FERMENTATION OF XYLOSE AND XYLANS BY KLUYVEROMYCES MARXIANUS IMB STRAINS

By

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FERMENTATION OF XYLOSE AND XYLANS BY

KLUYVEROMYCES MARXIANUS IMB STRAINS

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CHAPTER 1

INTRODUCTION

Bioethanol is the most abundantly used renewable transportation fuel and corn starch is currently the main source for bioethanol in the United States (Mosier et al. 2005b). About 9 billion gallons of bioethanol were produced in 2008 in the United States according to the Renewable Fuels Association (2009). Unfortunately, this technology together with reduced crop acres, droughts and increasing petroleum costs contributed to the enormous rise in the price of corn in 2008 (Anderson et al. 2008). Since corn is not available in sufficient quantities to replace petroleum based fuel, alternative substrates need to be found for the bioethanol industry.

Various substrates are subject for research as alternatives to corn for bioethanol production. Perennial crops are of interest since they can be harvested multiple times per year and do not need to be planted every year. Annual costs for establishing and managing perennial energy crops are lower in comparison to other feedstocks (Monique et al. 2003). An example of a perennial energy crop is switchgrass, which is composed of about 30% dry weight cellulose, 20% dry weight hemicellulose, and 18% dry weight lignin (Wiselogel et al. 1996). The main constituent of hemicelluloses in perennial energy crops and wood residues is xylan; a polymer of xylose molecules. Since the amount of xylan can be more than 20%, it is important to find

ways to utilize this component for ethanol production to increase the overall conversion yield. The utilization of xylose is not only important for fuel production but also for other products, such as xylitol, which is of interest to the food industry.

Mosier et al. (2005a) optimized controlled liquid hot water pretreatment of corn stover. In their study, 40% of corn stover xylan was solubilized and present in the prehydrolyzate (the liquid remaining after pretreatment), which could be used as feedstock for further fermentation. This requires the optimization of xylose fermentation, since xylose is the major carbohydrate in the prehydrolyzate. Dien et al. (2008) also pointed out the need for optimization of xylose fermentation. The researchers investigated enzyme characterizations for hydrolysis of AFEX (ammonia fiber explosion) and liquid hot water pretreated distillers' grains (DDGS) and their conversion to ethanol. The xylan content of DDGS was 40% and with a mixture of four enzyme preparations, a xylose yield of 81% was achieved. Again, this shows the great potential of increasing the overall conversion efficiency by enzymatic hydrolysis of xylan to xylose followed by fermentation.

Enzymatic hydrolysis (also known as saccharification) and fermentation can be executed in two different modes; simultaneous saccharification and fermentation (SSF) and separated hydrolysis and fermentation (SHF). Both methods show advantages and disadvantages depending on yeast strain, location and annual average temperature, ethanol tolerance and temperature optima of yeast and enzymes. Usually, the enzymes used for hydrolysis have thermophilic temperature optima (40–50°C), whereas, yeast often have mesophilic temperature optima (20-30°C). This favors the separated hydrolysis and fermentation where the optimal temperature can be used in each step. A

disadvantage to separate hydrolysis and fermentation is the resulting high sugar concentration after enzymatic hydrolysis that can cause product inhibition to the enzyme. In this case, SSF is favorable because the yeast is converting the hydrolyzed sugar immediately, and no inhibiting concentration of sugar can be built up (Savarese and Young 1978).

Since enzymes have higher temperature optima than common yeast strains used for alcohol fermentation, it would be beneficial to find strains with higher temperature optima to optimize simultaneous saccharification and fermentation (SSF) of xylan. Some potential candidates for such yeast are the *Kluyveromyces marxianus* IMB strains (Banat and Marchant 1995). Five *K. marxianus* strains, IMB1, 2, 3, 4, and 5, were found in an Indian distillery (Banat et al. 1992). These strains showed the ability to grow on glucose at higher temperatures (52°C) than *Saccharomyces cerevisiae* and to ferment xylose to ethanol at temperatures up to 45°C. IMB4's optimum growth rate, for instance, was found at 40°C and a value of 0.99 h^{-1} with glucose as substrate (Banat and Marchant 1995). Little research with thermotolerant yeast capable of fermenting xylose has been done.

Another product of xylose fermentation is xylitol. Xylitol is a sugar alcohol and can be used as a sweetener (Guo et al. 2006). Researchers have found that xylitol has an anti-ketonic (Kinami and Kitagawa 1969) and anti-infection effect (Brown et al. 2004). It was also found that the use of xylitol-containing chewing gum reduced dental plaque (Larmas et al. 1976; Scheinen et al. 1975), which was confirmed by Lynch and Milgrom (2003) who also found an anti-cariogenic effect. Since xylitol does not increase blood sugar, it can be used as a substitute for sugar in food for diabetics without changing

chemical, physical and sensory characteristics (Bakr 1997). Wilkins et al. (2008) found that IMB2, IMB4, and IMB5 were xylitol producers under anaerobic conditions. The yields ranged at 40°C between 0.05 g/g and 0.25 g/g with IMB4 and IMB5, respectively. It is not known how the IMB strains utilize xylose under microaerobic conditions, which is what is generally used for successful xylose fermentation.

Another way to improve efficiency of biomass conversion to biofuels is the use of a co-culture system. Advantages shown in previous studies, include better resistance to contamination (Harrison 1978) and increase in biomass yield, which was observed with a co-culture of *Candida kefyr* LY496 and *Candida valida* LY497 (Carlotti et al. 1990). *Pichia stipitis* has been found to be a xylose assimilating yeast strain. It produced up to 5.9 g/l ethanol in a media with 20 g/l xylose at 25°C (Toivola et al. 1984) and 21.46 g/l with 50 g/l xylose at 30°C (Du Preez and Prior 1985). *P. stipitis* in co-culture with either *S. cerevisiae* or *K. marxianus* was used for fermentation of a glucose/xylose mixture (Rouhollah et al. 2007). It was found that the co-culture of *P. stipitis* and *S. cerevisiae* did not improve ethanol production in comparison to fermentation with the respective monocultures. The researchers concluded there were adverse effects on each other, which they did not define. On the other hand, co-culture fermentation with *P. stipitis* and *K. marxianus* showed an increase in ethanol yield. An ethanol yield of 0.42 g/g was achieved by the co-culture as opposed to yields of 0.40 g/g and 0.36 g/g with monocultures of *P. stipitis* and *K. marxianus*, respectively. The media contained glucose and xylose in equal parts (30 g/l) along with other common sugars occurring in hemicellulose. It is still unknown how a co-culture with *P. stipitis* and *K. marxianus* ferments xylose as the sole carbon source.

In this study, five *K. marxianus* IMB strains were analyzed for fermentation of xylose at 40°C and 45°C under microaerobic conditions. Ethanol and xylitol production by the strains was measured. Based on these results, *K. marxianus* IMB2 was used in a SSF system with xylan as the sole carbon source and Multifect Xylanase as the xylan-hydrolyzing enzyme at 40°C and 45°C under microaerobic conditions. The second part of this study involved the use of IMB2 and *Pichia stipitis* in a co-culture system using xylose as the sole carbon source at 30°C under microaerobic conditions. Ethanol and xylitol production were the performance parameters measured.

CHAPTER 2

OBJECTIVES

The objectives of this research are:

1) Determine the abilities of *K. marxianus* IMB1, IMB2, IMB3, IMB4 and IMB5 to ferment xylose and produce ethanol and xylitol at 40 and 45°C.
2) Investigate the production of ethanol and xylitol from xylose using SSF on xylan with the IMB strain that showed best performance in the first objective.
3) Improve the production of ethanol from xylose using co-culture fermentation with *P. stipitis* and the IMB strain that showed the best performance in the first objective.

CHAPTER 3

LITERATURE

3.1 Feedstocks

Feedstocks can also be classified in terms of raw material type. Sugar, starch, and cellulose are the three raw materials used for bioethanol fermentation. Sugar is available as a main constituent of sugar cane, sweet sorghum and sugar beets. Crops rich in starch are grains, such as corn and milo, and root crops, such as potatoes and cassava. Cellulose is available in wood and herbaceous crops (Lin and Tanaka 2006). Switchgrass (*Panicum virgatum*), herbaceous crop, is native to the central and eastern US, including Oklahoma, and shows great potential as a substrate for bioethanol production (Sanderson et al. 1996). It is primarily composed of cellulose, hemicellulose, and lignin. It requires a different process to produce bioethanol than starch-based substrates. Switchgrass is composed of 31% dry weight cellulose, 20.4 % dry weight hemicellulose, and 17.6 % dry weight lignin (Wiselogel et al. 1996). The main constituent of switchgrass hemicelluloses and hemicelluloses in other grasses is xylan (Kormelink and Voragen 1993), which is a polymer of xylose molecules. Since grasses can obtain up to 20% xylan, it is very important to find methods to utilize this component for ethanol production or for other valuable products. To utilize xylose as a carbon source in fermentation, xylose fermenting yeast strains need to be found or constructed by genetic engineering since the

most commonly used ethanol producing yeast, *S. cerevisiae*, is not able to utilize xylose (Kreger-Van Rij 1984)

3.2 Bioethanol production

The bioethanol production process can be separated into four parts: pretreatment, hydrolysis, fermentation, and product purification. Prior to the processing, the substrate is of course harvested. The time of harvest is important since mature feedstocks show increased lignification, whereas, immature commodities lack in sufficient carbohydrates. For example, switchgrass has developed 90% of its dry matter by August (Gettle et al. 1996; Koshi et al. 1982). Figure 1 shows an overview of an ethanol production process based on cellulosic biomass. In this example the enzymatic hydrolysis and fermentation are combined in a mode known as simultaneous saccharification and fermentation (SSF). The pretreatment starts with mechanical size reduction by a hammer mill to increase the surface area, so that enzymatic and microbial attack is enhanced. A particle size of 0.2 to 2 mm can be achieved after milling (Sun and Cheng 2002). The power required for this process increases with decreasing final particle size and may be a limiting factor. Product purification is usually done by distillation together with molecular sieves.

3.2.1 Pretreatment of biomass

Lignocellulosic material needs pretreatment prior to enzymatic hydrolysis because of its structure. The carbohydrates necessary for fermentation are cellulose and hemicelluloses. Unfortunately, these polysaccharides are surrounded by a lignin structure that is highly resistant to microbial and/or enzymatic attack and is not fermentable

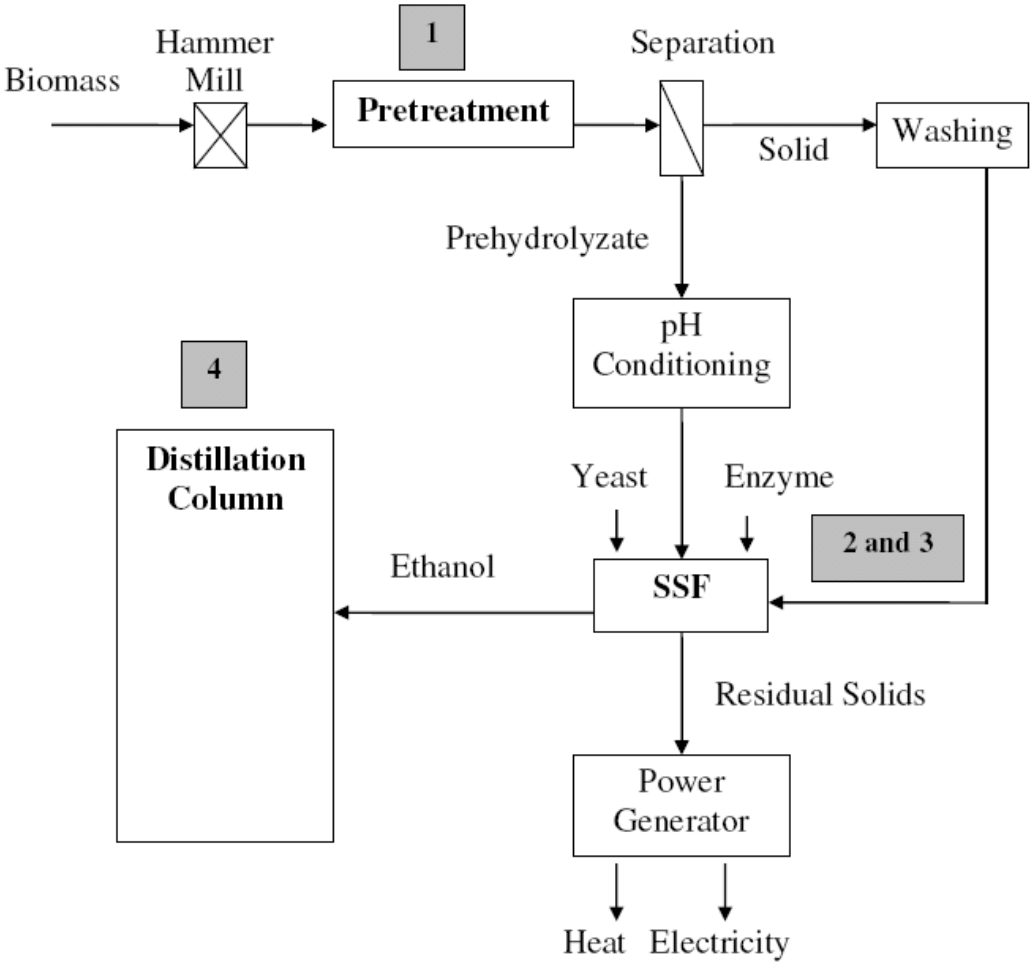

Figure 1: Process overview of ethanol production based on lignocellulosic material with SSF, with permission from Suryawati (2007). 1 = Pretreatment, 2 and 3 = simultaneous saccharification and fermentation, 4 = product purification.

(Hsu et al. 1980; Mosier et al. 2005b). Pretreatment methods disrupt and pull apart the lignin structure to make cellulose and hemicellulose available for enzymatic hydrolysis. The hemicellulose is typically dissolved in the liquid fraction, called prehydrolyzate, during pretreatment. The prehydrolyzate can also be used for fermentation. Mosier at al. (2005b) illustrated the effect of pretreatment on biomass (Figure 2). Due to an enhanced convertibility, process efficiency can be increased and costs decrease (Kohlmann et al. 1995; Lee et al. 1994; Lynd et al. 1996; Mosier et al. 2003a; Mosier et al. 2003b).

Steam explosion (autohydrolysis). During steam explosion, the biomass is first heated with high-pressure steam. Then the biomass undergoes an explosive decompression when the pressure is reduced rapidly to atmospheric pressure. Typical temperature conditions for steam explosion are 160 to 260°C. These conditions are held for a time ranging from seconds to a few minutes (Sun and Cheng 2002). Optimal conditions for solubilization and hydrolysis of hemicellulose of wood chips were found either at 270°C for 1 min or 190°C for 10 min (Duff and Murray 1996). One disadvantage of this process is the degradation of xylan and subsequent formation of inhibitory compounds for microbial populations.

Ammonia fiber explosion. Ammonia fiber explosion (AFEX) is similar to steam explosion pretreatment, but with the addition of liquid ammonia. High pressure and temperature is applied. No inhibitory compounds are produced and the particle size does not affect AFEX. One to two kg of ammonia/kg dry biomass is used with a temperature of 90°C for 30 min (Sun and Cheng 2002). It was observed that no solubilization of

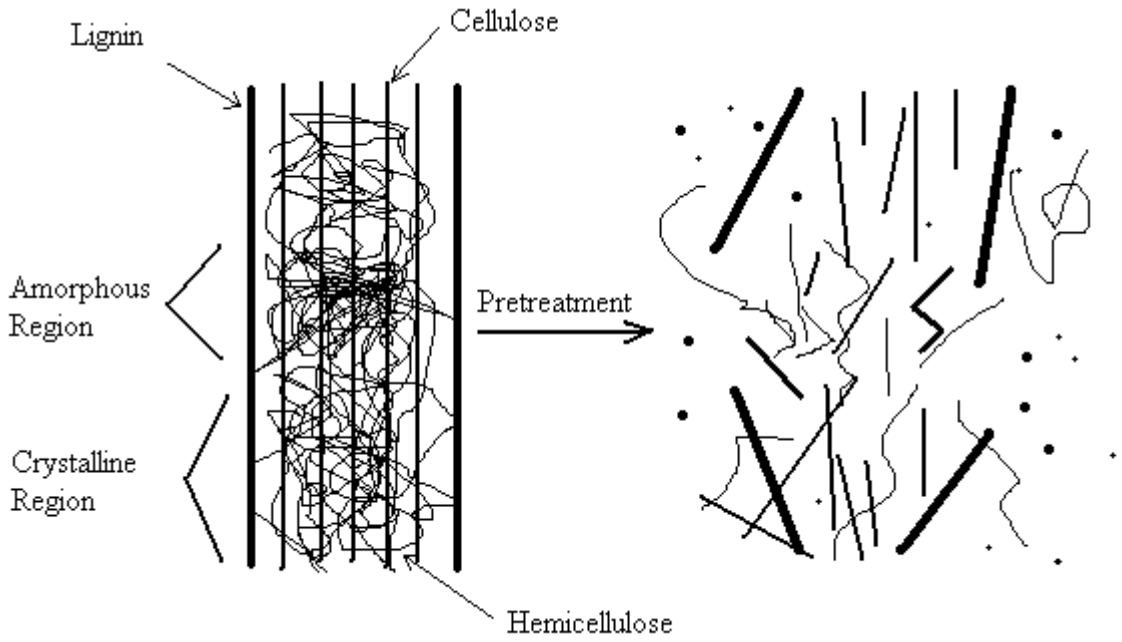

Figure 2: Effect of pretreatment on biomass (adapted from Hsu et al. (1980))

hemicellulose occurs with AFEX. The composition after pretreatment is almost the same as before. It was also stated that lignin-rich substrates are poorly hydrolyzed. Ammonia must to be recycled for environmental protection purposes.

CO_2 explosion. CO_2 explosion is similar to steam explosion and AFEX. It is hypothesized that CO_2 forms carbonic acid during the process (Sun and Cheng 2002). With the formation of acid, the rate of hydrolysis can be increased. It was found that the yields of enzymatic hydrolysis after CO_2 explosions are lower than with steam explosion, but CO_2 explosion is more cost effective (Zheng et al. 1998). Alfalfa pretreated with 4 kg CO_2/kg fiber and 5.62 MPa showed a release of 75% of the theoretical glucose after enzymatic hydrolysis (Dale and Moreira 1982). No inhibitor formation occurs with CO_2 explosion (Sun and Cheng 2002).

Liquid hot water pretreatment. In this pretreatment, water is maintained as a liquid at elevated temperatures due to pressure. Forty to sixty percent of the biomass is dissolved (Mosier et al. 2005b). It does not require costly chemicals that need to be neutralized after pretreatment. Since no corrosive chemicals are used, cheaper reactor equipment can be chosen (Mosier et al. 2005a). For batch treatment, a steel reactor is filled with biomass and water is added (Suryawati et al. 2008). The reactor is equipped with a stirring device, temperature and pressure control, and after filling it is completely sealed. The reactor is heated and the pressure increases. When the desired temperature is reached, they are held constant for a given time. After hydrolysis, temperature and pressure are reduced to normal. The pH of pure water drops to 5 when heated to 200°C

(Weil et al. 1998). With a high dielectric constant, ionic substances are dissociated. For corn stover the optimal conditions found by Mosier et al. (2005b) were 190°C for 15 min. Ninety percent of the cellulose was converted to glucose after cellulose treatment in that study. Suryawati et al. (2008) conducted a SSF of Kanlow switchgrass pretreated with hot water pretreatment. The researchers set the conditions to 200°C for 10 min with a 10% dry biomass loading. Under these conditions 43.9% of the dry solids were solubilized. The glucan content was increased from 36.6% to 56.6%. The conversion efficiency from cellulose to ethanol was 52%. Hemicellulose can be dissolved completely. However, xylan can be further degraded to furfural (Mosier et al. 2005b).

Acid hydrolysis. Concentrated acid (H_2SO_4 or HCl) may be used to hydrolyze cellulose. This process has several disadvantages since acid is corrosive, toxic, and hazardous. A development to improve acid hydrolysis is dilute acid hydrolysis. Lower temperatures with milder conditions also improve the xylan conversion to xylose. Dilute acid hydrolysis can be separated into two types, high and low temperature. High temperature (above 160°C) is used for low solid content in continuous processes. Low temperature (below 160°C) is suitable for high solid content and batch processes (Sun and Cheng 2002). Dien et al. (2006) investigated different forage plants for dilute acid hydrolysis and subsequent enzymatic hydrolysis and fermentation. Non-glucose polysaccharide degradation at 150°C (2 g plant samples mixed with 18 ml 0-2.5% wt/vol sulfuric acid) was on average 12% lower than glucose polysaccharide degradation. This could be related to a greater degradation of non-glucose sugars. In comparison to steam explosion or AFEX, dilute acid hydrolysis is cost intensive and neutralization of pH

increases the complexity. Not only are monosaccharides produced, but furfural and 5-hydroxylmethylfurfural as well.

Lime pretreatment. In comparison to acid pretreatment, alkali pretreatment is a time intensive process, but the reaction conditions are mild. Alkali pretreatment can operate at ambient temperatures (Mosier et al. 2005a). Saponification of intermolecular ester bonds crosslinking xylan hemicelluloses with other compounds is believed to be the mechanism by which alkaline hydrolyses work (Sun and Cheng 2002). Dilute NaOH treatment of lignocellulosic material was shown to decrease crystallinity and degree of polymerization and increase internal surface area. Furthermore, linkages between lignin and carbohydrates are broken and the lignin structure is disrupted (Fan et al. 1987). Playne (1984) improved the digestibility of cellulose from 20% to 72% at room temperature by treating sugarcane bagasse with lime. The cellulose degradation needed 192 h. Ammonia can also be used for delignification. A test on a corn cobs and stover mixture and switchgrass showed up to 80% and 85% reduction of lignin, respectively (Iyer et al. 1996). Lignin content is important for dilute NaOH treatment efficiency. Digestibility increased from 14% to 55% for decreasing lignin content (55% to 20%) in hardwood, whereas, NaOH treatment showed no effect on softwood with a lignin content above 26% (Millet et al. 1976). Wheat straws with a lignin content of 10% to 18% are not susceptible to dilute NaOH treatment (Bjerre et al. 1996).

Biological pretreatment. Fungi present in forests can degrade cellulose and lignin. These are brown-rot, white-rot, and soft-rot fungi. White-rot fungi were found to be

favorable for treatment of biomass. The enzymatic activities of these organisms are very specific. However, the reaction time is very slow (Sun and Cheng 2002).

