responsible for NAD^+ specifity of *S. stipitis* XDH were replaced with NADP-recognition sequence of *Thermoanaerobium brockii* alcohol dehydrogenase (~30% homology to *S. stipitis XYL2*). With this modification approximately a 9-fold increase in K_m for NAD^+ was obtained, however, the mutant enzymes had decreased XDH activity and still preferred NAD^+ over $NADP^+$. (Watanabe et al., 2007a; Metzger and Hollenberg, 1995) Watanabe *et al.* (2005) were able to create a novel XDH with coenzyme specifity toward $NADP^+$ using sorbitol dehydrogenase ($NADP^+$ preferring) as a reference enzyme. The

mutant enzyme was co-expressed with *wt* XR of *S. stipitis* in *S. cerevisiae*, resulting in better maintenance of intracellular redox balance, and furthermore, leading to improved ethanol production and lowered xylitol excretion (Watanabe et al., 2005; Watanabe et al., 2007b).

Based on these studies, among others, it seems a biochemical fact that complete anaerobic conversion of xylose into ethanol can only happen when recycling of cofactors is balanced, in other words, XR and XDH have matching coenzyme specifities. (Lee et al., 2003; Watanabe et al., 2007a; Watanabe et al., 2005; Dmytruk et al., 2008; Petschacher and Nidetzky, 2008) The xylose pathway from *S. stipitis,* as well as in many other organisms, has been under enthusiastic studies over the last few decades, unfortunately, the assembly of a chimeric pathway in which XR and XDH show exactly matching utilization of NADP(H) and NAD(H) appears to be difficult to achieve.

Xylose isomerase Pathtway. Besides oxidoreductive pathway, xylose isomerase expression has been an attractive target of metabolic engineering since the enzyme converts xylose directly to xylulose that *S. cerevisiae* can metabolize. Moreover, employing XI instead of XR/XDH cofactor imbalance and xylitol accumulation can be avoided (Wenger et al., 2010). The first fuctionally expressed *xylA* genes in *S. cerevisiae*, resulting in xylose fermentation, were from *Piromyces sp.* strain E2 and *Thermus thermophilus* (Kuyper et al., 2003; Walfridsson et al., 1996). The *xylA* gene from *T. thermophilus* was expressed in *S. cerevisiae* in an active form, but the activity at 30 °C was very low since the temperature optimum of the protein is 85 °C (Lonn et al., 2002; Walfridsson et al., 1996; Walfridsson et al., 1995). Lonn and co-workers (2002) were able to create a cold-adapted version of *T. thermophilus* XI by random PCR mutagenesis. The enzyme exhibited up to 9 times higher k_{cat} for xylose than the wild-type enzyme at 60 °C, however, in moderate temperatures (30–40 °C) the fermentation rates were not compatible with industrial processes. The XI from *Piromyces* sp. was expressed in yeast with high activity; however the strong inhibition by xylitol was its major drawback (Kuyper et al., 2003). Later Kuyper and co-authors (2005) were able to construct a haploid yeast strain expressing *xylA* of *Piromyces* sp. strain E2 which exhibited fast anaerobic growth (0.18 h^{-1}) on xylose and had also high ethanol production rates.

Moreover, Brat *et al.* (2009) screened XIs from 14 organisms exhibiting different phylogenetic affiliations from 17% to 60% identities to the *Piromyces* XI. A codon-optimized *xylA* from *Piromyces* sp. strain E2 (YEp-opt.*XI*-Piro) was used as a positive control in the screening system. As a result only the yeast transformants expressing the XI of *Clostridium phytofermentans* (52% identity to the XI from *Piromyces* sp. strain E2) and the positive control YEp-opt.*XI*-Piro could grow on the xylose medium. Importantly, the XI from *C. phytofermentans* had significantly lower sensitivity to inhibition by xylitol (K_i, 14.51 ± 1.08 mM) than the XI from *Piromyces* (K_i, 4.67 ± 1.77 mM) that may be advantageous during xylose conversion to ethanol with industrial strains. (Brat et al., 2009)

Many other attempts to express prokaryotic XI enzymes in *S. cerevisiae* have failed probably because of the different optima in temperature and/or pH. For example *xylA* genes from *E. coli*, *Bacillus subtilis*, *Lactobacillus pentosus* and *Clostridium thermosulfurogenes* have all resulted in *S. cerevisiae* strains either unable to express the gene or synthesize an active enzyme. In further studies mutagenesis might be the solution to overcome the obstacles, unless new enzymes with better temperature and pH optima are found. Furthermore, mixed sugar utilization of glucose and xylose was recently achieved by evolutionary engineering of recombinant XI-expressing yeast strains (Brat et al., 2009).

1.6.3 <u>P</u>entose <u>P</u>hosphate <u>P</u>athway (PPP) and approaches to engineer the intacellular redox balance

In addition to glycolysis, the pentose phosphate pathway is another main pathway of glucose catabolism in the cytosol of living organisms. The PPP consists of an irreversible oxidative part and of a reversible non-oxidative part (Qian et al., 2008). In the oxidative part the majority of NADPH needed for different reductive reactions in the cell is generated. In the non-oxidative part *TAL1* (transaldolase) and *TKL1* (transketolase), synthesise 4-, 5-, 6-, 7-carbon sugar phosphates. TAL converts sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate to erythrose-4-phosphate and fructose-6-phosphate. TKL catalyzes the conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate. (Walfridsson et al., 1995) Figure 1 shows

the steps how the glycolytic and xylose pathways are connected to the pentose phosphate pathway; via two reactions xylulose-5-phosphate is converted to glyceraldehyde 3-phosphate and fructose 6-phosphate, which are intermediates of glycolysis. Additionally, in the PPP ribose-5-phosphate is converted to phosphoribosylpyro-phosphate to build up purine and pyrimide nucleotides, components of nucleic acids and many co-factors. Erythrose-4-phosphate is a building block of aromatic amino acids (tryptophan, phenylalanine and tyrosine) as well as aromatic vitamins and in pyridoxine biosynthesis.

The enzyme xylulokinase phosphorylates D-xylulose (after XR-XDH and XI reactions) to xylulose-5-phosphate and in that way provides it for PPP. The work of Ho and co-workers (1998) showed that the overexpression of an endogenous *XKS1* gene with *XYL1* and *XYL2* enabled the *S. cerevisiae* strain effectively ferment high concentrations of xylose almost completely to ethanol with only small xylitol concentrations. Additional studies further suggest that the overexpression of *S. cerivisae*'s endogenous *XKS1* results in more efficient conversion of xylose to ethanol (Matsushika et al., 2008; Dmytruk et al., 2008; Eliasson et al., 2000b; Toivari et al., 2001). However, controversial results of uncontrolled *XKS1* overexpression have been published, *e.g* Johansson *et al.* (2001) demonstrated that overexpression of *XKS1* decreased xylose utilization although the ethanol yield was improved. The overexpressed *XKS1* was suggested to overrule the possible feedback control of xylulose phosphorylation leading to slow-down of cellular metabolism due to xylulose-5-phosphate accumulation and/or ATP depletion (Jin et al., 2003). Therefore, successful metabolic engineering requires alteration of multiple gene expression levels to overcome the rate-limiting steps, since a single enzyme has only a partial control of the total flux (Walfridsson et al., 1995; Johansson et al., 2001; Lu and Jeffries, 2007). Thus efficient PPP reactions linking xylose to xylulose 5-phosphate and to glycolysis are also needed to enhance xylose fermentation.

Several studies suggest that in the nonoxidative PPP especially transaldolase activity limits the growth on xylose; the overexpression of *TAL1* greatly improved growth on xylose whereas the overexpression of both *TKL1* and *TAL1* had the same effect as overexpression of *TAL1* only (Jin et al., 2005; Walfridsson et al., 1995; Hasunuma et al., 2011). Using a novel multiple-gene-promoter-shuffling (MGPS) method to overexpress the *TAL1*, *TKL1*

and *PYK1* (pyruvate kinase) genes, Lu *et al.* (2007) also showed that ethanol yield was sensitive to the overexpression of *TAL1* and *PYK1* but less sensitive to that of *TKL1* (Lu and Jeffries, 2007).

Besides manipulation of the nonoxidative PPP, lowering the flux through the NADPH-producing oxidative PPP has been studied. The oxidative part was blocked either by lowering phosphoglucose isomerase activity (PGI), by disruption of the *GND1* gene, one of the two isogenes encoding 6-phosphogluconate dehydrogenase, and by disruption of the *ZWF1* gene, which encodes glucose 6-phosphate dehydrogenase (G6PDH). All the modifications resulted in lower xylitol yield and higher ethanol yield compared with the control strains (Jeppsson et al., 2002). Additionally, in a previous study, lowering the PGI activity by the deletion of its promoter increased the ethanol yield with 15%. The lower activity of PGI results in accumulation of fructose 6-phosphate and fructose 1,6-diphosphate (FDP), which are required for induction of the ethanologenic enzymes, pyruvate decarboxylase and alcohol dehydrogenase, as well as for inactivation of the gluconeogenic fructose 1,6-bisphosphatase. Likewise, the deletion of gluconate 6-phosphate dehydrogenase encoding gene (*GND1Δ*), and trehalose 6-phosphate synthase encoding gene (*TPS1Δ*) together with trehalose 6-phosphate phosphatase encoding gene (*TPS2Δ*) increased the ethanol yield by 30% and 20%, respectively (Eliasson et al., 2000a).

Wahlbom and coworkers (2003), on the other hand, improved the uptake and growth rate on xylose with higher expression of the genes *HXT5, XKS1* and 6-phosphogluconolactonase (*SOL3*) and *GND1*, as well as, but to a lesser extent, by overexpression of *TKL1* and *TAL1*. The enhanced flux, however, could partly have been due to the glucose in the growth medium (Wahlbom et al., 2003). In another study, NADP$^+$-utilizing glyceraldehyde 3-phosphate dehydrogenase (GADPH) from *Kluyveromyces lactis* was expressed in *S. cerevisiae.* Additionally *ZWF1* was deleted in the strain in order to block the NADPH regenerating oxidative PPP coupled to CO_2 production. As a result of these genetic modifications, xylitol and CO_2 production decreased and ethanol yield from xylose improved (Verho et al., 2003).

Furthermore, Sonderegger *et al.* (2004) provided acetoin as an external NADH sink to *S. stipitis* cells; this reduced the extensive cytosolic NADH accumulation and thus enabled a higher flux on oxidoreductase reaction. Furthermore, Roca *et al.* (2003) deleted NADPH-dependent glutamate dehydrogenase gene (*GDH1*) and overexpressed either the NADH-dependent glutamate dehydrogenase or glutamate synthase (*GLT1*) and glutamine synthetase (*GLN1*) (together known as the GS-GOGAT system) to improve cofactor use in recombinant *S. cerevisiae*. The system assimilates ammonia into glutamate, using NADH as a cofactor, hereby increasing the conversion of xylitol to xylulose. In addition, due to the change in cofactor use, NADPH is used less for glutamate synthesis and can be directed to the use by the XR in the conversion of xylose to xylitol increasing the consumption rate by 15%. (Roca et al., 2003) Additionally, a group of different promoters has been tested in order to regulate the expression levels of the central enzymes in the pentose metabolism (Nevoigt, 2008). Since glycerol biosynthesis (besides ethanol production) serves as the dominant mechanism for NADH reoxidation under anaerobiosis other means must be exploited to prevent NADH accumulation and increase the NAD^+ availability for XDH.

1.7   The purpose of the study

The thesis consists of three separate studies that all aimed at improving the xylose metabolism of recombinant *S. cerevisiae* yeast expressing XR and XDH encoding genes of *S. stipitis*. The first study relates to the xylose transport and the second and third study to the intracellular redox imbalance.
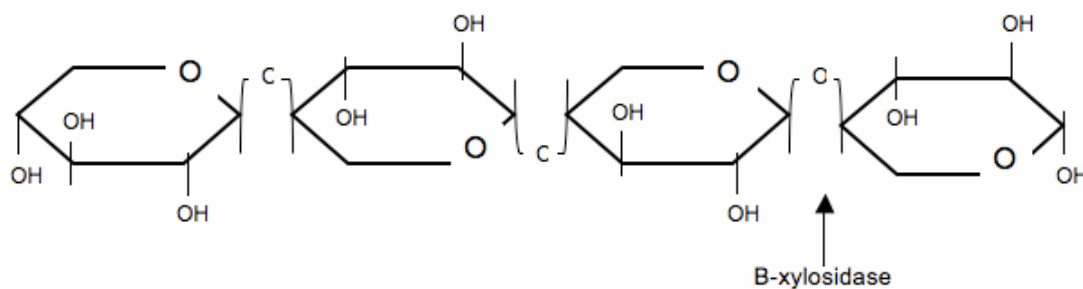
1.7.1 Xylose transporter assay

Figure 3. Enzymatic hydrolysis of xylan by T. reesei β-xylosidase

In this study, the aim was to build a high through-put assay for screening of specific xylose transporters. The development was based on β-xylosidase BXL1 of the filamentous ascomycete *T. reesei* (*Hypocrea jecorina*) and a commercially available xylose analogue p-nitrophenyl-β-D-xylopyranoside (pNPX). The extracellular enzyme BXL1 hydrolyses xylooligosaccharides such as xylobiose and xylan releasing xylose from the non-reducing end (La Grange et al., 2001; Margolles-Clark et al., 1996). The predicted sequence of BXL1 encoding gene *bxl1* is 785 amino acids long and contains a putative signal sequence of 20 amino acids with a predicted cleavage site after Ala-20. The molecular mass of the protein is 80.4 kDa. The BXL1 sequence does not show similarity to any other β-xylosidases classified in glycoside hydrolase (EC 3.2.1.37) families 39, 43 and 52 (Henrissat and Bairoch, 1993). However, the amino acid sequence shows similarities to the β-glucosidases of family 3 despite it β-glucosidase activity (Margolles-Clark et al., 1996).

Bxl1 was expressed in *S. cerevisiae* with the oxidoreductive xylose pathway. The 5' region of the gene encoding the putative signal sequence of *bxl1* (20 amino acids) was deleted in order to keep the enzyme intracellular. As demonstrated with *E. coli* (Chen et al., 2009), intracellular β-xylosidase cleaves the chromogenic group p-nitrophenol (pNP) from the xylose analogue pNPX creating a bright yellow colour that is proportional to the amount of pNPX taken into the cells (Margolles-Clark et al., 1996; Chen et al., 2009). The intesity of the colour can be measured by a spectrophotometer; the brighter the colour the stronger the uptake activity

1.7.2 Fumarate reductase expression

Fumarate reductases catalyze the reduction of fumarate to succinate in the citric acid cycle. The known enzymes can be divided into two classes (Fig. 5) depending on the mechanism of the electron transfer. The FRDs characterized to date are mostly membrane bound enzymes consisting of three to four subunits and they are structurally similar to succinate dehydrogenases (Coustou et al., 2006; Camarasa et al., 2007). An example of this type of FRD is that of *E. coli*. The other class of FRDs consists of soluble monomeric enzymes that catalyse succinate production by transferring electrons to fumarate from a non-covalently bound cofactor such as NADH or $FADH_2/FMNH_2$. The soluble enzymes are also suggested to be involved in maintenance of intracellular redox balance in anaerobic conditions. This type of enzymes has been identified among others from *S. cerevisiae*, several *Shewanella* species and *T. brucei*. (Miura et al., 2008)
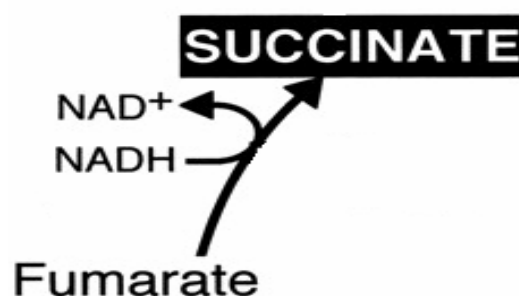
Figure 4. The reduction of fumarate to succinate by NADH dependent fumarate reductase in T. brucei (The figure modified from Besteiro et al. 2002).

