AN ABSTRACT OF THE THESIS OF

Ragothaman Avanasi Narasimhan for the degree of Master of Science in Biological and Ecological Engineering presented on November 18, 2010.

Title: Hemicellulose Utilization by Yeast for Ethanol Production- Adaptation, Effect of Inhibitors and a Flux Balance Based Analysis.

Abstract approved:_____

Ganti S. Murthy

A major challenge in ethanol production using lignocellulosic feed stock is inefficient utilization of hemicellulose, which accounts for 30-40% of lignocellulosic biomass. Xylose, comprising >60% of recoverable sugars from hemicellulose is a major product of the hemicellulose hydrolysis. Utilization of this carbon source would significantly increase the ethanol yield from an estimated 60 gal/dry ton to 90 gal/dry ton. While xylose is not consumed and fermented efficiently by industrial yeast *Saccharomyces cerevisiae*, xylulose can be fermented by it. Isomerizing xylose to xylulose can provide an alternative to genetic modification of yeasts for xylose utilization.

A method to isomerize xylose to xylulose utilizing commercially available xylose isomerase was used. A synthetic media replicating hemicellulose in composition was designed. Two yeast strains with (*Schizosaccharomyces pombe*) and without (*Saccharomyces cerevisiae*) specialized xylose transport capability were grown in the defined media. The two strains were adapted to the hemicellulose environment by growing them in chemostat for 40 days (1000hrs). The batch fermentations of the adapted and original yeast strains were compared. High pressure liquid chromatography was used to measure substrates and products of fermentation. Results indicate that the unadapted *Saccharomyces cerevisiae* performed better than the other three strains with respect to xylulose utilization and ethanol yield. A total of 53.1% of xylulose was utilized by the strain by the end of 120 hrs of fermentation producing 0.41 Cmoles of ethanol/ Cmole of total sugar.

Furfural is an important inhibitor formed in the pretreatment process of lignocellulosic ethanol production. Furfural, mainly formed from xylose in the hemicellulose is inhibitory to the fermentation by yeasts. Effect of furfural concentration on the fermentation of hemicellulose hydrolyzate was studied in a synthetic media using the strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* which were adapted to the hemicellulose like environment. Two different concentrations of furfural (0.5 and 2 g/L) were used in the medium. Also the effect of high inoculum on the fermentation of xylulose was verified by batch experiments with high initial inoculums of the original *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* strains. Performances of different strains under different conditions were compared in

terms of xylulose utilization in batch growth. In *Saccharomyces cerevisiae* fermentations, an intermediate concentration of 0.5 g/L of furfural had highest ethanol yield of 0.375Cmoles/Cmoles of total sugar and lowest glycerol yield of 0.041Cmoles/Cmoles of total sugar. In *Schizosaccharomyces pombe*, absence of any furfural in the medium had highest xylulose utilization of 51.84% over 120 hrs. Higher inoculum concentrations did not improve batch xylulose utilization in both the yeast strains but the utilization rates were improved.

A constraint based metabolic model of *Saccharomyces cerevisiae* metabolism was used to perform a flux balance based analysis of xylulose and furfural metabolism. The model outputs were comparable to the experimental production rates of products. Under the experimental conditions, the model gives an increase of 1.036 mmol/hr. g biomass of ethanol production by the consumption of xylulose. The model output rates of products suggested that, to detoxify furfural under normal metabolic functioning would reduce the specific growth rate to 0.00043 g/hr. g of biomass from 0.048 g/hr. g of biomass.

Hemicellulose Utilization by Yeast for Ethanol Production- Adaptation, Effect of Inhibitors and a Flux Balance Based Analysis

by

Ragothaman Avanasi Narasimhan

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APPROVED:

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Major Professor, representing Biological and Ecological Engineering

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Head of the Department of Biological and Ecological Engineering

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Ragothaman Avanasi Narasimhan, Author

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CONTRIBUTION OF AUTHORS

Dr. Ganti S. Murthy assisted in the concept and implementation of the whole thesis research. Christopher Beatty played important role in idea and implementation behind chapter 2. Dr.Frank W. R. Chaplen assisted in the concept, methodology and implementation in the chapter 3.

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DEDICATION

To my parents and my brother

Hemicellulose Utilization by Yeast for Ethanol Production- Adaptation, Effect of Inhibitors and a Flux Balance Based Analysis

Chapter 1

General Introduction

Cellulosic ethanol as a sustainable fuel alternative

Bio-fuel produced from biomass is increasingly being favored over usage of fossil derived fuel as it is an environmentally friendly alternative (Bai et al., 2010, Lynd 1996, Singh et al., 2010). Ethanol production from cellulose rich biomass is considered to be the second generation bio-ethanol. The main advantage of cellulosic ethanol is that it does not compete with food for feedstock, as is the case with ethanol from corn (First generation bio-ethanol) (Hamelinck et al., 2005, Lynd et al., 1991, Wyman, 2007). Cellulosic ethanol has potential to reduce green house gas emissions (produced due to combustion of fuel) as the amount of CO_2 that is returned upon combustion of ethanol and process residues is the same as the photosynthetically fixed CO_2. Also, the emissions of other green house gases such as methane, carbon monoxide are reduced when compared to the use of petroleum for the transportation sector (Lynd, 1996). A variety of biomass sources can be used in the process of converting cellulose to ethanol including forest derived biomass and agriculture derived biomass (Galbe and Zacchi, 2002).

Process steps involved in cellulosic ethanol production

The main steps in cellulosic ethanol production (Fig 1.1) include pretreatment of biomass, hydrolysis of cellulose, fermentation of hexose and pentose sugars to ethanol, recovery of ethanol by distillation and use of co-product lignin in combustion for power generation (Cardona and Sanchez, 2007). Pretreatment is an important step in the cellulosic ethanol process (Wooley et al., 1999, Wyman et al., 2005, Yang and Wyman, 2008). The main purpose of the pretreatment step is to remove hemicellulose and lignin, reduce the crystallinity of cellulose (Sun and Cheng, 2002).Many different pretreatment technologies are used for pretreatment including dilute acid catalyzed pretreatment, hot water catalyzed pretreatment, ammonia explosion and alkali catalyzed pretreatment (Wyman et al., 2005). Inhibitor (inhibitors of further processing of biomass to ethanol) formation is a result of the pretreatment process. The pretreated biomass is separated into solids and liquid fractions and most of the hemicelluloses component of the biomass is in the liquid fraction. Pretreatment is followed by enzymatic/acid hydrolysis of the cellulose fibers to its monomer glucose for fermentation (Philippidis et al., 1993). Resulting hexose and pentoses are fermented by microorganisms to ethanol. While industrial yeast *Saccharomyces cerevisiae*, is mainly used for ethanol fermentation, other yeasts and bacteria can also be used for the fermentation of the available sugars to ethanol. Ethanol is recovered by distillation and lignin co-product is usable for power generation by combustion.

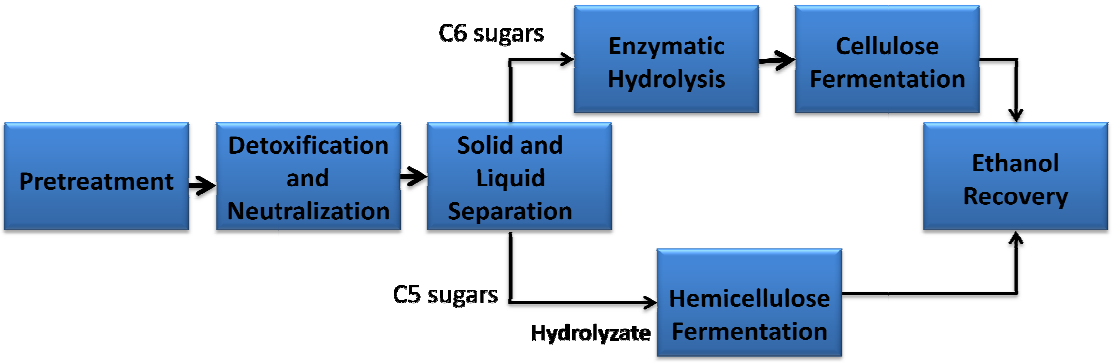

Fig 1.1.Process steps in cellulosic ethanol production

Challenges in lignocellulosic ethanol production

Many challenges are to be met in order to have cellulosic ethanol as a main source of fuel (Hagerdal et al., 2006, Wyman, 2003, Lynd, 1996, Zaldivar et al., 2001). One of the main challenges in the production of ethanol from cellulosic biomass is the utilization of pentose sugars which are the main components of the hemicellulose component of biomass (Bertilsson et al., 2008, Jefferies et al., 2003, Chandrakant and Bisaria, 2000). The hemicellulose comprises 30-40% of the biomass depending on the type of biomass. Xylose, the chief pentose sugar, accounts for almost 60% of the components in hemicellulose part of the biomass. Xylose is not efficiently utilized for fermentation by many of the microorganisms used for fermentative ethanol production (Gardonyi et al., 2003, Hamacher et al., 2002, Bertilsson et al., 2008). Some naturally occurring yeast strains like *Pichia stipitis* and *Candida shehatae* have the capability of utilizing xylose but are not suitable for industrial production of ethanol due to low yields or low tolerance to ethanol. Also, inhibitors formed during pretreatment methods such as dilute acid pretreatment pose an important challenge for developing a

sustainable cellulosic ethanol production process (Palmqvist et al., 2000a, 2000b). Development of pentose utilizing, ethanol producing strains with high tolerance to ethanol is required for efficient utilization of xylose.