3.2.2 Effect of inhibitors on fermentation organisms

The choice of pretreatment depends on process costs, speed of reaction, and inhibitor formation. Furfural, hydroxyl-methyl-furfural, and acetic acid, as well as phenolics commonly found in biomass, have inhibitory effects on yeast growth and ethanol formation. In physico-chemical treatments, xylose can be converted to furfural. High temperature and low pH enhance this process. It was shown that furfural inhibits certain key enzymes required for ethanol production. These are hexokinase, triosephosphate dehydrogenase and alcohol dehydrogenase (Banerjee et al. 1981), as well as aldehyde dehydrogenase and pyruvate dehydrogense (Modig et al. 2002). The citric acid cycle and ethanol production are blocked, and acetaldehyde accumulates to toxic levels. Triosephosphate dehydrogenase was the enzyme most affected by furfural. A furfural concentration of 2mg/ml leads to complete inhibition of triosephosphate dehydrogenase activity and probably leads to an inhibition of glycolysis. This result was confirmed by Sanchez and Bautista (1988) who tested the effect of furfural and 5-hydroxymethalfurfural on *S. cerevisiae* and *Candida guilliermondii*.

Cell growth is also affected by furfural, which results in an extended lag phase. Cell growth is slowed because the yeast needs time to express the necessary enzymes for furfural degradation (Boyer et al. 1992). Yeast are able to convert furfural to furfuryl alcohol, which is less toxic. This behavior can also be used to adapt yeast to mildly toxic conditions produced by furfural before use in fermentation (Liu et al. 2004).

It was also shown that inhibition occurs to a greater extent when toxic compounds are added in combination rather than individually. In other words, the sum of inhibition of the single compounds is less than the inhibition of the compounds in combination. Oliva et al. (2006) tested the effects of combinations of acetic acid, furfural and catechol on *K. marxianus* using glucose. The compounds significantly affected growth and ethanol fermentation. The lag phase was increased and no growth was observed until furfural was converted to furfuryl alcohol, but the interaction of all three components strongly affected the ethanol and biomass yield. A cumulative effect of toxic compounds was also described by Lohmeier-Vogel et al. (1998) with furfural, 5-hydroxymethylfurfural, and acetic acid as inhibitors of *P. stipitis*.

3.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis is necessary since yeast cannot hydrolyze or utilize cellulose or hemicellulose. Cellulose is composed of glucose molecules with a β-1,4-glycosidic bond. This bond can be hydrolysed via acids or enzymes. The disadvantages of acid hydrolysis were stated previously. Enzymes are proteins that catalyze reactions with high specificity and with mild reaction conditions. Most enzymes have a optimum activity at 45°C to 50 °C (Duff and Murray 1996). Cellulases hydrolyze the cellulose structure by adding one water molecule. The resulting mass in glucose is 0.51 times the mass of the cellulose before hydrolysis. Hemicellulose, which is mainly composed of xylan and some other pentose and hexose sugars, must be hydrolyzed as well for subsequent fermentation. Xylan is a polymer of xylose molecules. The structure of xylan depends on the plant material from which the xylan was obtained (Matsuo et al. 1991;

Teleman et al. 2001). The xylan structure can be hydrolyzed with xylanases. When xylan is hydrolyzed, the resulting xylose is 1.01 times the mass of xylan.

3.2.4 Fermentation

After the polysaccharides are hydrolyzed to monomers, yeast can start the fermentation process. Fermentation is the anaerobic conversion of carbohydrates (sugars) to ethanol, carbon dioxide, and other minor metabolites. While glucose can be utilized by all yeast, xylose can only be fermented by some species, *P. stipitis* for instance. The net reaction equation for glucose fermentation is:

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

Fermentation occurs under exclusion of oxygen (anaerobic). Glucose enters the glycolysis pathway and is in a sequence of chemical reactions converts to pyruvate. Pyruvate is further converted to ethanol by two nicotinamide adenine dinucleotide (NADH) that were produced in glycolysis. Under anaerobic fermentation only 1/18 of the chemical energy (in the form of ATP) is produced compared to aerobic conditions (Nelson and Cox 2005). The optimal fermentation temperature depends on the yeast strain used. It can vary between 20°C with *S. cerevisiae* (Madigan and Martinko 2006) to 45°C with *K. marxianus* (Banat and Marchant 1995). Yeast growing at temperatures between 20°C and 45°C are classified as mesophilic, whereas, yeast growing between 45°C and 70°C are classified as thermophilic (Fritsche 2002). Figure 3 shows the metabolic pathway for fermentation of glucose in yeast. Xylose fermentation is explained in more detail in a later section.

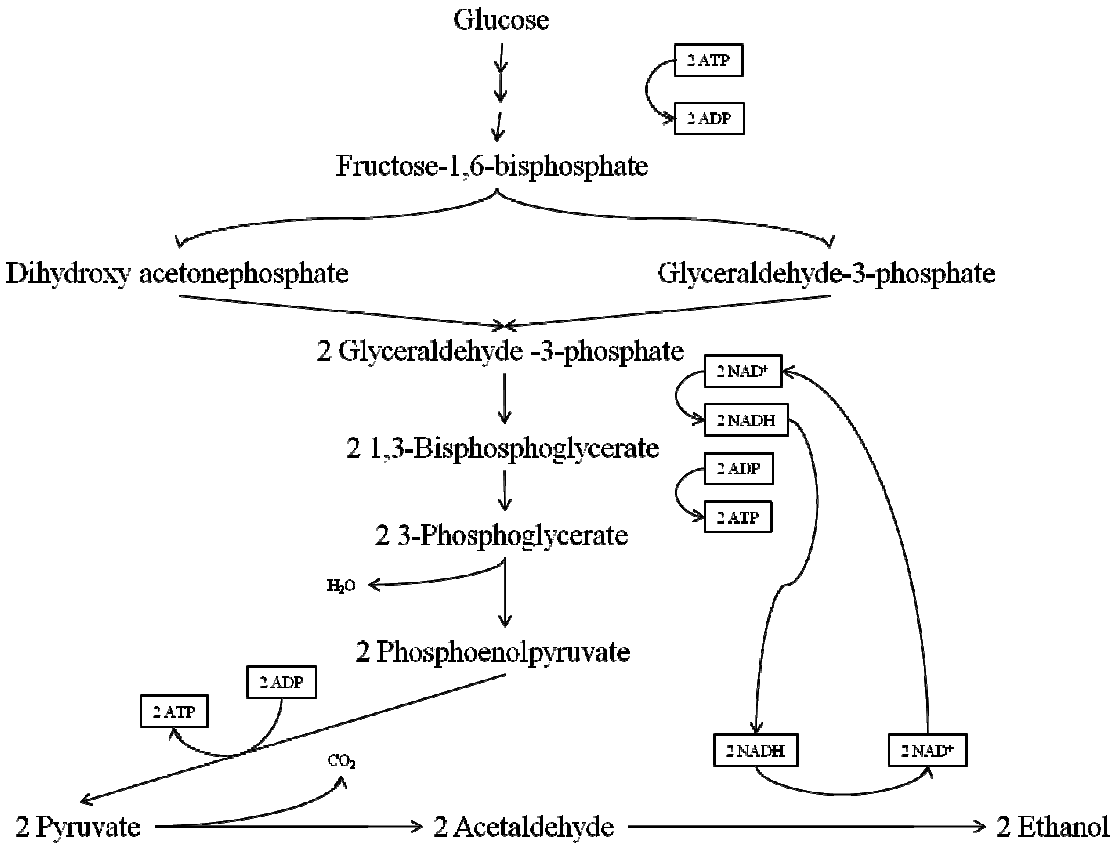

Figure 3: Glycolysis with anaerobic ethanol formation (adapted from Fritsche 2002).

3.2.4.1 SSF versus SHF

Enzymatic hydrolysis, also known as saccharification, and fermentation can be executed in two different modes. These are SSF and SHF. Both methods show advantages and disadvantages depending on yeast strain, location, annual average temperature, ethanol tolerance and temperature optima of yeast and enzymes. Usually, the enzymes used have thermophilic temperature optima (40°C to 50°C), whereas, yeast often have mesophilic temperature optima (20°C to 30°C). This favors SHF. In each step the optimal temperature is used and high yields can be obtained. However, the high sugar concentration after enzymatic hydrolysis can cause product inhibition to the enzyme. The use of two separate reactors also increases capital costs. In this case, SSF would be favorable because the yeast is immediately converting the hydrolyzed sugar and no inhibiting concentration of sugar can be built up (Savarese and Young 1978). Various tests with wood, grasses and agricultural residues in SSF mode have been done (Chang et al. 2001; Grohmann 1993; Mosier et al. 2005b; Wyman et al. 1992). A test on herbaceous feedstock with *S. cerevisiae* showed a higher ethanol yield by using SSF instead of the conventional method of separating saccharification and fermentation (Wyman et al. 1992). Blotkamp et al. (1978), Szczodrak and Targonski (1988) and Spindler et al. (1988) are looking for thermophilic yeast strains that can ferment at temperatures up to 50°C to have optimal temperatures for both enzymes and yeast with the advantage of avoiding product inhibition.

3.2.5 Xylose fermentation

Xylose, as a pentose sugar, cannot be utilized by all yeast. Yeast in the genera *Kluyveromyces, Pichia, Brettanomyces, Candida, Clavispora, Pachysolen* and *Schizosaccharomyces* have been studied for fermenting xylose (Skoog and Hahn-Hägerdal 1988). The enzymes necessary for xylose fermentation, xylose reductase (XR) and xylitol dehydrogenase (XDH), must be activated by the presence of xylose. XR and XDH are not available during glucose fermentation (Smiley and Bolen 1982). Xylose is reduced to xylitol, catalyzed by XR, and further oxidized to xylulose, catalyzed by XDH. Xylulose enters the pentose phosphate cycle and is eventually converted to ethanol (Chiang and Knight 1960; Fritsche 2002; Ligthelm et al. 1988). The overall reaction equation for xylose fermentation is:

$3\ C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5\ CO_2$

Figure 4 shows the pentose phosphate cycle.

3.2.5.1 Oxygen requirement for xylose fermentation

The role of oxygen in xylose fermentation has been reported often. Some yeast tend to produce mainly xylitol and others ethanol. This is caused by different co-factor regeneration systems. Figure 5 shows the pathway from xylose to xylitol and further to xylulose. It is known that yeast like *P. stipitis*, which produce ethanol, can use NADH as a co-factor for xylose reductase, whereas, xylitol producers primarily use NADPH (Yablochkova et al. 2004). Xylitol dehydrogenase uses NAD^+ as a co-factor in all xylose fermenting yeast (Yablochkova et al. 2004). In ethanol producing yeast, a regeneration cycle for NADH is established and xylulose production is favored. In xylitol producing

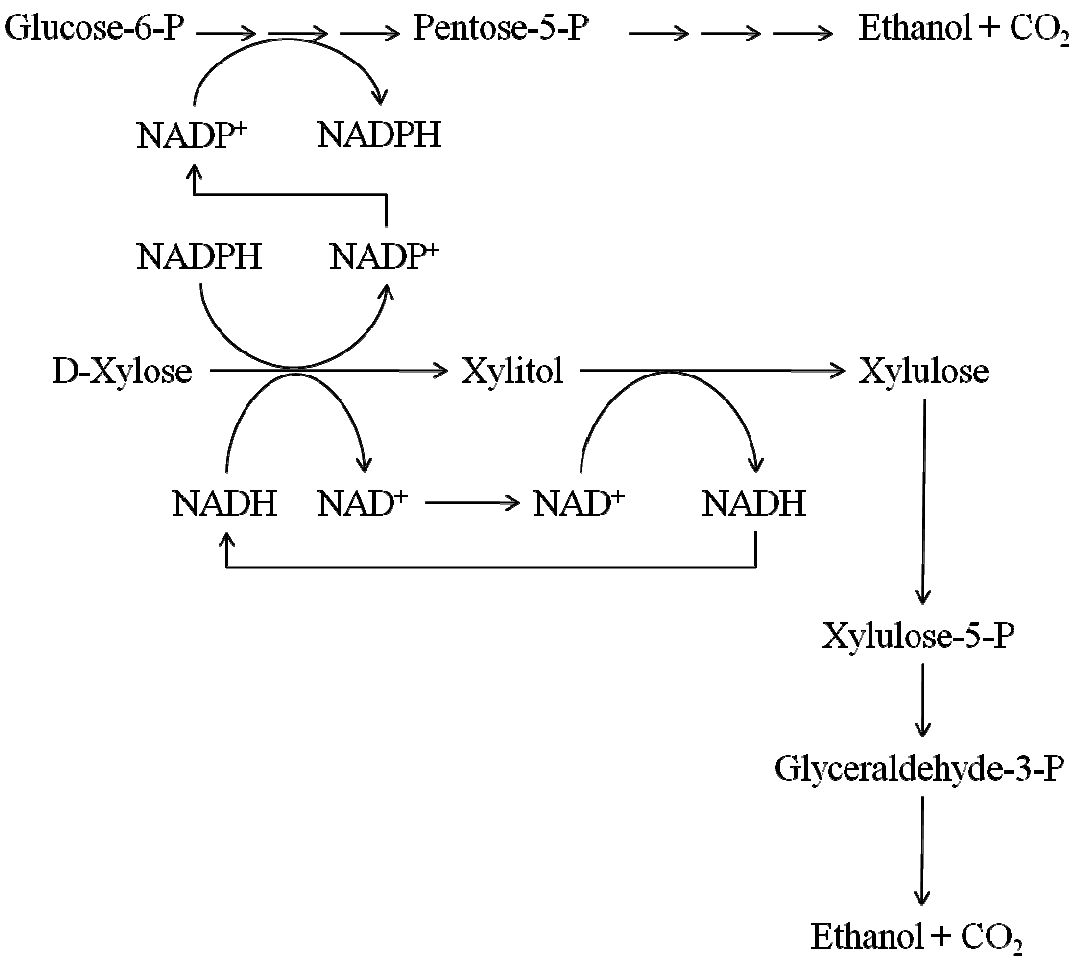

Figure 4: Xylose metabolism with pentose phosphate pathway (adapted from Bruinenberg 1986).

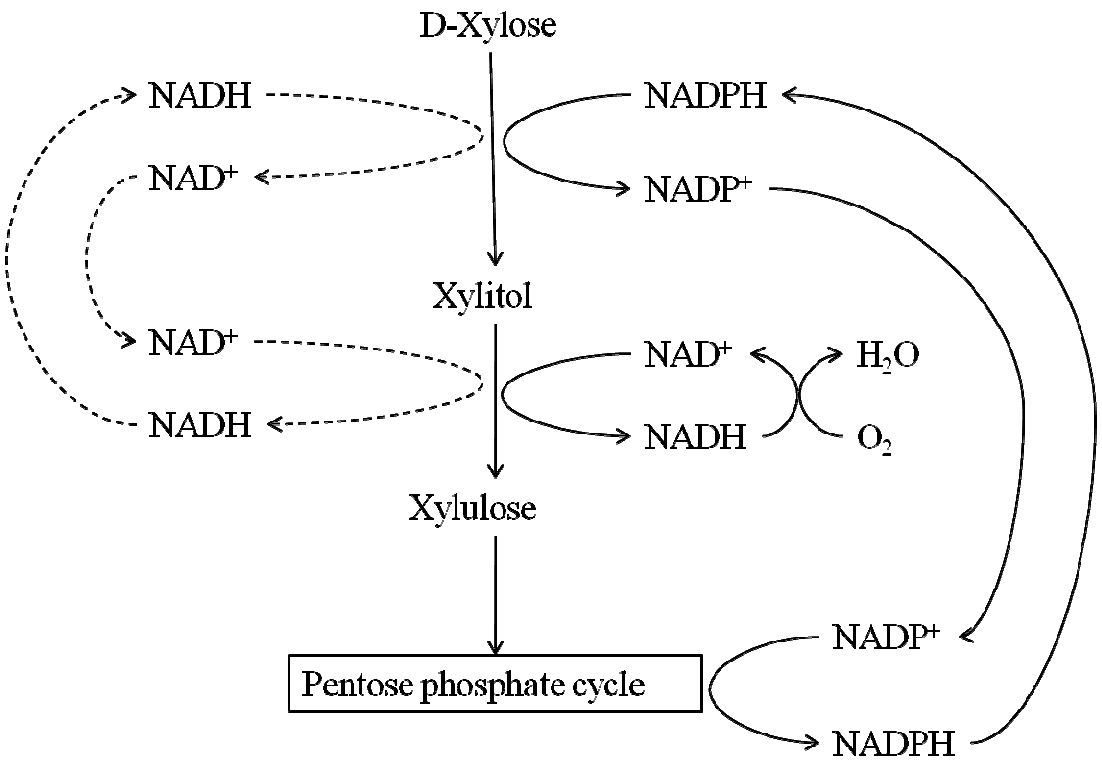

Figure 5: NAD^+ recycle during xylulose formation, adapted from Yablochkova et al. (2004).

yeast, NADPH is regenerated with the subsequent pentose phosphate cycle, but NADH accumulates and xylitol dehydrogenase is inhibited, resulting in xylitol accumulation. This process only occurs under anaerobic or microaerobic conditions since NAD^+ is regenerated by oxygen (Yablochkova et al. 2004).

3.3 Properties of xylitol

Xylitol is a by-product of xylose fermentation and is a sugar alcohol (Guo et al. 2006). Xylitol can be used as a sweetener. Researchers have found that xylitol prevents dental cavities (Lynch and Milgrom 2003) and has an anti-ketonic (Kinami and Kitagawa 1969) and anti-infection effect (Brown et al. 2004). Xylitol can be produced by catalytic hydrogenation of xylose (Aminoff et al. 1978). Among other yeast, *Candida mogii* was found to produce xylitol with a high yield ($Y_{P/S} = 0.62$) (Sirisansaneeyakul et al. 1995). They also found that initial xylose concentration, co-factor regeneration and oxygen transfer are key factors for xylitol production. The same researchers analyzed a *K. marxianus* and *P. stipitis* strain. *P. stipitis* produced negligible amounts of xylitol, but was shown in other studies to produce ethanol with high yields (Agbogbo and Wenger 2007; Du Preez and Prior 1985). *K. marxianus*, on the other hand, produced xylitol with a yield of 0.26 g/g and was found to produce ethanol (Banat and Marchant 1995; Wilkins et al. 2008). Guo et al. (2006) conducted a xylitol producing yeast screening and found *C. guiiliermondii* Xu280 and *Candida maltos* Xu316 as promising xylitol producers. Their conclusion is based on a two step evaluation with growth characteristics on xylose and ability to assimilate xylose as the first criteria, followed by xylitol production. Figure 6 shows the key reactions of xylose metabolism during xylitol production. For large scale

ethanol production, it needs to be decided if xylitol as a product is desired or not. This decision leads to the correct choice of microorganism. This depends, of course, on the market situation and the separation costs after fermentation.

3.4 Yeast

3.4.1 Kluyveromyces marxianus

Since enzymes have higher temperature optima than common yeast strains, it is necessary to find strains with higher temperature optima to optimize SSF of xylan. Five *K. marxianus* strains, IMB1, IMB2, IMB3, IMB4, and IMB5, were found in an Indian distillery and showed the ability to grow at high temperatures using glucose (52°C) and ferment xylose as a carbon source at temperatures up to 45°C. Their optimum growth rates were found at 40°C and a value of 0.93 h^{-1} with glucose as substrate (Banat et al. 1992). The fermentation with xylose showed low ethanol production. Only 0.98 g/l ethanol was produced with 10 g/l xylose (Banat and Marchant 1995). The researchers mentioned a high xylitol production under these conditions, but didn't state the values. Wilkins et al. (2008) carried out tests by using *K. marxianus* IMB 2, IMB4, and IMB5 with different pH values and different temperatures. IMB4 had the greatest ethanol production at 40°C and an initial pH of 4.5. The maximum ethanol concentration was 0.53 g/l with 10 g/l xylose. IMB5 had the greatest xylitol production at 40°C and an initial pH of 4.5 with a value of 1.33 g/l. The researchers used anaerobic conditions.

3.4.2 Pichia stipitis

P. stipitis is another xylose assimilating yeast strain. It produced up to 5.9 g/l ethanol in a media with 20 g/l xylose at 25°C (Toivola et al. 1984) and 21.46 g/l ethanol with 50 g/l xylose at 30°C (Du Preez and Prior 1985). Tests on complex media were also conducted. *P. stipitis* fermented corn stover hemicellulose hydrolyzate with 25 g/l xylose and 6.3 g/l glucose and produced a maximum ethanol concentration of 13 g/l. The hydrolyzate contained the inhibitors 5-hydroxylmethylfurfural, furfural and acetic acid in low concentrations. The ethanol yield on substrate of 0.44 g/g sugar was promising. The conditions of the fermentation were 30°C, 150 rpm, and an initial pH of 6 (Agbogbo and Wenger 2007). *P. stipitis* needs a microaerobic environment for xylose fermentation since the cofactor involved for xylose reductase and xylitol dehydrogenase, NADH, needs oxygen as an electron acceptor (Yablochkova et al. 2004). Therefore *P. stipitis* produces ethanol with a high yield and reduced xylitol production only under microaerobic conditions.

3.5 Co-culture fermentations

To increase the efficiency of fermentation, researchers have tried to use two different cultures in one reactor, which is known as co-culture fermentation. Certain advantages have been shown in previous studies. There was better resistance to contamination (Harrison 1978) and an increase in biomass yield was observed with a co-culture of *Candida kefyr* LY496 and *Candida valida* LY497 (Carlotti et al. 1990). One application for co-culture was ethanol production from a mixture of pentose and hexose. Taniguchi et al. (1997) used a co-culture of *P. stipitis* and *S. cerevisiae* for the conversion

of a glucose/xylose mixture. *S. cerevisiae* is a glucose fermenting yeast, whereas, *P. stipitis* ferments xylose and glucose. As expected, the glucose was consumed by *S. cerevisiae* with a high ethanol yield of 0.46 g/g. *P. stipitis* showed no growth and poor ethanol production. Xylitol production was observed, which led to the conclusion that *S. cerevisiae* consumed the oxygen in the reactor, which created an anaerobic environment. It was previously explained that *P. stipitis* needs microaerobic conditions for ethanol production using xylose. Ward et al. (1995) used *K. marxianus* IMB3 and *Talaromyces emersonii* CBS 814.70 for conversion of starch containing media to ethanol at 45°C. Their hypothesis was that *T. emersonii*, which showed amylolytic activity, would degrade starch to glucose that would be further consumed by *K. marxianus* and converted to ethanol. The result was a conversion of starch to ethanol at a 59% theoretical yield. This result was lower than a similar test where the researchers conducted a SSF including only the enzyme from *T. emersonii* (Ward et al. 1995). This test showed a conversion with 75% of the maximum theoretical yield. The difference could be in the different energy demand. The co-culture system requires more carbon for biomass production. Golias et al. (2002) conducted an experiment by using *Klebsiella oxytoca* P2 with several other yeast. *K. oxytoca* P2 is a modified strain with integrated pyruvate decarboxylase and alcohol dehydrogenase genes. This strain was combined with more ethanol-tolerant and thermotolerant yeast strains. *Saccharomyces pastorianus*, *K. marxianus*, and *Zymomonas mobilis* produced in combination with *K. oxytoca* more ethanol than when used as a single culture. *P. stipitis* in co-culture with either *S. cerevisiae* or *K. marxianus* was used to ferment a glucose/xylose mixture (Rouhollah et al. 2007). It was found that the coculture with *P. stipitis* and *S. cerevisiae* did not improve ethanol production in

comparison to fermentation with monocultures. The researchers concluded the yeast had adverse effects on each other, but they did not explain what those were. On the other hand, the co-culture fermentation with *P. stipitis* and *K. marxianus* showed an increase in ethanol yield. A yield of 0.42 g ethanol/g carbohydrate was achieved by the co-culture. *P. stipitis* alone had a yield of 0.40 g/g and *K. marxianus* had a yield of 0.36 g/g. The media contained glucose and xylose in equal parts (30 g/l) as well as other common sugars occurring in hemicellulose. It is still unknown how a co-culture with *P. stipitis* and *K. marxianus* ferments xylose as the only substrate. It is known that *K. marxianus* produces xylitol as a by-product of xylose fermentation with a rather low ethanol yield.