T. brucei is an African trypanosome causing sleeping sickness in humans. It possesses two distinct NADH-dependent fumarate reductases; *FRDm1*, which encodes a mitochondrial protein and is responsible for 30% of the total cellular NADH-FRD activity, and *FRDg* encoding a glycosomal protein responsible for 70% of the cellular NADH-FRD activity (Besteiro et al., 2002). *FRDg*, first identified by Klein *et al.* (1975), encodes an enzyme 35% identical to *S. cerevisiae* fumarate reductases (corresponding to the amino acids from position 406 to 839) (Fig. 5). In this study the fumarate reductase gene *FRDg* was overexpressed in *S. cerevisiae* strain H3675 with the oxidoreductive xylose pathway. The aim was to increase the intracellular NAD^+ availability for the reaction catalysed by XDH

and by this way alleviate the intracellular redox imbalance and to increase the ethanol yield from xylose.
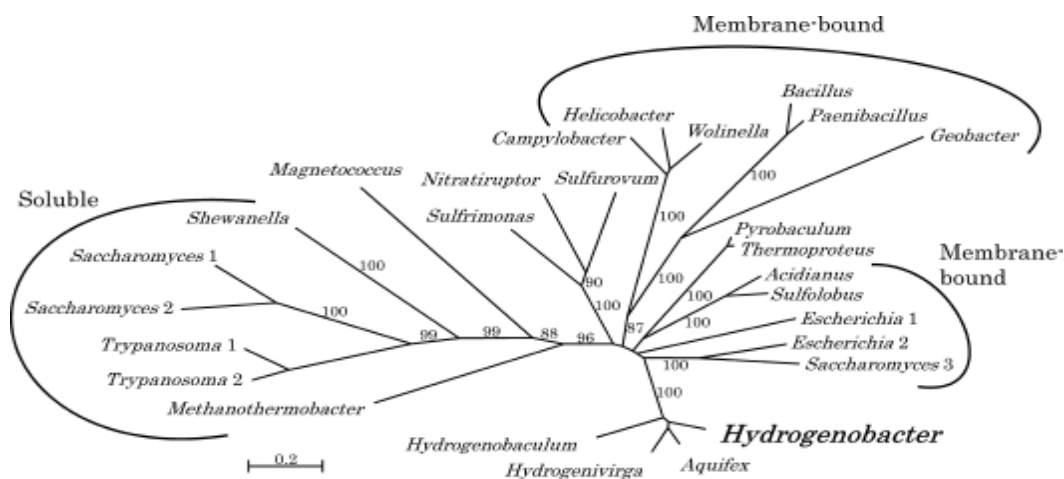
Figure 5. Phylogenetic tree of fumarate reductase catalytic subunits. The numbers at the nodes represent the values expressed as percentages of 1,000 bootstrap replications. The order of divergence is presumed to be reliable only when the bootstrap values were above 80. The scale bars represent 0.2 estimated changes per nucleotide (The figure modified from Miura et al., 2008).

Additionally, *POS5* encoding NADH -kinase of *S. cerevisiae* and MMP1489 encoding bifunctional NADP$^+$-phosphatase/NAD$^+$-kinase of *Methanococcus maripaludis* were expressed together with FRDg in H3675 (strains H3675/FRDg+POS5 and H3675/FRDg+MMP1489). NAD$^+$/NADH -kinases phosphorylate NAD$^+$/NADH to form NADP$^+$/NADPH. Conversely, NADP$^+$ phosphatases dephosphorylate NADP$^+$ to produce NAD$^+$ (Kawai and Murata, 2008). *S. cerevisiae* NAD$^+$ -kinase (*POS5*) encodes the mitochondrial NADH kinase, the sole source of NADPH in this organelle (Outten and Culotta, 2003). It is known that the regulation of the intracellular balance of NAD(H) and NADP(H) is the key function of these enzymes. In addition, Pos5p protects cells under the oxidative stress (hyperoxia) taking part in detoxification of reactive oxygen species (ROS) in mitochondria by maintaining the mitochondrial supply of NADP$^+$ and NADPH (Kawai and Murata, 2008; Outten and Culotta, 2003; Strand et al., 2003).

M. maripaludis, first isolated from salt marshes, belongs to the kingdom *Euryarchaeota* in the domain of *Archaea* growing preferably at 85 °C and 37 °C. This hydrogenotrophic methanogen contains 1722 protein-coding genes in a single circular chromosome of 1 661 137 bps. As a hydrogenotroph, *M. maripaludis* uses H$_2$ as an electron donor to reduce CO$_2$

to methane. (Kawai et al., 2005; Kawai et al., 2005; Hendrickson et al., 2004) $NADP^+$-phosphatase/NAD^+-kinase (MMP1489) of *M. maripaludis* has a function in maintaining cofactor balance of NAD^+/$NADP^+$ generating intracellular $NADP^+$ (Kawai et al., 2005).

1.7.3   XR and XDH activity screening

Xylose utilization pathways have been studied in various organisms, however, larger scale screening of xylose reductase and xylitol dehydrogenase activities has not been done recently. It has been shown that high activity of both XR and XDH is important for generating an efficient xylose-fermenting recombinant *S. cerevisiae,* but also a higher level of XDH activity relative to XR activity has been shown to decrease the xylitol excretion (Matsushika et al., 2008; Eliasson et al., 2000b). Moreover, due to the redox, the cofactor specificity of XR and XDH plays an important role in xylose fermentation of recombinant *S. cerevisiae* (Petschacher and Nidetzky, 2008). The aim of the study was to find new, interesting XR and XDH enzymes that could be applied in metabolic engineering of xylose-fermenting *S. cerevisiae* strains. In particular, the interest was either XRs with high NADH-specific activity, XDHs with high $NADP^+$-specific activity or enzymes with the high specific activity with either of the cofactors.

2   MATERIALS AND METHODS

2.1   Strains, plasmids and media

2.1.1   Strains and plasmids

For bacterial transformations *E. coli* strains DH5α (F-, *end*A1, *hsd*R17, *rec*A1, *gyr*A96, *rel*A1, φ80dΔ*lacZ*M15) and TOP10 Electrocomp (Invitrogen, USA) were used. All *S. cerevisiae* strains and plasmids used in this work are presented in Tables 1 and 2.

Table 1. The S. cerevisiae strains used in this study.

Strains	Description	Reference
CEN.PK2-1D (H1346)	*MATa,, leu2-3/112, ura3-52, trp1-289, his3Δ1, MAL2-8ᶜ, SUC2*	Boles *et al.* 1996
H2217	*CEN.PK2-1D; ura3::XYL1 XYL2 his3::XKS1 kanMX*	Richard *et al.* 2002
H3675	*CEN.PK2113-1A; HIS3. LEU2. TRP1. MAL2-8c. SUC2 ura3::XYL1 XYL2 xks1::XKS1*	VTT strain collection
H3488	*MATα , his3Δ 200, ura3-52, leu2Δ 1, lys2Δ 202, trp1Δ 63 for construction of a plasmid with homologous recombination in yeast*	VTT strain collection
H2217/B435BXL1	*H2217 and B435 with BXL1*	This study
H2217/B1184BXL1	*H2217 and B1184 with BXL1*	This study
H3675/B1181	*H3675 and empty B1181*	This study
H3675/FRDg	*H3675 and B1181 with FRDg*	This study
H3675/FRDg+MMP1489	*H3675/B1181FRDg and pLS1*	This study
H3675/FRDg+POS5	*H3675/B1181FRDg and pLS6*	This study
H3675/p1181+pKK27-1	*H3675/B1181 and pKK27-1*	This study
H3675/FRDg+pKK27-1	*H3675/B1181FRDg and pKK27-1*	This study

Table 2. The plasmids used in this study.

Plasmid	Description	Reference
pRS315	*LEU2, fi ori, T7 promoter, LacZ, MCS, T3 promoter, CEN6, ARSH4*	Sikorski *et al*. 1989
YEplac181	*pUC19, LEU2, Amp (Shuttle vector)*	Gietz and Sugino 1988
YEplac195	*pUC19, URA3, Amp (Shuttle vector)*	Gietz and Sugino 1988
pKK27-1	*Kan between TEF promoter and terminator, negative control plasmid for pLS1 and pLS6*	VTT plasmid collection
B435	*pRS315 with PGK1 promoter and terminator*	VTT plasmid collection
B1181	*YEplac195 with PGK1 promoter and terminator, URA3, Amp*	VTT plasmid collection
B1184	*YEplac181 with PGK1 promoter and terminator, LEU2, Amp*	VTT plasmid collection
pLS1	*MMP1489 under TPI promoter and ADH1 terminator, Kan*	VTT plasmid collection
pLS6	*POS5Δ17 under TPI promoter and ADH1 terminator, Kan*	VTT plasmid collection
pTOPO_BXL1	*pCR$^®$ 2.1-TOPO with BXL1*	This study
B435BXL1	*B435 with BXL1*	This study
B1184BXL1	*B1184 with BXL1*	This study
B1181FRDg	*B1181 with FRDg*	This study

2.1.2   Media and growth conditions

Luria-Bertani broth (LB-broth) medium (1 w/v Bacto-tryptone, 0.5 w/v Bacto-yeast extract (BD, USA) and 0.5 w/v NaCl) was used in *E. coli* cultivations. For plasmid selection 100 µg/ml ampicillin (Sigma-Aldrich, Germany) was added into the media. Bacteria were grown in glass test tubes or in 250 ml erlenmeyer flasks over night at 37 °C and shaken at 250 rpm. SOC medium (2 w/v Bacto-tryptone (BD, USA), 0.5 w/v Bacto-yeast extract (BD, USA), 10 mM NaCl, 2 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 0.4 w/v D-glucose (Sigma-Aldrich, Germany)) was used for the recovery of *E. coli* cells after transformation by electroporation.

For *S. cerevisiae* the rich growth medium (Yeast peptone dextrose, YPD) was used as a general growth medium and the synthetic complete (SC) media were used for the selection and screening. YPD medium contained 1 w/v Bacto-yeast extract (BD, USA), 2 w/v Bacto-

peptone (BD, USA) and 2 w/v D-glucose (Sigma-Aldrich, Germany). YPX medium was similar to YPD medium except D-glucose was replaced with D-xylose (Sigma-Aldrich, Germany) as a carbon source. SC media contained 6.7 g/l yeast nitrogen base (YNB) without amino acids (Sigma-Aldrich, Germany). The SC-medium with 200 μg/ml Geneticin (G418) (Sigma-Aldrich, Germany) was prepared from YNB without amino acids and ammonium sulphate. In stead of ammonium sulphate 1 g/l of Monosodium Glutamate was used as a nitrogen source in the medium.

Amino acid/nucleotide stock was added to YNB to get SC medium with following concentrations of amino acids: 0.1 mM L-adenine, 2 mM L-arginine, 2 mM L-aspartic acid, 0.4 mM L-histidine, 0.2 mM myo-inositol, 4 mM L-isoleucine, 2 mM L-leucine, 0.6 mM L-lysine, 1 mM L-methionine, 0.5 mM L-phenylalanine, 1 mM L-serine, 1 mM L-threonine, 0.4 mM L-tryptophan, 0.2 mM L-tyrosine, 0.2 mM uracil and 1 mM L-valine. Cells with plasmids with *URA3* or *LEU3* markers were grown in SC- medium lacking either uracil or L-leucine. SCD-media contained 2% w/v of glucose and SCX-media 2% w/v xylose as a carbon source. Solidified media were supplemented with 2% w/v of Bacto Agar (BD, USA). Yeasts were grown at 30 °C with 250 rpm shaking over night.

Table 3. The cultivation media and the duration of growth periods.

Aerobic cultivations

Strain	Precultivation			Cultivation	
	Media	Time, day	OD$_{init.}$	Media	Time, days
Yeast from VTT Culture Collection	YPX	1	-	YPX	1
CEN.PK2-1D (H1346)	YPD	1	-	YPD	1
H2217	YPD	1	-	YPD	1
H3675	YPD	1	-	YPD	1
H3488	YPD	1	-	YPD	1
H2217/B435BXL1	SCD-leu	1	-	SCX-leu	1
H2217/B1184BXL1	SCD-leu	1	-	SCX-leu	1
H3675/B1181	SCD-ura *OR* YNB/L-glutamate-ura+Glc	1	0.4	SCX-ura *OR* YNB/L-glutamate-ura+Xyl	6
H3675/FRDg	SCD-ura *OR* YNB/L-glutamate-ura+Glc	1	0.4	SCX-ura *OR* YNB/L-glutamate-ura+Xyl	6
H3675/FRDg+MMP1489	YNB/L-glutamate-ura+Glc	1	0.4	YNB/L-glutamate-ura+Xyl	6
H3675/FRDg+POS5	YNB/L-glutamate-ura+Glc	1	0.4	YNB/L-glutamate-ura+Xyl	6
H3675/B1181+pKK27-1	YNB/L-glutamate-ura+Glc	1	0.4	YNB/L-glutamate-ura+Xyl	6
H3675/FRDg+pKK27-1	YNB/L-glutamate-ura+Glc	1	0.4	YNB/L-glutamate-ura+Xyl	6

Anaerobic cultivations

Strain	Precultivation			Cultivation	
	Media	Time, day	OD$_{init.}$	Media	Time, days
H3675/B1181	SCD-ura *OR* YNB/L-glutamate-ura+Glc	1	5	SCX-ura *OR* YNB/L-glutamate-ura+Xyl	14
H3675/FRDg	SCD-ura *OR* YNB/L-glutamate-ura+Glc	1	5	SCX-ura *OR* YNB/L-glutamate-ura+Xyl	14
H3675/FRDg+MMP1489	YNB/L-glutamate-ura+Glc	1	5	YNB/L-glutamate-ura+Xyl	14
H3675/FRDg+POS5	YNB/L-glutamate-ura+Glc	1	5	YNB/L-glutamate-ura+Xyl	14
H3675/B1181+pKK27-1	YNB/L-glutamate-ura+Glc	1	5	YNB/L-glutamate-ura+Xyl	14
H3675/FRDg+pKK27-1	YNB/L-glutamate-ura+Glc	1	5	YNB/L-glutamate-ura+Xyl	14

2.2   Recombinant DNA techniques

2.2.1   DNA purification, amplification and electrophoresis

E. coli plasmid purifications were carried out by using QIAprep Spin Miniprep Kit (Qiagen GmbH, Germany). All primers (Table 4) used in this work were manufactured by Sigma-Genosys Ltd. (Germany). The used restriction enzymes (*Bgl*II, *Sal*I*, and EcoR*I*)* were acquired from New England BioLabs (USA) and the T4 ligase from Promega (USA). To remove the 5' phosphates from the digested vectors the Calf intestinal phosphatase from Finnzymes (Finland) was used. The DNA polymerases used in the reactions were Phusion™ high fidelity polymerase (Finnzymes, Finland), DyNAzyme II DNA Polymerase (Finnzymes, Finland) and its optimized mixture DyNAzyme EXT (Finnzymes, Finland). The enzymes were used according to manufacturers' instructions. PCR programmes and concentrations in the reaction mixtures were also the ones recommended by the manufacturers.