Utilization of xylose in yeast

While utilization of xylose in yeast primarily occurs in the pentose phosphate pathway, xylose is transported into the cells by two different mechanisms in yeasts. In xylose consuming yeasts such as *Schizosaccharomyces pombe*, *Pichia stipitis* and *Candida sp.,* xylose is transported by Proton symport (PS) (Fig 1.2), while in other yeasts such as *Saccharomyces cerevisiae*, a facilitated diffusion system (FDS) (Fig 1.3) is present (Lucas and van Uden, 1986, Lastick et al., 1989, Kotter and Ciriacy, 1993). The PS system is a high affinity transport system which is highly specific to xylose transport. The FDS is a low affinity system used to transport both hexoses and pentoses. Transport of xylose by FDS is prevented in the presence of even trace amounts of hexose. Metabolism of xylose in yeasts involves conversion of xylose to xylitol and then to xylulose. The xylulose is then phosphorylated and enters the pentose phosphate pathway.

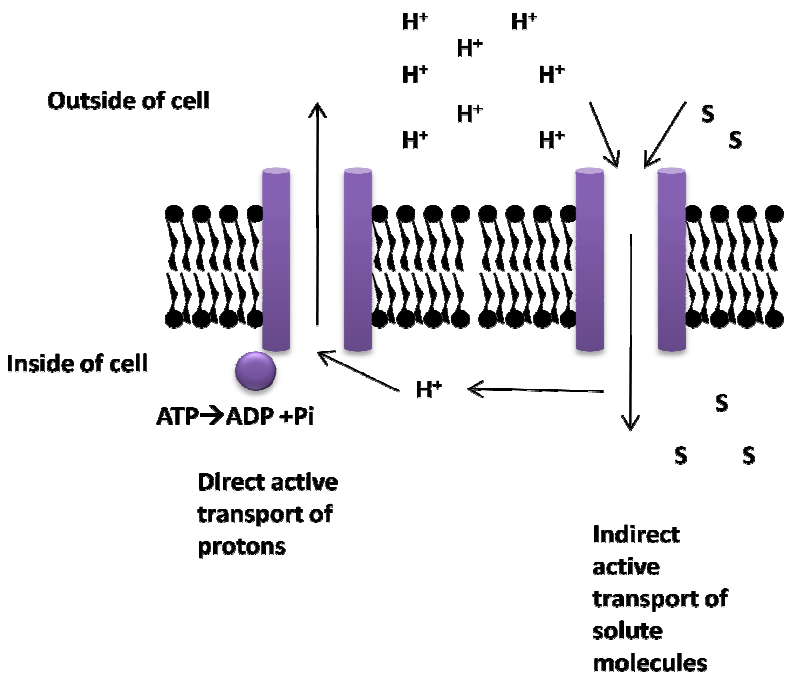

Fig 1.2. Proton symport

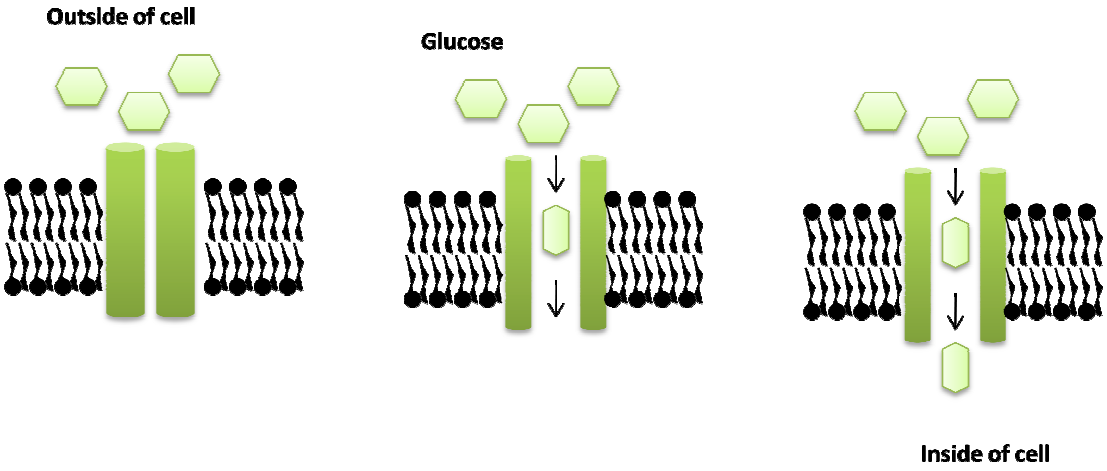

Fig 1.2.Facilitated Diffusion System

In yeasts, the conversion of xylose to xylitol is catalyzed by xylose reductase (XR). This reaction uses NADH or NADPH as a cofactor depending on the availability of

oxygen. Most of the time under anaerobic/ micro aerobic conditions, NADH is the cofactor for this reaction. Conversion of xylitol to xylulose is catalyzed by xylitol dehydrogenase (XD). In most yeast, xylose is readily converted to xylitol but is not further converted to xylulose due to redox imbalance (Kotter and Ciriacy, 1993).In addition to this limitation, other challenges have been identified in xylulose utilization for ethanol production due to competition for intermediates glyceraldehyde 3-phosphate and fructose 6-phosphate between the glycolysis and pentose phosphate pathways (Jeffries et al., 2004) (Fig 1.4). Overexpression of xylose reductase and xylitol dehydrogenase have been tried to increase utilization of xylose for ethanol production (Kotter and Ciriacy, 1993). Also, overexpression of xylulokinase (Ho et al., 1999, Lee at al., 2003) and other genes of the pentose phosphate pathway have been used (Eliasson et al., 2000).

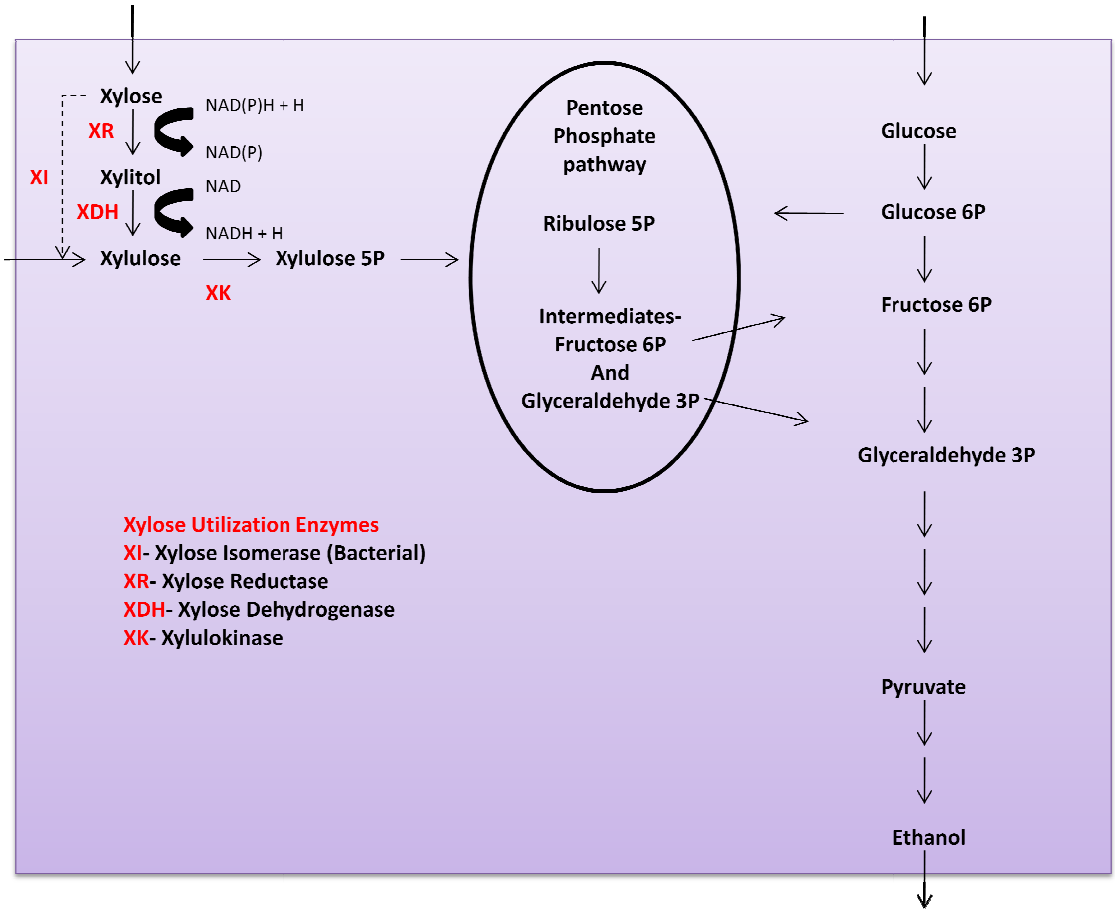

Fig 1.4.Metabolic pathways for pentose utilization

Other alternative strategies to utilize xylose

Bacteria have xylose isomerase gene encoding for the enzyme xylose isomerase, which converts the xylose to xylulose in a single step. Expression of this xylose isomerase has been tried in yeasts (Kuyper et al., 2005a). Chandrakant and Bisaria (2000) have successfully carried out simultaneous isomerization of xylose in the culture medium using compatible xylose isomerase and co-fermentation of xylose with glucose. Kuyper et al. (2005b) have showed improved xylose utilization by

evolutionary engineering of yeast strains by adapting them to the desired environmental conditions.