CHAPTER 4

METHODOLOGY

4.1 Preparation of seed culture

Prior to the test, all glassware, stoppers, and other equipment used in direct contact to the organisms was sterilized via autoclaving. A biological safety cabinet was used to transfer cells and for taking samples. Cultures were stored in slants on solid YPD media in a refrigerator. A seed culture, which can be stored for a few weeks was prepared in media containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l xylose. Sterilization was achieved by using a filter assembly with a 0.45 µm filter. A loopful of each *K. marxianus* IMB strain was added to 100 ml of seed media in 250 ml baffled flasks. The flasks were closed with a stopper containing a filter to allow air exchange and prevent microbial contamination (Bug Stopper, Whatman Inc., Florham Park, NJ, USA). Picture 1 shows flasks with stoppers. The seed culture was incubated for 18h at 45°C. Aeration was achieved by shaking at 220 rpm.

4.2 Preparation of inocula for IMB screening

The inoculum media contained 10 g/l yeast extract, 20 g/l peptone, and 22 g/l glucose monohydrate. It was sterilized with a filter assembly using a 0.45 µm filter.

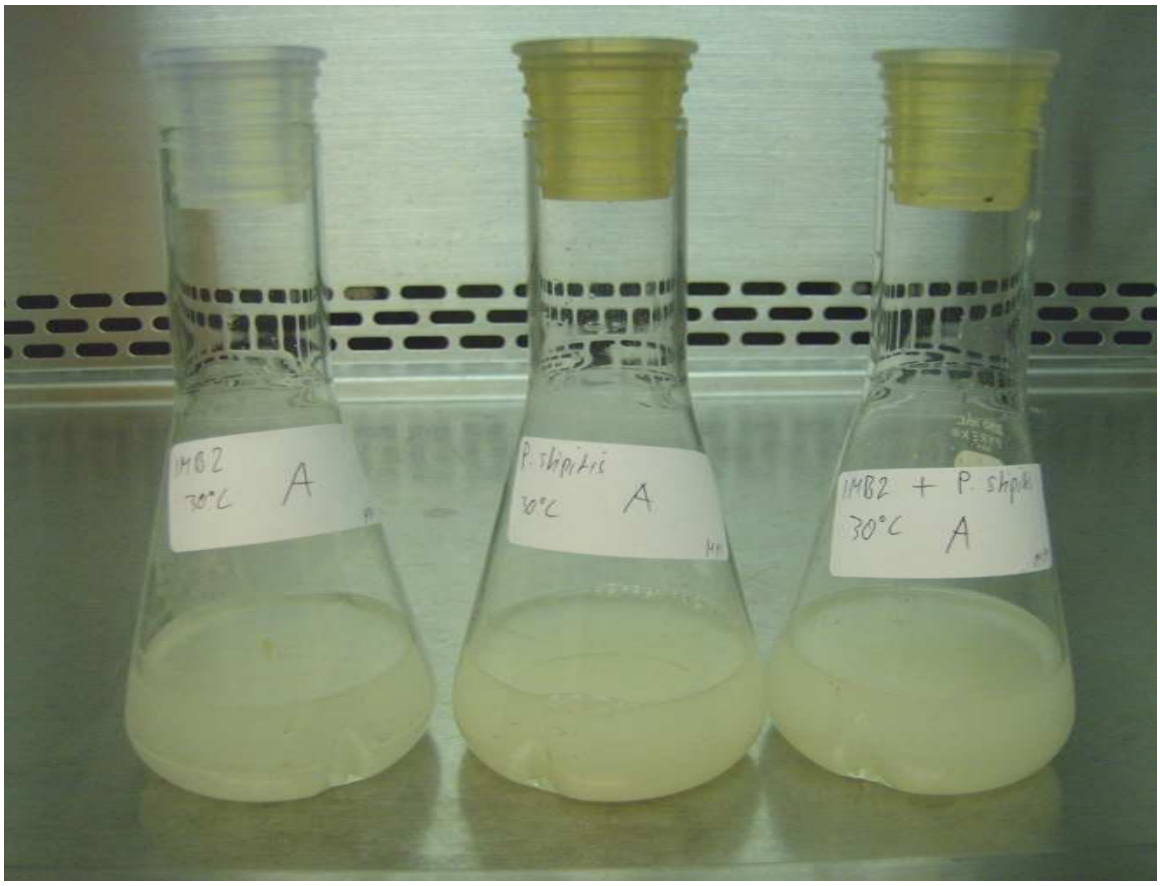

Figure 6: Baffled flasks with stoppers with filter to allow air exchange. Here are the three different cultures for co-culture fermentation.

Baffled flasks (250ml) were filled with 100 ml of inoculum media and inoculated with 1ml of seed culture containing one of the *K. marxianus* strains and closed with the stopper previously described. Conditions were the same as for the seed culture (45°C, 18 hours, 220 rpm).

4.3 Preparation of fermentation media for IMB screening

To maintain pH during fermentation, a 50 mM sodium citrate buffer at pH 5.5 was used to dissolve the media components. The fermentation media contained 0.56 g/l yeast extract, 2.22 g/l KH_2PO_4, 1.11 g/l $(NH_4)_2SO_4$, 1.11 g/l $MgSO_4*7H_2O$, 0.11g/l $MnSO_4$, and 22.22 g/l xylose.

4.4 Preparation of fermentation culture for IMB screening

After 18 h the optical density (OD) of the inocula at a wave length of 660 nm was determined using UV visible spectrophotometer (Varian, Palo Alto, CA, USA). An initial OD of 0.5 was set for each fermentation culture by mixing centrifuged (5 min, 4°C, and 3750 rpm), DI water washed cells from the inocula with fermentation media (Dowe and McMillan 2001). The determination of the correspondent cell mass concentration to OD is explained later. The flasks were enclosed with the previously described stoppers. The growth conditions for fermentation were 40 or 45°C with shaking at 100 rpm. According to Yablochkova et al. (2004), 100 rpm results in an oxygen transfer rate of 5 mmol/(l*h) and leads to a microaerobic environment for ethanol and xylitol production from xylose. Constant temperature was achieved by using an incubator (MaxQ Mini 4450) from. Barnstead International (Dubuque, IA, USA) (Picture 2). Samples were taken at 0, 1, 2, 3,

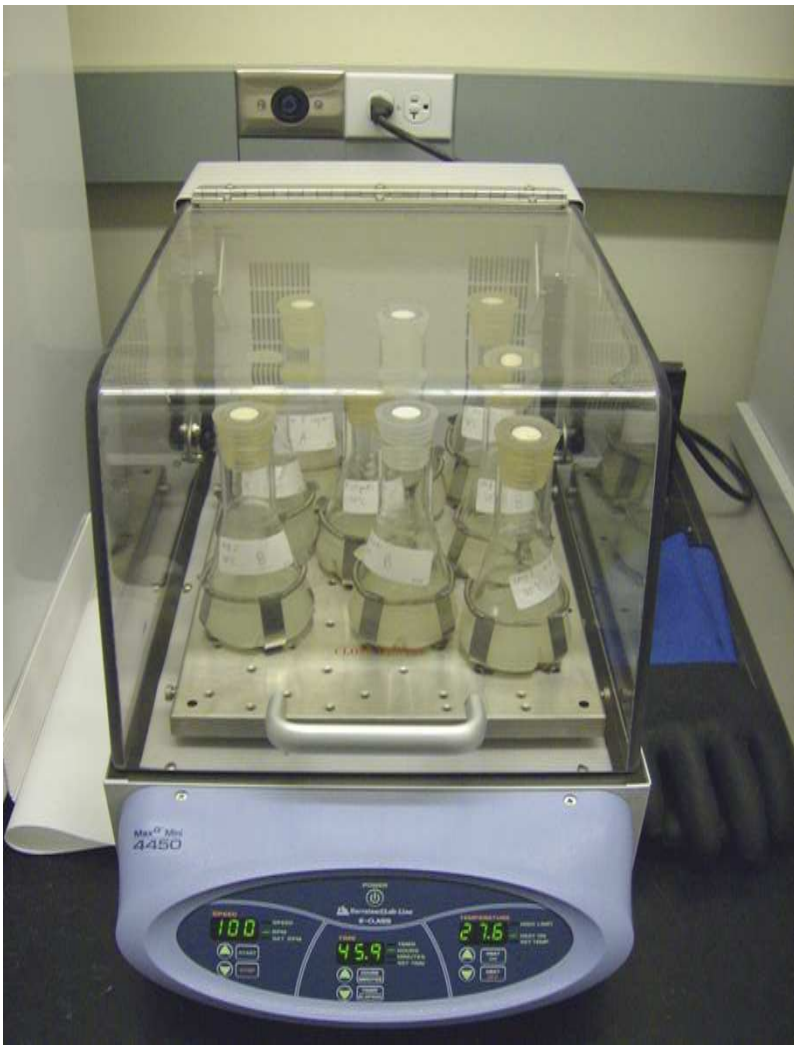

Figure 7: MaxQ Mini incubator from Barnstead International, Dubuque, IA, USA.

4, 5, 6, 24, 48, 72, 96, 120, 144 h. Optical density was measured via UV Vis and samples were filtered through a 0.2 µm filter from Fisher Scientific (Pittsburgh, Pa., USA). The permeate was used for high performance liquid chromatography (HPLC) analysis, which is described later. Every IMB strain was tested twice at each temperature.

Determination of growth rates. To determine the growth rates, OD had to be converted to cell mass concentration. A calibration curve of known OD vs. cell mass concentration was created by filtering 100ml of cell suspensions with through a 0.45 µm filter. The filters with cells were dried overnight in an oven at 100°C. The dry cell mass was measured with an analytical balance. Finally, a linear regression of OD versus concentration was performed. For each sample at each time point the cell mass concentration was calculated. For each time point the natural logarithm of cell mass concentration over initial cell mass concentration (Equation 1) was calculated and plotted over time. The slope of the linear section represents the growth rate.

$$\ln\left(\frac{X}{X_0}\right) = \mu_{net} \cdot t \qquad (1)$$

Where X is the cell mass concentration; X_0 is the initial cell mass concentration; μ_{net} is the growth rate and t is the time.

4.5 Enzyme screening to determine enzyme preparation with highest xylanase activity

The following enzyme solutions were tested: NS 50012 and Pectinex Ultra SP/L from Novozymes (Lyngby, Denmark), Rapidase PNS from DSM Enzymes (Parsippany, NJ), and Multifect Xylanase and Multifect Pectinase FE from Genencor (Palo Alto, CA). For each enzyme a sterile xylan solution was prepared by mixing 5 g/l xylan into 1l DI-

water and following autoclaving. Xylan from birch wood (≥ 90% xylose residues) was obtained from Sigma-Aldrich Co., St. Louis, MO. 50 µl enzyme solution was added to each flask containing 100 ml xylan solution to obtain an enzyme concentration of 500 µl/l. The flasks were incubated at 45°C for 72 h. The incubator described earlier was used to maintain constant temperature. Samples were taken at 0, 24, 48 and 72 h, and samples centrifuged for 10 min at 13,000 rpm, filtered through a 0.2 µm filter (Fisher Scientific, Pittsburgh, Pa., USA) and analyzed for xylose concentration via HPLC, as described later.

4.6 Simultaneous saccharification and fermentation (SSF)

The seed culture and inocula were prepared in the same way as for the IMB screening. An initial OD of 5 (1.4 g cells/l) was set for each fermentation (Dowe and McMillan 2001). The fermentation media contained 0.56 g yeast extract, 2.22 g KH_2PO_4, 1.11 g $(NH_4)_2SO_4$, 1.11 g $MgSO_4*7H_2O$, 0.11 g $MnSO_4$, and 20 g xylan dissolved into 1 L of citric acid buffer at pH 5.5. Enzyme concentration was set to 1, 2, or 3 µl/ml. SSF conditions were either 40 or 45°C with shaking at 100 rpm to maintain a microaerobic environment as previously described. Constant temperature was achieved as described earlier. Samples were taken at 0, 6, 12, 24, 48, 72, 96, 120 and 144 h. Samples were prepared for HPLC analysis described in a latter section.

Estimation of enzyme activity. To estimate the performance of the enzyme during SSF the xylose concentration was obtained. The xylose used for ethanol and xylitol production was calculated and added to the xylose concentration measured with HPLC. The total xylose concentration was plotted over time. By linear regression of the first 12 h

the slope and enzyme activity in g xylose/(l*h) was obtained. With the molecular weight and amount of enzyme used, a unit conversion was done to get IU/ml enzyme.

4.7 Preparation of inoculum for xylose fermentation with co-culture

The inoculum media contained 10 g/l yeast extract, 20 g/l peptone, and 22 g/l xylose. It was sterilized with a filter assembly using a 0.45 µm filter. Baffled flasks (250 ml) were filled with 100 ml of inoculum media and inoculated with 1 ml of seed culture of *K. marxianus* IMB2 or *P. stipitis* and closed with the stopper previously described. Conditions of growth were 30°C for 18 h at 220 rpm.

4.8 Preparation of fermentation media for co-culture

An initial pH was set at pH 6 with 2 M NaOH. The fermentation media contained 0.56 g yeast extract, 2.22 g KH_2PO_4, 1.11 g $(NH_4)_2SO_4$, 1.11 g $MgSO_4*7H_2O$, 0.11 g $MnSO_4$, and 45 g xylose dissolved into 1 liter DI water sterilized via filtration with 0.45 µm filters.

4.9 Preparation of fermentation of co-culture

After 18 h, the OD of the inocula at a wave length of 660 nm was determined. An initial OD representing a cell mass of 1.4 g cells/L was set for *K. marxianus* IMB2 and *P. stipitis* as monocultures. Co-cultures were prepared by mixing inocula containing equal cell mass concentrations of each strain together by mixing with DI water washed and centrifuged (10 min, 4°C, and 3750 rpm) cells from the inocula with fermentation media (Dowe and McMillan 2001). Hence, the cell mass concentration of each strain in the co-

culture is equal to 0.7 g/l to get a final concentration of 1.4 g/l. The flasks were enclosed with the previously described stoppers. The growth conditions for fermentation were 30°C with shaking at 100 rpm. The same incubator was used as described earlier. Samples were taken at 0, 6, 12, 24, 48, 72, 96 h. OD was measured and samples were filtered through a 0.45 µm filter system from Fisher Scientific (Pittsburgh, Pa., USA). The permeate was used for HPLC analysis. Each yeast and the co-culture were tested in triplicate.

4.10 Yield and conversion efficiency calculation

For all tests the yields and conversion efficiencies are calculated. For the SSF experiment no yields were calculated, since the substrate concentrations are unknown during simultaneous substrate utilization. For the calculation of product yields, following equation was used:

$$Y_{P/S} = \frac{\left(C_{P\_\max} - C_{P\_0}\right)}{\left(C_{S\_0} - C_S\right)} \qquad (2)$$

Where $Y_{P/S}$ is the product yield; C_{P_max} is the maximum product concentration; C_{P_0} is the initial product concentration at t = 0 h; C_{S_0} is the initial substrate concentration at t = 0 h; and C_S is the substrate concentration at the time point of C_{P_max}.

For the conversion efficiency, following equation was used:

$$CE = \frac{C_{P\_theo}}{C_{P\_\max}} \cdot 100\% \qquad (3)$$

Where CE is the conversion efficiency; C_{P_theo} is the theoretical maximum product concentration; and C_{P_max} is the maximum product concentration.

Where C_{P_theo} is:

$$C_{P\_theo} = C_{S\_0} \cdot 0.51 \quad \text{for ethanol as product, and} \tag{4}$$

$$C_{P\_theo} = C_{S\_0} \cdot 1.01 \quad \text{for xylitol as product.} \tag{5}$$

The factors 0.51 and 1.01 for theoretical maximum ethanol and xylitol concentration, respectively, are based on the molecular weights of substrate and product.

4.11 HPLC analysis

Concentrations of xylose, xylitol, ethanol, acetic acid, and glycerol were analyzed on an HPX-87H column (Bio-Rad, Sunnyvale, Ca.). The eluent was 0.01 N H_2SO_4 with a flow rate of 0.6 ml/min at 60°C, and a refractive index detector (1100 Series Agilent, Santa Clara, CA, USA) was used (Sluiter et al. 2006).

4.12 Statistical Analysis

A two factorial test was performed with yeast strain and temperature as the independent variables for yeast screening and enzyme concentration and temperature as the independent variables for SSF. For the co-culture experiment, the type of culture was the factor tested. Ethanol and xylitol concentration were the dependent variables for all tests. The yeast screening and SSF experiments, each combination of the independent variables were performed in duplicate. For the co-culture fermentation experiment, each type of culture was performed in triplicate. An analysis of variance was calculated using SAS Release 9.1 (SAS, Carey, NC). If the independent variable was found to be significant, means for each variable were separated by Fisher's protected least significant difference analysis at $P < 0.05$.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 IMB screening

Five yeast strains, *K. marxianus* IMB1, IMB2, IMB3, IMB4, and IMB5, were tested for their capability to ferment xylose as their primary carbon source to produce ethanol and xylitol at 40 and 45°C. Tables 1 and 2 show an overview of the primary results, including product yields and conversion efficiencies. Figures 6 and 7 show the growth curves of the IMB strains at 40°C and 45°C, respectively. From the growth curves, the growth rates were calculated (Figure 8). The growth rate differed between the two temperatures. In general the growth rate was higher at 40°C. The increase between 40°C and 45°C in growth rate varied between 29% and 137% with IMB2 and IMB4, respectively. At 40°C the highest growth rate of 0.2 h^{-1} was achieved with IMB4. IMB2 showed the highest growth rate of 0.12 h^{-1} at 45°C with a value. Banat and Marchant (1995), who investigated the IMB strains, found growth rates on xylose at 45°C between 0.18 h^{-1} and 0.26 h^{-1} for IMB5 and IMB4, respectively. IMB2 had in their study a growth rate of 0.19 h^{-1}. The higher growth rates can be explained by the difference in aeration. In this study microaerobic conditions were used, whereas, Banat and Marchant (1995) showed the growth rates for aerobic conditions.

Table 1: Overview of most important findings of IMB yeast screening at 40°C based on the average of duplicates (# between 24 and 72 h). C_{EtOH_max} = maximum ethanol concentration, C_{Xtol_max} = maximum xylitol concentration, $Y_{E/X}$ = ethanol yield, $Y_{X/X}$ = xylitol yield, CE = conversion efficiency.

Product	**Strain**	**C_{EtOH_max} [g/l]**	**Time [h]**	**$Y_{E/X}$ [g/g]**	**CE [%]**	**ethanol productivity [g/(l*h)]#**
Ethanol	IMB1	1.64	144	0.08	16.1	0.013
	IMB2	1.38	144	0.07	13.5	0.012
	IMB3	1.61	120	0.08	15.8	0.018
	IMB4	1.09	120	0.06	10.7	0.010
	IMB5	1.26	120	0.06	12.4	0.016
Product	**Strain**	**C_{Xtol_max} [g/l]**	**Time [h]**	**$Y_{X/X}$ [g/g]**	**CE [%]**	**xylitol productivity [g/(l*h)]#**
Xylitol	IMB1	7.24	96	0.41	35.8	0.120
	IMB2	7.84	96	0.42	38.8	0.130
	IMB3	5.27	72	0.34	26.1	0.093
	IMB4	6.10	72	0.4	30.2	0.100
	IMB5	5.52	72	0.32	27.3	0.099

Table 2: Overview of most important findings of IMB yeast screening at 45°C based on the average of duplicates (# between 24 and 72 h). C_{EtOH_max} = maximum ethanol concentration, C_{Xtol_max} = maximum xylitol concentration, $Y_{E/X}$ = ethanol yield, $Y_{X/X}$ = xylitol yield, CE = conversion efficiency.

Product	**Strain**	**C_{EtOH_max} [g/l]**	**Time [h]**	**$Y_{E/X}$ [g/g]**	**CE [%]**	**ethanol productivity [g/(l*h)]#**
Ethanol	IMB1	0.45	96	0.03	4.4	0.0075
	IMB2	0.69	120	0.04	6.8	0.0096
	IMB3	0.57	96	0.05	5.6	0.0075
	IMB4	0.61	120	0.04	6.0	0.0054
	IMB5	0.52	96	0.05	5.1	0.0070
Product	**Strain**	**C_{Xtol_max} [g/l]**	**Time [h]**	**$Y_{X/X}$ [g/g]**	**CE [%]**	**xylitol productivity [g/(l*h)]#**
Xylitol	IMB1	5.54	120	0.36	27.4	0.065
	IMB2	7.7	120	0.42	38.1	0.100
	IMB3	4.35	144	0.31	21.5	0.048
	IMB4	6.73	144	0.41	33.3	0.053
	IMB5	4.40	144	0.29	21.8	0.049

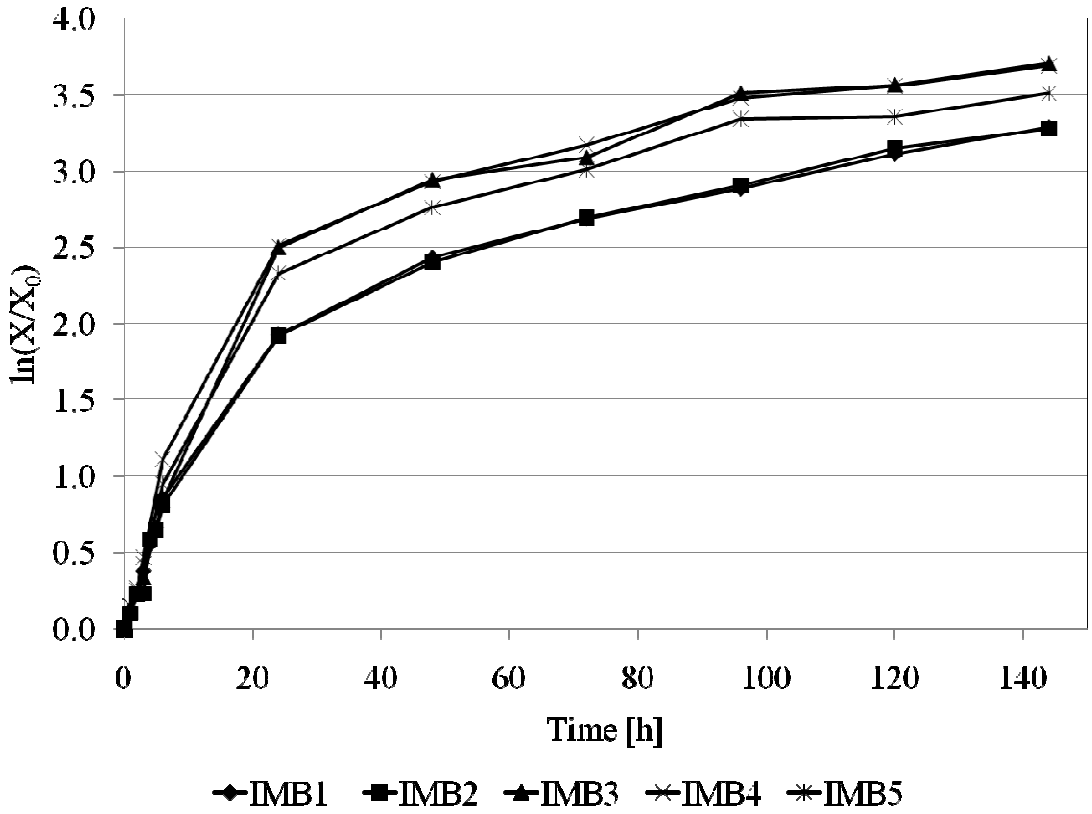

Figure 8: Growth curves for IMB strains at 40°C. X = cell mass concentration, X_0 = initial cell mass concentration.