Plasmids, digested DNA fragments and PCR products, were separated by electrophoresis in 1% agarose gel (SeaKem LE agarose by BMA, USA). GeneRuler™ 1 kb DNA Ladder (Fermentas, USA) was used as a standard size marker. TD loading buffer [20 v/v Ficoll 400 (Amersham Biosciences, USA); 0.0125 v/v bromophenol blue (Merck, Germany); 0.1 v/v xylene cyanol ff (Chroma-Gesellschaft, Germany), 250 mM Tris [pH 8.0] (MP Biomedicals, USA), 25 mM boricacid (Sigma-Aldrich, Germany), 125 mM EDTA [pH 7.5] (Sigma-Aldrich, Germany); 0.5 v/v SDS (Sigma-Aldrich, Germany)] was used at 1/5 of the volume of the DNA samples. In some DNA samples loading buffer from Fermentas (USA) was used at 1/6 of the total volume of the sample. When needed, the DNA fragments were isolated from the gel using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany).

Table 4. The primers used in this work.

Name	Sequence (5´to 3´)	Used for	Strain
TrBXL1BglII frw	CGCAGATCTACAATGCA GAACAATCAAACATAC	*BXL1* amplification	*T. reesei* bank in *E.coli*
TrBXL1BglII rev	CCGAGATCTTTATGCGTC AGGTGTAGCAT	*BXL1* amplification	*T. reesei* bank in *E.coli*
BXL1 revII	ATGGCGTCGAAACCGAGACG	Sequencing	H2217/p435BXL1, H2217/p1184BXL1
M13 Universal primer frw	GACCGGCAGCAAAATG	Sequencing	pTOPO_BXL1
M13 Universal primer rev	CAGGAAACAGCTATGAC	Sequencing	pTOPO_BXL1
TbFRDg frw	CGCGCGCCCGGGGAAGTAAT	*FRDg* amplification	Optimized FRDg
TbFRDg rev	TTAATTAACCCGGGAATA ATTCC	*FRDg* amplification	Optimized FRDg
TbFRDg frw3	GCTGATGATAAGCCATTGAC	Sequencing	H3675/FRDg
TbFRDg frw4	TCATAGAGCACCAGATAAGA	Sequencing	H3675/FRDg
PGK promoter	TCAAGTTCTTAGATGCTT	Sequencing	H2217/B435BXL1, H2217/B1184BXL1, H3675/FRDg
PGK terminator	TAGCGTAAAGGATGGGG	Sequencing	H2217/B435BXL1, H2217/B1184BXL1, H3675/FRDg
Kan+158 rev	TGTTTCAGAAACAACTCTGG	Colony PCR	H3675/B1181+PKK27-1, H3675/FRDg+PKK27-1, H3675/FRDg+POS5
TPI1 promoter	TCTATTGATGTTACACCTGG	Colony PCR	H3675/FRDg+POS5
pTEF rev	GGGTGTTTTGAAGTGGTACG	Colony PCR	H3675/B1181+PKK27-1, H3675/FRDg+PKK27-1, H3675/FRDg+MMP1489
ADH1 oligo1	CTGGAGTTAGCATATCTACA	Colony PCR	H3675/FRDg+MMP1489

2.2.2 Sequencing

The samples for DNA sequencing were prepared according to the instructions of Big Dye[®] sequencing kit (Applied Biosystems, USA) manual. Capillary electrophoresis was used to analyse the reactions by ABI Prism[®] 3100 Genetic Analyser (PE/Applied Biosystems, Perkin Elmer, USA). The resulted DNA sequences were compared to the original ones

using sequence analysis software Chromas version 2.13 (Technolysium Pty Ltd., Australia).

2.2.3 Transformations

E. coli. The *E. coli* cells were transformed by electroporation (GenePulser™, BioRad, USA) with the following settings: 25 µF, 200 Ω and 2.50 kV. The transformation mixture included 40 µl of electrocompetent DH5α *E. coli* cells (thawed on ice) and 2 µl of plasmid DNA. The mixture was transferred into cold 0.2 cm electroporation cuvette (BioRad, USA). After the pulse the cells were immediately resuspended in 1 ml SOC medium and inoculated on LB plates with 30 µg/ml ampicillin (LB_{amp}) (Sigma-Aldrich, Germany).

After transformations with TOPO TA Cloning® kit (Invitrogen, USA), the LB_{amp} plates were additionally supplemented with 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactorytanoside), which was used for blue/white screening to distinguish recombinant colonies. In the TOPO vector the *LacZ* gene encodes β-galactosidase hydrolysing X-gal resulting in an intense blue precipitate in the vector containing cells. The cloned fragment interrupts the *LacZ* gene and the colonies remain white. The cells were incubated over night at 37 °C.

S. cerevisiae. The yeast transformations were carried out with Lab Transformation Kit (Molecular Research Reagents Inc.USA), the method introduced by Gietz *et al.* (1992). After transformation the cells were inoculated on SCD or SCX plateslacking either uracil or leucine for plasmid selection. For selection of the vector containing the geneticin resistance gene, the Ymin glutamine mediumwas supplemented with 200 µg/ml geneticin (G418) (Sigma-Aldrich, Germany). The incubation temperature for yeast strains was 30 °C and the time from two to three days.

2.3   Plasmid construction

2.3.1   Cloning of *BXL1*

The *bxl1* gene was amplified from *T. reesei* cDNA bank with TrBXL1BglIIfrw and TrBXL1BglIIrev primers. The Phusion™ polymerase (Finnzymes, Finland) was used and concentrations of deoxynucleotides, reaction buffer and oligonucleotide primers in the reaction mixture were as recommended by the manufacturer. The PCR programme (Bioer XP cycler, Bioer Technology CO. Ltd., China)was as follows: initial denaturation 98 °C 30 sec, 25 x (denaturation 98 °C 10 sec; annealing 62 °C 30 sec; extension 72 °C 1 min) and final extension 72 °C 10 min. After amplification the DNA product was purified by QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) and the 3' A-overhang was attached to the fragment in accordance with the TOPO TA Cloning® Kit (Invitrogen, USA) manual.

The fragment was cloned into a pCR®2.1®-TOPO vector (Invitrogen, USA) and transformed into the TOP10 (Invitrogen, USA) *E. coli* strain. White colonies were picked from LB plates containing ampicillin and the plasmid isolation was carried out by the QIAprep Spin Miniprep Kit (Qiagen GmbH, Germany). The inserted gene was sequenced with M13 universal primers before the *bxl1* gene fragment was released by *Bgl*II (New England BioLabs, USA) and then ligated into the *Bgl*II sites of the B435 and B1184 yeast expression vectors (Fig. 6) with T4 ligase (Promega, USA) over night at 16 °C. Prior to transformation into yeast, both vectors were digested with *Bgl*II (New England Biolabs, USA) and *Sac*I (New England Biolabs, USA) restriction enzymes to verify the correct orientation of the gene.
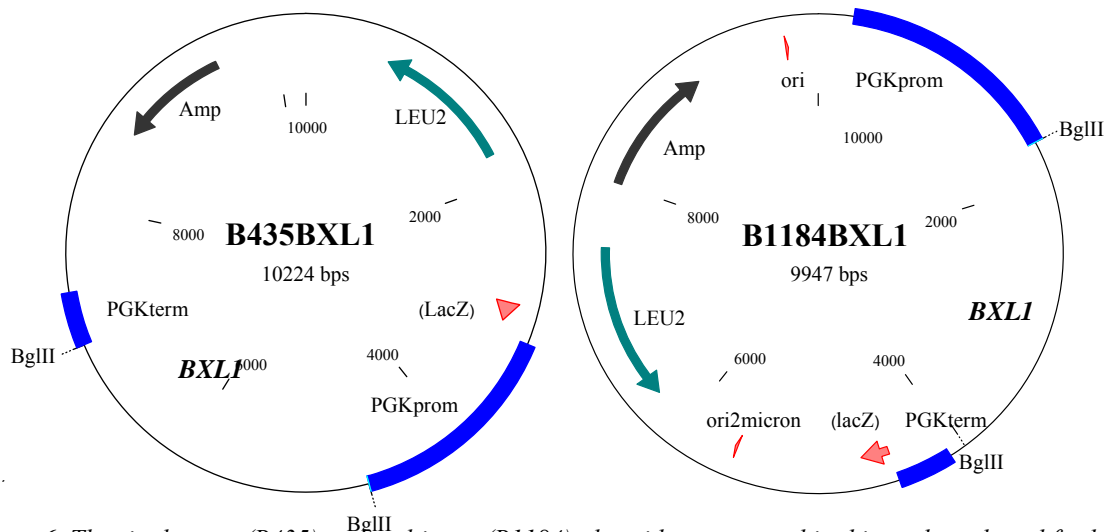
Figure 6. The single copy (B435) and multicopy (B1184) plasmids constructed in this study and used for bxl1 expression in S. cerevisiae.

The vectors were transformed into *S. cerevisiae* strain H2217 by Gietz Lab Transformation Kit (Molecular Research Reagents Inc., USA) and grown on SCD medium lacking leucine. The clones were screened using the colony PCR method designed particularly for yeast. Yeast colonies were picked from the plates into Eppendorf tubes. The cell walls were disrupted by incubating cells ten minutes at room temperature with zymolyase (1 mg/ml) (Sigma-Aldrich, Germany) and subsequently the cells were centrifuged with tabletop centrifuge (5415D, Eppendorf AG, Germany) for 1 minute at 2300 g. The cell pellets were heated five minutes at 95 °C and resuspended in 20 µl of DDIW. The amount of template used in 50 µl PCR reaction was 10 µl, otherwise the PCR mixture was prepared according to the manufacturer's recommendations for Dynazyme II (Finnzymes, Finland) DNA polymerase. The programme used in PCR with *PGK* promoter and terminator primer pair is described in Table 5. After PCR, the samples were loaded in an agarose gel. GeneRuler™ 1 kb DNA Ladder (Fermentas, USA) was used as a standard size marker.

Table 5. The PCR programme used for PGK promoter and terminator primer pair. The steps 2 to 4 were repeated 30 times.

Step	Temperature °C	Time
1. Initial denaturation	94	3 min
2. Denaturation	94	30 sec
3. Annealing	55	30 sec
4. Elongation	72	3 min
5. Final elongation	72	10 min
6. Hold	4	∞

2.3.2 Cloning of *FRDg*

The *FRDg* from *T. brucei* was codon optimized for *S. cerevisiae* and synthesized by GENEART (Regensburg, Germany). The synthesised gene was amplified by PCR (Bioer XP cycler, Bioer Technology CO. Ltd., China) using Phusion™ DNA polymerase (Finnzymes, Finland) and primer pair TbFRDgPGKflank promoter and TbFRDgPGKflank terminator, which included homologous sequences to *PGK1* promoter and terminator as well as sites for *Bgl*II restriction enzyme. The PCR reaction was prepared in accordance with the manual also adding 50 mM of $MgCl_2$ into the mixture. The PCR programme included the following steps: initial denaturation 30 s at 98 °C, 30 x (denaturation 10 s at 98 °C, alignment 30 s at 65 °C, elongation 1 min. and 30 s at 72 °C), final elongation 10 min at 72 °C. After amplification, a small amount of the product was run in 1% agarose gel to make sure the size of the fragment produced was right. The rest of the product was purified by QIAquick PCR purification kit (Qiagen GmbH, Germany).

The fragment with the flanking regions for *PGK* promoter and terminator was transformed into the *S. cerevisiae* strain H3488 together with *Bgl*II (New England BioLabs, USA) digested vector B1181 and yeast was plated on SCD plates lacking uracil. The obtained colonies were pooled and the whole plasmid pool was isolated according to the plasmid rescue method of Hoffmann and Winston (1987). The cells were lysed by vortexing with glass beads in a plasmid release solution (2 v/v Triton X-100, 1 v/v SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM Na_2•EDTA), phenol and chloroform-isoamyl alcohol (24:1). The DNA was precipitated with cold 70% ethanol and resuspended in TE [pH 7.0]. The isolated plasmids were transformed to the DH5α *E. coli* strain and grown on LB_{amp} plates. *E. coli* transformants were screened by PCR using the *PGK* promoter and terminator primer pair. The bacterial colonies were suspended in the PCR mixture and the PCR programme was as described in Table 5. After electrophoresis, the vectors were digested with *Eco*RI (New England Biolabs, USA) to confirm the orientation, and the whole gene was sequenced with the *PGK* promoter and terminator primer pair as well as with the TbFRDg frw3 and TbFRDg frw4 primers. After sequencing, the plasmid with the correct *FRDg* gene was transformed into *S. cerevisiae* strain H3675. The control strain carried the empty B1181 plasmid, respectively.
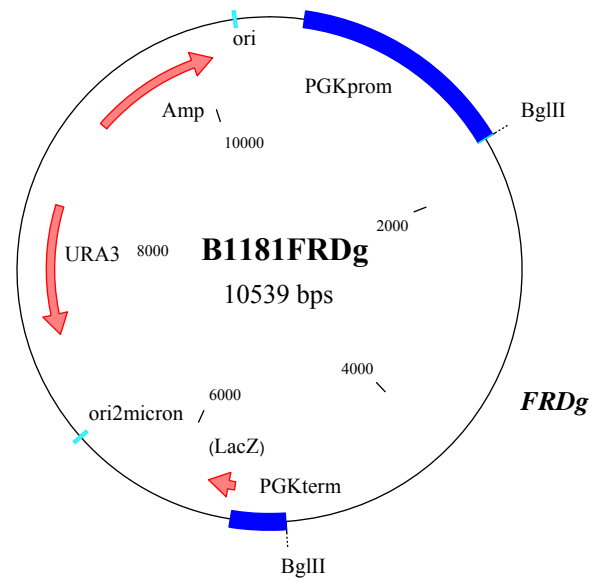
Figure 7. The constructed vector for FRDg expression in yeast S. cerevisiae strain H3675.