Direction of this research

In this research, we tried to adapt yeast strains *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* having specific and non-specific xylose transporters to better utilize xylulose in the presence of glucose (Fig 1.5). Development of such strains would result in a single step for fermentation of hexose and pentose sugars, thus reducing cost and improving efficiency of the process step. Also, another aim of the adaptation was to reduce xylitol production. This would prevent loss of sugar as a by-product directing more sugar towards ethanol production. The xylose was isomerized *in vitro* using commercially available xylose isomerase. Also the effect of inhibitors on the fermentation by the strains was studied. A constraint based model of the metabolism of the yeast *Saccharomyces cerevisiae* was used and experimental data was used to perform a flux balance based analysis.

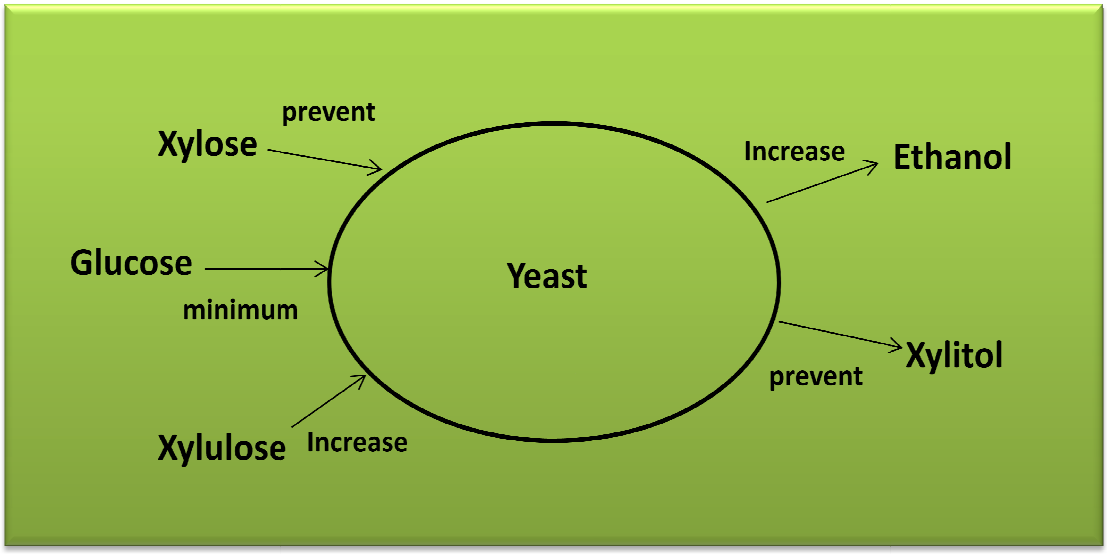

Fig 1.5.Goals of adaptation in hemicellulose media

This document covers the work performed aimed towards better utilization of hemicellulose component of the energy rich lignocellulosic biomass for ethanol production. Chapter 2 details the strategies, methods and the results of adaptation of yeast strains. Chapter 3 describes the basic principles and methodologies that were used to perform a constraint based analysis of the *Saccharomyces cerevisiae* metabolism. The results suggest a good agreement between the experiment and the model simulations encouraging further utilization of the model tools for other analyses. In chapter 4 the results of the study of the effect of furfural inhibitor and a high inoculum: sugar ratios on the fermentation process are presented. Finally, a comparison of the different fermentation experiments is performed.

Chapter 2

Hemicellulose fermentation by industrial yeast *Saccharomyces cerevisiae*

Presented at the

2010 ASABE Annual International Meeting

Paper Number: 1009508 (Avanasi and Murthy, 2010)

Abstract

A major challenge in ethanol production using lignocellulosic feed stock is inefficient utilization of hemicellulose, which accounts for 30-40% of lignocellulosic biomass. Xylose, comprising >60% of recoverable sugars from hemicellulose is a major product of hemicellulose hydrolysis. Utilization of this carbon source would significantly increase the ethanol yield from an estimated 60 gal/dry ton to 90 gal/dry ton. While xylose is not consumed and fermented efficiently by industrial yeast *Saccharomyces cerevisiae*, xylulose can be fermented by it. Isomerizing xylose to xylulose can provide an alternative to genetic modification of yeasts for xylose utilization. The objective of this research was to increase xylulose utilization during the fermentation process.

A method to isomerize xylose to xylulose utilizing commercially available xylose isomerase was used. A synthetic media replicating hemicellulose in composition was designed. Two yeast strains with (*Schizosaccharomyces pombe*) and without (*Saccharomyces cerevisiae*) specialized xylose transport capability were grown in the defined media. The two strains were adapted to the hemicellulose environment by growing them in chemostat for 40 days (1000hrs). The batch fermentations of the adapted and original yeast strains were compared. High pressure liquid chromatography was used to measure substrates and products of fermentation. Results indicate that the unadapted *Saccharomyces cerevisiae* performed better than the other three strains with respect to xylulose utilization and ethanol yield. A total of 53.1% of

xylulose was utilized by the strain by the end of 120 hrs of fermentation producing 0.41 Cmoles of ethanol/ Cmole of total sugar.

Keywords. Lignocellulosic ethanol, hemicellulose, xylulose utilization, *Saccharomyces cerevisiae*, chemostat adaptation, high pressure liquid chromatography

Introduction

A major challenge in ethanol production using lignocellulosic feed stocks is inefficient utilization of hemicellulose component, which accounts for 30-40% of lignocellulosic biomass (Table2.1). Hemicellulose is mainly comprised of pentose sugars (Sun and Cheng, 2005). Xylose comprises more than 60% of the recoverable sugars derived from hemicelluloses. So utilization of this carbon source would significantly increase the ethanol yield (Bertilsson et al., 2008, Jefferies et al., 2003, Chandrakant and Bisaria, 2000). Xylose is the major pentose sugar obtained upon hydrolysis of hemicellulose, and its bioconversion presents a biochemical challenge especially when present with glucose (Chandrakant and Bisaria, 2000).

Table2.1 Composition of feedstocks (BFIN Bio-energy Feedstock Characteristics)

Bio-energy feedstocks / composition	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn Stover	35	28	16-21
Sweet sorghum	27	25	11
Sugarcane bagasse	32-48	19-24	23-32
Hardwood	45	30	20
Softwood	42	21	26
Hybrid poplar	42-56	18-25	21-23
Bamboo	41-49	24-28	24-26
Switch grass	44-51	42-50	13-20
Miscanthus	44	24	17

Many researchers have focused on utilization of xylose as potentially the ethanol yield increases from 60 gal/dry ton to 90 gal/dry ton on complete hemicellulose utilization (Gardonyi et al., 2003, Hamacher et al., 2002, Bertilsson et al., 2008). Utilization of xylose by yeasts is dependent on xylose transport systems. Two types of transport systems are present in yeasts for xylose transport; proton symport (PS) and facilitated diffusion system (FDS) (Lucas and van Uden, 1986, Lastick et al., 1989, Kotter and Ciriacy, 1993). Facilitated diffusion system is a low affinity system for transporting glucose and xylose. *Saccharomyces cerevisiae* has a common FDS for xylose transport and glucose transport and it favors glucose transport over xylose transport in presence of even small amounts of glucose (near zero concentration). The PS system on the other hand is specific for xylose uptake and is found in yeasts such as *Schizosaccharomyces pombe*. *Saccharomyces cerevisiae* lacks a PS system for xylose

transport. This property can be utilized to prevent uptake of xylose by *Saccharomyces cerevisiae*.

In yeasts, D-xylose is reduced to xylitol and xylitol is then oxidized to xylulose. Xylulose enters the pentose phosphate pathway of catabolism. But most yeasts convert xylose readily to xylitol but not further to xylulose due to redox imbalance. Bacteria have an enzyme xylose isomerase to convert xylose directly to xylulose. Isomerization of the available xylose to xylulose using commercially produced xylose isomerase *in vitro* could be one of the approaches to increase conversion of xylose to xylulose in yeasts (Chiang et al., 1981, Chandrakant and Bisaria, 2000). The resulting xylulose can then be utilized by the yeast. However since xylitol is a repressor of xylose isomerase, xylitol production has to be reduced by repressing xylose uptake for optimal utilization of xylulose. Simultaneous isomerization and co-fermentation (SICF) of a glucose/xylose mixture by *Saccharomyces cerevisiae* utilizing xylose isomerase was reported by Chandrakant and Bisaria (2000).

Xylulose fermentation is slow when compared to glucose fermentation (Chiang et al., 1981, Eliasson et al., 2000, Jeppsson et al., 1996). Reason for the slower fermentation with xylulose is that glycolytic pathway competes with pentose phosphate pathway for an intermediate glyceraldehyde-3-phosphate during co-utilization of glucose and xylulose (Eliasson et al., 2000). Adaptation of yeasts to specific environmental conditions has been tried by researchers to improve substrate utilization (Kuyper et al., 2005b). Adaptation refers to the process of directed evolution in which

microorganisms are grown in defined environmental conditions and are allowed to evolve to best suit the environment.

The yeast *Saccharomyces cerevisiae* (Fig.2.1) is preferred for ethanol production from lignocellulose hydrolyzates because of its successful exploitation in the fermentation industry, proven ability to produce high ethanol concentrations rapidly and high-level resistance to inhibitors found in lignocellulose hydrolyzates (Jeppsson and Hagerdal, 1996, Kuyper et al., 2005b). *Schizosaccharomyces pombe* (Fig.2.1) is fission yeast which also has potential to be used for lignocellulosic ethanol production.