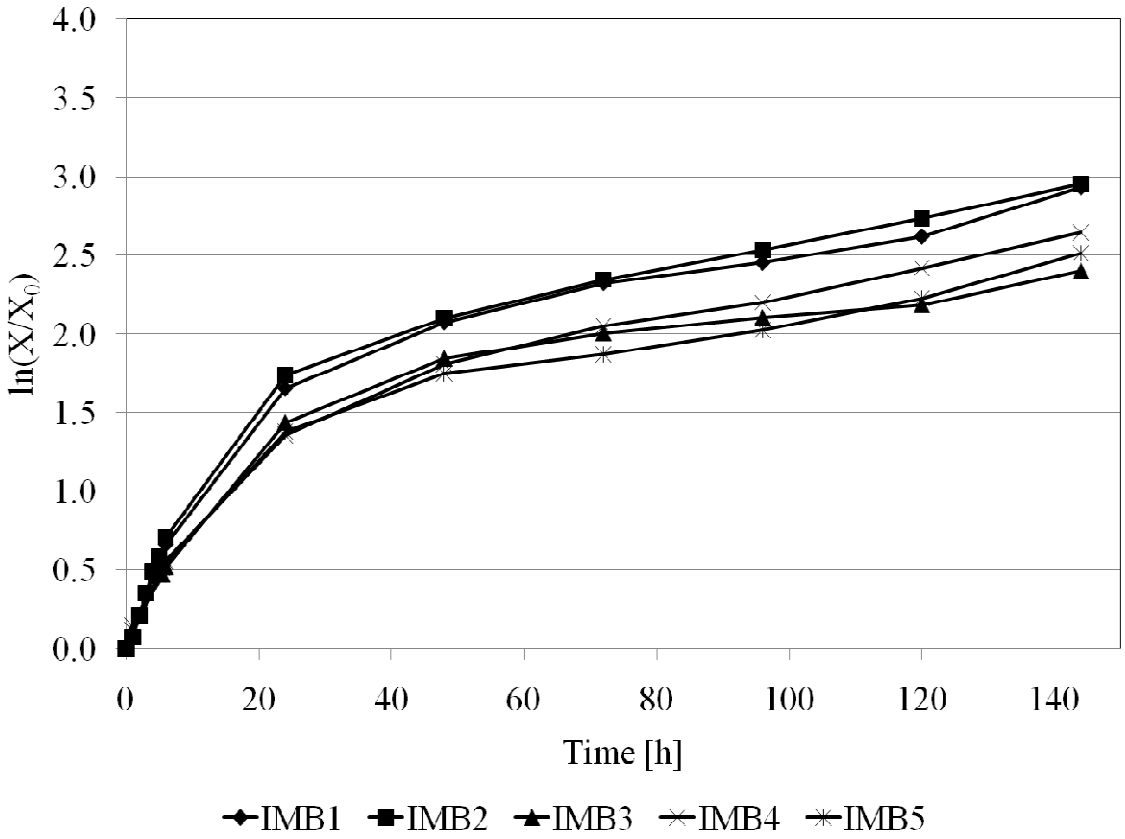

Figure 9: Growth curves for IMB strains at 45°C. X = cell mass concentration, X_0 = initial cell mass concentration.

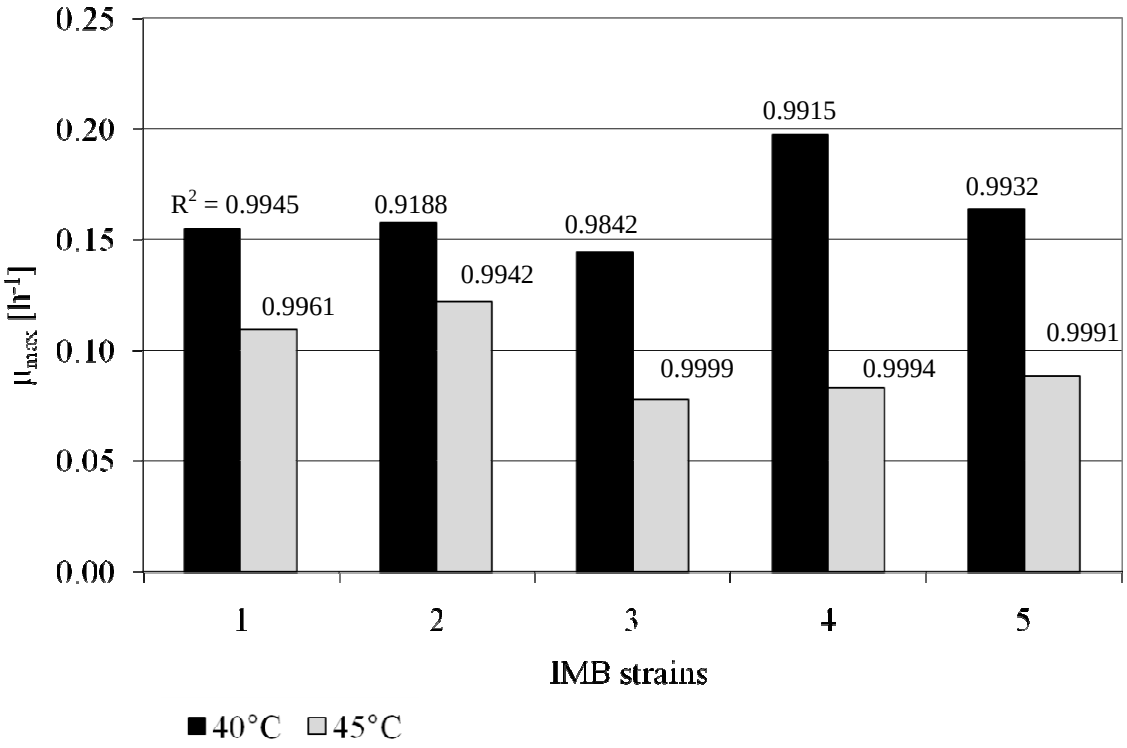

Figure 10: Maximum rowth rates of IMB strains at 40 and 45°C based on the average of duplicates. The R^2 values from linear regression are given above the columns.

Microaerobic conditions can be achieved by aeration with oxygen concentrations below the oxygen concentration of air. Aerobic conditions lead to better growth in biomass, whereas, microaerobic conditions lead to more alcoholic fermentation.

The xylose consumption varied with temperature as well. At 40°C all strains consumed almost all of the xylose available, whereas, at 45°C after 144 h, xylose still remained in the media. The amount varied from 6 g/l with IMB4 to 0.2 g/l with IMB2 (Figures 9 and 10). Similar characteristics where shown in a previous study that was conducted with IMB2, IMB4, and IMB5 (Wilkins et al. 2008). According to the figures shown in their work, IMB4 did not utilize xylose completely at 45°C with a value of about 10 g/l after 120 h. In that study it was concluded that IMB4 produced the highest ethanol concentration. However, that study was under anaerobic conditions, whereas, this study was conducted under microaerobic conditions. The influence of oxygen as a fermentation parameter is discussed later in this section.

Ethanol production from xylose depended on temperature. At 45°C the maximum ethanol concentration was significantly lower ($P < 0.05$) (Table 3) and the production rate was not as great as at 40°C. The yeast strain had an effect on ethanol production as well ($P < 0.05$). Taking into account both temperatures, IMB2 and IMB3 had higher ethanol concentrations at 120 h than did IMB1, IMB4 and IMB5 ($P < 0.05$). After 144 h, IMB2 had the highest ethanol concentration, whereas, IMB1, IMB3, and IMB5 were similar and IMB4 had the lowest ethanol concentration ($P < 0.05$). Figures 11 and 12 show ethanol concentrations over time for all five IMB strains. IMB1 produced the highest ethanol concentration with a value of 1.63 g/l after 144 h at 40°C. IMB4 produced the lowest ethanol concentration at 40°C with a value of 1.10 g/l. This represents 16.0% and 10.7%

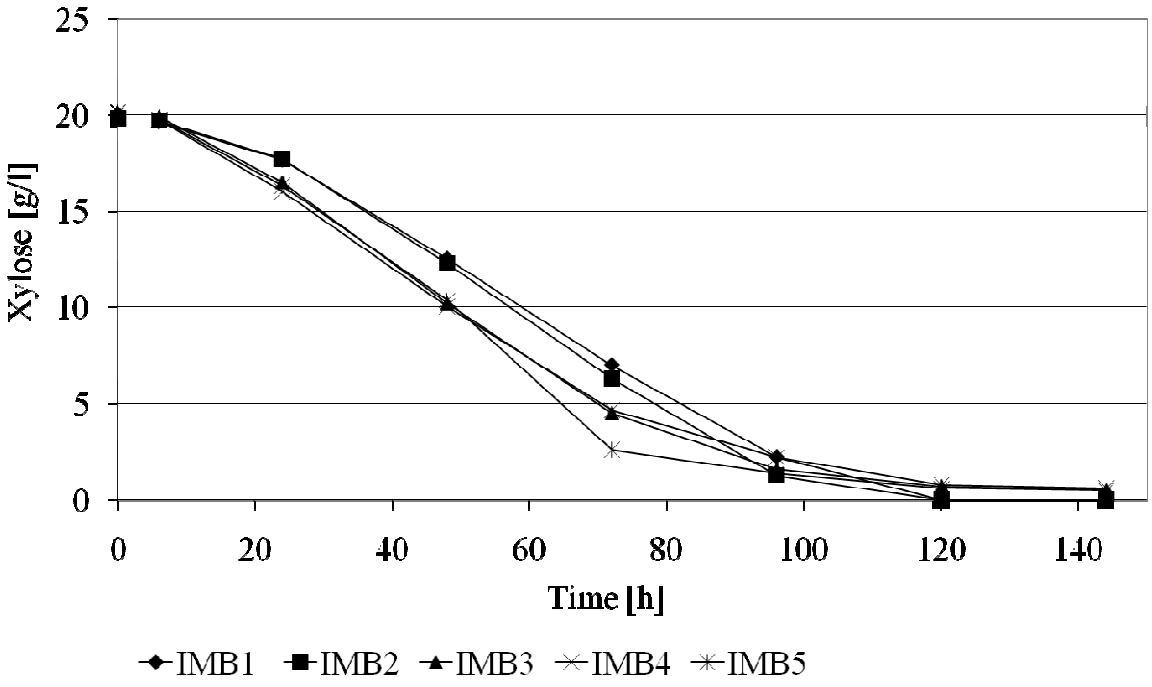

Figure 11: Xylose consumption over time by IMB strains at 40°C.

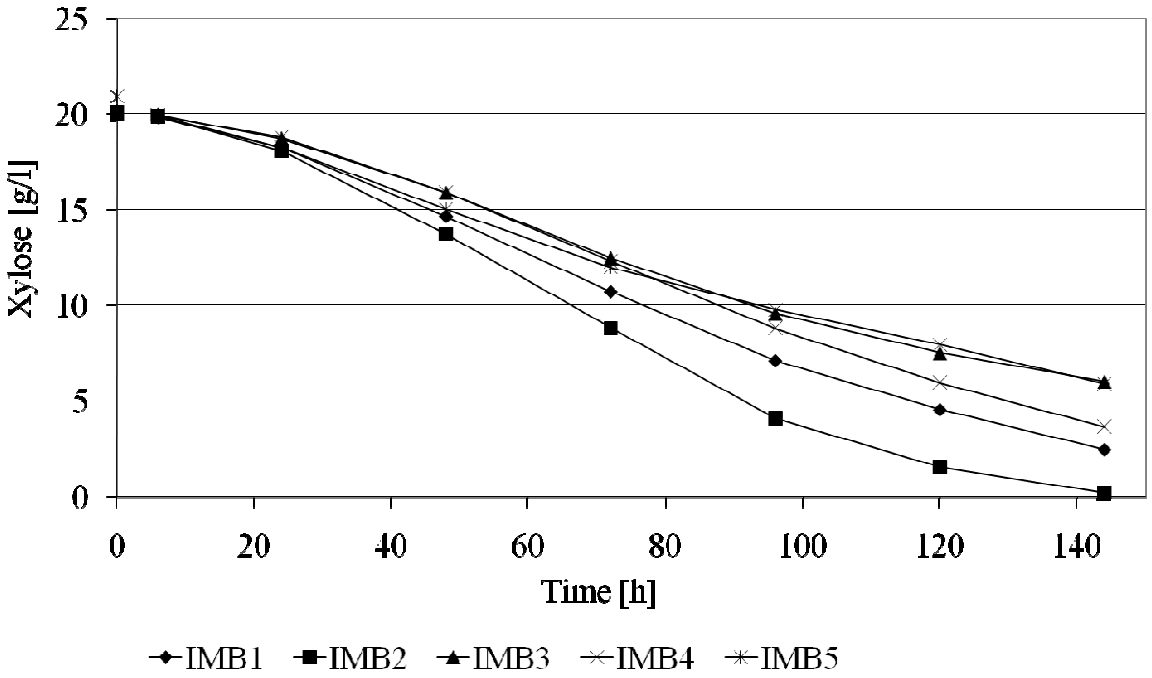

Figure 12: Xylose consumption over time by IMB strains at 45°C.

Table 3: Mean values of ethanol concentration [g/l] during IMB screening (bold). Values with the same letter for each column were not significantly different.

Time [h]		24	48	72	96	120	144
Strain	IMB1	**0.000^{C}**	**0.219^{B}**	**0.487^{B}**	**0.735^{C}**	**0.917^{B}**	**0.949^{AB}**
	IMB2	**0.005^{C}**	**0.262^{B}**	**0.525^{B}**	**0.867^{AB}**	**1.018^{A}**	**0.988^{A}**
	IMB3	**0.118^{B}**	**0.427^{A}**	**0.719^{A}**	**0.963^{A}**	**1.082^{A}**	**0.845^{AB}**
	IMB4	**0.178^{A}**	**0.378^{A}**	**0.556^{B}**	**0.787^{BC}**	**0.853^{B}**	**0.764^{B}**
	IMB5	**0.111^{B}**	**0.406^{A}**	**0.658^{A}**	**0.829^{BC}**	**0.866^{B}**	**0.795^{AB}**
Temperature [°C]	40	**0.077^{A}**	**0.388^{A}**	**0.736^{A}**	**1.122^{A}**	**1.346^{A}**	**1.304^{A}**
	45	**0.087^{A}**	**0.289^{B}**	**0.442^{B}**	**0.551^{B}**	**0.549^{B}**	**0.432^{B}**
P values	Strain	0.000	0.000	0.001	0.016	0.000	0.171
	Temperature	0.151	0.000	0.000	0.000	0.000	0.000
	Str. + Temp.	0.077	0.031	0.010	0.033	0.000	0.019

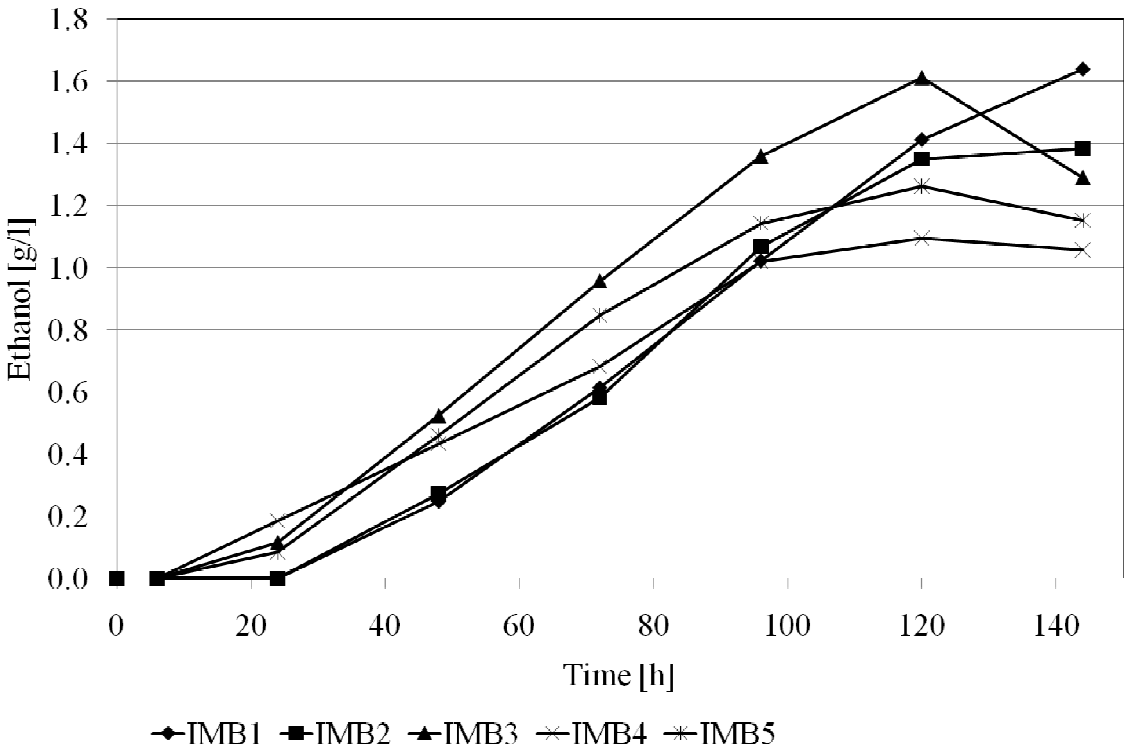

Figure 13: Ethanol production over time with IMB strains at 40°C.

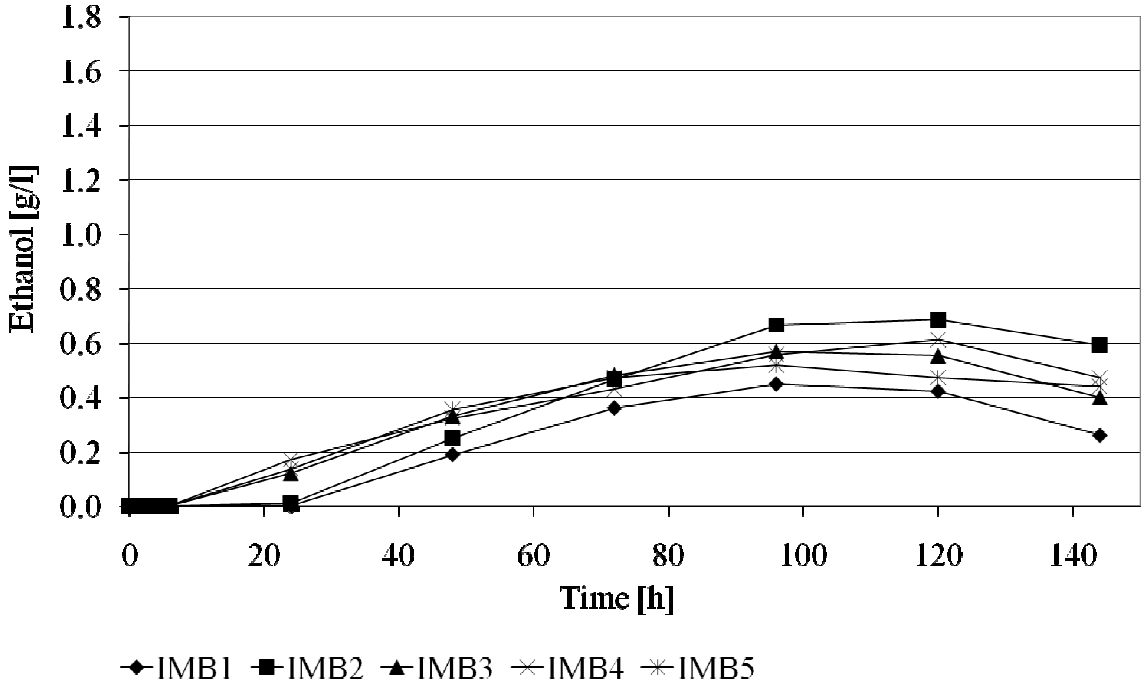

Figure 14: Ethanol production over time with IMB strains at 45°C.

of the theoretical maximum yield for IMB1 and IMB4, respectively. The highest ethanol concentrations at 45°C were achieved by IMB2 and IMB4 with 0.69 g/l and 0.61 g/l and 6.8% and 6.0% theoretical yield, respectively. However, IMB2 achieved only a yield of 0.04 g/g due to high xylose consumption. The low ethanol production at 45°C may be due to reduced activity of pentose phosphate cycle enzyme, but the activities of these enzymes were not analyzed. Banat and Marchant (1995) found IMB4 to be the most effective strain for ethanol production on xylose. In their study, IMB4 showed an ethanol concentration of 1.2 g/l, which represented 23.5% of the theoretical maximum and IMB2 showed a conversion efficiency of 21.6%. It is unknown what starting cell mass concentration the researchers used (Banat and Marchant 1995).

The ethanol production of all strains in this study was lower compared to tests with other native or genetically modified yeast. A recombinant *S. cerevisiae* expressing bacterial enzymes for xylose fermentation showed conversion yields up to 0.42 g ethanol/g xylose at 40°C (Lönn et al. 2003). *Pachysolen tannophilus* was found to produce ethanol with a conversion efficiency of 27% of the theoretical yield (Zhao et al. 2008). The fermentation was carried out at 30°C in aerobic conditions. A thermophilic yeast capable of fermenting xylose was developed by Voronovsky et al. (2005). They included genes for the expression of xylose isomerases from *Escherichia coli* and *Streptomyces coelicor* into *Hansenula polymorpha*. *H. polymorpha* Δ*xyl1* #4 (pScoel) #12 was reported to have an ethanol productivity of 0.81 mg/(l*h) at 37°C using xylose as sole carbon source. In the present study IMB3 had the highest ethanol productivity at 40°C (18 mg/(l*h)). Based on the results presented here, it is concluded that ethanol production from xylose by IMB yeast is too low for large scale production.