The H3675/FRDg strain was further transformed with pLS1 and pLS6 vectors containing *POS5* (NADH kinase of *S. cerevisiae*) and MMP1489 (NADP+-phosphatase/NAD+-kinase with NADH-kinase activity from *M. maripaludis*) genes and with emty control vector pKK27-1 resulting in strains H3675/FRDg+POS5, H3675/FRDg+MMP1489, H3675/FRDg+pKK27-1 and H3675/B1181+pKK27-1. Fresh plate cultures were used for the transformation. The master mix for transformation included 100 µl 1 M LiAc, 400 µl PEG- 4000 50% and 3.9 µl 2-mercaptoethanol. Per one yeast colony 100 µl of master mix, 3 µl denatured herring sperm [10 mg/ml] (BioRad, USA) and 1 µg of plasmid was added. The cells were incubated at 37 °C for 30 minutes and pelleted 800 g for 1 minute. The pellets were resuspended in DDIW and plated on SCDplates lacking uracil and supplemented with 200 µg/ml of G418.

2.4   Cultivations

2.4.1   Aerobic shake flask cultivations

Precultures were started from fresh plate cultures. Pre-cultivations were carried out in 4 ml of liquid media in plastic test tubes at 30 °C with 250 rpm shaking over night. All cultivations were carried out in 50 ml of medium in 250 ml Erlenmeyer flasks. If the inoculum volume was bigger than 1 ml, the cells were first collected with a flying rotor centrifuge (3000 g and 5 min) (Eppendorf centrifuge 5810 R, Eppendorf AG, Germany), the pellet was washed with 100 mM sodium phosphate buffer [pH 7.0] and the cells were resuspended in the cultivation media. The media for the cultivationsare presented in Table 3.The *FRDg* strain cultivations were started from OD_{600} (Optical Density) 0.4 and the cell growth was followed by measuring the OD_{600} with Ultrospec 2100 pro – UV/Visible spectrophotometer (Biochrom, UK) twice a day during the whole cultivation time. The samples for HPLC measurement were also taken once a day. After the growth period the plasmid stability was studied by plating 10^{-3} and 10^{-4} dilutions on solidified media of YPD, SCD-ura (H3675/B1181 and H3675/FRDg) and YPD with 200 μg/ml G418 for what strains?.

2.4.2   Anaerobic shake flask cultivations

Anaerobic cultivations were carried out with all of the *FRDg* strains and their control strains. To prevent as much aeration as possible the cultivations were carried out in a volume of 100 ml in 100 ml erlenmeyer flasks with glycerol-locks and shaken by 100 rpm. The media (table 3) were also sparged with nitrogen prior to use.

Pre-cultivations were carried out in two steps. The first step was performed as described in subsection entitled "aerobic shake flask cultivations". The second stage of pre-cultivations was carried out by inoculating cells from the previous culture in 100 ml of the same medium in 250 ml flasks. From this culture the cells were harvested by centrifugation

(3000 g, 5 min) and washed with 0.9% NaCl before resuspension into the medium sparged with nitrogen and supplemented with 20 g/L D-xylose as a carbon source. Prior to sealing the flasks with glycerol-locks, the medium was sparged again with nitrogen, and samples for the OD_{600} and HPLC measurements were taken. Xylose fermentation was followed by weighing the flasks daily. The efficiency of xylose fermentation could be followed as the weight of the flasks decreases when CO_2 formed in the fermentation escapes. After the cultivation new samples for OD_{600} measurements and HPLC were taken. Also the plasmid stability was verified after the cultivation.

2.4.3 Anaerobic cultivations on solidified media

Inoculation for the anaerobic plate cultures was done from fresh yeast cultures. The yeast strains were inoculated in 50 µl of YP medium in microtitre plate wells with a glass rod. The fumarate reductase strains and their control strains were inoculated in 0.9% NaCl. Carefully resuspended cells were replicated on plates with xylose as a carbon source. The yeast strains from VTT Culture Collection were grown on YPXplates. The plates were put in a sealed jar with AnaeroGen (OXOID Ltd., UK) sachet to generate anaerobic growth conditions. The AnaeroGen sachet absorbs the atmospheric oxygen with simultaneously generating CO_2. The cells were cultured in the jar at 30 °C for 4 days.

2.5   Enzyme assays

Enzyme activity measurements were carried out by Konelab Arena version 6.5 (Thermo, Finland) at 30 °C. The reaction mixture contained the sample and cofactor (NADH or NADPH) in 100 mM sodium phosphate buffer [pH 7.0]. The oxidation of NADPH/NADH cofactor was followed as decrease in absorbance at 340 nm before (background activity) and after the addition of the start reagent (substrate). The molar absorptivities (ε) of NADH (Roche, USA) and NADPH (Roche, USA) at the wavelength of 340 nm are 6.22 mM^{-1cm-1}. The enzyme activity was calculated from $\Delta A = \Delta A_2 - \Delta A_1$, as

shown in equation 1, the activity as U/ml. The Konelab reduces the sample blank value automatically from ΔA.

$$Volumetric\_activity = \frac{V_{tot}}{V_{sample} * \varepsilon * b} * \frac{\Delta A(\min^{-1})}{60s/\min} \qquad (1)$$

Where V_{tot} = total volume of the reaction (μl)

V_{sample} = volume of the sample (μl)

b = length of the light pathway (1 cm)

ε = molar absorptivity of NADH/NADPH ($6.22 \cdot 10^3$ M^{-1} cm^{-1})

ΔA = change in absorbanse

Protein concentrations from the crude cell extracts were measured with Konelab Arena analyzer by the method introduced by Bradford (1976) by using immunoglobulin (IgG) [1.54 mg/ml] (BioRad, USA) as a standard. Bio-Rad Protein Assay (BioRad, USA) is based on Coomassie Brilliant Blue G-250 dye that binds to proteins and causes a change in the absorption maximum of the dye from 465 nm to 595 nm. The increase in absorption is measured at 595 nm in Konelab. The specific activity was calculated by dividing the volumetric activity by the protein concentration of the crude extract:

$$Specific\_activity = \frac{Activity(U/ml)}{[C]_{prot}(mg/ml)} \qquad (2)$$

Crude cell extract preparation. For each enzyme activity measurement 30 ml of log-phase yeast cultivation was harvested by centrifugation 3000 g for 5 min. The cell pellets were washed with 100 mM sodium phosphate buffer [pH 7.0] or with 0.9% NaCl and resuspended in 1 ml of the lysis buffer (100 mM sodium phosphate buffer with Complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche, USA)). The cells were disrupted by FastPrep^{TM} FP120 homogenizer (Q-BIOgene, USA) with 500 μl of (∅ 0.5 mm) glass beads (Braun Biotech International GmbH, Germany) 3 times 30 seconds at the speed of 6.5 g. Between every cycle (30 seconds) the samples were chilled on ice. The cell lysates

were centrifuged (18 000 g, 20 min) at 4 °C and the supernatants were collected and used for the enzyme activity measurements.

2.5.1 β-xylosidase activity measurements

Crude cell extracts were assayed for BXL1 activity. The crude extract of strain H2217 was used as a negative control and the purified BXL1 enzyme from *T. reesei* as a positive control. The p-nitrophenyl-β-d-xylopyranoside (pNPX) (Sigma-Aldrich, Germany) was dissolved in 100 mM sodium phosphate buffer [pH 7.0] at different concentrations (5-40 mM). 100-175 µl of cell extract or 10 ug of β-xylosidase was added into the reaction buffer and incubated at 30 °C for 15 min. After incubation 125 mM NaOH in final concentration was added to stop the reaction and to develop the yellow colour due to the cleavage of the chromogenic group *p*-nitrophenol (pNP) from the β-d-xylopyranoside. The absorbance to correspond the BXL1 activity level was measured by the UV-spectrophotometer (Ultrospec 2100 pro – UV/Visible spectrophotometer (Biochrom, UK) at 405 nm.

2.5.2 Fumarate reductase activity measurements

Activity measurements were carried out with Konelab analyzer at 340 nm with different amounts of crude cell extract (10 to 100 µl) in a total volume of 215 µl according to the assay described by Mracek *et al.* (1991). Concentrations of fumarate (Sigma-Aldrich, Germany) varied between 1-50 mM and NADH concentration was 150 µM or 200 µM. 50 mM potassium phosphate [pH 7.0] or 100 mM sodium phosphate [pH 7.0] were used as buffer. Nine absorbance values were recorded during the 240 s reaction time.

2.5.3   XR and XDH activity measurements

The purpose of the study was to find new, interesting XR and XDH enzymes that could be applied in metabolic engineering of xylose-fermenting *S. cerevisiae* strains. In particular, the interest was either XRs with high NADH-specific activity, XDHs with high $NADP^+$-specific activity or enzymes with the high specific activity with either of the cofactors.

The measurements were carried out by Konelab analyzer from the crude cell extracts of yeast strains selected from the VTT Culture Collection. The absorbance was recorded at nine points during the 240 second measurement time. Assays were carried out in 100 mM sodium phosphate buffer [pH 7.0] containing 200 µM NADH or NADPH as cofactors in total volume of 215 µl (XR) or 200 µl (XDH).

In XR activity measurements 10 µl of cell extract was added as a sample and 200 mM D-xylose in final concentration (Sigma-Aldrich, Germany) was used to start a reaction. XDH activity was determined by measuring the reduction of xylulose to xylitol and by following the oxidation of NADH to NAD^+ or NADPH to $NADP^+$. The reaction was started with 200 µM D-xylulose in final concentration (Sigma-Aldrich, Germany). The reaction time was 300 seconds and twelve absorbance values were recorded during this time. The activity was measured with both cofactors NADPH and NADH.

2.6   <u>H</u>igh <u>P</u>erformance <u>L</u>iquid <u>C</u>hromatography (HPLC)

The extracellular metabolites and xylose consumption were measured by HPLC from the supernatant samples of the anaerobic and aerobic cultivations of *S. cerevisiae* FRDg strains. The measurements were carried out by Waters HPLC (High performance liquid chromatography) system. The apparatus consisted of a 510 pump, 717 autosampler, column oven, 410 refractive index (RI) detector and 2487 dual λ UV detector and a Fast Acid Analysis Column (100 mm x 7.8 mm, BioRad Laboratories, Hercules, CA) linked to an Aminex HPX-87H column (BioRad Labs, USA) at 55 °C with 5 mM H_2SO_4 as eluent

and a flow rate of 0.5 ml min^{-1}. The data processing was carried out with the Waters Millennium software.

The 1 ml samples from the cultures were centrifuged for 10 min at 15 700 g to remove all possible cell material. The supernatants were stored at -20 °C and right before the measurements they were diluted 1/5 in 25 mM H_2SO_4. Three different standards were run together with the samples: STDI (maltose, glucose, succinate, glycerol and propionate), STDII (citrate, acetate, fructose, lactic acid and ethanol) and STDIII (acetate, xylitol and xylose).

2.7   Northern analysis

Northern analysis was carried out by using the method first described by Alwine *et al.* (1977). The *S. cerevisiae* strains H2217/B435BXL1 and H2217/B1181BXL1 were grown in 50 ml SCD-leu medium supplemented with 20 $_g$ L^{-1} D-glucose. The RNA extraction was carried out with a Trizol reagent (Life Technologies Inc., USA) according to the manufacturer's instructions. The total amount of extracted RNA was measured with NanoDrop ND-1000 spectrophotometer (Thermo scientific, USA). For one sample 5 μg of RNA in a volume of 3.7 μl was used in the analysis. The concentration in each sample was measured with NanoDrop ND- 1000 spectrophotometer (Thermo scientific, USA). In all, per one sample 2.7 μl of 6 M glyoxal; 8.0 μl dimethylsulfoxide and 1.6 μl of 1.2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) -NaH_2PO_4 [pH 7.0] was added. The mixtures were incubated at 50 °C for one hour and then cooled to 20 °C. The samples were loaded to the RNA agarose gel (10 mM DMPC- NaH_2PO_4 [pH 7.0], DMPC-DDIW up to a total volume of 350 ml, and SYBR Green II gel stain (BMA Biomedicals, Switzerland) was added 12 μl/100 ml). All equipment and solutions used for RNA work were di-methylpropyl carbonate (DMPC) (Sigma-Aldrich, Germany) -treated to inactivate RNAses.

The RNA agarose gel was blotted over night onto a nitrocellulose filter (Hybond N membrane, Amersham Biosciences, USA) using capillary method and 20x saline-sodium

citrate (SSC) [pH 7.0] (17.2% w/v NaCl, 8.8% w/v trisodiumcitratedihydrate) solution as a transfer buffer. The RNA was cross-linked to the Hybond N membrane (Amersham Biosciences, USA) by UV light. The membrane was prehybridized at 42 °C for one hour in 20 ml of 50% v/v deionised formamide, 10% w/v dextran sulphate, 1 M NaCl, 1% v/v SDS (Sigma-Aldrich, Germany) supplemented with 10 mg/ml herring sperm DNA. The prehybridization mixture was boiled in a water bath for 10 minutes before the use.
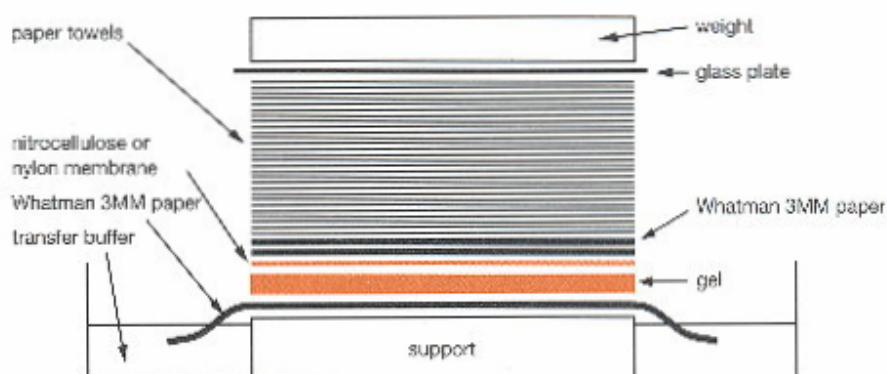

Figure 8. The illustration of the RNA gel blotting.

The probe for the hybridization was amplified by PCR (Bioer XP cycler, Bioer Technology CO. Ltd., China) from the TOPO-*bxl1* vector with the primers TrBXL1BglIIfrw and TrBXL1revII acquired from Sigma-Aldrich (Germany). The Phusion™ polymerase (Finnzymes, Finland) was used in the reaction prepared according to the manufacturer's recommendations and the PCR programme included following steps: 98 °C 30 s, 30x (98 °C 10 s, 69 °C 30 s, 72 °C 30 s) and 72 °C 10 min. The probe was extracted from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) and labelled with [α-32P]dCTP (Amersham Biosciences, USA) using the Randomly primed DNA labelling kit (Roche, Switzerland). Sephadex® G-50 (Amersham Biosciences, USA) gel filtration resin was used for the probe purification and 1x TE buffer [pH 7.5] (100 mM Tris and 50 mM EDTA) as an eluent. The incubation was carried out over night at 42 °C.

After hybridization the membrane was washed with 5x, 1x and 0.1x SSPE solutions (20 x SSPE: 17.4 w/v NaCl, 2.8 w/v NaH$_2$PO$_4$ • H$_2$O, 37 g EDTA). Autoradiography was

analysed with Typhoon Scanner (Amersham Biosciences, USA) and the image was processed by Image Quant software (Amersham Biosciences, USA).