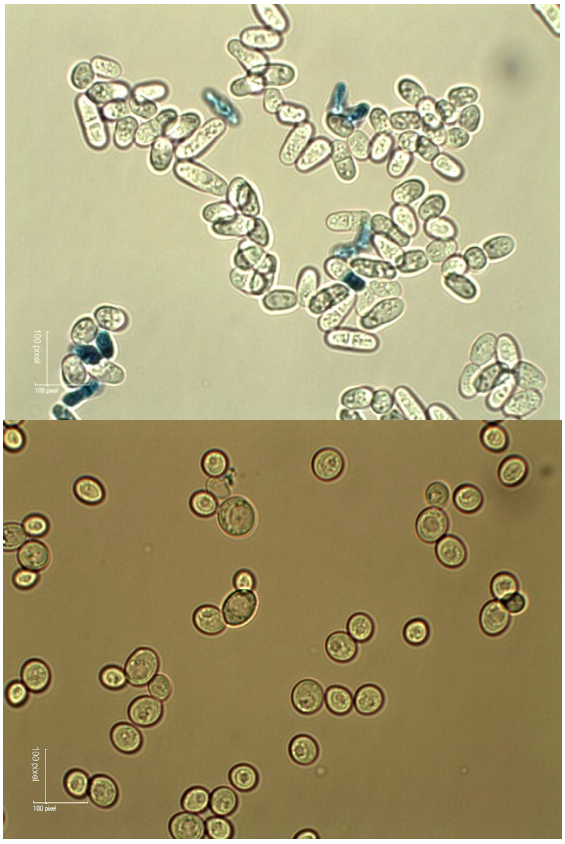

Figure 2.1.*Schizosaccharomyces pombe* (top) and *Saccharomyces cerevisiae* (bottom)

In this research our aims were to adapt the yeasts *Saccharomyces cerevisiae* (Red star) and *Schizosaccharomyces pombe* (ATCC 2476) to improve xylulose utilization by growing them in an environment similar to hemicellulose hydrolyzate (after isomerization) for 1000hrs (approximately 500 generations), prevent utilization of xylose, prevent formation of xylitol and increase ethanol yield (Fig 1.5).

Materials and Methods

Hemicellulose media design and components

A synthetic media similar in composition to hemicellulose hydrolyzate was designed (Table 2.2). The composition of hemicellulose media was based on the study by Sun and Cheng (2005). Urea was used as a buffer to maintain the pH and nitrogen source. Tween 80 was supplemented as a source of oleic acid. Oleic acid is responsible for ethanol tolerance and is not synthesized under anoxic conditions (You et al., 2003, Domberk et al., 1986). Ergosterol and fatty acids are not synthesized by *Saccharomyces cerevisiae* in absence of oxygen. Lipid supplementation has improved fermentation (Murthy et al., 2006). Yeast extract is supplied as a source of vitamins (Belviso et al., 2004). GENSWEET SGI (Genencor, Palo Alto, CA) was used for the process of isomerization. The enzyme requires 36 ppm of magnesium ion as a cofactor for optimal activity. Additionally, it has been found that boron helps in shifting the equilibrium towards xylulose. Boron was supplied in the form of Sodium Borate.

The medium was prepared, autoclaved, isomerized and used for culturing. The medium components xylose, GENSWEET SGI, tetracycline, magnesium sulphate and

tween 80 were added after autoclaving by filter sterilization to prevent caramelization and denaturation of the components.

Table 2.2. Hemicellulose media components

Component	Concentration (g/L)
Xylose	22
Glucose	0.5
Urea	1.5
Tween80	0.4
Yeast Extract	1.5
Sodium Borate	2
Magnesium Sulphate	0.6
Tetracycline	10 e-3
Gensweet SGI	3

Isomerization of xylose to xylulose

The enzyme glucose isomerase GENSWEET SGI was used for isomerization of xylose to xylulose. The optimal conditions for the activity of the enzyme are a temperature of 60°C and pH of 7.5. Isomerisation was carried out for seven hrs at the above conditions.

Chemostat adaptation

BiostatM (B.Braun) laboratory fermenters with a working volume of 750mL were used for adaptation. Peristaltic pumps were used for inflow and outflow. An initial batch growth was allowed till the culture reached stationery phase (216 hrs for *S.cerevisiae* and 192 hrs for *S.pombe*) and was followed by chemostat growth. A dilution rate of 0.08 hr^{-1} (1 mL/min flow rate) was used to maintain the yeast strains at steady state. Isomerized hemicellulose media was used as the feed. Adaptation was carried out for 40 days and the pH was controlled at 5.0 (adjusted using 1N HCl and

1N NaOH) and a temperature of 30°C was maintained throughout. Agitation was maintained at 300 rpm. The adapted cells were preserved in glycerol stocks (15% glycerol) at -80°C for further fermentation studies. Care was taken to prevent contamination by sterilizing the fermenters and by using hydrogen peroxide to disinfect any exposed surfaces. Samples were taken each day and preserved. Optical density was measured and correlated to cell concentration. Cell counts were done using a haemocytometer and an optical microscope.

Batch fermentation studies in simulated hydrolyzate

The adapted and original cultures were grown in isomerized hemicellulose medium with high sugar concentrations (glucose 45g/L and xylose 45g/L). The medium was bubbled with nitrogen gas to remove dissolved oxygen. The experiments were performed in batch mode in shake flasks of 225mL volume. An initial inoculum level of 0.5g/L was used. Fermentation was carried out at 30°C and pH 5 with an agitation of 100rpm for 120 hrs. Samples and weight loss measurements were taken at 0, 4, 6, 9, 12, 24, 48, 72, 96 and120 hrs. Samples were filtered through a 0.2µm membrane filter to prevent cell growth during storage and handling. The samples were stored at 4°C until used for HPLC analysis. All experiments were performed in triplicates.

HPLC

HPLC analyses were done using the Agilent 1200 series HPLC system. Aminex HPX-87H (Bio-Rad, Hercules, CA) column was used for analyzing the samples. Glucose, xylose, xylulose, glycerol, xylitol and ethanol were detected using the refractive index

detector (Model G1362A). Each sample was filtered using 0.2 µm filters and an injection volume of 20 µl was used per sample. The column was maintained at 65°C and each sample was run for 30 minutes using $0.005MH_2SO_4$ as the mobile phase. The data was processed using the Chemstation software package.

Results

The isomerization process was carried out at the specified conditions over a period of 10 hrs. Results indicate that isomerization was quick to reach equilibrium and there was little increase in xylulose after 7hrs (Fig.2.2). All isomerization were carried out for seven hours.

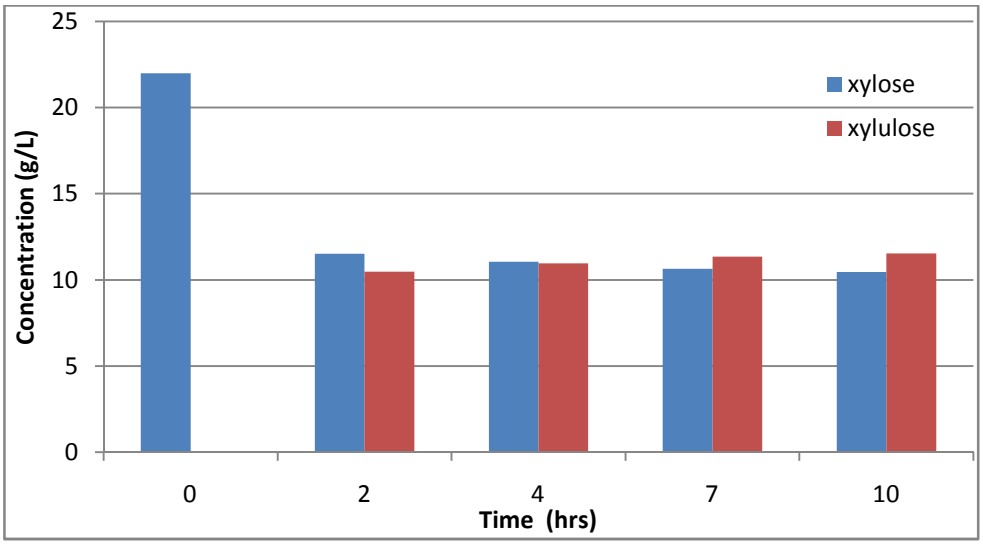

Figure 2.2.Isomerization of xylose to xylulose

Chemostat growth curves show initial batch growth followed by a long stationary phase representing the long steady state phase of growth (Fig.2.3). Batch fermentation

results by the adapted and unadapted strains show consumption of glucose, xylulose, production of ethanol, glycerol and xylitol (Fig.2.4).