The IMB yeast showed a greater capability for xylitol production in comparison to ethanol production. Like ethanol production, xylitol production was higher at 40°C than at 45°C ($P < 0.05$) (Table 4). The yeast strain also affected the xylitol production. IMB2 had the highest xylitol yield of all of the strains with the same value at both temperatures of 0.42 g xylitol/g xylose. The lowest production at both temperatures was by IMB5 with yields of 0.32 g/g and 0.29 g/g for 40 and 45°C, respectively (Figures 13 and 14). That represented 27% and 22% of the theoretical maximum yield, respectively. All strains decreased xylitol production at 45°C except IMB2 and IMB4. IMB4 achieved 30% of the theoretical maximum yield at 40°C and 33% at 45°C. It should be noted that the xylitol concentration for all IMB strains at 40°C decreased rapidly after reaching the maximum concentration. At 45°C this pattern was not observed. Within xylose metabolism xylitol is an intermediate that can be further converted to ethanol. Since ethanol production is enhanced at 40°C, more xylitol is used. Therefore, the concentration decreases as ethanol increases.

Yablochkova et al. (2004) described the difference in ethanol and xylitol producing yeast on xylose. For the metabolism of xylose, xylose reductase and xylitol dehydrogenase are the key enzymes. Those enzymes need cofactors to be functional. Xylose reductase, which catalyzes the reduction of xylose to xylitol, has higher activity using NADPH than it does using NADH as a cofactor in xylitol producing yeast. In ethanol producing yeast, xylose reductase activity using NADH as a cofactor is comparable to the activity using NADPH. The oxidation of xylitol to xylulose, catalyzed by xylitol dehydrogenase, uses NAD^+ as cofactor, which can be regenerated under aerobic conditions since oxygen acts as an electron acceptor. However, when ethanol is

Table 4: Mean values of xylitol concentration [g/l] during IMB screening (bold). Values with the same letter for each column were not significantly different.

Time [h]		24	48	72	96	120	144
Strain	IMB1	**0.094^{E}**	**1.986^{C}**	**4.402^{BC}**	**5.931^{B}**	**6.119^{B}**	**4.945^{B}**
	IMB2	**0.192^{D}**	**2.707^{AB}**	**5.687^{A}**	**7.462^{A}**	**7.454^{A}**	**6.111^{A}**
	IMB3	**0.622^{C}**	**2.494^{B}**	**4.002^{C}**	**4.226^{C}**	**3.333^{C}**	**2.292^{C}**
	IMB4	**0.888^{A}**	**2.905^{A}**	**4.612^{B}**	**5.564^{B}**	**5.189^{B}**	**4.059^{B}**
	IMB5	**0.713^{B}**	**2.644^{AB}**	**4.256^{BC}**	**4.431^{C}**	**3.669^{C}**	**2.528^{C}**
Temperature [°C]	40	**0.568^{A}**	**3.208^{A}**	**5.705^{A}**	**6.244^{A}**	**4.730^{B}**	**2.423^{B}**
	45	**0.436^{B}**	**1.887^{B}**	**3.478^{B}**	**4.801^{B}**	**5.572^{A}**	**5.551^{A}**
P values	Strain	0.000	0.001	0.000	0.000	0.000	0.000
	Temperature	0.000	0.000	0.000	0.000	0.011	0.000
	Str. + Temp.	0.000	0.000	0.005	0.060	0.018	0.001

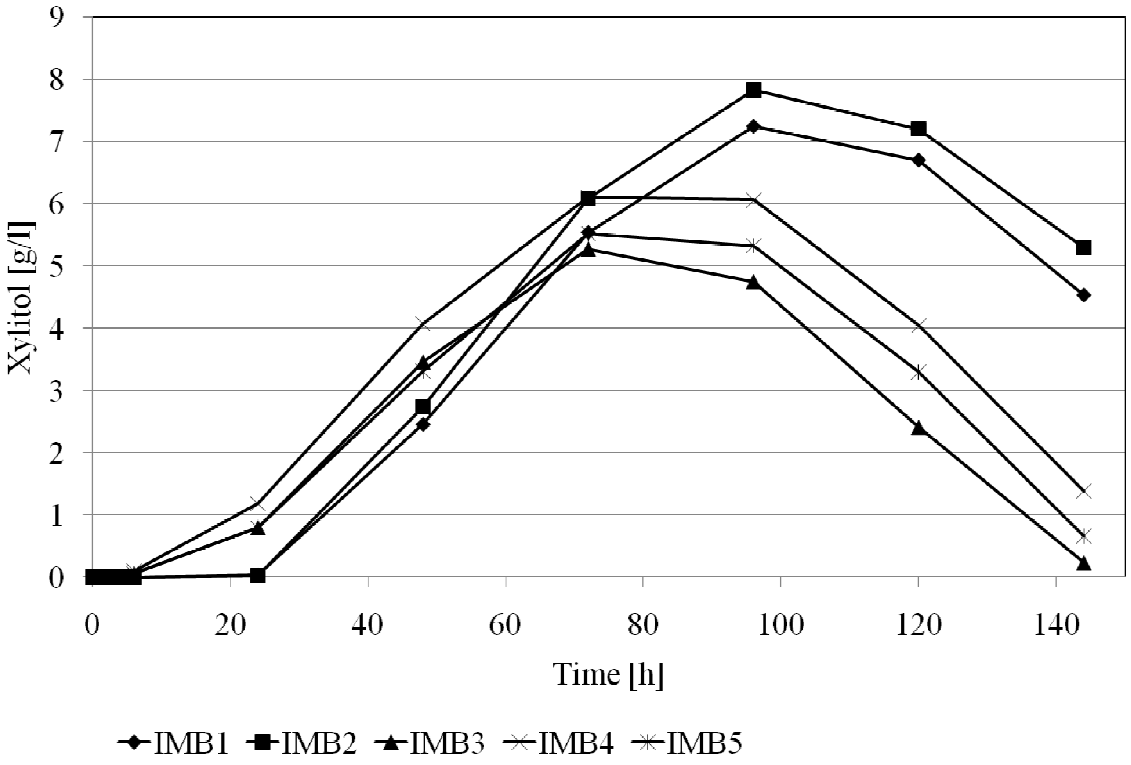

Figure 15: Xylitol production over time with IMB strains at 40°C.

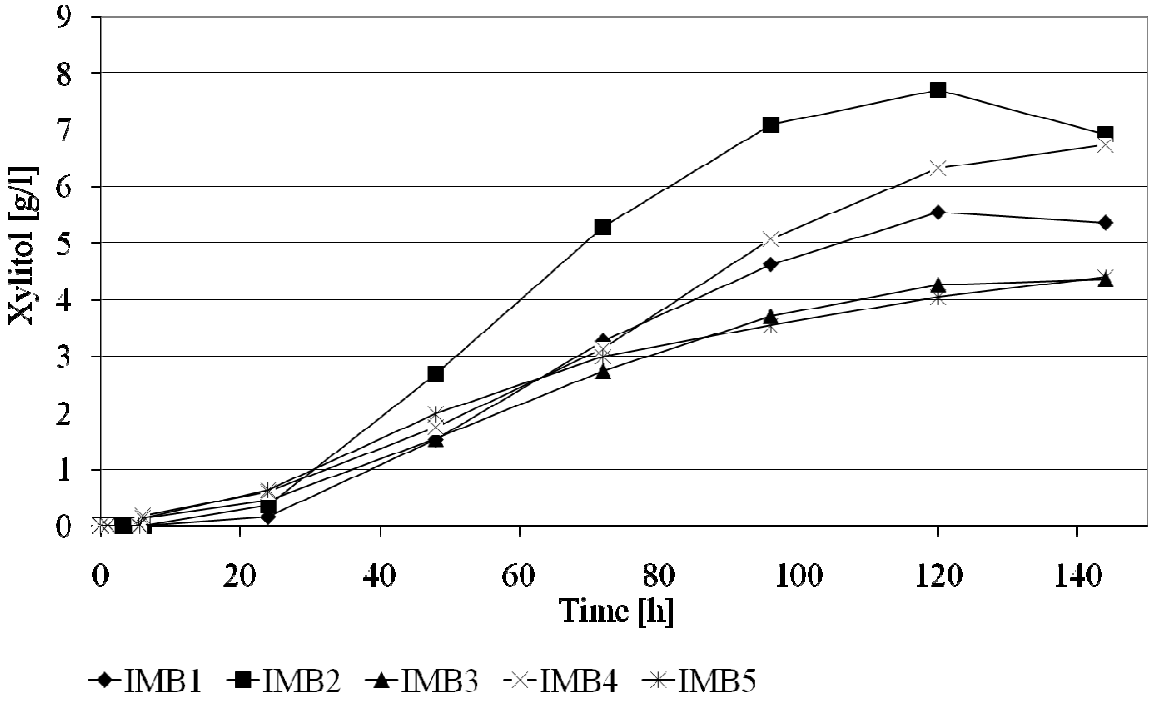

Figure 16: Xylitol production over time with IMB strains at 45°C.

the desired product, microaerobic or anaerobic conditions are required. NAD^+ is not regenerated fast enough and accumulates due to low oxygen supply if xylose reductase cannot use NADH. Subsequently, xylitol dehydrogenase is inhibited, xylulose formation is reduced and xylitol accumulates. The results of this study show that IMB yeast produce high amounts of xylitol as compared to ethanol. Therefore, an imbalance of cofactors caused by microaerobic conditions is probably present. The cofactor specificity of xylose reductases in IMB yeast need to be measured to confirm this, but *K. marxianus* has previously been shown to have low xylose reductase activity while using NADH as cofactor (Yablochkova et al. 2004).

5.2 SSF of xylan with IMB2 and Multifect Xylanase

SSF was shown to increase the conversion efficiency by avoiding a product inhibition of the enzyme (Blotkamp et al. 1978). A fermentation test on xylan in SSF simulates more likely the conditions in a large scale ethanol production than the fermentation of xylose. The utilization of xylose as pure substrate is too expensive. For the utilization of xylan in SSF, an activity test of different enzymes was conducted to identify the most effective enzyme. Since manufacturers use different units to identify the activity of their enzyme, a simple test was performed to see which enzyme produced the highest xylose concentration in the shortest time. Table 5 shows the experimental design with the conditions for enzymatic hydrolysis. Since SSF was to be performed at 45°C, that was the temperature chosen. Figure 15 shows the xylose concentration over time when using each enzyme mixture. Multifect Xylanase produced the highest xylose concentration at 48 h with a value of 2.2 g/l ($P < 0.05$).

Table 5: Experimental design for enzyme screening.

Substrate	Temperature [°C]	Enzyme concentration [µl/ml]	Enzyme
Xylan [5 g/l]	45	0.5	NS 50012
			Rapidase PNS
			Multifect Xylanase
			Multifect Pectinase FE
			Pectinex Ultra SP/L

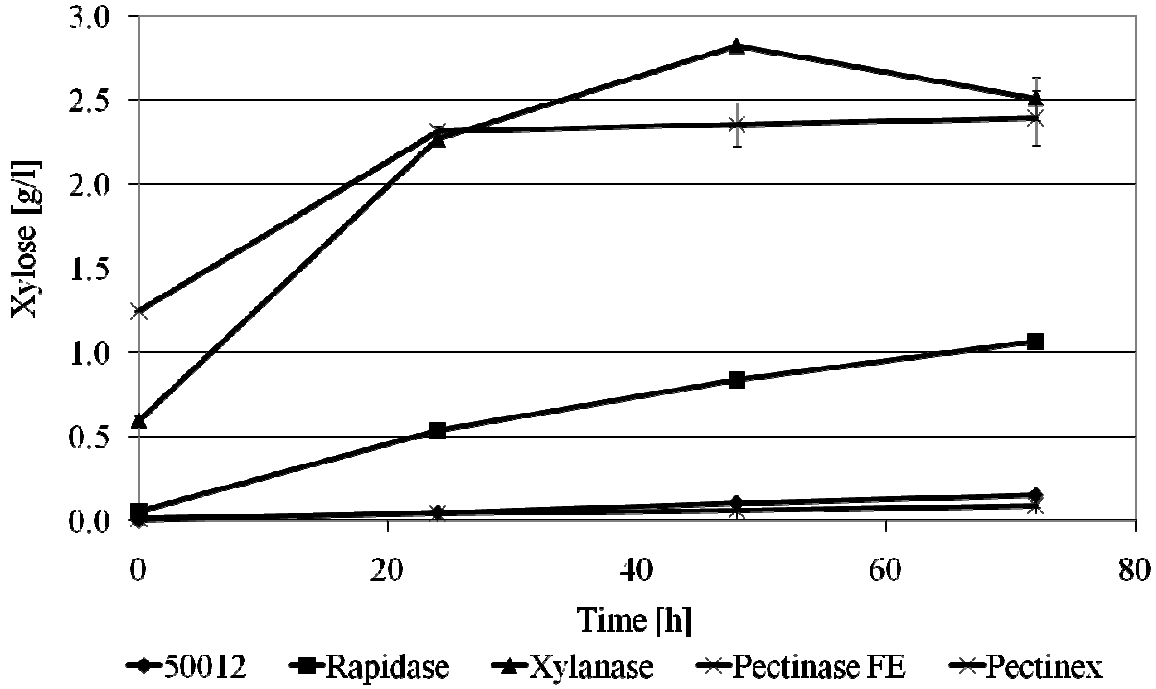

Figure 17: Enzyme screening. Xylose concentration over time with different enzyme mixtures.

This concentration was calculated by subtracting the initial value from the maximum. The presence of initial xylose can only be explained by delayed sampling after injecting the enzyme. The corrected concentration represents 40% of the theoretical maximum yield. The temperature in this test was lower than the optimum temperature of the enzyme, which may explain the relatively low xylose yield. Since using Multifect Xylanase resulted in the most xylose produced, it was chosen for the SSF tests.

Based on the findings from the previous IMB screening, *K. marxianus* IMB2 was used with Multifect Xylanase for SSF of xylan at 40 and 45°C with three different enzyme concentrations (1, 2, and 3 µl/ml). The enzyme activity, which was estimated for the first 12 h of the test, was higher at 45°C than at 40°C ($P < 0.05$), but varied depending on the enzyme concentration (Table 6). Figure 16 shows the xylose concentration over time released by the enzyme. The xylose concentration is the sum of xylose in the media and xylose used for ethanol and xylitol production. Biomass and other metabolites are not taken into account, which leads to a decrease in xylose concentration after 48 h. An enzyme concentration of 1, 2, and 3 µl/ml at 40°C showed 90%, 83% and 78% of the activity at 45°C, respectively (Table 6). The xylose production during the first 12 h showed significant differences with enzyme concentration ($P < 0.05$). As mentioned earlier, the optimum temperature of xylanase is higher than the test temperatures and, therefore, the increase of activity from 40°C to 45°C was expected. At 45°C the highest activity was 3222 IU/ml enzyme with 1 µl/ml enzyme concentration. The lowest enzyme concentration has the highest activity since the amount of released xylose is not doubled with doubled enzyme concentration. However, a higher initial enzyme concentration results in a faster conversion of xylan to xylose.

Table 6: Results of simultaneous saccharification and fermentation experiment based on the average of duplicates. Far upper right column shows activity of the enzymes in IU/ml enzyme (= µmol/(min*ml)) ([#] between 12 and 48 h). C_{EtOH_max} = maximum ethanol concentration, C_{Xtol_max} = maximum xylitol concentration, CE = conversion efficiency.

Product	Temperature [°C]	Enzyme concentration [µl/ml]	C_{EtOH_max} [g/l]	Time [h] with max. ethanol conc.	CE [%]	ethanol productivity [g/(l*h)][#]	Enzyme productivity [g/(l*h)]	Enzyme activity [IU/ml enzyme]
Ethanol	40	1	1.31	120	12.6	0.0079	0.26	2889
		2	1.69	96	16.2	0.0120	0.38	2111
		3	1.94	96	18.6	0.0190	0.42	1556
	45	1	0.50	72	4.8	0.0082	0.29	3222
		2	0.22	72	2.1	0.0022	0.46	2556
		3	0.43	72	4.1	0.0072	0.54	2000
Product	**Temperature [°C]**	**Enzyme concentration [µl/ml]**	**C_{Xtol_max} [g/l]**	**Time [h] with max. xylitol conc.**	**CE [%]**	**xylitol productivity [g/(l*h)][#]**		
Xylitol	40	1	2.37	72	11.5	0.040		
		2	3.26	72	15.8	0.065		
		3	2.74	48	13.3	0.072		
	45	1	1.70	96	8.2	0.021		
		2	2.25	96	10.9	0.035		
		3	3.47	120	16.8	0.060		

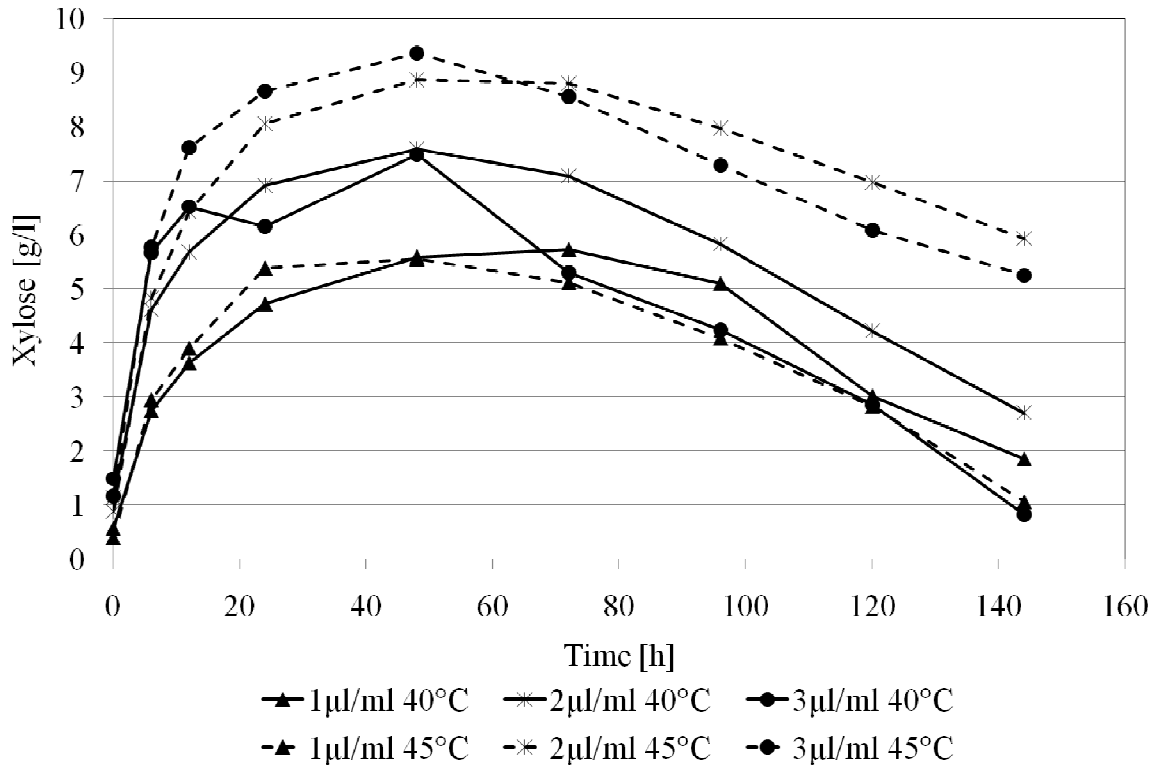

Figure 18: Xylose production over time with Multifect Xylanase during SSF at 40°C and 45°C with three different enzyme concentrations.

An enzyme concentration of 2 µl/ml had an xylose production of 0.46 g/(l*h) as compared to 0.29 g/(l*h) with 1µl/ml. Therefore, a 100% increase in enzyme concentration led to a 58% increase in xylose production.

Ethanol production was higher at 40°C than at 45°C ($P < 0.05$) (Figure 17, Table 7). The ethanol yield at 45°C ranged from 2.1% to 4.8% of the theoretical maximum yield, with 2 µl/ml and 1 µl/ml enzyme concentration, respectively. The yield at 40°C ranged from 12.6% to 18.6% of the theoretical maximum yield with 1 µl/ml and 3 µl/ml enzyme concentration, respectively. The maximum ethanol concentration at 40°C was achieved after 96 h for 2 and 3µl/ml enzyme concentration and 120 h for 1 µl/ml enzyme concentration. However, statistical analysis showed no significant difference in ethanol production with different enzyme concentrations ($P > 0.05$). The reduced ethanol production at 45°C was previously observed during the IMB screening experiment. IMB2 achieved in the screening a conversion efficiency of 13.5% at 40°C, whereas, 18.6% was achieved during SSF with the highest enzyme concentration at the same temperature. The xylan used in this experiment is not only composed of xylose but also of other sugars (≤10%) that might be responsible for the higher conversion efficiency. In comparison to *P. stipitis*, which produced 21.46 g/l ethanol on 50 g/l xylose at 30°C (Du Preez and Prior 1985), IMB2 was a poor ethanol producer using xylan and SSF at 40 and 45°C.

With enzyme concentrations of 1µl/ml and 2µl/ml, a higher xylitol yield was achieved at 40°C than at 45°C (Figure 18). An increased xylitol yield was observed with a temperature of 45°C with 3µl/ml xylanase concentration as compared to 40°C. The highest xylitol yield was achieved after 120 h at 45°C and 3 µl/ml xylanase concentration

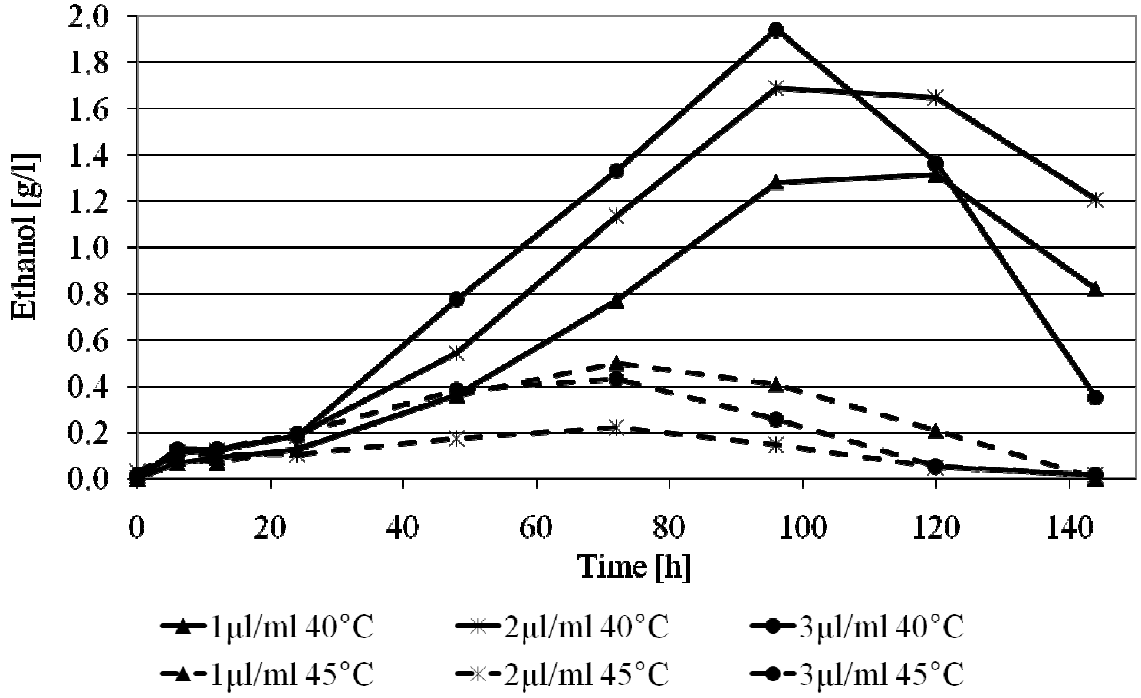

Figure 19: Ethanol production over time with *K. marxianus* IMB2 during SSF at 40°C and 45°C with three different enzyme concentrations.