3   RESULTS

3.1   Development of a transporter assay for screening of novel xylose-specific transporters

3.1.1   Enzyme activities

To build up the xylose transporter assay the β-xylosidase encoding gene *bxl1* was amplified by PCR from the *T. rees*ei cDNA bank in *E. coli*. The native BXL1 protein in *T. reesei* is excreted from the cell. To prevent this in yeast, the putative signal sequence (59 bps) encoding the N-terminal 20 amino acids of BXL1 was excluded from the amplified gene. The PCR product was ligated via pCR$^{®}$2.1$^{®}$-TOPO vector (Invitrogen, USA) into the single copy plasmid B435 and to the multicopy plasmid B1184. These plasmids were subsequently transformed into the *S. cerevisiae* strain H2217. The enzyme activity of BXL1 was measured from crude cell extracts using pNPX as a substrate in the reaction.

Regardless of the concentration of pNPX, the amount of cell extract, temperature (30-60 °C), pH (3.5-6.0) and incubation time (5-30 min.), the difference in activities between the negative control (H2217) and the single- and multicopy *bxl1* strains were undetectable. The positive control on the other hand showed relatively higher activity, the absorbance above 1.00 ± 32.

3.1.2 Northern analysis

To study the transcription of the *bxl1* gene in the strains with either singlecopy or multicopy vectors, a Northern blot analysis was carried out. Figure 9 shows the hybridization of [α-32P]dCTP labelled *bxl1* probe to the mRNA of *bxl1* gene expressed in the cells.
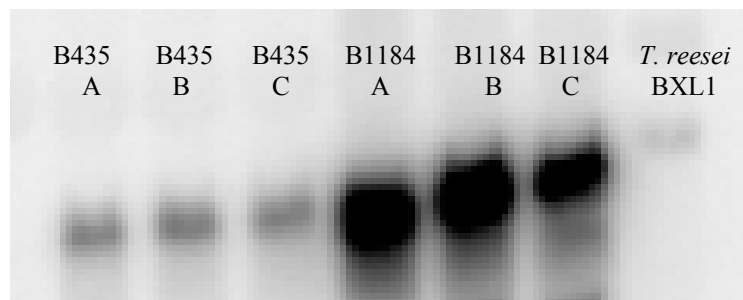

| B435 A | B435 B | B435 C | B1184 A | B1184 B | B1184 C | *T. reesei* BXL1 |

Figure 9. The bxl1 expression was studied by Norther analysis. The hybridization of the [α-32P]dCTP labelled probe with mRNA of bxl1 expressed from single- (B435) and multicopy (B1184) vectors in H2217 S. cerevisiae strain. T. reesei total RNA was hybridized as a positive control (right-most lane in the figure).

Figure 9 shows that the *bxl1* gene was expressed from both vectors. The transcription level was notably higher in the multicopy strain H2217/B1181BXL1 compared to the single copy strain H2217/B435BXL1, as expected. As a positive control, a total RNA sample from *T. reesei* was used. The Northern blot analysis clearly shows the expression of *bxl1,* but since the activity could not be measured in the transformed cells, it can be assumed that the enzyme was either not produced at all, or at least it was not active without the signal sequence.

3.2   Expression of fumarate reductase encoding gene in xylose-utilising *S.*
**   *cerevisiae* to alleviate the redox imbalance**

3.2.1   Enzyme activities

The NADH-dependent fumarate reductase encoding gene (*FRDg*) of *T. brucei* was expressed in the *S. cerevisiae* strain H3675 (with *Ss*XR-XDH pathway) in order to increase the NAD^+ pool for the XDH reaction, and moreover, to enhance fermentation of xylose to ethanol. Furthermore, the vectors containing Pos5p (NADH kinase of *S. cerevisiae*) and MMP1489 (NADP+-phosphatase/ NAD+-kinase with NADH-kinase activity from *M. maripaludis*) encoding genes were transformed into the H3675/FRDg strain. The resulting strains and the controls (empty B1181 and B1181/FRDg+pKK27-1) were grown on xylose as a carbon source. Fumarate reductase activity measurements were performed with the strain H3675/FRDg and the H3675/B1181 served as a reference.

The activity was measured as oxidation of NADH since fumarate reductase uses NADH as an electron donor to reduce fumarate to succinate. Different concentrations of fumarate and cofactor NADH, as well as variable amounts of cell extract were used in the assay. However, the enzyme activity could not be detected.

3.2.2   Aerobic cultivations

Two independent clones of the strains constructed in this study were cultivated in aerobic shake flask cultures on SCX-ura or on YNB/L-glutamate + xylose media to better maintain *POS5*, MMP1489 and pKK27-1 plasmids with the G418 selection. 200 µg/ml of G418 as the final concentration was added to the cultures of the strains H3675/FRDg+POS5, H3675/FRDg+MMP1489, H3675/FRDg+pKK27-1 and H3675/B1181+pKK27-1 The cultivations were started from OD_{600} 0.4 and the biomass production was measured daily at OD_{600} with a spectrophotometer.
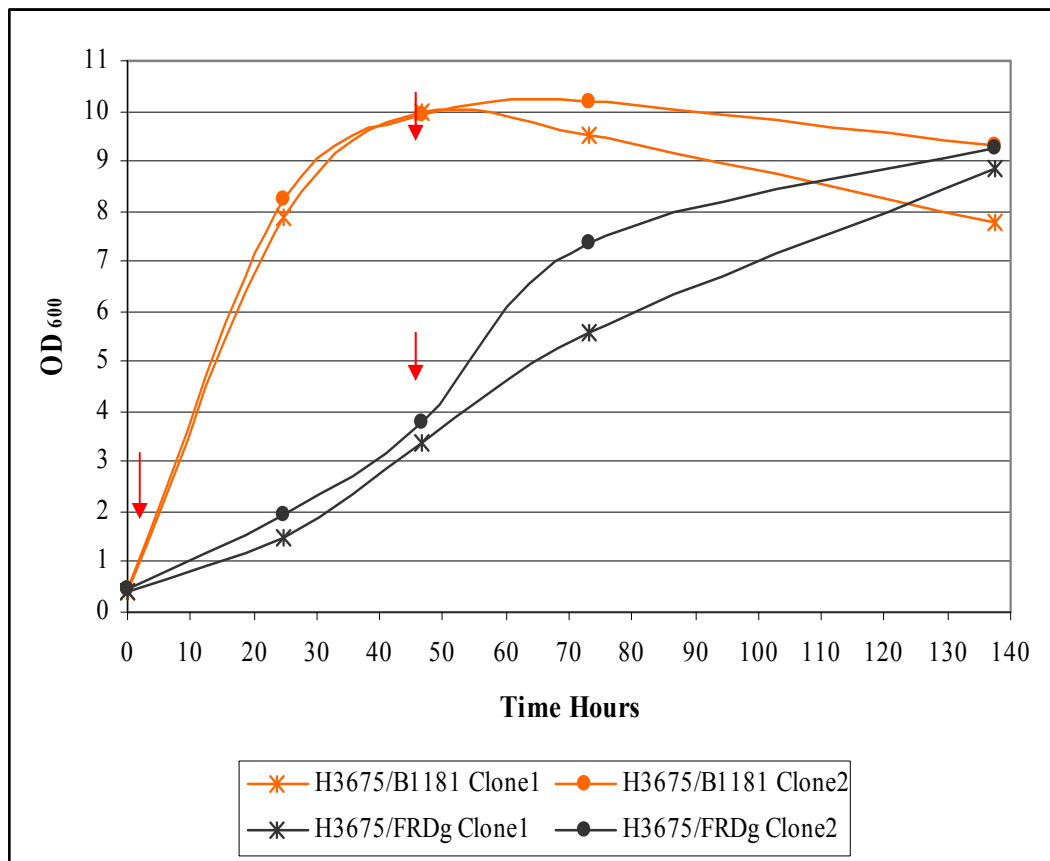
Figure 10. Aerobic growth of strains H3675/FRDg and H3675/B1181 on SCX-ura cultivation medium.The arrows are marked in the Figure to show the period (0-48 h) that growth rates and xylose consumption rates were calculated.

Figure 10 shows that both H3675/FRDg and H3675/B1181 strains grew on xylose under aerobic conditions due to the integrated genes encoding the XR and XDH enzymes. However, the strains expressing *FRDg* grew at a considerably lower rate compared to the control strain. The result suggests that some kind of an extra load is created in the cells due to *FRDg* expression, despite the fact that the activity could not be detected by the assay used.

The rest of the constructed strains additionally carried plasmids with a G418 resistance encoding gene. Hence, the aerobic growth of these strains was carried out on YNB/glutamate + G418 medium (Figure 11).
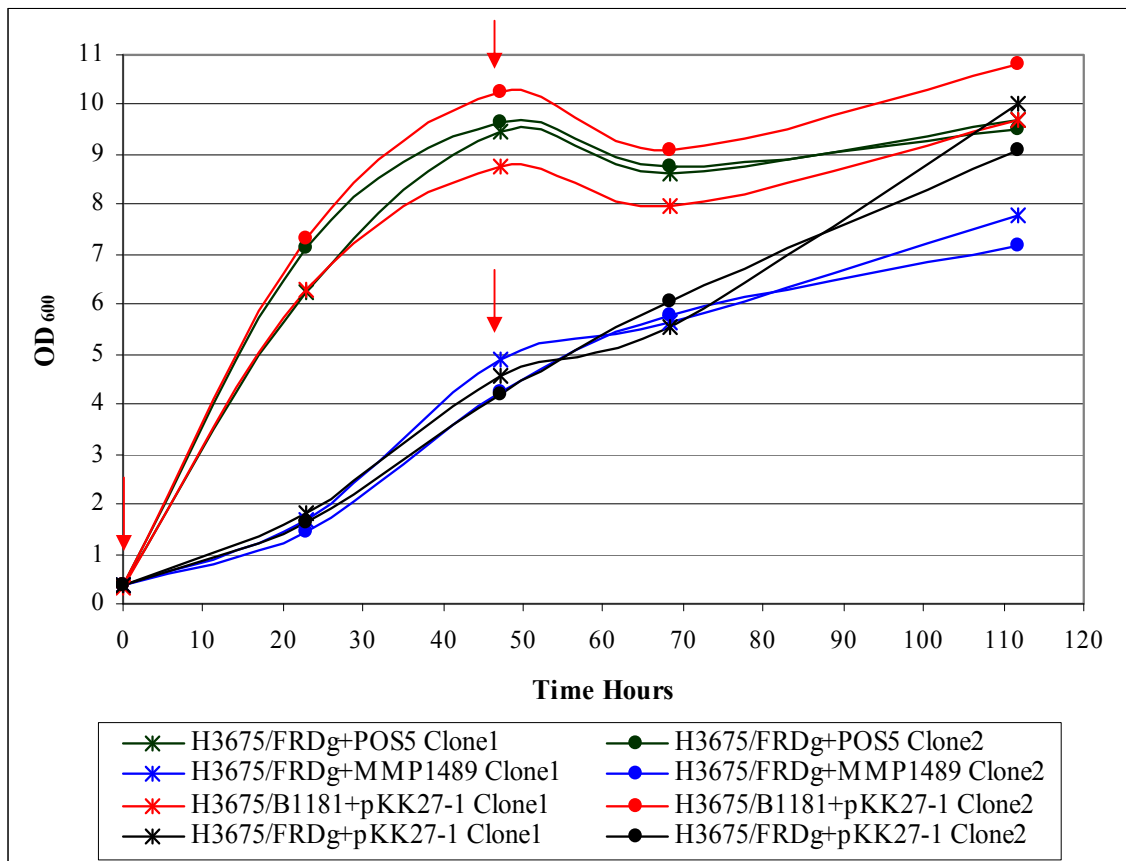
Figure 11. Aerobic growth of strains containing expression vectors based on G418 selection in the YNB/L-glutamate+2% xylose medium. The arrows are marked in the Figure to show the period (0-48 h) that growth rates and xylose consumption rates were calculated.

When *POS5* was expressed together with *FRDg*, the growth rate was restored to the level of the control strain B1181-pKK27-1. As seen in Figure 10, the strains carrying only the *FRDg* gene had a significantly lower initial growth rate compared to the control strain. Although the FRDg + pKK27-1 and MMP1489 strains had a slower growth rate (Table 6) compared to the FRDg + POS5 strain, at the end of the cultivation the FRDg+pKK27-1 strain had a comparable final biomass to the B1181+pKK27-1 and FRDg + POS5 strains, while the FRDg + MMP1489 strain lagged slightly behind reaching only OD_{600} of 8.

Table 6 summarizes the growth rates (OD/h) and xylose consumption rates (g/h) calculated from the first 24 hours of the cultivation. The xylose concentration (g/l) in the media at the end of the cultivations is also shown. The values are given as averages of two independent clones in duplicate cultivations.

Table 6. The growth rates and xylose consumption rates (0-48 h) and the xylose concentration in the medium at the end of the cultivation.

Strain	Growth rate on xylose OD/h	Xylose consumption g/l/h	Xylose concentration g
H3675/B1181	0.26 ± 0.08 (1.)	0.29 ± 0.01 (1.)	0.08 ± 0 (1.)
H3675/FRDg	0.07 ± 0.00 (5.)	0.02 ± 0.00 (5.)	0.66 ± 0.12 (2.)
H3675/FRDg+POS5	0.20 ± 0.00 (3.)	0.25 ± 0.03 (2.)	0.67 ± 0.07 (2.)
H3675/FRDg+MMP1489	0.09 ± 0.01 (5.)	0.06 ± 0.01 (4.)	1.97 ± 0 (4.)
H3675/B1181+pKK27-1	0.19 ± 0.02 (2.)	0.25 ± 0.01 (2.)	0.78 ± 0.21 (3.)
H3675/FRDg+pKK27-1	0.09 ± 0.00 (4.)	0.07 ± 0.01 (3.)	2.12 ± 0.11 (5.)

The data (Figures 10 and 11, and Table 6) show that the growth rates were proportional to the xylose consumption rates; the better the growth rate the better the xylose consumption was. The strains B1181, FRDg + POS5 and B1181 + pKK27-1 had only minor differences in their growth rates and xylose consumption. However, regarding the total xylose consumed, the control strain B1181 had the best capacity to utilize xylose over the entire cultivation, but the FRDg + POS5 and FRDg strains consumed almost equal amounts of xylose, in spite of the fact that the FRDg strain had a much lower growth rate. The growth rate of H3675/FRDg + MMP1489 strain was comparable with the H3675/FRDg strain, but the amount of xylose consumed (g/l) was significantly lower. The B1181 + pKK27-1 and FRDg + pKK27-1 consumed less xylose compared to the B1181 and FRDg strains; this may be due to additional plasmid burden caused by pKK27-1 or the different growth media used.

Overall, the results show that the expression of *POS5* gene in the H3675/FRDg strain restored the growth rate of the *FRDg* expressing strain to the level of the control strain H3675/B1181+pKK27-1 (Figure 11). Perhaps, under aerobic conditions NADPH provided by the Pos5p reaction was used for biomass production. The expression of fumarate reductase encoding gene decreased the biomass production rate compared to the control strains, suggesting that the enzyme was produced in the cells creating an extra burden. Although the FRD activity *in vitro* was not detected, it may still have *in vivo* activity in the

cells affecting the xylose consumption and growth. However, HPLC analysis did not show any succinate excretion by the strains. Thus, if active, it likely did not carry out the reduction of fumarate to succinate or succinate was rapidly used by some other reactions in the metabolism.