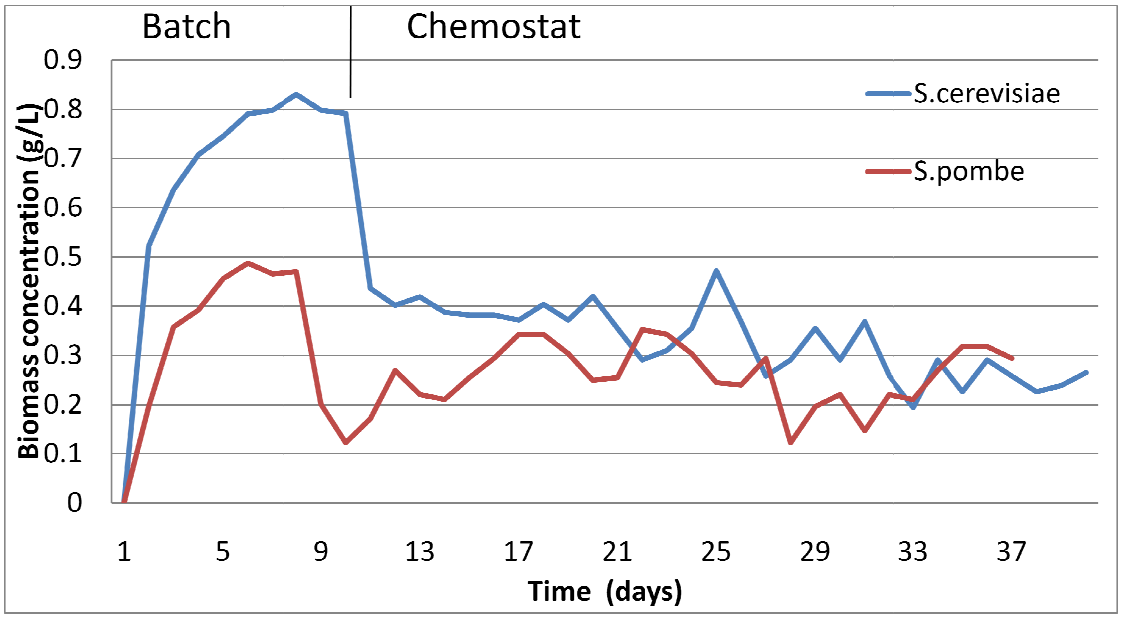

Figure 2.3. Chemostat growth curves

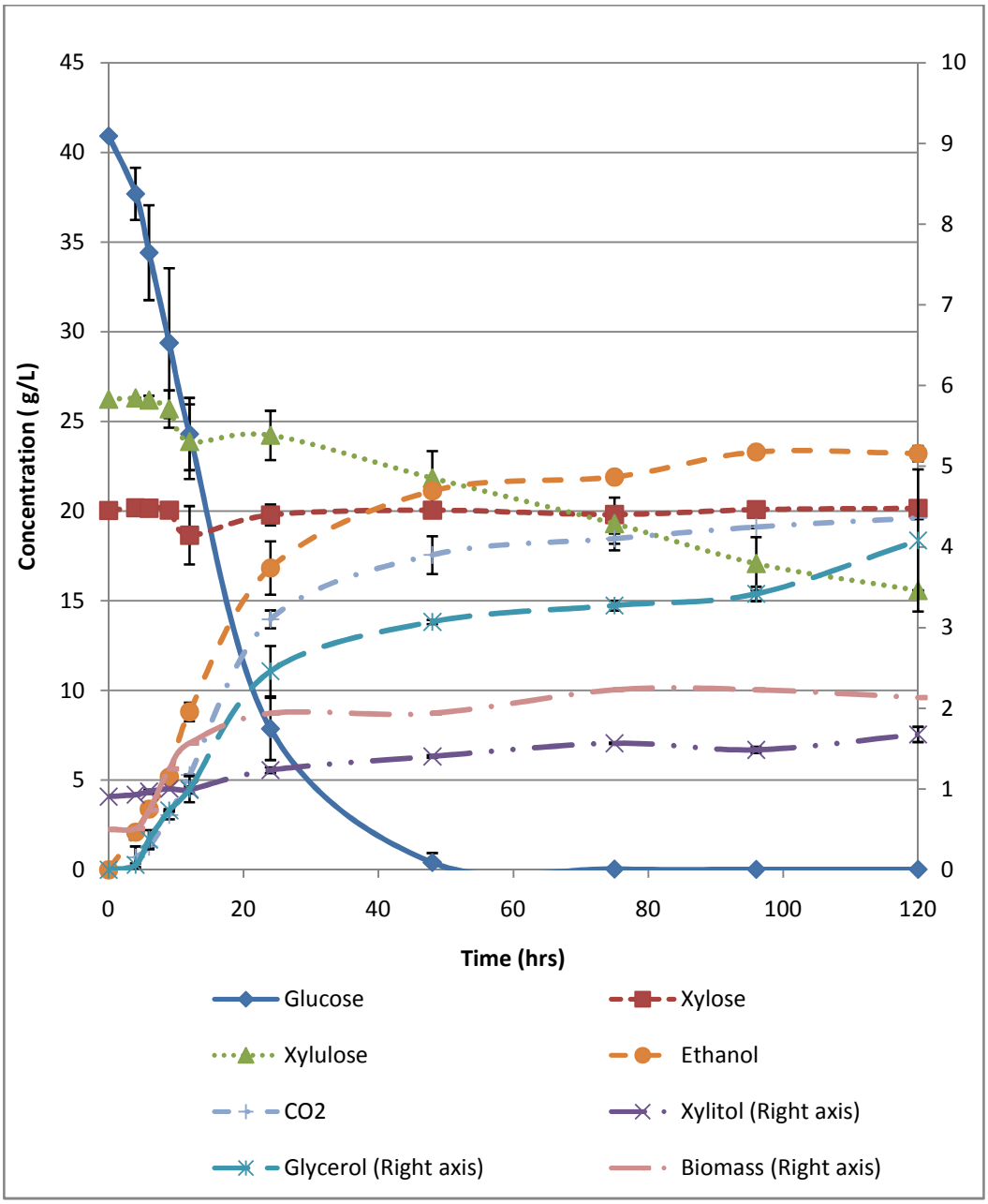

Figure 2.4. Batch fermentation by adapted *S.cerevisiae*

Xylose levels remain constant over the period of time and weight loss measurements account for the release of CO_2 during fermentation (Smits et al., 1996) (Fig.2.5). The

sum of carbon moles of all reactants and products remain constant throughout the fermentation period indicating mass balance closure.

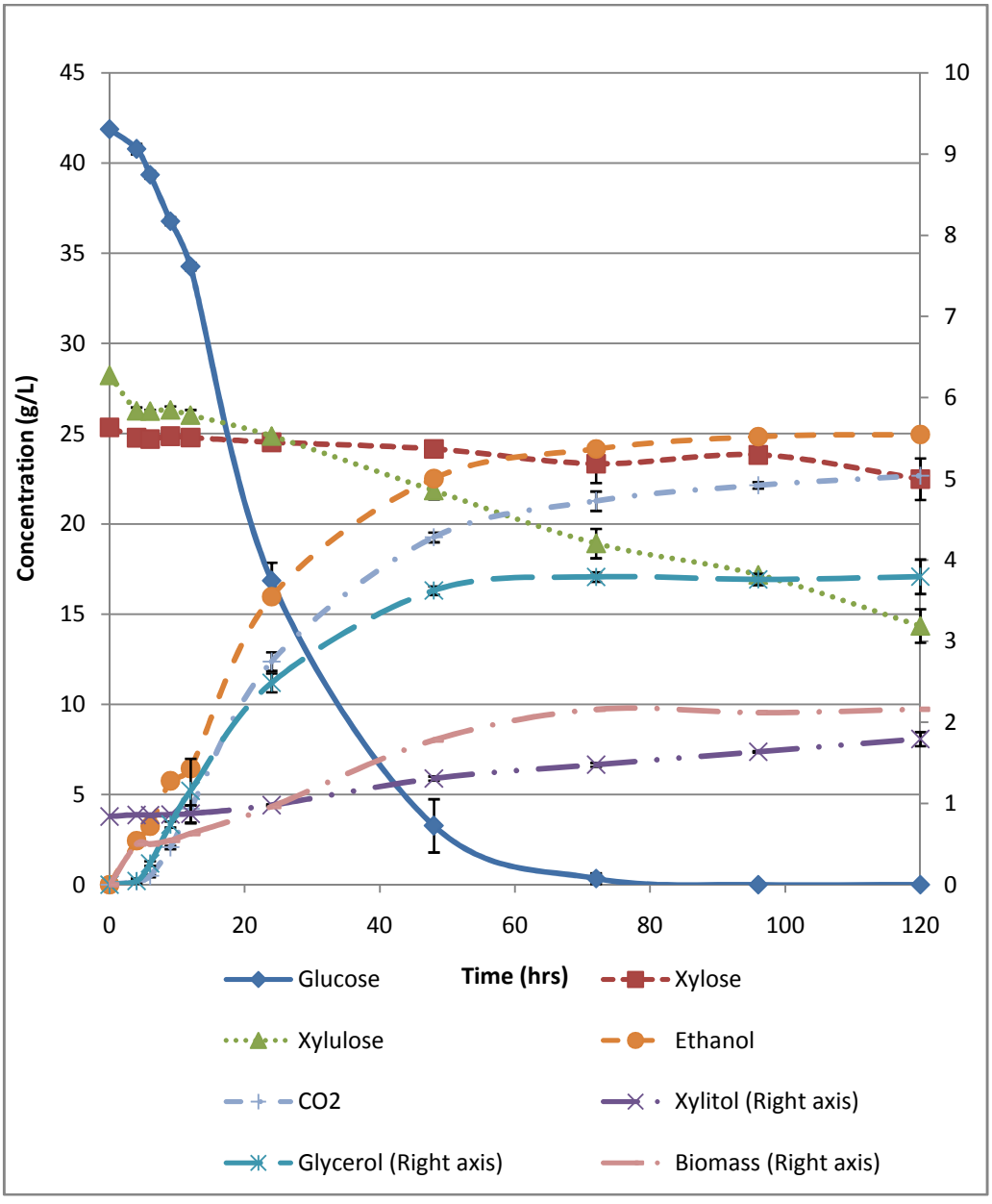

Figure 2.5. Batch fermentation by adapted *S.pombe*

Batch fermentation characteristics by the original strains of *S.cerevisiae* and *S.pombe* are shown in Fig.2.6 and Fig.2.7 respectively.