Table 7: Mean values of ethanol and xylitol concentration [g/l] during SSF (bold). Values with the same letter for each column were not significantly different.

Ethanol								
Time [h]			24	48	72	96	120	144
Enzyme concentration [µl/ml]		1	**0.125^{B}**	**0.359^{B}**	**0.879^{A}**	**0.843^{A}**	**0.759^{A}**	**0.409^{A}**
		2	**0.144^{B}**	**0.358^{B}**	**0.679^{A}**	**0.917^{A}**	**0.848^{A}**	**0.610^{A}**
		3	**0.187^{A}**	**0.578^{A}**	**0.634^{A}**	**1.097^{A}**	**0.707^{A}**	**0.182^{A}**
Temperature [°C]		40	**0.163^{A}**	**0.559^{A}**	**1.078^{A}**	**1.636^{A}**	**1.441^{A}**	**0.791^{A}**
		45	**0.141^{A}**	**0.305^{B}**	**0.384^{B}**	**0.268^{B}**	**0.102^{B}**	**0.009^{B}**
P values	Enzyme		0.015	0.030	0.118	0.173	0.320	0.165
	Temperature		0.112	0.004	0.000	0.000	0.000	0.003
	Enz. + Temp.		0.038	0.056	0.035	0.031	0.073	0.164
Xylitol								
Time [h]			24	48	72	96	120	144
Enzyme concentration [µl/ml]		1	**0.505^{C}**	**1.303^{C}**	**1.969^{B}**	**1.758^{A}**	**0.735^{B}**	**0.243^{C}**
		2	**0.760^{B}**	**2.073^{B}**	**2.699^{A}**	**2.100^{A}**	**1.449^{A}**	**1.150^{B}**
		3	**0.966^{A}**	**2.729^{A}**	**2.617^{AB}**	**1.792^{A}**	**1.754^{A}**	**1.689^{A}**
Temperature [°C]		40	**0.681^{B}**	**2.319^{A}**	**2.511^{A}**	**1.293^{B}**	**0.261^{B}**	**0.055^{B}**
		45	**0.806^{A}**	**1.751^{B}**	**2.345^{A}**	**2.474^{A}**	**2.364^{A}**	**1.999^{A}**
P values	Enzyme		0.000	0.000	0.073	0.737	0.020	0.000
	Temperature		0.028	0.000	0.490	0.022	0.000	0.000
	Enz. +Temp.		0.002	0.008	0.007	0.020	0.013	0.000

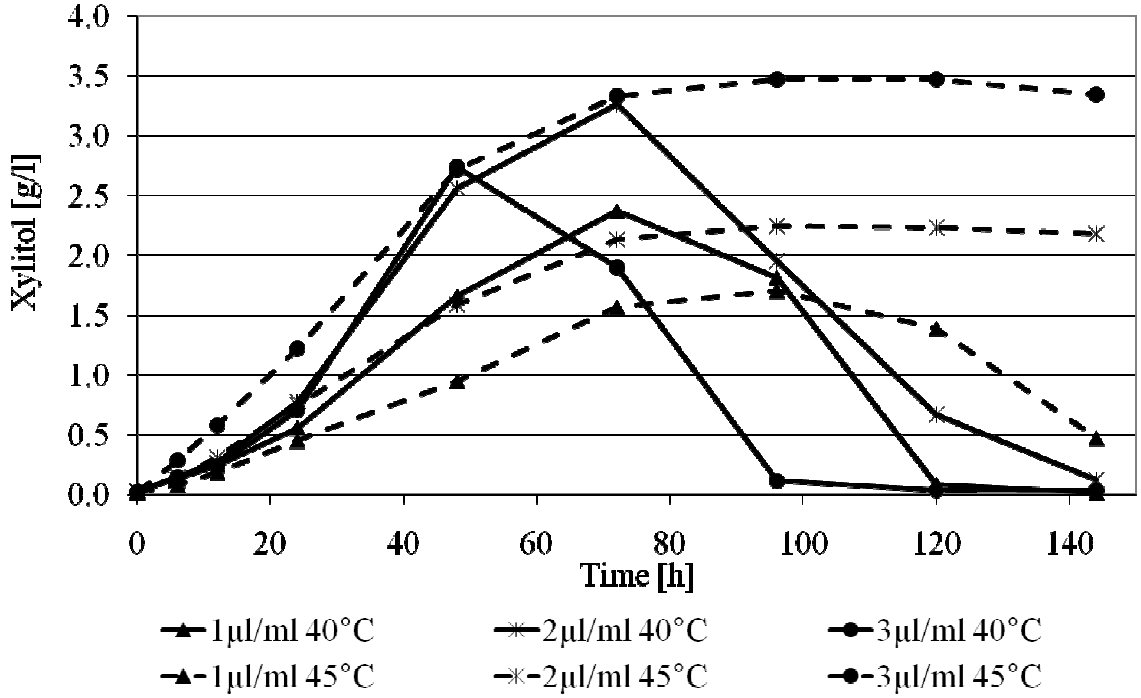

Figure 20: Xylitol production over time with *K. marxianus* IMB2 during SSF at 40°C and 45°C with three different enzyme concentrations.

with 16.8% of the theoretical maximum yield. Xylitol concentrations after 120 h using 2 µl/ml and 3 µl/ml enzyme concentrations were similar ($P > 0.05$). *C. mogii* ATCC 18364 was found to produce xylitol with a high yield of 0.62 g/g (Sirisansaneey et al. 1995) and Guo et al. (2006) found *Candida guilliermondii* Xu264 with a yield of 0.64 g/g. The lower yield in the present study may be based on the temperature conditions. The values found in the literature were conducted at 30°C, whereas, this study was conducted at thermophilic conditions for SSF purposes. Enzyme concentration and temperature as well as an interaction of enzyme concentration with temperature were observed as significant factors ($P < 0.05$). The lower xylitol yield at 40°C is based on the active ethanol production. The xylitol concentration reaches its maximum at 48 h (for 3 µl/ml enzyme concentration) and 72 h (for 1 and 2µl/ml enzyme concentration) and decreases rapidly to almost zero. The increase in ethanol production at 72 h is probably due to xylitol being further converted to ethanol via the pentose phosphate pathway, as was previously discussed. In contrast, xylitol concentration at 45°C remains constant after reaching its maximum. These findings confirm the results in the IMB screening test where all strains except IMB2 showed no decrease in xylitol concentration after 96 h at 45°C.

Xylose consumption was different at both temperatures. At 40°C almost all xylose was consumed, whereas, at 45°C xylose remained at the end of the test. With 1, 2, and 3 µl/ml xylanase concentration, 0.6 g/l, 3.8 g/l, and 1.9 g/l xylose are still remaining in the media at 45°C, respectively. It is not known why the yeast stop producing xylitol at 45°C after 72 h. Suryawati at al. (2008) observed that the IMB strains stopped fermenting glucose after 72 h at 45°C. The thermophilic conditions may affect cell growth and lead

to death of the cells. The incomplete xylose utilization was also found in the IMB screening.

5.3 Co-culture fermentation of xylose with IMB2 and *P. stipitis*

The IMB screening and SSF tests showed poor ethanol production on xylose and xylan, respectively. To enhance the production of ethanol from xylose with IMB2, a co-culture experiment with *P. stipitis* was conducted.

The *P. stipitis* monoculture and the co-culture grew faster than the *K. marxianus* IMB2 monoculture and reached a maximum cell concentration after 24 h. *K. marxianus* as monoculture reached the cell mass concentration maximum after 72 h (Figure 19). The maximum cell mass concentrations for *P. stipitis* and *K. marxianus* as monocultures were 3.16 g/l and 2.73 g/l, respectively. The cell mass concentration for the co-culture could not be obtained by spectroscopy since both strains have different factors to convert optical density to cell mass concentration.

Xylose was utilized most quickly by *P. stipitis* and only 0.1 g/l of xylose remained at the end of fermentation ($P > 0.05$). IMB2 and the co-culture had final xylose concentrations of 11.7 g/l and 6.7 g/l, respectively (Figure 20).

P. stipitis as a monoculture produced the highest ethanol concentration of all treatments ($P < 0.05$) (Table 8). It reached 79.6% of the theoretical maximum yield with a productivity of 0.37 g/l*h during the linear production phase (Table 9 and Figure 21). The maximum ethanol concentration of 16.2 g/l was reached after 72 h with a yield of 0.43 g/g. This result confirms data found in the literature. Agbogbo et al. (2006) showed

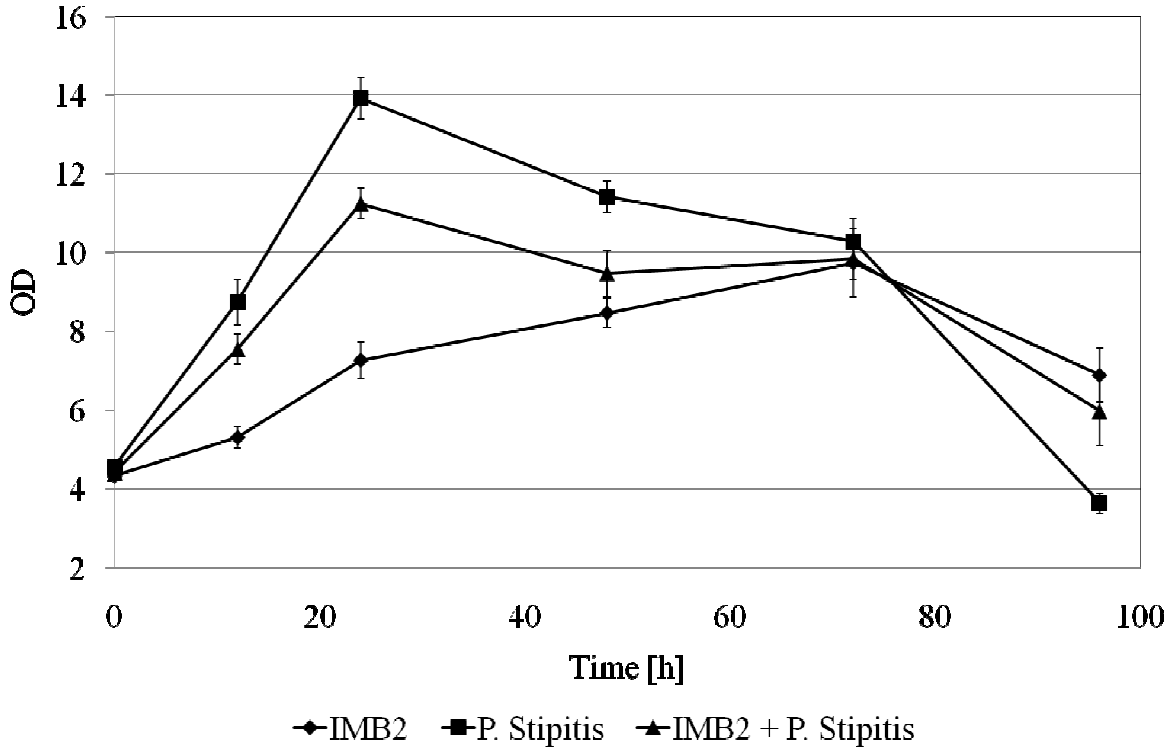

Figure 21: Optical density over time with IMB2, *P. stipitis* and co-culture. The vertical bars represent standard deviation.

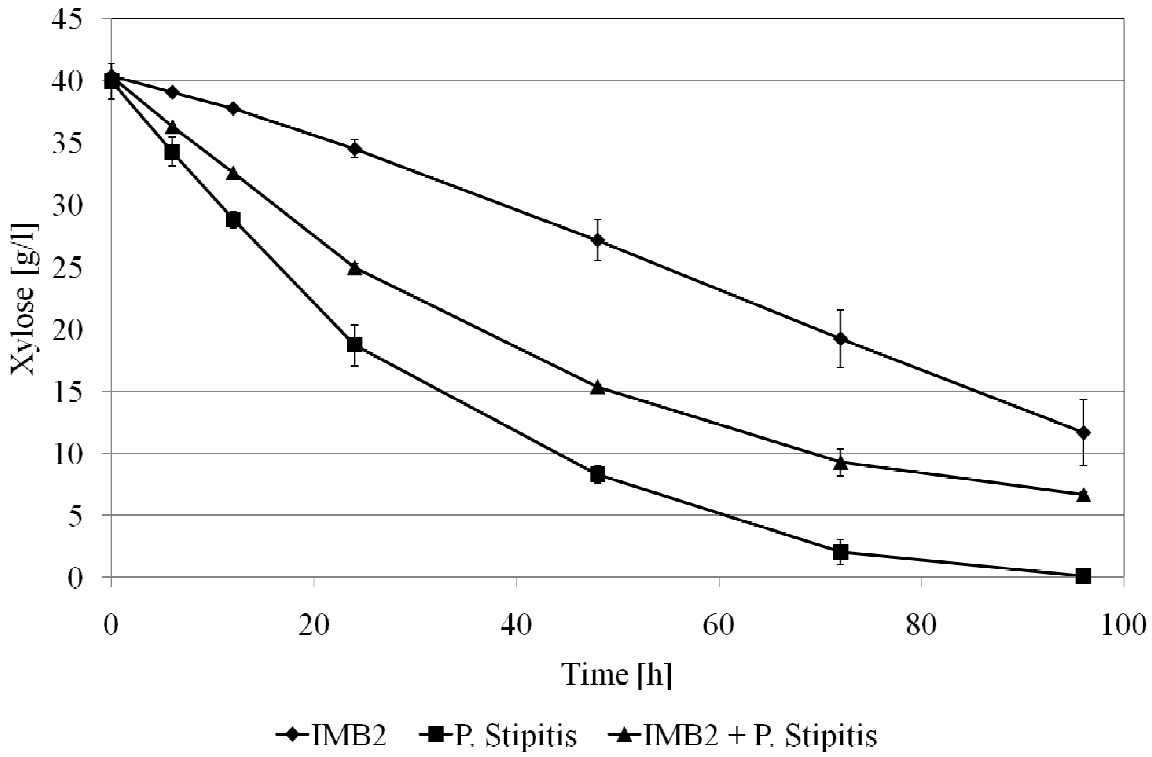

Figure 22: Xylose consumption over time with IMB2, *P. stipitis* and co-culture. The vertical bars represent standard deviation.

Table 8: Mean values of ethanol and xylitol concentration [g/l] during co-culture fermentation. Values with the same letter for each column were not significantly different.

Ethanol					
Time [h]		24	48	72	96
Culture	IMB2	0.374^{C}	0.700^{C}	1.065^{C}	1.480^{C}
	P. stipitis	8.861^{A}	14.151^{A}	16.235^{A}	15.638^{A}
	Co-culture	5.512^{B}	8.058^{B}	8.748^{B}	10.027^{B}
Xylitol					
Time [h]		24	48	72	96
Culture	IMB2	3.766^{A}	8.646^{A}	14.093^{A}	19.095^{A}
	P. stipitis	0.500^{C}	0.676^{C}	0.679^{C}	0.666^{C}
	Co-culture	2.493^{B}	4.268^{B}	4.540^{B}	5.002^{B}

Table 9: Results of co-culture experiment based on the average of triplicates ($^{\#}$ in the first 24 h). C_{EtOH_max} = maximum ethanol concentration, C_{Xtol_max} = maximum xylitol concentration, $Y_{E/X}$ = ethanol yield, $Y_{X/X}$ = xylitol yield, CE = conversion efficiency.

Product	**Culture**	**C_{EtOH_max} [g/l]**	**Time [h] with max. ethanol conc.**	**$Y_{E/X}$ [g/g]**	**CE [%]**	**ethanol prod. [g/(l*h)]$^{\#}$**
Ethanol	IMB2	1.48	96	0.05	7.3	0.014
	P. stipitis	16.24	72	0.43	79.6	0.370
	IMB2 + *P. stipitis*	10.03	96	0.30	49.2	0.230
Product	**Culture**	**C_{Xtol_max} [g/l]**	**Time [h] with max. xylitol conc.**	**$Y_{X/X}$ [g/g]**	**CE [%]**	**xylitol prod. [g/(l*h)]$^{\#}$**
Xylitol	IMB2	19.10	96	0.660	47.1	0.160
	P. stipitis	0.68	72	0.018	1.7	0.019
	IMB2 + *P. stipitis*	5.00	96	0.150	12.3	0.100

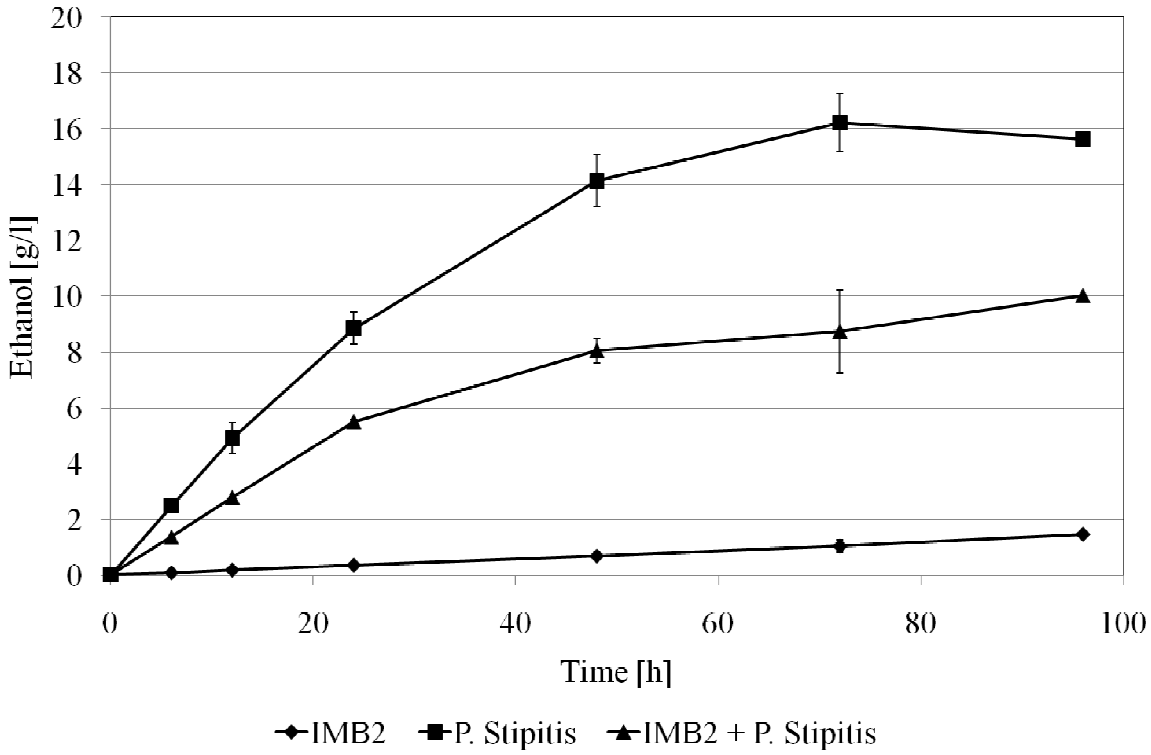

Figure 23: Ethanol production over time with IMB2, *P. stipitis* and co-culture. The vertical bars represent standard deviation.

an ethanol yield with *P. stipitis* based on xylose of 0.44 g/g. They used microaerobic conditions at a temperature of 30°C. A test on corn stover hemicellulose hydrolyzate showed an ethanol yield of 0.44 g/g (Agbogbo and Wenger 2007). The hydrolyzate also contained glucose (6.3 g/l) and the yeast inhibitors acetic acid, furfural and hydroxylmethylfurfural with concentrations of 6.1 g/l, 0.63 g/l, and 0.76 g/l, respectively. Nigam (2001) achieved an ethanol yield of 0.43 g/g with *P. stipitis*. The media used in this test was a wheat straw hemicellulose hydrolyzate that also contained sugars other than xylose in small amounts. IMB2 as a monoculture showed much lower ethanol production than the co-culture or the *P. stipitis* monoculture ($P < 0.05$). IMB2 reached only 7.3% of the theoretical maximum yield with a productivity of 0.014 g/l*h and a maximum ethanol concentration of 1.5 g/l. The ethanol production found in the IMB screening was higher. At 40°C IMB2 showed 13.5% conversion efficiency. This indicates that the optimum temperature for ethanol production with IMB2 is between 30°C and 40°C. The co-culture experiment had an ethanol productivity of 0.23 g/l*h with 49% of the maximum theoretical yield and a maximum ethanol concentration of 10 g/l. Since IMB2 monoculture performed poorly in ethanol production, the ethanol production using co-culture was probably dominated by *P. stipitis*.