3.2.3 Anaerobic cultivations

The *S. cerevisiae* can not grow either aerobically or anaerobically on xylose, since it lacks the sufficient enzyme activities needed for its metabolism. The genetically modified strains can metabolise xylose, but with a much lower rate than glucose, due to most likely the redox imbalance created by the reactions converting xylose to xylulose. The anaerobic cultivations were carried out on SC-ura (Fig. 12) and YNB/glutamate media (Fig. 13) with 20 $_g$ L^{-1} of xylose starting from an initial OD_{600} of 4. The fermentation rate was followed by measuring daily the weight loss of the flasks. At the end of fermentation the extracellular metabolites were analysed from samples of the culture medium by HPLC.

The fermentation rates as weight loss (mg) due to the concominant production (and loss) of CO_2 are presented in Figures 12 and 13. The strains H3675/B1181 and H3675/FRDg are presented separately in Figure 12 as they lack the geneticin resistance plasmid, and therefore were cultivated in a different medium than the rest of the strains.
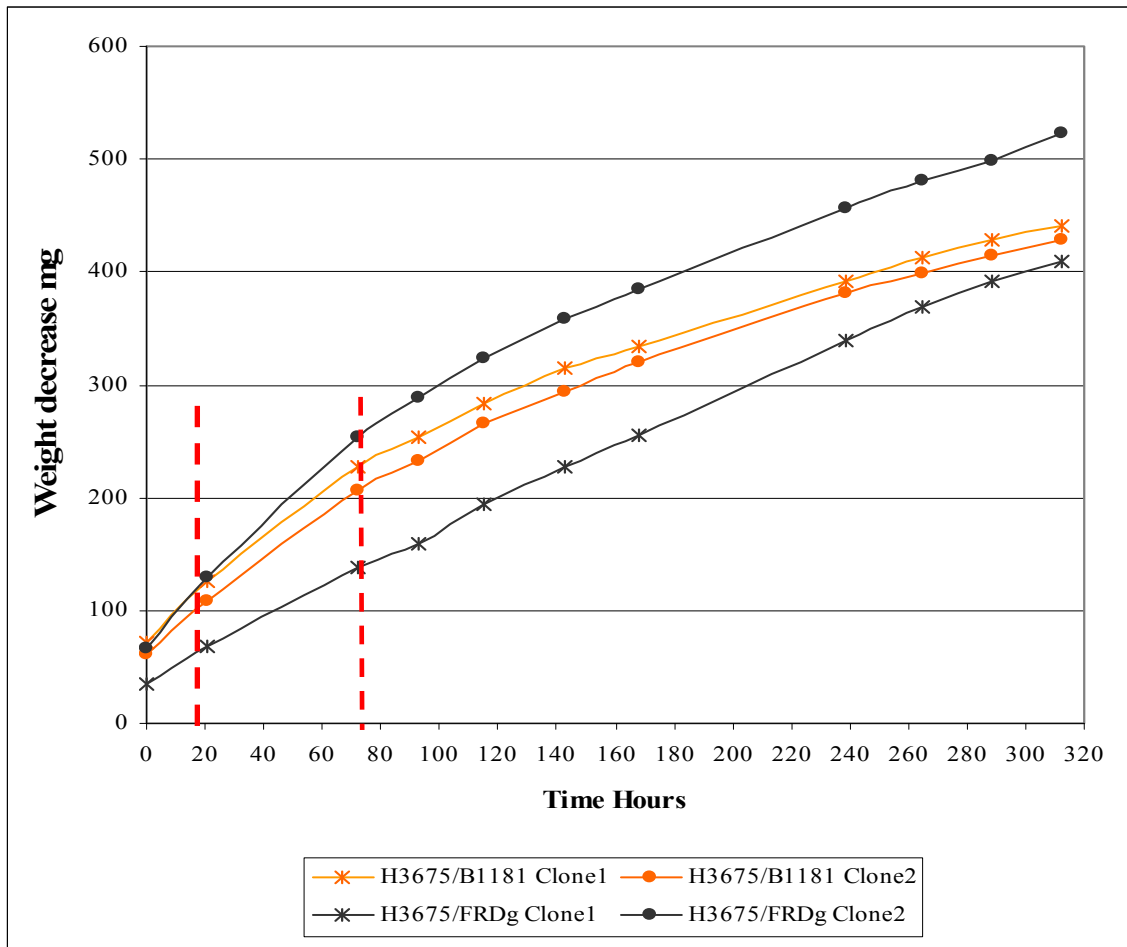
Figure 12. The fermentation rates of the strains H3675/B1181 and H3675/FRDg, shown as weight loss, in anaerobic shake flask cultivations.The fermentation rates were calculated from the period (20-72 h) marked in the fugure with dashed lines.
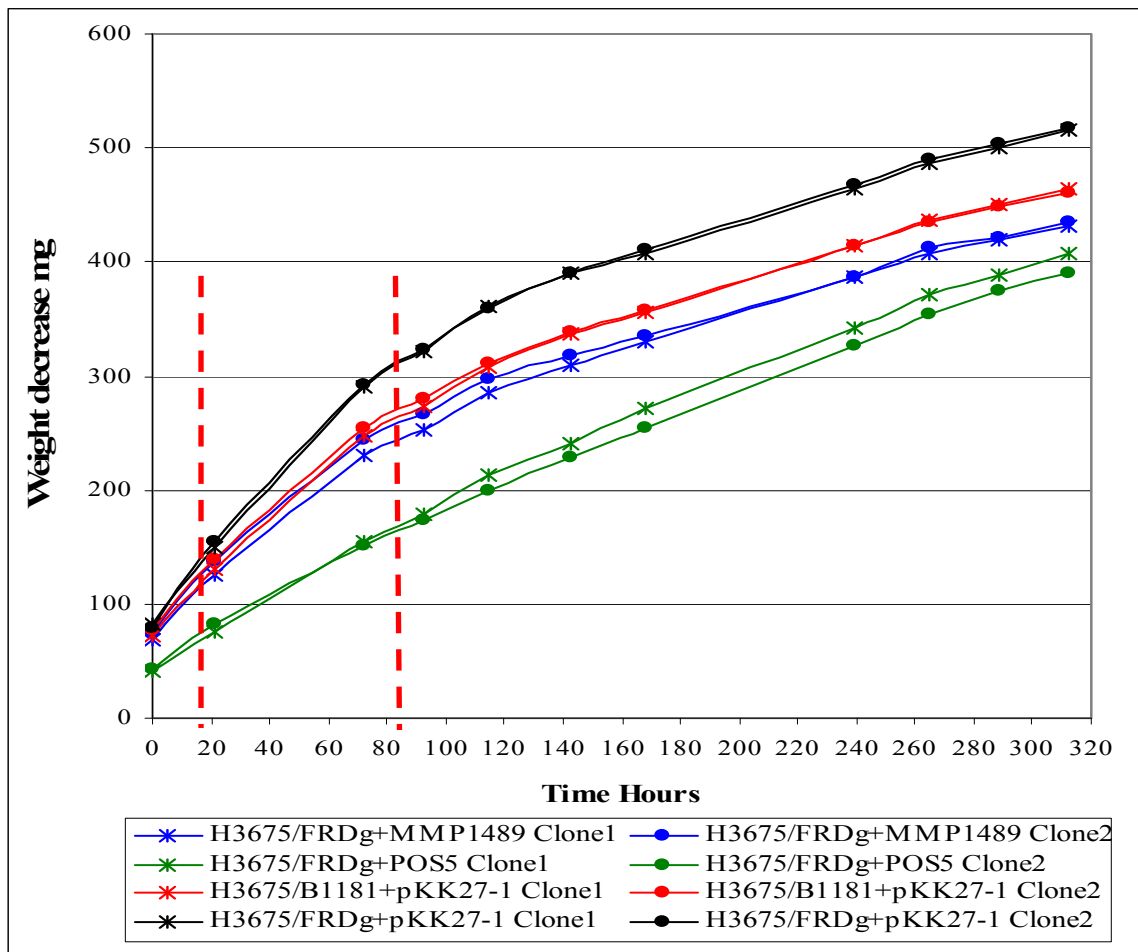
Figure 13. The figure shows the fermentation rates as weight loss of FRDg strains with the plasmid carryingeither POS5 or MMP1489, or the empty PKK27-1 plasmid, with the geneticin resistance gene on 20 g L^{-1} xylose. The fermentation rates were calculated from the period (0-72 h) marked with dashed lines.

The fermentation rate, [C-mmol ethanol/h], of each strain is represented in in Table 7. In anaerobic xylose cultivations the fermentation slows down, due to increasing redox imbalance. Taking this, and the relatively long culticvation time, to account the fermentation rates (Table 6) were calculated between 0-72 hours, marked in Figures 12 and 13 with dashed lines.

Table 7. The fermentation rates calculated from the loss of CO_2 (0-72 h) and the total [C-mmol ethanol/h] produced during the anaerobic cultivation.

Strain	[C-mmol ethanol/h]		Total [C-mmol ethanol]
	Clone 1	Clone 2	
H3675/B1181	0.045	0.044	9.85 ± 0.18
H3675/FRDg	0.031	0.055	10.58 ± 1.81
H3675/FRDg+POS5	0.035	0.031	9.05 ± 0.26
H3675/FRDg+MMP1489	0.047	0.048	9.83 ± 0.05
H3675/B1181+pKK27-1	0.052	0.051	10.49 ± 0.08
H3675/FRDg+pKK27-1	0.063	0.061	11.72 ± 0.03

Table 7 shows that FRDg + pKK27-1 strains had the fastest and the strains with FRDg + POS5 had the slowest fermentation rate. The other strains had comparable fermentation rates, between 0,044 and 0,052. Unfortunately, the 2 clones of H3675/FRDg strain were not comparable, clone 1 having a significantly lower rate than clone 2.

The xylose consumption rate, ethanol and xylitol production rates in anaerobic cultivations are summarised in Table 8 below. Moreover, the succinate production is shown. The metabolites were measured from the growth medium samples before and after the fermentation.

Table 8. The xylose consumption rate and ethanol and xylitol production rates during the anaerobic cultivation (h=312) on 20 g/L xylose. Succinate production is shown as grams per cultivation.

Strain	Total xylose consumed g/L/h	EtOH production g/L/h	Xylitol production g/L/h	Total succinate produced g
H3675/B1181	0.055 ± 0	0.009 ± 0	0.031 ± 0	0.05 ± 0
H3675/FRDg	0.057 ± 0.02	0.011 ± 0.001	0.025 ± 0	0.04 ± 0.02
H3675/FRDg+POS5	0.044 ± 0.01	0.006 ± 0	0.028 ± 0.001	0.04 ± 0.02
H3675/FRDg+MMP1489	0.055 ± 0.01	0.009 ± 0	0.032 ± 0.001	0.04 ± 0.02
H3675/B1181+pKK27-1	0.057 ± 0.01	0.010 ± 0	0.032 ± 0	0.04 ± 0.02
H3675/FRDg+pKK27-1	0.057 ± 0	0.012 ± 0	0.028 ± 0	0.04 ± 0.02

All the strains consumed comparable amounts of xylose, ecxept the FRDg + POS5 strain which clearly showed the lowest xylose consumption rate. The production rates of ethanol were highest with FRDg, FRDg + pKK27-1 and B1181+pKK27-1 strains. In all strains the xylitol production rate was higher than that of ethanol. Even though FRDg was expected to increase succinate production there were no differences in the amounts excreted.

The yields [(g) EtOH or xylitol produced/(g) xylose consumed] shown in Table 9 give a better understanding of the differences between the strains regardind the ethaol and xylitol production. The ratio shows how much xylitol was produced over ethanol in each strain.

Table 9. The table represents the ethanol and xylitol yields over the xylose consumed (g/g). The yields are calculated from total ethanol/xylitol produced and divided by the total xylose consumed during the fermentation.

Strain	EtOH Yield g/g	Xylitol Yield g/g	Ratio Xylitol/EtOH
H3675/B1181	0.17 ± 0	0.56 ± 0.01	3.3 ± 0.1
H3675/FRDg	0.20 ± 0	0.43 ± 0.01	2.2 ± 0.1
H3675/FRDg+POS5	0.13 ± 0	0.64 ± 0.02	4.7 ± 0.0
H3675/FRDg+MMP1489	0.16 ± 0	0.59 ± 0	3.6 ± 0.0
H3675/B1181+pKK27-1	0.17 ± 0.01	0.57 ± 0.01	3.3 ± 0.1
H3675/FRDg+pKK27-1	0.21 ± 0.01	0.49 ± 0.01	2.3 ± 0.1

The yields show that besides ethanol all strains produced significant amounts of xylitol as a side product. However, FRDg and FRDg+pKK27-1 strains produced clearly less xylitol compared with the other strains and had the lowest ratio of xylitol over ethanol, which suggests an improved NAD^+ availability and redox balance compared to the other strains; *e.g.* in the control strains B1181 and B1181+pKK27-1 the respective value was 3.3. The strains with FRDg and either POS5 or MMP1489 had the highest ratio of xylitol over ethanol; 3.6 and 4.7, respectively.

The results from the anaerobic shake flask cultivations on xylose show that the *FRDg* expression improved the ethanol yield from xylose by about 15%. Under anaerobic conditions *POS5* expression did not give any advantage; in contrast the *POS5* expressing strain produced less ethanol compared with the control strains.

3.3   XR and XDH activity screening

From the 150 yeast species and strains selected from the VTT Culture collection, overall 44 yeast species/strains showed growth on solidified YPX medium under microaerobic conditions. These yeast species/strains were selected for the XR and XDH enzyme activity measurements. Besides these, *S. stipitis* strain CBS6054 with endogenous XR and XDH enzymes was included in the study as a control. The activities were measured at least twice from all of the chosen strains grown on YP + 2% w/v xylose and the results are reported as the means, and additionally, the standard error of the mean was calculated. As described in the Materials and methods section, the XDH activity was measured in the reverse direction, from xylulose to xylitol. Thus the results are presented as XDH NADH and NADPH activities. The XR activities were measured in the forward direction. Only a few of the yeast assayed had interesting XR and XDH activities and for this reason only selected data is presented.