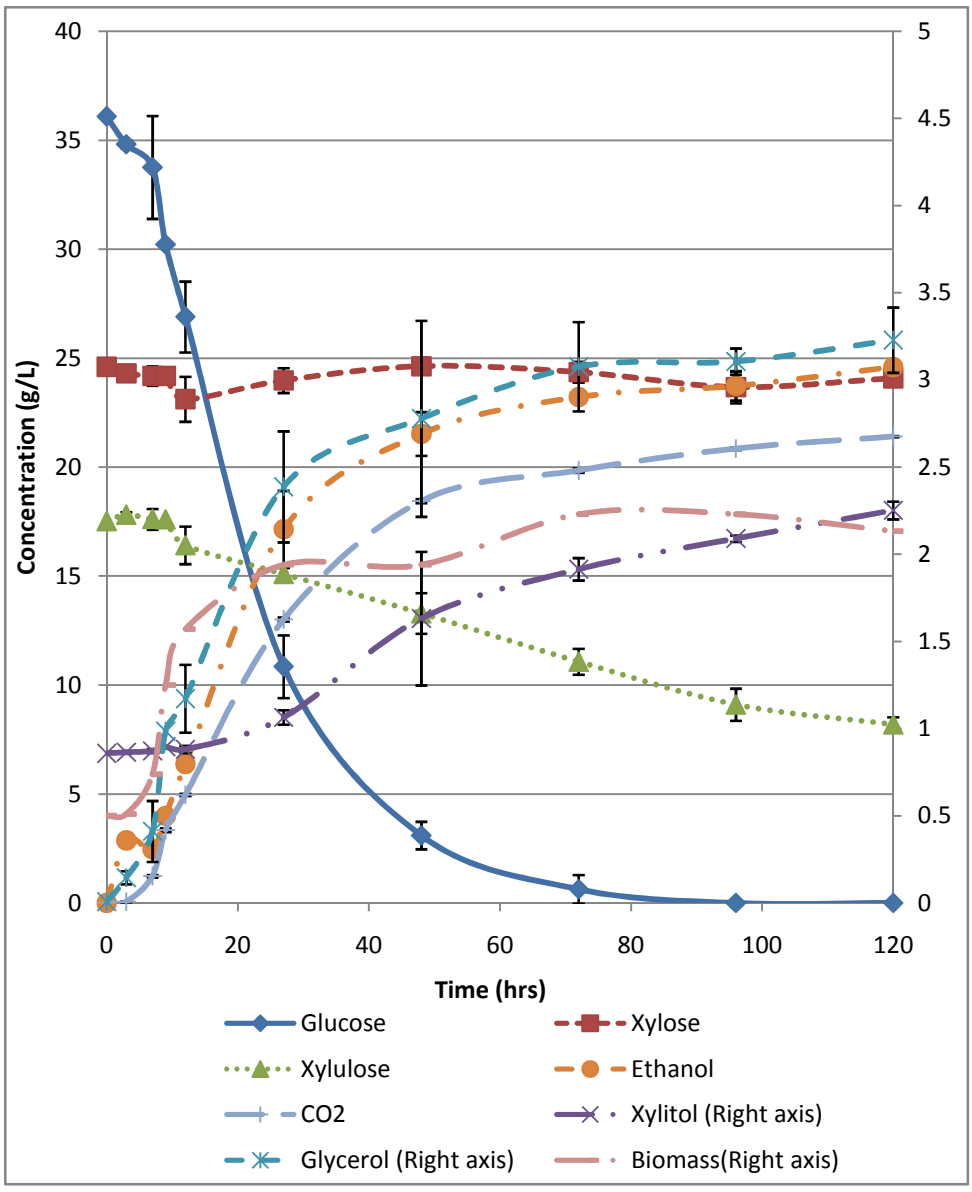

Figure 2.6. Batch fermentation by original *S.cerevisiae*

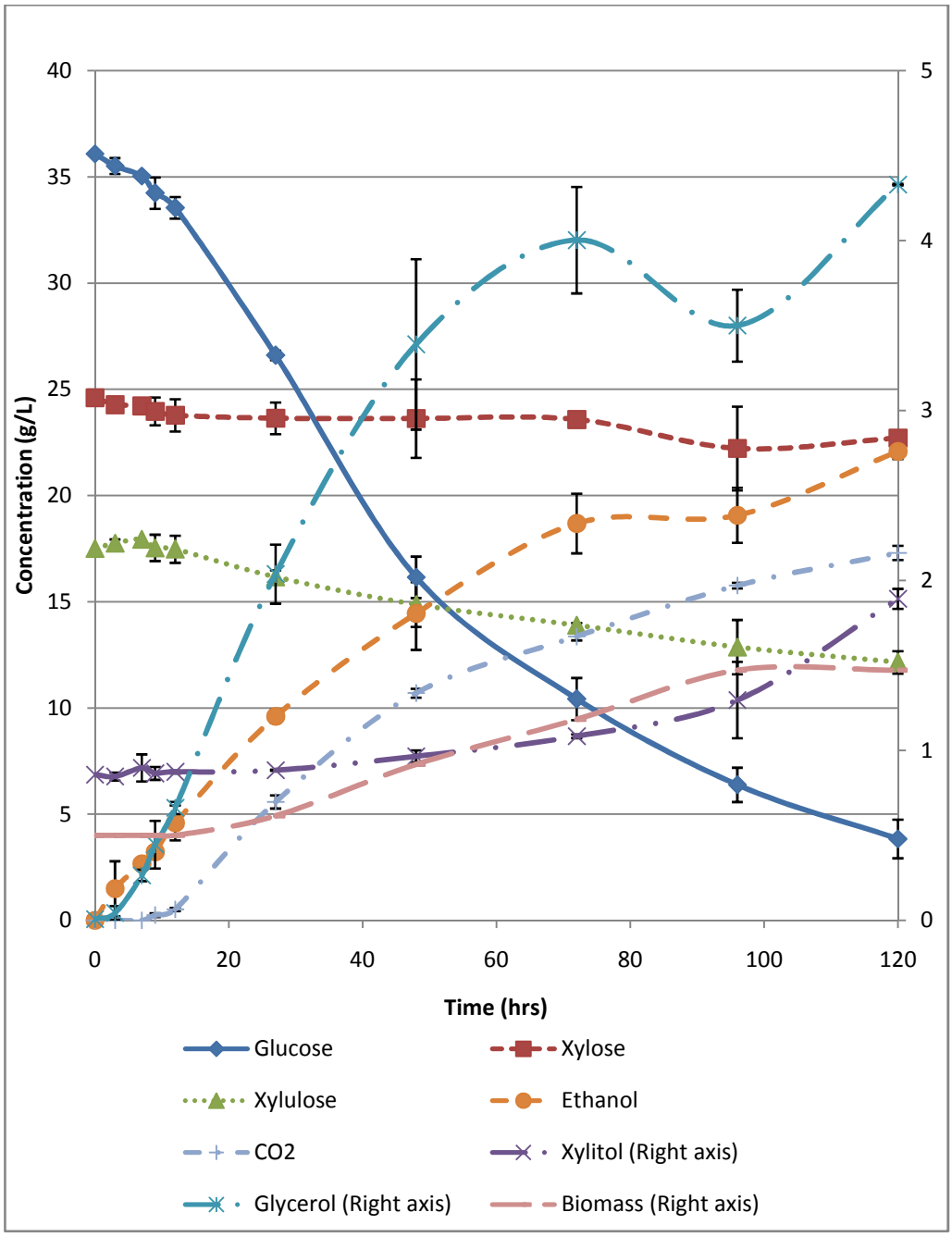

Figure 2.7. Batch fermentation by original *S.pombe*

The glucose utilization rate was highest in the unadapted *S.cerevisiae* and was not completed in the unadapted *S.pombe* by the end of 120 hrs (Fig.2.8).

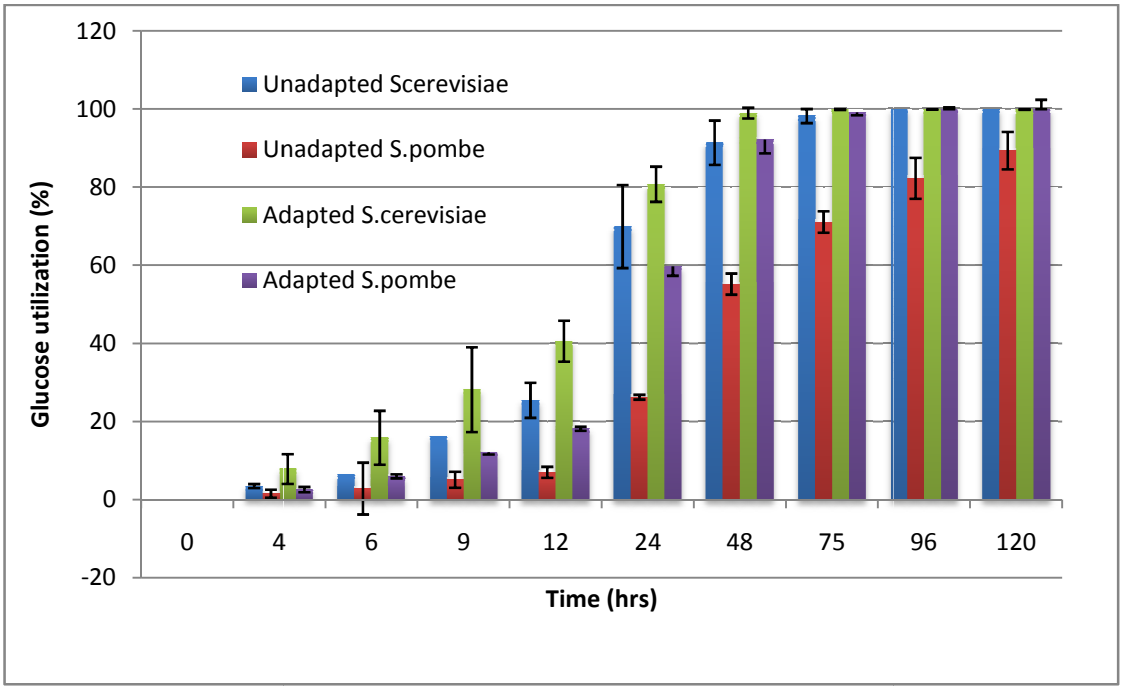

Figure 2.8. Comparison of glucose utilization

Xylulose utilization was highest in the unadapted *S.cerevisiae* fermentation where 53.1% of xylulose present initially was utilized by the end of 120 hrs (Fig.2.9). A final xylulose utilization of 30.6, 40.6 and 49.1% were seen in fermentations of unadapted *S.pombe,* adapted *S.cerevisiae* and adapted *S.pombe* respectively.