IMB2 had the highest xylitol concentration of all of the treatments ($P < 0.05$). After 96 h 19.1 g/l xylitol was produced with a yield of 0.66 g/g, which was 47% of the theoretical maximum yield (Table 8 and Figure 22). The concentration may not have been the maximum since xylose was still left in the media and the xylitol production curve had a positive slope at the end of the experiment, although, in the IMB screening,

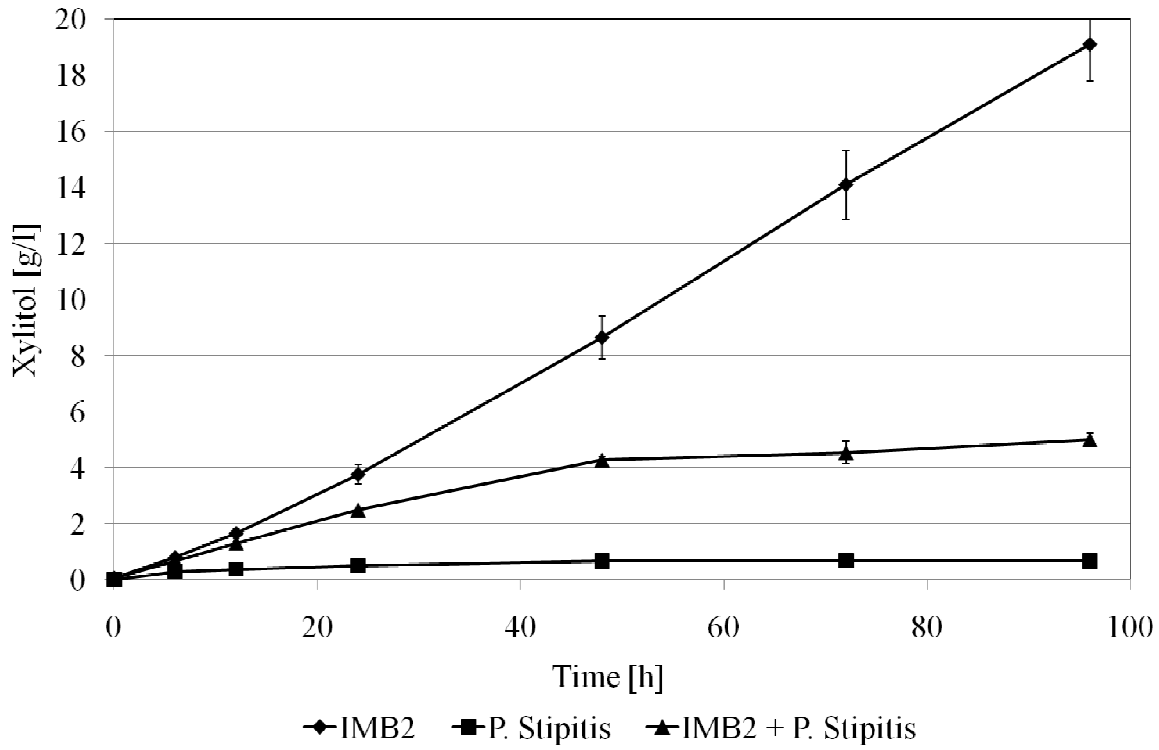

Figure 24: Xylitol production over time with IMB2, *P. stipitis* and co-culture. The vertical bars represent standard deviation.

xylitol quickly decreased after 96 h after reaching a maximum. Since the OD of the IMB2 monoculture decreased after 72 h, it was assumed the fermentation stopped and the test was terminated at 96 h. The xylitol yields for IMB2 favorably compare to *C. mogii* ATCC 18364 (0.62 g/g) (Sirisansaneey et al. 1995) and *Candida guilliermondii* Xu264 (0.64 g/g) (Guo et al. 2006), which were discussed earlier. In comparison, *P. stipitis* produced only 0.7 g/l xylitol. This was expected since *P. stipitis* was found in the literature to be an ethanol producing yeast with high yield. *P. stipitis* can use NADH as a co-factor for xylose reductase, as well as NADPH (Yablochkova et al. 2004). Therefore, the xylitol production in the co-culture is dominated by IMB2. The xylitol yield in the co-culture represented 12% of the theoretical maximum yield. A lower initial cell mass of IMB2 results in a lower xylitol production. All three treatments started with the same cell mass concentration. No synergistic effect between the two yeast for ethanol or xylitol production was observed. The results obtained in this experiment are contradictory to the findings of Rouhollah et al. (2007), which observed an improved efficiency with a co-culture of *K. marxianus* and *P. stipitis* on a xylose/glucose mixture as opposed to their respective monocultures. The co-culture reached 80% of the theoretical maximum ethanol yield. According to the authors, the high yield could be obtained because *K. marxianus* continued utilizing the xylose after *P. stipitis* stopped due to low ethanol tolerance. No xylitol production was mentioned in their findings.

When the SSF experiment and the IMB2 monoculture from the co-culture experiment are compared, a difference in xylitol production can be observed. Only 16% of the theoretical maximum yield was obtained by SSF, whereas, 47% of the theoretical maximum yield was reached on xylose at 30°C. SSF was carried out at 40°C which

shows that the optimum temperature for xylitol production is below 40°C. The differences in ethanol yield may also be based on the different temperatures used in both experiments. SSF had yields at 40°C of 18.6% of the maximum theoretical yield, whereas, at 30°C (IMB2 monoculture experiment with co-culture conditions) only 7.3% of the theoretical maximum yield was obtained. This shows again that the optimum temperature for ethanol production with IMB2 is between 30°C and 40°C.

CHAPTER 6

CONCLUSION

K. marxianus IMB yeast were poor ethanol producers at thermophilic conditions with xylose as the sole carbon source during normal sugar batch fermentation as well as during SSF. This is due to the accumulation of NADH that results from low NADH activity of xylose reductase (Yablochkova et al. 2004). However, IMB yeast produced higher ethanol concentration at high temperatures and IMB2 had highest ethanol and xylitol production compared to other IMB strains with mean ethanol and xylitol concentration values of 1.02 g/l and 7.45 g/l, respectively (Table 3 and 4).

Simultaneous saccharification and fermentation on xylan showed no improvement in ethanol production. The mean values for ethanol and xylitol concentration are 0.88 g/l and 2.70 g/l, respectively (Table 7).

A co-culture of IMB2 and *P. stipitis* was better than IMB2 alone in terms of ethanol production. However, the co-culture was not better than a monoculture of *P. stipitis*, the model xylose fermenter. No synergetic effects could be observed.

It was shown that the IMB yeast have promise for biological xylitol production with the mean xylitol concentration of 19.1 g/l (Table 8). IMB2 showed great conversion efficiencies at 30°C, which should be analyzed in more detail in future work.

CHAPTER 7

FUTURE WORK

Since *K. marxianus* IMB2 performed poorly in ethanol production but showed excellent xylitol production, future work with this yeast strain should concentrate on xylitol production at mesophilic conditions with xylose as the sole carbon source. A variable for fermentation should be temperature. This test found good results at 30°C but to determine the temperature optimum, xylitol production at 25°C and 35°C should be tested. The aeration rate could have a significant effect on xylitol production. Yablochkova et al. (2004) found the highest specific NADPH dependant XR activity for *K. marxianus* Y-488 under anaerobic conditions with a value of 0.34 µmol/(mg*min). Under microaerobic conditions the specific NADPH dependant XR activity is only 0.16 µmol/(mg*min). The low xylitol dehydrogenase activity at anaerobic conditions favors the xylitol production.

Additionally, complex substrates that are rich in hemicellulose, such as wood residues or herbaceous crops, should be included in the research. Using xylose from these substrates for xylitol production is in direct conflict to its use for ethanol production, but when xylitol gets more established in the market (e.g. diabetics' food), it can be a high value product. The substrates can be used for ethanol production with xylose as residue used for xylitol production.

Concerning the ethanol production with xylose as the carbon source, future work should concentrate on optimizing the fermentation with *P. stipitis*. The yeast showed excellent conversion efficiencies. A fermentation parameter that should be focused on should be inhibitor concentrations. Depending on the pretreatment method, prehydrolyzates can have high concentrations of furfural, 5-hydroxylmethylfurfural, acetic acid and other yeast inhibitors. An adaptation of the yeast to these conditions would be beneficial and necessary to use these substrates for increasing the overall conversion efficiency of lignocellulosic substrates. Further research is needed to find yeast capable of growing at thermophilic conditions for SSF purposes.

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APPENDIX

SAS program for IMB screening:

```
options ls=74 ps=60;
data IMB24;
infile "c:\Documents and Settings\michael.mueller\Desktop\SAS files\IMB24.csv"
dlm=",";
input temp strain$ OD xyl xtol gly aa etol;
cards;
run;
data IMB48;
infile "c:\Documents and Settings\michael.mueller\Desktop\SAS files\IMB48.csv"
dlm=",";
input temp strain$ OD xyl xtol gly aa etol;
cards;
run;
data IMB72;
infile "c:\Documents and Settings\michael.mueller\Desktop\SAS files\IMB72.csv"
dlm=",";
input temp strain$ OD xyl xtol gly aa etol;
cards;
run;
data IMB96;
infile "c:\Documents and Settings\michael.mueller\Desktop\SAS files\IMB96.csv"
dlm=",";
input temp strain$ OD xyl xtol gly aa etol;
cards;
run;
data IMB120;
infile "c:\Documents and Settings\michael.mueller\Desktop\SAS files\IMB120.csv"
dlm=",";
input temp strain$ OD xyl xtol gly aa etol;
cards;
run;
data IMB144;
infile "c:\Documents and Settings\michael.mueller\Desktop\SAS files\IMB144.csv"
dlm=",";
input temp strain$ OD xyl xtol gly aa etol;
cards;
run;
```

```
proc glm data=IMB72; class strain temp;
model etol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB48; class strain temp;
model xtol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB72; class strain temp;
model xtol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB48; class strain temp;
model etol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB24; class strain temp;
model etol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB96; class strain temp;
model etol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB120; class strain temp;
model etol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB144; class strain temp;
model etol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB24; class strain temp;
model xtol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB96; class strain temp;
model xtol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB120; class strain temp;
model xtol = strain|temp;
means strain temp/lsd;
run;
proc glm data=IMB144; class strain temp;
model xtol = strain|temp;
```

means strain temp/lsd;
run;

SAS output for IMB screening:

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: etol 72h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	0.65688501	0.07298722	23.06	<.0001
Error	10	0.03164455	0.00316445		
Corrected Total	19	0.68852956			

R-Square	Coeff Var	Root MSE	etol Mean
0.954040	9.549771	0.056253	0.589056

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	0.14920133	0.03730033	11.79	0.0008
temp	1	0.43181403	0.43181403	136.46	<.0001
strain*temp	4	0.07586965	0.01896741	5.99	0.0100

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	0.14920133	0.03730033	11.79	0.0008
temp	1	0.43181403	0.43181403	136.46	<.0001

strain*temp	4	0.07586965	0.01896741	5.99 0.0100

The GLM Procedure

t Tests (LSD) for etol 72h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.003164
Critical Value of t	2.22814
Least Significant Difference	0.0886

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	0.71910	4	3
A			
A	0.65800	4	5
B	0.55636	4	4
B			
B	0.52512	4	2
B			
B	0.48669	4	1

The GLM Procedure

t Tests (LSD) for etol 72h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.003164
Critical Value of t	2.22814
Least Significant Difference	0.0561

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.73599	10	40
B	0.44212	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: xtol 48h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	13.76337030	1.52926337	35.32	<.0001
Error	10	0.43301592	0.04330159		
Corrected Total	19	14.19638622			

R-Square	Coeff Var	Root MSE	xtol Mean
0.969498	8.169705	0.208090	2.547097

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	1.91937397	0.47984349	11.08	0.0011
temp	1	8.72653017	8.72653017	201.53	<.0001
strain*temp	4	3.11746616	0.77936654	18.00	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	1.91937397	0.47984349	11.08	0.0011
temp	1	8.72653017	8.72653017	201.53	<.0001
strain*temp	4	3.11746616	0.77936654	18.00	0.0001

The GLM Procedure

t Tests (LSD) for xtol 48h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.043302
Critical Value of t	2.22814
Least Significant Difference	0.3279

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	2.9046	4	4
A			
B A	2.7066	4	2
B A			
B A	2.6438	4	5
B			
B	2.4941	4	3
C	1.9864	4	1

The GLM Procedure

t Tests (LSD) for xtol 48h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05

Error Degrees of Freedom 10
Error Mean Square 0.043302
Critical Value of t 2.22814
Least Significant Difference 0.2074

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	3.20765	10	40
B	1.88655	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read 20
Number of Observations Used 20

The GLM Procedure

Dependent Variable: xtol 72h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	34.36049746	3.81783305	41.64	<.0001
Error	10	0.91682784	0.09168278		
Corrected Total	19	35.27732530			

R-Square	Coeff Var	Root MSE	xtol Mean
0.974011	6.594309	0.302792	4.591712

Source	DF	Type I SS	Mean Square	F Value	Pr > F

strain	4	6.79057822	1.69764456	18.52 0.0001
temp	1	24.79566125	24.79566125	270.45 <.0001
strain*temp	4	2.77425799	0.69356450	7.56 0.0045

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	6.79057822	1.69764456	18.52	0.0001
temp	1	24.79566125	24.79566125	270.45	<.0001
strain*temp	4	2.77425799	0.69356450	7.56	0.0045

The GLM Procedure

t Tests (LSD) for xtol 72h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.091683
Critical Value of t	2.22814
Least Significant Difference	0.4771

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	5.6874	4	2
B	4.6116	4	4
B			
C B	4.4015	4	1
C B			
C B	4.2563	4	5
C			
C	4.0018	4	3

The GLM Procedure

t Tests (LSD) for xtol 72h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.091683
Critical Value of t	2.22814
Least Significant Difference	0.3017

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	5.7052	10	40
B	3.4783	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: etol 48h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	0.20225042	0.02247227	22.80	<.0001
Error	10	0.00985677	0.00098568		
Corrected Total	19	0.21210720			

R-Square	Coeff Var	Root MSE	etol Mean
0.953529	9.281505	0.031395	0.338259

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	0.13704477	0.03426119	34.76	<.0001
temp	1	0.04875992	0.04875992	49.47	<.0001
strain*temp	4	0.01644573	0.00411143	4.17	0.0305

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	0.13704477	0.03426119	34.76	<.0001
temp	1	0.04875992	0.04875992	49.47	<.0001
strain*temp	4	0.01644573	0.00411143	4.17	0.0305

The GLM Procedure

t Tests (LSD) for etol 48h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.000986
Critical Value of t	2.22814
Least Significant Difference	0.0495

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	0.42711	4	3
A			
A	0.40625	4	5
A			
A	0.37767	4	4
B	0.26175	4	2
B			
B	0.21852	4	1

The GLM Procedure

t Tests (LSD) for etol 48h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.000986
Critical Value of t	2.22814
Least Significant Difference	0.0313

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.38763	10	40
B	0.28888	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: etol 24h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	0.09869669	0.01096630	53.35	<.0001

Error	10	0.00205564	0.00020556
Corrected Total	19	0.10075234	

R-Square	Coeff Var	Root MSE	etol Mean
0.979597	17.45813	0.014338	0.082125

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	0.09579246	0.02394812	116.50	<.0001
temp	1	0.00049736	0.00049736	2.42	0.1509
strain*temp	4	0.00240687	0.00060172	2.93	0.0767

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	0.09579246	0.02394812	116.50	<.0001
temp	1	0.00049736	0.00049736	2.42	0.1509
strain*temp	4	0.00240687	0.00060172	2.93	0.0767

The GLM Procedure

t Tests (LSD) for etol 24h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.000206
Critical Value of t	2.22814
Least Significant Difference	0.0226

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	0.17773	4	4
B	0.11768	4	3
B			

B	0.11050	4	5
C	0.00471	4	2
C			
C	0.00000	4	1

The GLM Procedure

t Tests (LSD) for etol 24

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.000206
Critical Value of t	2.22814
Least Significant Difference	0.0143

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.087112	10	45
A			
A	0.077138	10	40

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: etol 96h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	1.83878111	0.20430901	35.69	<.0001
Error	10	0.05725250	0.00572525		
Corrected Total	19	1.89603361			

R-Square	Coeff Var	Root MSE	etol Mean
0.969804	9.046451	0.075665	0.836410

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	0.11943409	0.02985852	5.22	0.0156
temp	1	1.62652096	1.62652096	284.10	<.0001
strain*temp	4	0.09282606	0.02320652	4.05	0.0331

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	0.11943409	0.02985852	5.22	0.0156
temp	1	1.62652096	1.62652096	284.10	<.0001
strain*temp	4	0.09282606	0.02320652	4.05	0.0331

The GLM Procedure

t Tests (LSD) for etol 96h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.005725
Critical Value of t	2.22814
Least Significant Difference	0.1192

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	0.96332	4	3
A			
B A	0.86737	4	2
B			
B C	0.82925	4	5
B C			
B C	0.78736	4	4
C			
C	0.73474	4	1

The GLM Procedure

t Tests (LSD) for etol 96h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.005725
Critical Value of t	2.22814
Least Significant Difference	0.0754

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.12159	10	40
B	0.55123	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read 20

Number of Observations Used 20

The GLM Procedure

Dependent Variable: etol 120h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	3.55721950	0.39524661	238.75	<.0001
Error	10	0.01655497	0.00165550		
Corrected Total	19	3.57377447			

R-Square	Coeff Var	Root MSE	etol Mean
0.995368	4.295199	0.040688	0.947285

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	0.15837526	0.03959382	23.92	<.0001
temp	1	3.17544375	3.17544375	1918.12	<.0001
strain*temp	4	0.22340049	0.05585012	33.74	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	0.15837526	0.03959382	23.92	<.0001
temp	1	3.17544375	3.17544375	1918.12	<.0001
strain*temp	4	0.22340049	0.05585012	33.74	<.0001

The GLM Procedure

t Tests (LSD) for etol 120h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.001655
Critical Value of t	2.22814
Least Significant Difference	0.0641

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	1.08209	4	3
A			
A	1.01840	4	2
B	0.91645	4	1
B			
B	0.86625	4	5
B			
B	0.85323	4	4

The GLM Procedure

t Tests (LSD) for etol 120h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.001655
Critical Value of t	2.22814
Least Significant Difference	0.0405

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.34575	10	40
B	0.54882	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values

strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: etol 144h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	4.32868119	0.48096458	25.46	<.0001
Error	10	0.18891993	0.01889199		
Corrected Total	19	4.51760112			

R-Square	Coeff Var	Root MSE	etol Mean
0.958181	15.83586	0.137448	0.867955

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	0.15078445	0.03769611	2.00	0.1713
temp	1	3.80809166	3.80809166	201.57	<.0001
strain*temp	4	0.36980508	0.09245127	4.89	0.0190

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	0.15078445	0.03769611	2.00	0.1713
temp	1	3.80809166	3.80809166	201.57	<.0001
strain*temp	4	0.36980508	0.09245127	4.89	0.0190

The GLM Procedure

t Tests (LSD) for etol 144h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.018892
Critical Value of t	2.22814
Least Significant Difference	0.2166

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	0.98803	4	2
A			
B A	0.94863	4	1
B A			
B A	0.84475	4	3
B A			
B A	0.79450	4	5
B			
B	0.76387	4	4

The GLM Procedure

t Tests (LSD) for etol 144h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.018892
Critical Value of t	2.22814
Least Significant Difference	0.137

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.30431	10	40
B	0.43160	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: xtol 24h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	2.50074732	0.27786081	126.39	<.0001
Error	10	0.02198425	0.00219842		
Corrected Total	19	2.52273156			

R-Square	Coeff Var	Root MSE	xtol Mean
0.991286	9.342586	0.046887	0.501867

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	1.88113585	0.47028396	213.92	<.0001
temp	1	0.08779718	0.08779718	39.94	<.0001
strain*temp	4	0.53181429	0.13295357	60.48	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	1.88113585	0.47028396	213.92	<.0001
temp	1	0.08779718	0.08779718	39.94	<.0001
strain*temp	4	0.53181429	0.13295357	60.48	<.0001

The GLM Procedure

t Tests (LSD) for xtol 24h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.002198
Critical Value of t	2.22814
Least Significant Difference	0.0739

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	0.88762	4	4
B	0.71325	4	5
C	0.62237	4	3
D	0.19195	4	2
E	0.09415	4	1

The GLM Procedure

t Tests (LSD) for xtol 24h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.002198
Critical Value of t	2.22814
Least Significant Difference	0.0467

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp

A	0.56812	10	40
B	0.43561	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: xtol 96h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	39.94557099	4.43839678	24.65	<.0001
Error	10	1.80036613	0.18003661		
Corrected Total	19	41.74593712			

R-Square	Coeff Var	Root MSE	xtol Mean
0.956873	7.682790	0.424307	5.522827

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	27.19791035	6.79947759	37.77	<.0001
temp	1	10.40979863	10.40979863	57.82	<.0001
strain*temp	4	2.33786201	0.58446550	3.25	0.0596

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	27.19791035	6.79947759	37.77	<.0001
temp	1	10.40979863	10.40979863	57.82	<.0001

strain*temp	4	2.33786201	0.58446550	3.25	0.0596

The GLM Procedure

t Tests (LSD) for xtol 96h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.180037
Critical Value of t	2.22814
Least Significant Difference	0.6685

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	7.4615	4	2
B	5.9309	4	1
B			
B	5.5643	4	4
C	4.4313	4	5
C			
C	4.2262	4	3

The GLM Procedure

t Tests (LSD) for xtol 96h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.180037
Critical Value of t	2.22814
Least Significant Difference	0.4228

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	6.2443	10	40
B	4.8014	10	45

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: xtol 120h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	57.69219636	6.41024404	17.85	<.0001
Error	10	3.59103502	0.35910350		
Corrected Total	19	61.28323138			

R-Square	Coeff Var	Root MSE	xtol Mean
0.941403	11.63401	0.599252	5.150866

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	46.97351299	11.74337825	32.70	<.0001
temp	1	3.54442951	3.54442951	9.87	0.0105
strain*temp	4	7.17425386	1.79356347	4.99	0.0179

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	46.97351299	11.74337825	32.70	<.0001
temp	1	3.54442951	3.54442951	9.87	0.0105
strain*temp	4	7.17425386	1.79356347	4.99	0.0179

The GLM Procedure

t Tests (LSD) for xtol 120h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.359104
Critical Value of t	2.22814
Least Significant Difference	0.9441

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	7.4539	4	2
B	6.1194	4	1
B			
B	5.1789	4	4
C	3.6693	4	5
C			
C	3.3328	4	3

The GLM Procedure

t Tests (LSD) for xtol 120h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05

Error Degrees of Freedom	10
Error Mean Square	0.359104
Critical Value of t	2.22814
Least Significant Difference	0.5971

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	5.5718	10	45
B	4.7299	10	40

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	1 2 3 4 5
temp	2	40 45

Number of Observations Read	20
Number of Observations Used	20

The GLM Procedure

Dependent Variable: xtol 144h

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	104.4505136	11.6056126	35.21	<.0001
Error	10	3.2964458	0.3296446		
Corrected Total	19	107.7469594			

R-Square	Coeff Var	Root MSE	xtol Mean
0.969406	14.40023	0.574147	3.987066

Source	DF	Type I SS	Mean Square	F Value	Pr > F

strain	4	41.73284754	10.43321189	31.65 <.0001
temp	1	48.91399723	48.91399723	148.38 <.0001
strain*temp	4	13.80366881	3.45091720	10.47 0.0013

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	41.73284754	10.43321189	31.65	<.0001
temp	1	48.91399723	48.91399723	148.38	<.0001
strain*temp	4	13.80366881	3.45091720	10.47	0.0013

The GLM Procedure

t Tests (LSD) for xtol 144h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.329645
Critical Value of t	2.22814
Least Significant Difference	0.9046

Means with the same letter are not significantly different.

t Grouping	Mean	N	strain
A	6.1106	4	2
B	4.9454	4	1
B			
B	4.0588	4	4
C	2.5283	4	5
C			
C	2.2923	4	3

The GLM Procedure

t Tests (LSD) for xtol 144h

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.329645
Critical Value of t	2.22814
Least Significant Difference	0.5721

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	5.5509	10	45
B	2.4232	10	40