The measured XDH (NADH) activities are shown in Figure 14. Only one strain, 338 (*Cryptococcus victoriae*), had clearly higher XDH activity than *S. stipitis*. However, the strains 334 (*Cryptococcus macerans*) and 270 (*Candida parapsilosis*) showed comparable activities.
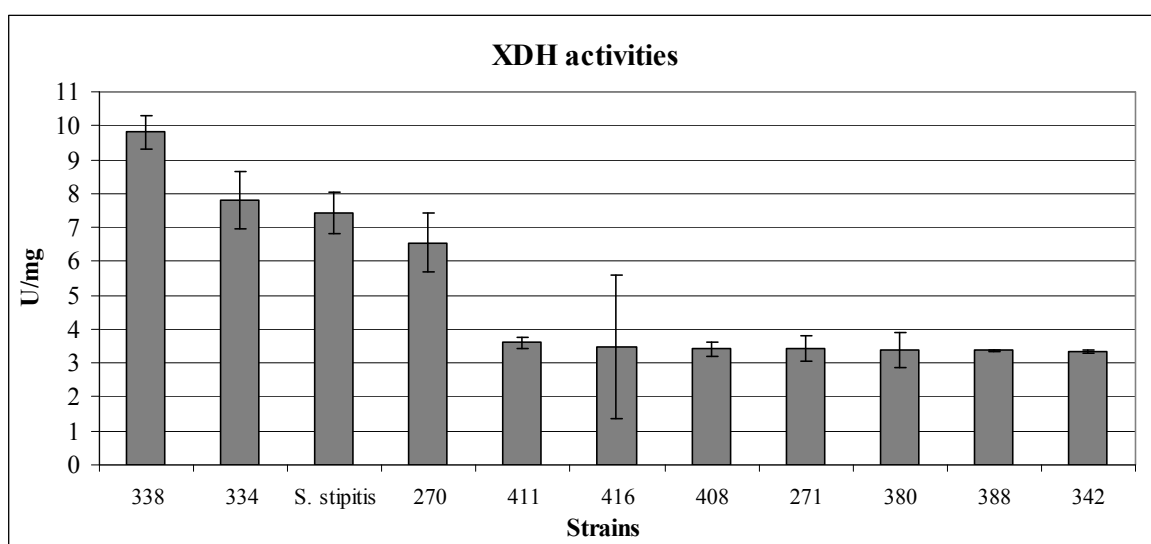

Figure 14. The highest XDH NADH activities are shown. The strains from left to right: 338; C. victoriae, 334; C. macerans; S. stipitis (CBS6045), 270; Candida intermedia, 411; Cryptococcus diffluens; 416; Lipomyces starkeyi, 408; S. stipitis, 271; C. parapsilosis, 380; K. fluxuum, 388; Pichia burtonii, 342; D. hungarica.

Also the XDH activity with NADPH was measured from the strains; however, the activities were much lower compared to the activity with NADH. Interesting though, the strains *C. victoriae* (338), *C. macerans* (334) and 503 (*Pichia mandshurica*) showed higher XDH NADPH activity than *S. stipitis* (Fig. 15). The NADPH/NADH ratios were 3.6 (338), 3.2 (334) and 2.1 (503) fold higher compared to that of *S. stipitis*.

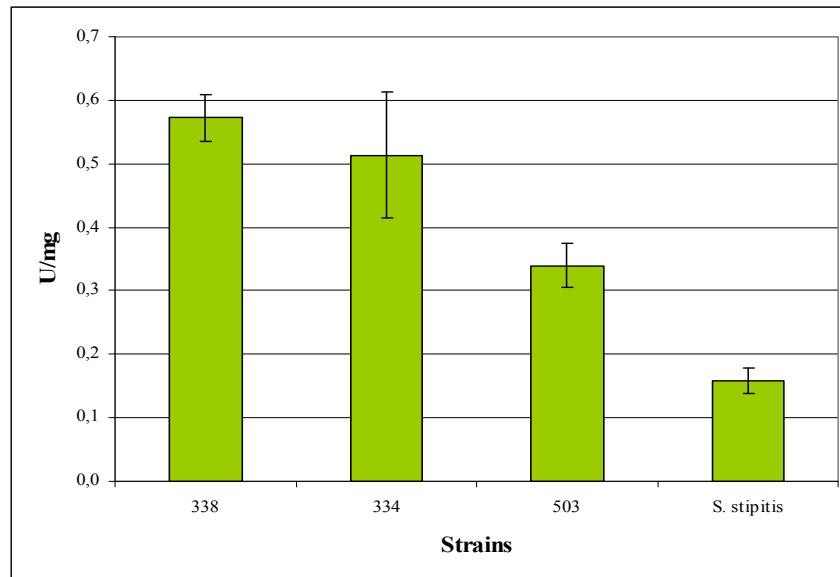*Figure 15. The XDH NADPH activities of the strains 338 (C. victoriae), 334 (C. macerans) and 503 (Pichia mandshurica).*

Figure 16 below shows the highest XR (NADPH) activities found within the strains studies. Overall, four strains showed comparable activities with *S. stipitis*. These strains were: 271; *C. parapsilosis*, 334; *C. macerans*, 338; *C. victoriae* and 342; *Dioszegia hungarica*.
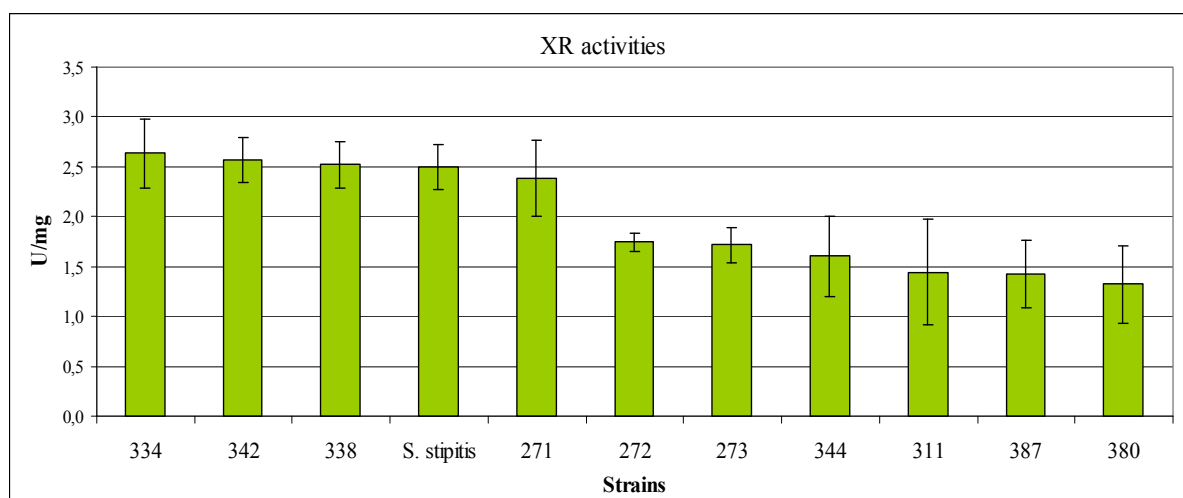
Figure 16. The highest XR activities measured are presented The strains are represented in order of decreasing activity: 334; C. macerans, 342; D. hungariga, 338; C. victoriae, S. stipitis, 271; C. parapsilosis, 272; Yarrowia lipolytica, 273; Candida parapsilosis, 344; Hanseniaspora uvarum, 311; S. stipitis, 387; Pichia burtonii, 380; Kregervanrija fluxuum.

Furthermore, the XR of strains 408 (*S. stipitis, unknown strain*), 413 (*Lipomyces tetrasporus*) and 416 (*Lipomyces starkeyi*) showed dual cofactor specifity, similar to *S. stipitis* (Fig. 17). *S. stipitis* control strain had an XR NADPH/NADH ratio of 1.5 and the ratios for the other strains were 4.1, 1.3 and 1.6, respectively. Although the total activities (U/mg) were much lower compared to *S. stipitis*, the results show that XR of *L. starkey* has even better, nearly equal, capability of using both cofactors than *S. stipitis.*
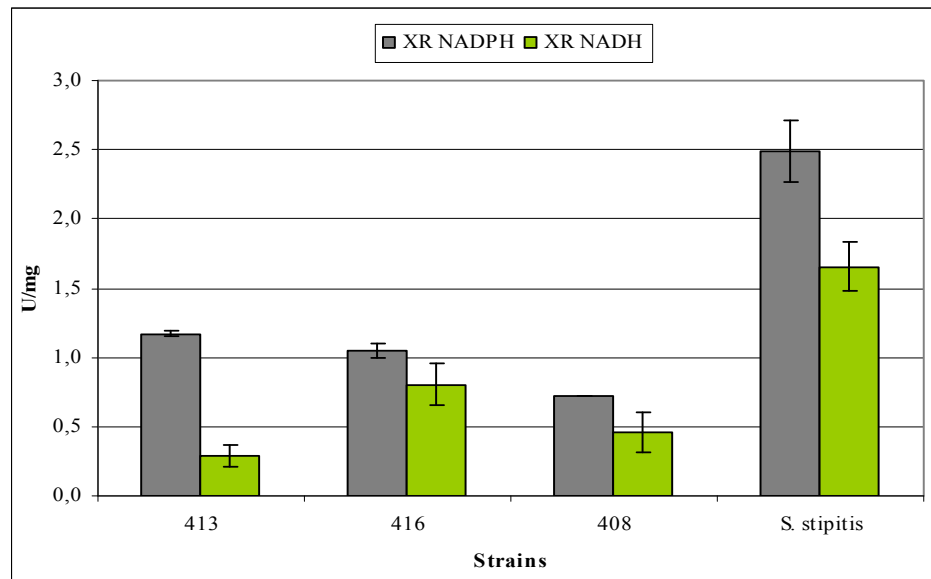
Figure 17. The dual cofactor specifities of XR from 413 (L. tetrasporus), 416 (L. starkey) and 408 (S. stipitis) and S. stipitis.

The majority of the strains had much higher XDH activities compared to the XR activity, but 12 yeasts showed higher XR than XDH activities (Figure 18). However, the activities are significantly lower compared to the levels measured and shown in Figure 14 for XDH and in Figure 16 for XR.
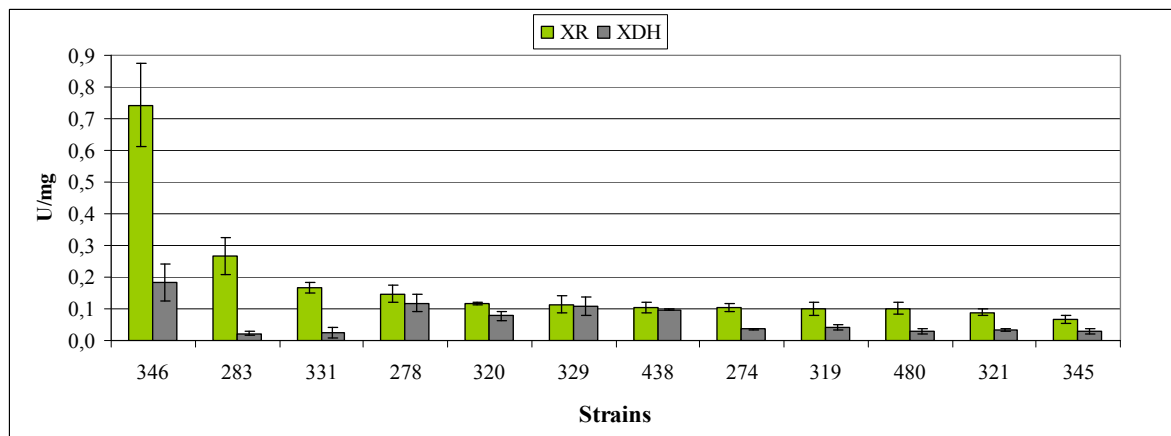
Figure 18. Strains with higher XR activity compared to XDH activity. The strains are represented in order from the highest XR activity to the lowest. Also the XDH activities are shown. The strains are 346; Rhodotorula pinicola, 283; Candida succiphila, 331; Candida solani, 278; Cryptococcus albidus, 320; Cryptococcus wieringae, 329; Candida picinguabensis, 438; Candida catenulate, 274; Debaryomyces hansenii, 319; Cryptococcus albidosimilis, 480; Cryptococcus laurentii, 321; Filobasidium globisporum, 345; Pichia fabianii.

The strains with the highest XDH/XR activity ratios are presented in the Figure 19 below. Additionally, the ratio of the strain 334 is shown, since it was equal to *S. stipitis*. The strain with the highest ratio, 332, is *Candida cylindracea*.

Figure 19. The XHD/XR activity ratios represented together with XR and XDH activities. The strains, in addition to S. stipitis, are 332;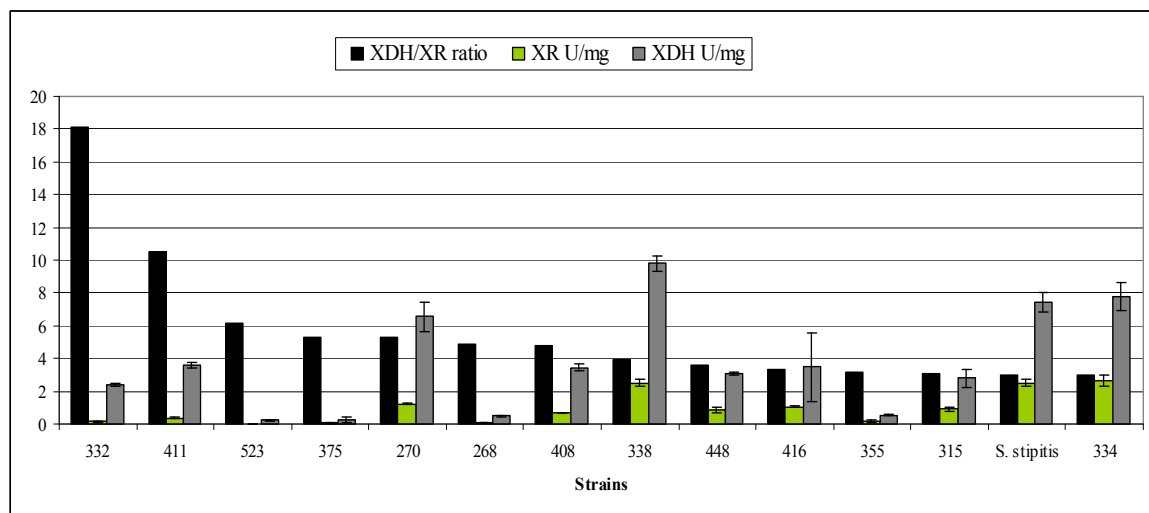 C. cylindracea, 411; C. diffluens, 523; Pichia cactophila, 375; Pichia fermentans, 270; C. intermedia, 268; Yarrowia lipolytica, 408; P. stipitis, 338; P. burtonii, 448; Pichia farinose, 416; L. starkeyi, 355; Pichia heedii, 315; Pichia angusta, 334; C. macerans.

As a summary, a few strains with higher or similar XDH NADH (Fig. 14) and XR NADPH (Fig. 16) activities compared to *S. stipitis* were identified. *C. victoriae* and *C. macerans* had the highest XDH activity with both cofactors NADH and NADPH. Additionally, *Pichia mandshurica* showed higher XDH NADPH activity than *S. stipitis*. The highest XR NADPH activities (Fig. 16) were measured from *C. macerans, D. hungariga and C. victoriae.* Furthermore, the highest XR NADH activities were found from *L. tetrasporus*, *L. strakey* and *S. stipitis*. In general, the XDH activities were much higher than XR activities; however, in 12 yeast the XR activities were higher (Fig. 18). Furthermore, in 12 yeast the ratio of XDH/XR activity (Fig. 19) was higher compared with *S. stipitis.*

4 DISCUSSION

4.1 Development of a transporter assay for screening of xylose-specific transporters

The aim of this study was to build up a xylose transporter assay for a high-throughput screening of xylose transporters from a variety of organisms. The strategy was based on the study of Chen *et al.* (2009) where an *in vivo* assay in *E. coli* expressing β-xylosidase (encoded by the *XynB* gene) from *Bacillus pumilus* was developed by using thechromogenic xylose analogue pNPX. The *XynB* was expressed alone or together with the *Zymomonas mobilis* glucose facilitator protein Glf (*glf*). Chen's group succeeded in developing a transport assay with the minimal pNPX transport activity being 0.2 nmol min^{-1} mg dw $^{-1}$, which is sufficient for high-throughput screening.