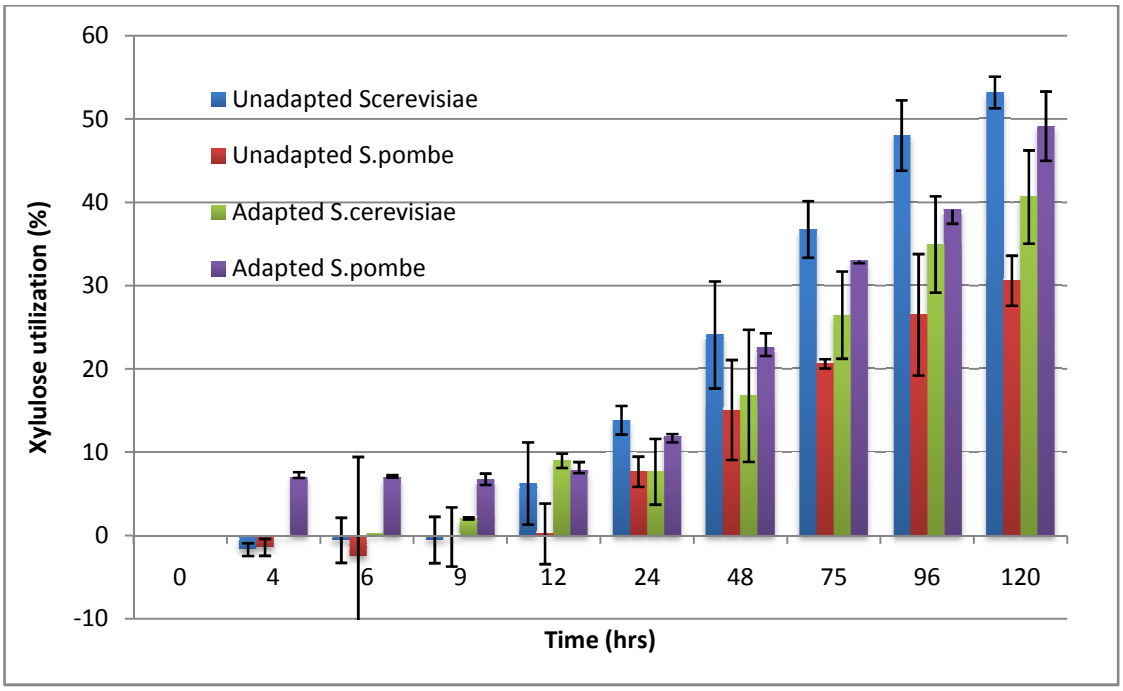

Figure 2.9.Comparison of xylulose utilization.

Comparison of the adapted and unadapted *S.cerevisiae* and *S.pombe* fermentation characteristics suggests that unadapted *S.cerevisiae* ferments with a highest ethanol yield of 0.41 Carbon mole of ethanol per Carbon mole of total sugars which is 61.5% of maximum theoretical conversion (Fig.2.10). An ethanol yield of 0.36, 0.34 and 0.34 Cmoles of ethanol per Cmoles of total sugar was obtained for the unadapted *S.pombe,* adapted *S.cerevisiae* and adapted *S.pombe* respectively.

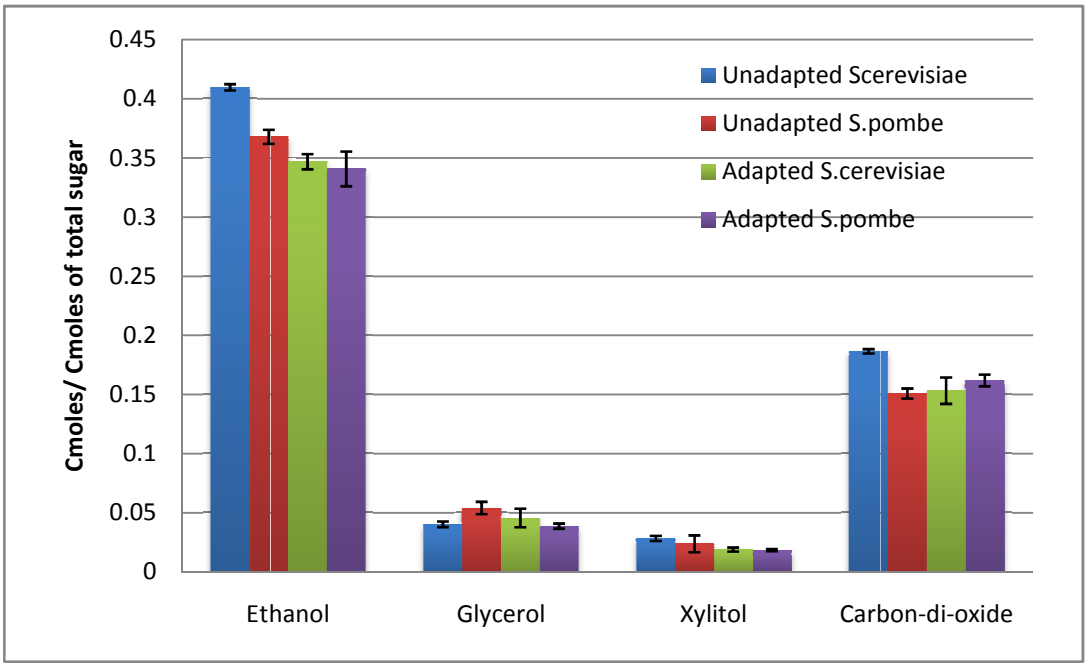

Figure 2.10. Product yields during the simulated hydrolyzate fermentation

Discussion

The medium designed to replicate hemicellulose hydrolyzate was successfully isomerized using commercially available xylose/glucose isomerase. The enzyme xylose isomerase is same as glucose isomerase commercially used for production of high fructose corn syrup (Bhosle et al., 1996). An isomerization ratio of 50:50, xylose: xylulose was achieved under the given conditions. An unknown precipitate formed in the medium during and after isomerization of xylose. Repeated heating of $MgSO_4$ during autoclaving and isomerization in the presence of phosphates and carbonates in yeast extract caused the precipitate. Magnesium ions formed salts with the phosphate and carbonate ions and precipitated out. Addition of magnesium sulphate after autoclaving the media prevented the formation of precipitate.

The concentration of glucose in the medium was reduced from 1.5g/L to 0.5g/L following experimental observation of glucose-only growth by the yeast. Lower glucose concentration was used during the chemostat adaptation so that the yeast starts to use xylulose for fermentation due to low availability of glucose. Presence of glucose prevented uptake of xylose as seen by the constant level of xylose over the course of fermentation. Adaptation of yeasts for utilization of xylose has been found to increase ethanol yield (Kuyper et al., 2005b). Our aim was to adapt the yeast strains to utilize xylulose more effectively.

The batch fermentation experiment was performed at high sugar concentrations to determine the characteristics of the adapted and unadapted strains suggest that the original *Saccharomyces cerevisiae* was better at utilizing xylulose and has higher ethanol yield. This suggests that, the regular industrial yeast *Saccharomyces cerevisiae* has evolved in best possible way constrained by its metabolic capabilities to utilize the hemicellulose component of lignocellulosic biomass. Also, isomerization of xylose in the hemicellulose hydrolyzate to xylulose and utilization of xylulose for fermentation reduces xylitol formation, which is a major problem in utilization of xylose directly for fermentation by yeasts. The data obtained from these experiments will be used to verify a constraint based model of yeast metabolic network.

Conclusion

A synthetic medium similar in composition to hemicellulose hydrolyzate was designed. A 50% isomerization of xylose to xylulose was achieved using

commercially available xylose isomerase. Adaptation of yeasts to this condition was performed for 1000 hrs in chemostat mode. Batch fermentation by the adapted and unadapted strains in high sugar concentrations determined that the original *Saccharomyces cerevisiae* was best in terms of xylulose utilization and ethanol yield. A total of 53.1% of xylulose was utilized by the strain at the end of 120 hrs fermentation producing 0.41 Cmoles of ethanol per Cmole of total sugar.

Acknowledgements

We would like to acknowledge ONAMI for funding the project, Dr. Christine Kelly Lab group for their support with equipment, Genencor for providing the enzymes for research and all members of Sustainable technologies lab for their support.