SAS program for SSF:

```
options ls=74 ps=60;
data ssf24;
infile "C:\Documents and Settings\michael.mueller\Desktop\SAS_files_SSF\ssf24.csv"
dlm=",";
input temp enz$ xtol etol;
cards;
run;
data ssf48;
infile "C:\Documents and Settings\michael.mueller\Desktop\SAS_files_SSF\ssf48.csv"
dlm=",";
input temp enz$ xtol etol;
cards;
run;
data ssf72;
infile "C:\Documents and Settings\michael.mueller\Desktop\SAS_files_SSF\ssf72.csv"
dlm=",";
input temp enz$ xtol etol;
cards;
run;
data ssf96;
infile "C:\Documents and Settings\michael.mueller\Desktop\SAS_files_SSF\ssf96.csv"
dlm=",";
input temp enz$ xtol etol;
```

```
cards;
run;
data ssf120;
infile "C:\Documents and Settings\michael.mueller\Desktop\SAS_files_SSF\ssf120.csv"
dlm=",";
input temp enz$ xtol etol;
cards;
run;
data ssf144;
infile "C:\Documents and Settings\michael.mueller\Desktop\SAS_files_SSF\ssf144.csv"
dlm=",";
input temp enz$ xtol etol;
cards;
run;
proc glm data=ssf24; class enz temp;
model etol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf24; class enz temp;
model xtol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf48; class enz temp;
model etol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf48; class enz temp;
model xtol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf72; class enz temp;
model etol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf72; class enz temp;
model xtol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf96; class enz temp;
model etol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf96; class enz temp;
model xtol = enz|temp;
means enz temp/lsd;
run;
```

```
proc glm data=ssf120; class enz temp;
model etol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf120; class enz temp;
model xtol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf144; class enz temp;
model etol = enz|temp;
means enz temp/lsd;
run;
proc glm data=ssf144; class enz temp;
model xtol = enz|temp;
means enz temp/lsd;
run;
```

SAS output for SSF:

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00520043	0.00104009	12.33	0.0041
Error	6	0.00050598	0.00008433		
Corrected Total	11	0.00570641			

R-Square	Coeff Var	Root MSE	etol Mean
0.911332	8.765155	0.009183	0.104768

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.00429277	0.00214638	25.45	0.0012
temp	1	0.00054116	0.00054116	6.42	0.0445
enz*temp	2	0.00036650	0.00018325	2.17	0.1950

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.00429277	0.00214638	25.45	0.0012
temp	1	0.00054116	0.00054116	6.42	0.0445
enz*temp	2	0.00036650	0.00018325	2.17	0.1950

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000084
Critical Value of t	2.44691
Least Significant Difference	0.0159

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.124523	4	300
A			
A	0.110508	4	200
B	0.079274	4	100

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000084
Critical Value of t	2.44691
Least Significant Difference	0.013

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.111484	6	40
B	0.098053	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.19963798	0.03992760	21.72	0.0009
Error	6	0.01103222	0.00183870		

Corrected Total 11 0.21067021

R-Square	Coeff Var	Root MSE	xtol Mean
0.947633	14.14740	0.042880	0.303095

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.07785583	0.03892791	21.17	0.0019
temp	1	0.03493225	0.03493225	19.00	0.0048
enz*temp	2	0.08684990	0.04342495	23.62	0.0014

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.07785583	0.03892791	21.17	0.0019
temp	1	0.03493225	0.03493225	19.00	0.0048
enz*temp	2	0.08684990	0.04342495	23.62	0.0014

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.001839
Critical Value of t	2.44691
Least Significant Difference	0.0742

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.40841	4	300
B	0.28804	4	200
C	0.21284	4	100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.001839
Critical Value of t	2.44691
Least Significant Difference	0.0606

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.35705	6	45
B	0.24914	6	40

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.01478962	0.00295792	6.72	0.0190
Error	6	0.00263932	0.00043989		

Corrected Total 11 0.01742894

R-Square	Coeff Var	Root MSE	etol Mean
0.848567	13.81099	0.020973	0.151861

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.00807552	0.00403776	9.18	0.0149
temp	1	0.00152349	0.00152349	3.46	0.1121
enz*temp	2	0.00519060	0.00259530	5.90	0.0383

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.00807552	0.00403776	9.18	0.0149
temp	1	0.00152349	0.00152349	3.46	0.1121
enz*temp	2	0.00519060	0.00259530	5.90	0.0383

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.00044
Critical Value of t	2.44691
Least Significant Difference	0.0363

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.18697	4	300
B	0.14353	4	200
B			
B	0.12508	4	100

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.00044
Critical Value of t	2.44691
Least Significant Difference	0.0296

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.16313	6	40
A			
A	0.14059	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.69922646	0.13984529	24.66	0.0006

Error	6	0.03403196	0.00567199
Corrected Total	11	0.73325843	

R-Square	Coeff Var	Root MSE	xtol Mean
0.953588	10.13134	0.075313	0.743363

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.42657454	0.21328727	37.60	0.0004
temp	1	0.04730050	0.04730050	8.34	0.0278
enz*temp	2	0.22535142	0.11267571	19.87	0.0023

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.42657454	0.21328727	37.60	0.0004
temp	1	0.04730050	0.04730050	8.34	0.0278
enz*temp	2	0.22535142	0.11267571	19.87	0.0023

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.005672
Critical Value of t	2.44691
Least Significant Difference	0.1303

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.96560	4	300
B	0.75983	4	200

C 0.50465 4 100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.005672
Critical Value of t	2.44691
Least Significant Difference	0.1064

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.80615	6	45
B	0.68058	6	40

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	5	0.41584080	0.08316816	8.61	0.0104
Error	6	0.05797210	0.00966202		
Corrected Total	11	0.47381290			

R-Square	Coeff Var	Root MSE	etol Mean
0.877648	22.76219	0.098296	0.431837

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.12794979	0.06397489	6.62	0.0303
temp	1	0.19443583	0.19443583	20.12	0.0042
enz*temp	2	0.09345519	0.04672759	4.84	0.0561

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.12794979	0.06397489	6.62	0.0303
temp	1	0.19443583	0.19443583	20.12	0.0042
enz*temp	2	0.09345519	0.04672759	4.84	0.0561

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.009662
Critical Value of t	2.44691
Least Significant Difference	0.1701

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.57787	4	300

B	0.35944	4	100
B			
B	0.35821	4	200

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.009662
Critical Value of t	2.44691
Least Significant Difference	0.1389

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.55913	6	40
B	0.30455	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: xtol

Sum of

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	5	5.51925504	1.10385101	54.27	<.0001
Error	6	0.12204969	0.02034162		
Corrected Total	11	5.64130473			

R-Square	Coeff Var	Root MSE	xtol Mean
0.978365	7.008730	0.142624	2.034948

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	4.07158049	2.03579024	100.08	<.0001
temp	1	0.96540720	0.96540720	47.46	0.0005
enz*temp	2	0.48226735	0.24113367	11.85	0.0082

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	4.07158049	2.03579024	100.08	<.0001
temp	1	0.96540720	0.96540720	47.46	0.0005
enz*temp	2	0.48226735	0.24113367	11.85	0.0082

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.020342
Critical Value of t	2.44691
Least Significant Difference	0.2468

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz

A	2.7285	4	300
B	2.0731	4	200
C	1.3032	4	100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.020342
Critical Value of t	2.44691
Least Significant Difference	0.2015

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	2.31859	6	40
B	1.75131	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.85294237	0.37058847	16.99	0.0017
Error	6	0.13090718	0.02181786		
Corrected Total	11	1.98384955			

R-Square	Coeff Var	Root MSE	etol Mean
0.934014	20.20809	0.147709	0.730938

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.13632709	0.06816354	3.12	0.1175
temp	1	1.44714484	1.44714484	66.33	0.0002
enz*temp	2	0.26947045	0.13473523	6.18	0.0350

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.13632709	0.06816354	3.12	0.1175
temp	1	1.44714484	1.44714484	66.33	0.0002
enz*temp	2	0.26947045	0.13473523	6.18	0.0350

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.021818
Critical Value of t	2.44691
Least Significant Difference	0.2556

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.8794	4	300
A			
A	0.6791	4	200
A			
A	0.6343	4	100

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.021818
Critical Value of t	2.44691
Least Significant Difference	0.2087

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.07821	6	40
B	0.38367	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	5.24617408	1.04923482	6.88	0.0180
Error	6	0.91543035	0.15257172		
Corrected Total	11	6.16160443			

R-Square	Coeff Var	Root MSE	xtol Mean
0.851430	16.08540	0.390604	2.428316

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	1.27845550	0.63922775	4.19	0.0726
temp	1	0.08282539	0.08282539	0.54	0.4890
enz*temp	2	3.88489318	1.94244659	12.73	0.0069

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	1.27845550	0.63922775	4.19	0.0726
temp	1	0.08282539	0.08282539	0.54	0.4890
enz*temp	2	3.88489318	1.94244659	12.73	0.0069

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.152572
Critical Value of t	2.44691
Least Significant Difference	0.6758

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	2.6987	4	200
A			
B A	2.6171	4	300
B			
B	1.9691	4	100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.152572
Critical Value of t	2.44691
Least Significant Difference	0.5518

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	2.5114	6	40
A			
A	2.3452	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	6.12761147	1.22552229	42.81	0.0001
Error	6	0.17177394	0.02862899		
Corrected Total	11	6.29938541			

R-Square	Coeff Var	Root MSE	etol Mean
0.972732	17.76762	0.169201	0.952300

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.13672322	0.06836161	2.39	0.1726
temp	1	5.61401300	5.61401300	196.10	<.0001
enz*temp	2	0.37687525	0.18843762	6.58	0.0307

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.13672322	0.06836161	2.39	0.1726
temp	1	5.61401300	5.61401300	196.10	<.0001
enz*temp	2	0.37687525	0.18843762	6.58	0.0307

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.028629
Critical Value of t	2.44691
Least Significant Difference	0.2928

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	1.0971	4	300
A			
A	0.9168	4	200
A			
A	0.8430	4	100

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.028629
Critical Value of t	2.44691
Least Significant Difference	0.239

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.63628	6	40
B	0.26832	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read 12

Number of Observations Used 12

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	11.66425089	2.33285018	5.25	0.0337
Error	6	2.66358939	0.44393157		
Corrected Total	11	14.32784028			

R-Square	Coeff Var	Root MSE	xtol Mean
0.814097	35.38109	0.666282	1.883158

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.28545596	0.14272798	0.32	0.7368
temp	1	4.18618690	4.18618690	9.43	0.0219
enz*temp	2	7.19260804	3.59630402	8.10	0.0197

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.28545596	0.14272798	0.32	0.7368
temp	1	4.18618690	4.18618690	9.43	0.0219
enz*temp	2	7.19260804	3.59630402	8.10	0.0197

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.443932
Critical Value of t	2.44691
Least Significant Difference	1.1528

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	2.1004	4	200
A			
A	1.7916	4	300
A			
A	1.7575	4	100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.443932
Critical Value of t	2.44691
Least Significant Difference	0.9413

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	2.4738	6	45
B	1.2925	6	40

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	5.54174920	1.10834984	75.69	<.0001
Error	6	0.08786295	0.01464382		
Corrected Total	11	5.62961215			

R-Square	Coeff Var	Root MSE	etol Mean
0.984393	15.69145	0.121012	0.771195

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.04060229	0.02030114	1.39	0.3199
temp	1	5.37852270	5.37852270	367.29	<.0001
enz*temp	2	0.12262422	0.06131211	4.19	0.0727

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.04060229	0.02030114	1.39	0.3199
temp	1	5.37852270	5.37852270	367.29	<.0001
enz*temp	2	0.12262422	0.06131211	4.19	0.0727

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.014644

Critical Value of t 2.44691
Least Significant Difference 0.2094

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.84767	4	200
A			
A	0.75921	4	100
A			
A	0.70671	4	300

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha 0.05
Error Degrees of Freedom 6
Error Mean Square 0.014644
Critical Value of t 2.44691
Least Significant Difference 0.171

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.44068	6	40
B	0.10171	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values
enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	18.16129648	3.63225930	26.72	0.0005
Error	6	0.81551134	0.13591856		
Corrected Total	11	18.97680782			

R-Square	Coeff Var	Root MSE	xtol Mean
0.957026	28.08527	0.368671	1.312686

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	2.19183294	1.09591647	8.06	0.0199
temp	1	13.26805291	13.26805291	97.62	<.0001
enz*temp	2	2.70141063	1.35070532	9.94	0.0125

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	2.19183294	1.09591647	8.06	0.0199
temp	1	13.26805291	13.26805291	97.62	<.0001
enz*temp	2	2.70141063	1.35070532	9.94	0.0125

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6

Error Mean Square 0.135919
Critical Value of t 2.44691
Least Significant Difference 0.6379

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	1.7542	4	300
A			
A	1.4494	4	200
B	0.7345	4	100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha 0.05
Error Degrees of Freedom 6
Error Mean Square 0.135919
Critical Value of t 2.44691
Least Significant Difference 0.5208

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	2.3642	6	45
B	0.2612	6	40

The GLM Procedure

Class Level Information

Class	Levels	Values

enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.57012951	0.51402590	6.93	0.0177
Error	6	0.44492017	0.07415336		
Corrected Total	11	3.01504968			

R-Square	Coeff Var	Root MSE	etol Mean
0.852434	68.02980	0.272311	0.400282

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	0.36714190	0.18357095	2.48	0.1645
temp	1	1.83440603	1.83440603	24.74	0.0025
enz*temp	2	0.36858158	0.18429079	2.49	0.1636

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	0.36714190	0.18357095	2.48	0.1645
temp	1	1.83440603	1.83440603	24.74	0.0025
enz*temp	2	0.36858158	0.18429079	2.49	0.1636

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.074153
Critical Value of t	2.44691
Least Significant Difference	0.4712

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	0.6098	4	200
A			
A	0.4094	4	100
A			
A	0.1817	4	300

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.074153
Critical Value of t	2.44691
Least Significant Difference	0.3847

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	0.7913	6	40
B	0.0093	6	45

The GLM Procedure

Class Level Information

Class	Levels	Values

enz	3	100 200 300
temp	2	40 45

Number of Observations Read	12
Number of Observations Used	12

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	19.71582903	3.94316581	145.01	<.0001
Error	6	0.16315584	0.02719264		
Corrected Total	11	19.87898487			

R-Square	Coeff Var	Root MSE	xtol Mean
0.991793	16.05269	0.164902	1.027254

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enz	2	4.27041480	2.13520740	78.52	<.0001
temp	1	11.33515610	11.33515610	416.85	<.0001
enz*temp	2	4.11025813	2.05512906	75.58	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enz	2	4.27041480	2.13520740	78.52	<.0001
temp	1	11.33515610	11.33515610	416.85	<.0001
enz*temp	2	4.11025813	2.05512906	75.58	<.0001

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.027193
Critical Value of t	2.44691
Least Significant Difference	0.2853

Means with the same letter are not significantly different.

t Grouping	Mean	N	enz
A	1.6888	4	300
B	1.1498	4	200
C	0.2431	4	100

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.027193
Critical Value of t	2.44691
Least Significant Difference	0.233

Means with the same letter are not significantly different.

t Grouping	Mean	N	temp
A	1.99916	6	45
B	0.05535	6	40

SAS program for co-culture fermentation:

```
options ls=74 ps=60;
data cocult 24;
```

```
infile "C:\Documents and
Settings\michael.mueller\Desktop\SAS_files_cocult\cocult24.csv" dlm=",";
input culture$ xyl xtol etol;
cards;
run;
data cocult48;
infile "C:\Documents and
Settings\michael.mueller\Desktop\SAS_files_cocult\cocult48.csv" dlm=",";
input culture$ xyl xtol etol;
cards;
run;
data cocult72;
infile "C:\Documents and
Settings\michael.mueller\Desktop\SAS_files_cocult\cocult72.csv" dlm=",";
input culture$ xyl xtol etol;
cards;
run;
data cocult96;
infile "C:\Documents and
Settings\michael.mueller\Desktop\SAS_files_cocult\cocult96.csv" dlm=",";
input culture$ xyl xtol etol;
cards;
run;
proc glm data=cocult24; class culture;
model etol = culture;
means culture /lsd;
run;
proc glm data=cocult24; class culture;
model xtol = culture;
means culture /lsd;
run;
run;
proc glm data=cocult48; class culture;
model etol = culture;
means culture /lsd;
run;
proc glm data=cocult48; class culture;
model xtol = culture;
means culture /lsd;
run;
proc glm data=cocult72; class culture;
model etol = culture;
means culture /lsd;
run;
proc glm data=cocult72; class culture;
model xtol = culture;
```

```
means culture /lsd;
run;
proc glm data=cocult96; class culture;
model etol = culture;
means culture /lsd;
run;
proc glm data=cocult96; class culture;
model xtol = culture;
means culture /lsd;
run;
```

SAS output for co-culture fermentation:

The GLM Procedure

Class Level Information

Class	Levels	Values
culture	3	IMB2 IMB2&Ps Pstipiti

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	272.2228022	136.1114011	382.36	<.0001
Error	6	2.1358680	0.3559780		
Corrected Total	8	274.3586702			

R-Square	Coeff Var	Root MSE	etol Mean
0.992215	7.812663	0.596639	7.636818

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culture	2	272.2228022	136.1114011	382.36	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culture	2	272.2228022	136.1114011	382.36	<.0001

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.355978
Critical Value of t	2.44691
Least Significant Difference	1.192

Means with the same letter are not significantly different.

t Grouping	Mean	N	culture
A	14.1512	3	Pstipiti
B	8.0597	3	IMB2&Ps
C	0.6996	3	IMB2

The GLM Procedure

Class Level Information

Class	Levels	Values
culture	3	IMB2 IMB2&Ps Pstipiti

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	95.60703588	47.80351794	227.76	<.0001
Error	6	1.25932429	0.20988738		
Corrected Total	8	96.86636017			

R-Square	Coeff Var	Root MSE	xtol Mean
0.986999	10.11360	0.458135	4.529886

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culture	2	95.60703588	47.80351794	227.76	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culture	2	95.60703588	47.80351794	227.76	<.0001

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.209887
Critical Value of t	2.44691
Least Significant Difference	0.9153

Means with the same letter are not significantly different.

t Grouping	Mean	N	culture
A	8.6464	3	IMB2

B	4.2675	3	IMB2&Ps
C	0.6757	3	Pstipiti

The GLM Procedure

Class Level Information

Class	Levels	Values
culture	3	IMB2 IMB2&Ps Pstipiti

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	345.6103997	172.8051998	157.22	<.0001
Error	6	6.5948396	1.0991399		
Corrected Total	8	352.2052393			

R-Square	Coeff Var	Root MSE	etol Mean
0.981276	12.07888	1.048399	8.679601

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culture	2	345.6103997	172.8051998	157.22	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culture	2	345.6103997	172.8051998	157.22	<.0001

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.09914
Critical Value of t	2.44691
Least Significant Difference	2.0946

Means with the same letter are not significantly different.

t Grouping	Mean	N	culture
A	16.2347	3	Pstipiti
B	8.7481	3	IMB2&Ps
C	1.0560	3	IMB2

The GLM Procedure

Class Level Information

Class	Levels	Values
culture	3	IMB2 IMB2&Ps Pstipiti

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	286.0994924	143.0497462	246.88	<.0001

Error	6	3.4766427	0.5794405
Corrected Total	8	289.5761351	

R-Square	Coeff Var	Root MSE	xtol Mean
0.987994	11.82572	0.761210	6.436903

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culture	2	286.0994924	143.0497462	246.88	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culture	2	286.0994924	143.0497462	246.88	<.0001

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.57944
Critical Value of t	2.44691
Least Significant Difference	1.5208

Means with the same letter are not significantly different.

t Grouping	Mean	N	culture
A	14.0925	3	IMB2
B	4.5398	3	IMB2&Ps
C	0.6785	3	Pstipiti

The GLM Procedure

Class Level Information

Class	Levels	Values
culture	3	IMB2 IMB2&Ps Pstipiti

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: etol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	304.9930023	152.4965011	3574.66	<.0001
Error	6	0.2559627	0.0426604		
Corrected Total	8	305.2489650			

R-Square	Coeff Var	Root MSE	etol Mean
0.999161	2.282682	0.206544	9.048305

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culture	2	304.9930023	152.4965011	3574.66	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culture	2	304.9930023	152.4965011	3574.66	<.0001

The GLM Procedure

t Tests (LSD) for etol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.04266
Critical Value of t	2.44691
Least Significant Difference	0.4127

Means with the same letter are not significantly different.

t Grouping	Mean	N	culture
A	15.6382	3	Pstipiti
B	10.0268	3	IMB2&Ps
C	1.4799	3	IMB2

The GLM Procedure

Class Level Information

Class	Levels	Values
culture	3	IMB2 IMB2&Ps Pstipiti

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: xtol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	557.0953229	278.5476614	471.14	<.0001
Error	6	3.5473385	0.5912231		
Corrected Total	8	560.6426614			

R-Square	Coeff Var	Root MSE	xtol Mean
0.993673	9.315515	0.768910	8.254083

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culture	2	557.0953229	278.5476614	471.14	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culture	2	557.0953229	278.5476614	471.14	<.0001

The GLM Procedure

t Tests (LSD) for xtol

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.591223
Critical Value of t	2.44691
Least Significant Difference	1.5362

Means with the same letter are not significantly different.

t Grouping	Mean	N	culture
A	19.0953	3	IMB2
B	5.0015	3	IMB2&Ps
C	0.6655	3	Pstipiti

VITA

Michael Mueller

Candidate for the Degree of

Master of Science

Thesis: FERMENTATION OF XYLAN AND XYLANS BY KLUYVEROMYCES MARXIANUS IMB STRAINS

Major Field: Biosystems Engineering

Biographical:

Personal Data: Born in Dernbach, Germany, on January 30, 1983
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Experience:
Internship at Königsbacher Brewery GmbH & Co KG, Koblenz, Germany, 1st to 26th July 2007.
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Title of Study: FERMENTATION OF XYLOSE AND XYLANS BY KLUYVEROMYCES MARXIANUS IMB STRAINS

Pages in Study: 155 Candidate for the Degree of Master of Science

Major Field: Biosystems Engineering

Scope and Method of Study: The first objective was to analyze five *Kluyveromyces marxianus* IMB strains on their ability to ferment xylose as sole carbon source at thermophilic conditions. Based on the findings a Simultaneous saccharification and fermentation (SSF) with IMB2, which performed the best in the first step, and Multifect Xylanase was performed at thermophilic conditions with xylan as substrate. Different enzyme concentrations were compared. To enhance ethanol production on xylose a co-culture fermentation with IMB2 and *Pichia stipitis* was conducted. Since *P. stipitis* has an optimum temperature at 30°C, the fermentation was performed at 30°C.

Findings and Conclusions: The screening of the IMB strains showed that IMB2 performed the best at 40°C on xylose fermentation with an ethanol yield of 0.07g/g and a xylitol yield of 0.42g/g. IMB2 showed in the SSF experiment the best ethanol production at 40°C with 18.6% of the theoretical maximum yield with no significant difference in enzyme concentration. Xylitol production was best at 40°C with 15.8% of the theoretical maximum yield and 2µl/ml enzyme concentration. The monoculture of IMB2 showed poor results in ethanol production at the co-culture conditions. The co-culture showed better results in ethanol production which was derived by *P. stipitis*. *P. stipitis* as monoculture had the highest conversion efficiency with 84%. An interesting finding was the high xylitol production by IMB2 as monoculture. The conversion efficiency was 65% and is comparable to other in the literature mentioned yeast strains. It can be concluded that the *K. marxianus* IMB strains are not suitable for ethanol production at thermophilic conditions with xylose as sole carbon source. *K. marxianus* was found as a potential strains for xylitol production.

ADVISER'S APPROVAL: Dr. Mark R. Wilkins