The above-mentioned assay was tried to be developed in *S. cerevisiae* with *T. reesei* BXL1 enzyme, which was previously produced in an active form by Margolles-Clark *et al.* (1996) in *S. cerevisiae.* However, contrary to Margolles-Clarck and coworkers, our aim was to express the protein intracellularly by deleting the signal sequence. Moreover, also the β-xylosidase from *Aspergillus oryzae* has been succesfully expressed in *S. cerevisiae* (Katahira et al., 2004). Dissapontingly, the positive control was the only one showing enzyme activity in the assay. In spite the Norther blot analysis showing expression of *bxl*, no enzyme activity could be detected. Therefore, the siqnal sequences with different lengths should have been tested to assess the effect of signal sequence deletion on the activity of the enzyme.

Xylanolytic enzymes are usually secreted or do not possess suitable pH and temperature optima for *S. cerevisiae* (Yanai and Sato, 2001). However, there is an extremely potential enzyme candidate, the intracellular β-xylosidase from *Candida utilis* reported by Yanai and Sato (2001). Unfortunately, the sequence encoding the enzyme is still unknown.

4.2 Expression of fumarate reductase encoding gene in the xylose-utilising *S. cerevisiae* to alleviate the redox imbalance

Despite close to three decades of studies, the industrial processes still lack the fully optimal organism for the economical 2nd generation bioethanol production. One of the bottlenecks of xylose fermentation by recombinant *S. cerevisiae* with the oxidoreductive pathway is the maintenance of intracellular redox balance. Numerous studies concerning this limiting step have been published and the strategies from protein engineering to evolutionary engineering have been reported in *S. cerevisiae* (Vemuri et al., 2007; Krahulec et al., 2010; Bengtsson, 2009; Sonderegger and Sauer, 2003; Jin et al., 2005; Petschacher and Nidetzky, 2008; Kuyper et al., 2003; Walfridsson et al., 1995; Toivari et al., 2001; Matsushika et al., 2009; Panagiotou et al., 2009). In this study the very same obstacle was in focus; the improvement of intracellular redox balance was assessed with *T. brucei* NADH-fumarate reductase in order to increase the NAD^+ availabity for the XDH reaction and thus decrease the xylitol excretion. Moreover, *POS5* encoding the NADH-kinase and the MMP1489 encoding the NADP+-phosphatase/NAD+-kinase were expressed together with the fumarate reductase encoding gene to additionally increase the NADPH availability for the XR reaction.

Under anaerobic conditions respiration is blocked, thus other means for continuous regeneration of NAD^+ are needed. Since mitochondria are not permeable to pyridine nucleotides, including NADH, some other way must exist to allow the reoxidation of reduced nucleotides in the compartment where they are produced (Enomoto et al., 2002). In fermentation the [NAD^+/NADH] ratio is exclusively regulated by the production of glycerol that acts as a primary electrol sink to eliminate NADH surplus (Bakker et al., 2000). Furthermore, the reoxidation of mitochondrial NADH is important for ethanol-acetaldehyde shuttle under anaerobic conditions (Bakker et al., 2000; Camarasa et al., 2007).

The *FRDg* gene from *T. brucei* was overexpressed in yeast under the *S. cerevisiae PGK1* promoter. In general, the African Trypanosomatids and their metabolism is relatively well studied for medical purposes, however, to our knowledge, *FRDg* has not been expressed

before in *S. cerevisiae*. In *T. brucei*, *FRDg* is expressed in the procyclic stage of the cells in the peroxisome-like organelles called glycosomes. The enzyme is responsible for 70% of the parasite's fumarate reduction and subsequent succinate production (Coustou et al., 2006). Furthermore, the enzyme participates in the redox balancing under anaerobic conditions (Mracek et al., 1991). Similar kind of FRDs have also been found from other Trypanosomatids *e.g.* from *Trypanosoma cruzi*, and additionally, from other parasites such as *Leishmania* and *Shewanella* species as well as from thermophilic chemolithoautotrophic bacterium, *Hydrogenobacter thermophilus* TK-6 (Miura et al., 2008; Chen et al., 2001).

Mracek *et al.* (1991) measured the specific activity of endogenous FRDg in *T. brucei* using 1 mM fumarate and 150 μM NADH, and the reported activity was 7 mU/mg at pH 7.0-7.4. Former studies also showed clear quantitative correlation between the FRD activity and the amount of excreted succinate (Coustou et al., 2006; Camarasa et al., 2007; Miura et al., 2008; Besteiro et al., 2002). Although, the same method was used in this study we were not able to detect increased FRDg activity or succinate production in *FRDg* expressing strains compared with the control strains. There are several possible reasons for this result. The reason/reasons could be in the assay setup; non-optimal concentrations of the substrate/cofactor/protein, temperature or pH. Additionally, protein misfolding could decrease the activity to a level difficult to measure. Besteiro and co-workers (2002) measured the activities of FRDg in *T. brucei* procyclic cells in different salt concentrations and showed that only in 300 mM KCl approximately 80% of the FRD activity was soluble, indicating that the protein needs a high ionic strength to be soluble. Furthermore, they tried to express *FRDg* gene in *E. coli* and in *Pichia pastoris*, but both failed to produce soluble recombinant proteins for functional assays, most likely due to too low intracellular ionic strength. Extra succinate production could not either be detected or succinate possibly produced was used in the cells, such as for the reoxidation of $FADH_2$ (Camarasa et al., 2007). Moreover, as glycosomes do not exist in yeast, protein mistargeting could take place, leading to *FRDg* ubiquitination. Ubiquination is a post-translational process which can influence the activity of some proteins or target them for proteasome-mediated degradation (Leach and Brown, 2011).

Despite the fact that FRDg activity was not detected, expression of its encoding gene had a physiological effect on aerobically and anaerobically growing *S. cerevisiae* cells on xylose. In aerobic cultivations the H3675/FRDg strain had lower growth rate compared to control implying that the enzyme probably was active and reduced either the ATP supply and/or affected negatively to the intracellular redox balance. Even if the ethanol yields (Table 8) remained remarkably under the theoretical level (0.51 g ethanol g consumed xylose^{-1}), the anaerobic cultivations gave promising results as the strain H3675/FRDg showed the the lowest xylitol to ethanol ratio, which implicates to the NADH oxidazing activity of FRDg and to the improved recycling of redox cofactors in the initial steps of pentose metabolism.

When NADH-kinase encoding gene *POS5* was expressed together with FRDg encoding gene the aerobic growth on xylose was restored back to the level of the control strain. Also previous studies have shown that overexpression of the NADH-kinase encoding gene in *Aspergillus nidulans* increased the maximum specific growth rate as well as biomass yield during aerobic glucose cultivations. This was proposed to demostrate the increased NADPH availability in the mitochondria (Panagiotou et al., 2009). NADH-kinase consumes in its reaction ATP to produce either NADPH or NADP$^+$, which suggests that the *FRDg* expressing cells were not depleted of ATP but of NADPH for the synthesis of biomass. For instance, if succinate was converted to isocitrate by isocitrate lyase, and further, to citrate by isocitrate dehydrogenase, NADPH would have been consumed in the latter reaction. However, neither citrate was detected in the HPLC measurements.

Under anaerobic conditions *POS5* expression increased the xylitol production at a cost of the ethanol titer. Hou *et al.* (2009) observed accordingly that overexpression of the cytosolic NADH kinase encoding gene decreased the specific growth rate and biomass yield as well as increased xylitol accumulation during anaerobic growth. This is likely due to an energetic limitation; in anaerobiosis there is less ATP available, also required for phosphorylation of xylulose to xylulose 5-phosphate.

The MMP1489 had only a minor positive effect on the aerobic growth rate of *FRDg* expressing strain, and its negative effect on the anaerobic fermentation rate was not as strong as the effect of *POS5* expression, suggesting that its NADH/NAD$^+$-kinase activity

was lower. NAD^+ kinases are strictly ATP dependent which could explain the decreased growth and poor ethanol fermentation rate (Kawai and Murata, 2008; Kawai et al., 2005). However, it was not studied whether the $NADP^+$-phosphatase or the NAD^+-kinase activity was predominant in our strains. In addition, the plasmid stability was not studied, so it is possible that the pLS1 (*MMP1489*) and the pLS6 (*POS5*) plasmids were lost at some point of the cultivation to decrease the burden in the cells. Although the phosphatase or kinase enzymes did not increase the rate of xylose consumption and fermentation in this study they might still provide an important source of redox cofactors in yeast, which can be useful for metabolic engineering strategies where the redox fluxes are manipulated (Hou et al., 2009).

Even though the aerobic growth rates remained low in H3675/FRDg, as well as in FRDg+pKK27-1, both strains showed the best fermentation ability among the strains studied. Thus our results suggest that the cofactor imbalance is slightly improved due to extra NADH oxidation, even if with relatively low activity. However, there is still an unfortunate cofactor imbalance dominating between the XR and XDH enzymes. The *POS5* expressing strains were able to utilize xylose for biomass production in aerobic conditions, but the fermentation rate remained relatively low and the amount of xylitol excreted was the highest of all the strains.

4.3   XR and XDH activity screening

In this study, we screened ~150 yeast species and strains from the VTT Culture Collection on aerobic and microaerobic plate cultrures with 20 g/L xylose. 44 of these yeast, including *S. stipitis*, showed growth on xylose also under microaerobic conditions. XR and XDH activities of the yeast were measured and compared with the XR and XDH activities of *S. stipitis* that is well known for its XR able to use both cofactors NADH and NADPH. In all, most of the yeast species studied are relatively unknown or at least their xylose metabolism and fermentation capability is not yet wellstudied. However, the species with the highest enzyme activities towards xylose and xylulose mainly included *Candida, Cryptococcus, Lipomyces* and *Pichia* species.

Even if in this study the yeast *C. parapsilosis* (271 and 273) showed only minor XR/NADH activity, Lee *et al.* (2003) reported ten times lower K_m for NADH than for NADPH, which is contradictory to our findings. However, we measured the activitywith a high cofactor concentration, which is why the results do not necessarily correlate to each other. The XR encoded by the *C. parapsilosis XYL1* gene is the first XR enzyme reported to prefer NADH over NADPH, since the enzyme carries an arginine instead of a lysine in the tetra-amino acid motif (Ile-Pro-Lys-Ser) which is conserved among the NADPH-dependent xylose reductases (Lee et al., 2003; Lee, 1998). It is possible that our isolate of the species has a slightly different metabolism or the cultivations were contaminated at some point and therefore significantly lower activities with NADH were measured. In addition, it has been repoted that some strains of *C. victoriae* are able to assimilate D-xylose, but lack the capability to ferment it (Thomas-Hall et al., 2002). Our *Cryptococcus* species, especially *C. victoriae* and *C. macerans* were able to compete in XR (NADPH) and XDH (NADH) activities with *S. stipitis*, but for more importantly, they showed clearly much higher XDH NADPH activities. Also *L. starkeyi* with the XDH NADPH activity has previously been shown to utilize xylose. However, the *Cryptococcus* as well as *Lipomyces* genera belong to the family of oil-rich yeast, which are usually able to utilize xylose, but they prefer producing oils over alcohols (Thomas-Hall et al., 2002; Fall et al., 1984). Up today, there are only a few reports on the yeast genus *Dioszegia*. In our study *D. hungarica* had one of the highest XR NADPH activities among the strains. It has previously been reported that this yeast is able to assimilate D-xylose (Takashima et al., 2001).

Only few strains, for example *S. stipitis*, *C. shehatae* and *C. tenuis* have been previously found to harbour an XR with dual coenzyme specificity; nevertheless, their XRs still tend to prefer NADPH over NADH (Verduyn et al., 1985; Lee et al., 2003; Ho et al., 1990; Kavanagh et al., 2003; Neuhauser et al., 1997). In this study, a few previously unreported XR dual specitifies were found; in *L. strakey* (416) the activity ratio of the cofactors was lower than in *S. stipitis*, 1.3 and 1.5, respectively. Unfortunately, strains with an XR preferring only NADH were not discovered. Since most of the strains had a strictly NADPH dependent XR, while being able to grow on xylose under nearly anaerobic conditions, these yeast must have another way to balance the redox cofactors or they prefer producing other compounds instead of ethanol.

Additionally, xylose isomerase activity was measured from a few species that lacked almost completely the XDH activity, but with negative results. To our knowledge, NADP$^+$ dependent XDH enzymes have not been identified yet. However, at the end, we were able to find yeast strains with this uncommon XDH NADPH activity; *C. victoriea* (338), *C. macerans* (334) and *Pichia mandshurica* (503). In the future, the identified enzymes could be used for metabolic engineering of yeast to produce bioethanol or xylitol.

5 FUTURE PROSPECTS

This thesis consists of three separate studies. In the first study, development of a transporter assay for screening of specific xylose transporters was attempted by using β-xylosidase from *T. reesei*.In the second study, optimized *T. brucei FRDg* was expressed in *S. cerevisiae* in order to alleviate the intracellular redox imbalance due to different cofactor specificities of XR and XDH enzymes.In the third study, XR and XDH activities were measured from microaerobically xylose utilising yeast species.

The xylose transporter assay study was hindered by the inactivity ofthe enzyme, most likely as a result of the deletion of the siqnal sequence. However, different lengths of deletions were not tried in the plasmid construction, which would be reasonable to be further examined. The other option is the expression of a natively intracellular β-xylosidase; however, few native intracellular β-xylosidases have been identified, the only one reported so far is that of *Candida utilis* (Yanai and Sato, 2001). Unfortunately, the gene has not been sequenced yet, but in the future it could be used in this kind of an assay.

In the case of expression of the fumarate reductase encoding gene, our results indicate an improved recycling of cofactors in the *FRDg* carrying strains under anaerobic conditions on xylose, furthermore leading to the lowered ratio of xylitol over ethanol produced. Hence, the studies have been continued; the plasmid B1181FRDg has been re-transformed into *S. cerevisiae* H3675, more transformants have been characterized and also more controlled cultivations in bioreactors have been performed. In shake flask cultures the parameters to be measured, such as pH and aeration can not be controlled and monitored constantly as in bioreactors, consequently leading to some differences between cultivations

in individual shake flasks. So far the results are in agreement with the ones presented in this study. Additionally, Northern blot analysis showed that FRDg is expressed in *S. cerevisiae* cells and the enzyme activity was measured from the crude cell extracts by an alternative assay.

Regarding the XR and XDH activity screening, the *C. victoriae* (338) NADP$^+$/XDH is now purified from the yeast. The next step is the cloning and expression of its encoding gene in the xylose utilising *S. cerevisiae*.

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