Chapter 3

A constraint based analysis of hemicellulose fermentation by industrial yeast *Saccharomyces cerevisiae*

Presented at the

2010 ASABE Annual International Meeting

Paper Number: 1009509 (Avanasi et al., 2010)

Abstract

A major challenge in ethanol production using lignocellulosic feed stocks is the inefficient utilization of hemicellulose, which accounts for 30-40% of the lignocellulosic biomass. Xylose is the major product of the hydrolysis of hemicellulose of lignocellulosic feed stock, which is not fermented by industrial yeasts. So, utilization of this carbon source would significantly increase the ethanol yield from 60 gal/dry ton to 90 gal/dry ton. One strategy for the utilization of xylose is by fermenting xylulose, an isomer of xylose. A flux balance based model can be used to study the xylulose metabolism. This approach relies on the stoichiometry of the yeast reaction network and the constraints determined by steady state growth of the organism in a defined media. A media replicating the hemicellulose hydrolyzate in composition was designed and isomerized. Sugars (glucose, xylose, xylulose), sugar alcohols (ethanol, xylitol and glycerol), yeast cell mass, was monitored during the growth. The data from fermentation experiments were used to conduct a flux balance analysis of the yeast using the *Saccharomyces cerevisiae* iND750 model. The model outputs were comparable to the experimental production rates of products. Under the experimental conditions, the model gives an increase of 1.036 mmol/hr. g biomass of ethanol production by the consumption of xylulose.

Keywords. Lignocellulosic ethanol, hemicellulose utilization, *Saccharomyces cerevisiae*, flux balance analysis, experimentally determined constraints

Introduction

Flux balance analysis is a mathematical modeling approach which brings metabolic engineering, bioinformatics and genomics together (Kauffman et al., 2003). It is used to quantitatively simulate microbial metabolism (Varma and Palsson, 1993a, 1993b). It is used to analyze the steady state behavior of biochemical networks. FBA is a constraint based modeling system, in which the possible solutions for a network are constrained by stoichiometry, direction of network reactions and maximum allowable flux values. The best solution is then computed by linear optimization. Basic assumption in finding optimized solution is that organisms (represented by biological network) have evolved to maximize the objective function representing microbial growth rate (Famili et al., 2004).

The series of steps involved in metabolic network reconstruction are (Rocha et al., 2008)

- Identification of reactions from databases and literature
- Determination of reaction stoichiometry and direction
- Identification of different compartments and the respective reactions
- Determination of biomass composition
- Fixing experimentally determined energy requirements

Bioinformatics databases and available literature can be referred to obtain information on reactions, stoichiometry of the reactions and compartmentation. Experimentally determined information on biomass composition and energy requirements can be

added to the stoichiometric model. Once the stoichiometric model is set up, mathematical tools such as flux balance analysis can be used for analyses. A steady-state approximation is applied, which reduces the mass balance ordinary differential equations to a set of linear homogeneous equations. The number of variables (reaction fluxes) are almost always greater than the number or reactions (metabolites), resulting in an under-determined system having multiple possible solutions. Constraints in the form of maximum and minimum allowable fluxes, experimentally determined reaction fluxes, and availability of nutrients reduce the number of possible solutions. Linear optimization can then be used to solve the set of reactions optimizing for a particular objective function. A simplex method can be used to find the optimal solution set for the given objective function. The feasible solution space can be considered to be a convex polytope. The simplex method starts at the origin and follows a path along the edges of the polytope to the vertex where the maximum occurs. The optimal solution will lie on a vertex of the convex polytope giving a unique set of values for the variables. It can also lie on an edge of the convex polytope. In this case, the optimal value of the objective function will be unique but the set of solution values of other variables giving the same optimal objective function value will not be unique. Alternate optimal solutions will be present.

A major challenge in ethanol production using lignocellulosic feed stocks is the inefficient utilization of hemicellulose component, which accounts for 30-40% of lignocellulosic biomass. Xylose comprises more than 60% of the recoverable sugars derived from hemicelluloses. So, the utilization of this carbon source would

significantly increase the ethanol yield (Van Vleet and Jeffries, 2009). Isomerization of the available xylose to xylulose using commercially produced xylose isomerase *in vitro* could be one of the approaches to increase conversion of xylose to xylulose in yeasts (Chiang et al., 1981, Chandrakant and Bisaria, 2000). The resulting xylulose can then be utilized by the yeast. Metabolic fluxes under experimental xylulose utilization conditions can be found using a flux balance based model. Our objectives were to

- To perform constraint based analysis of hemicellulose metabolism, furfural metabolism using the *Saccharomyces cerevisiae* iND750 model
- Use experimentally determined data and compare model predictions with experimental results

Materials and Methods

Metabolic Network Reconstruction

The metabolic network used in this study was the fully compartmentalized genome scale metabolic model of *Saccharomyces cerevisiae* iND750 that considered 750 genes and was developed by Natalie et al. (2004). The reconstructed network includes 646 metabolites and 1149 irreversible/reversible reactions occurring in 8 different compartments (extracellular space, cytosol, mitochondria, peroxisome, nucleus, golgi apparatus, endoplasmic reticulum and vacuole). Transport reactions representing the transport of metabolites from the extracellular to the cytosol (reversible/irreversible) and in between compartments are included in the network. Additional reactions for

xylulose uptake and metabolism of furfural were included in the network based on literature. Exchange reactions representing the input into the cell and output from the cells were represented. The fluxes of these reactions were used as the uptake rates of nutrients and secretion rates of products.

Biomass composition was represented as a drain of precursors or building blocks into biomass. The building blocks include amino acids, carbohydrates, ribonucleotides, deoxyribonucleotides, lipids, sterols, phospholipids and fatty acids (Forster et al., 2003). Additionally, a growth-associated ATP requirement of 59.2 mmole ATP/hr. g biomass was assumed to be required. Non-growth-associated ATP requirement of 6.58 mmole ATP/hr. g biomass was used. The reconstructed metabolic network was used to do a flux balance based analysis of *Saccharomyces cerevisiae* metabolism.

PERL programming

The reaction set used for the iND750 model was obtained and perl programming was used to declare the model in General Algebraic Modeling System (GAMS). The model was declared and optimization was performed in GAMS.

Model development

A steady state approximation was applied, converting the metabolic network to a set of coupled ordinary differential equations represented as the rate of change of each metabolite over time equal to zero. The equations were mathematically expressed as,

$$\sum_{j=1}^{n} Sij\, Vj = 0, i = 1 \ldots, m,$$

V_j Corresponds to j^{th} metabolic flux and S_{ij} is the stoichiometry of i^{th} metabolite in j^{th} reaction.

These equations were written in matrix form as

$$S.v = 0,$$

Where vector v stands for flux vector and the matrix S is the stoichiometric matrix. Each column in this matrix represents an individual reaction and each row refers to the steady-state mass balance for each metabolite.

Fixing of constraints

The main constraints were the reversibility/irreversibility of the reactions. All reversible reactions were represented as separate reactions (one in forward and the other in the reverse direction) and the fluxes were allowed to be between 0 and 1000.

$$0 \leq V_j \leq 1000$$

The fluxes of exchange reactions of all nutrients that are unavailable were constrained to zero.

$$V_j = 0,$$

$$j - exchange\ fluxes\ of\ unavailable\ nutrients$$

Optimization

The optimization problem was specified as,

$$Maximize\ Z = c.v$$

$$such\ that\ S.v = 0,$$

$$x \leq V_j \leq y$$

Where x - the lower limit of flux value, y - the upper limit of flux value

The objective function was to maximize the flux of biomass formation and it was declared as $c_{biomass} = 1$.

Experimental verification

Batch fermentation

The strain of *Saccharomyces cerevisiae* was grown in an isomerized hemicellulose medium (Table 2.2) with high sugar concentrations (glucose 45g/L and xylose 45g/L). The media was bubbled with nitrogen gas to remove the dissolved oxygen. The experiments were performed in batch mode in shake flasks of 225 mL volume. An initial inoculum level of 0.5 g/L was used. Fermentation was carried out at 30°C and pH 5 with an agitation of 100 rpm for 120 hrs. Samples and weight loss measurements were taken at 0, 4, 6, 9, 12, 24, 48, 72, 96 and 120 hrs. Weight loss measurements were considered as CO_2 measurements (Smits et al., 1996). Samples were filtered to remove any cells to prevent growth during storage and handling. The samples were stored at 4°C until used for HPLC analysis. All treatments were conducted in triplicates.

Sample Analysis

HPLC analyses were done using the Agilent 1200 series HPLC system. Aminex HPX-87H (Bio-Rad, Hercules, CA) column was used for analyzing the samples. Glucose, xylose, xylulose, glycerol, xylitol and ethanol were detected using the refractive index detector (Model G1362A). Each sample was filtered using 0.2 µm filters and an injection volume of 20 µl was used per sample. The column was maintained at 65°C and each sample was run for 30 minutes using 0.005 M H_2SO_4 as the mobile phase. The data was processed using the Chemstation software package.

Biomass growth rate

Growth of the biomass was monitored by measuring optical density at 600 nm at each time point (You et al., 2003). The absorbance values were correlated to dry cell weight. Specific growth rate was calculated using the data from the experiments.

$$\mu = \frac{\ln(m2/m1)}{(t_2 - t_1)}$$

Where m = biomass concentration, t = time

Model comparison

Experimentally determined flux values for the uptake of glucose and xylulose were fixed in the model and the output fluxes of the products such as ethanol, CO_2, glycerol were compared with experimentally determined production rates.

Elemental Composition Analysis

Biomass samples of *S.cerevisiae* from the experiments were used in the Elemental Combustion System ECS 4010 (Costech Analystical Technologies Inc, Valencia, CA, USA) to determine their elemental C: N ratio. These values were then compared with the C: N ratio of the metabolites forming the biomass in the model reaction set.

Results

The data obtained from the batch fermentation was used for determining the uptake rates of substrates and the output rates of products (Fig. 2.6). The exponential phase of growth was considered for obtaining uptake/secretion rates. A glucose uptake rate of 5.256 ± 0.172 (mmol/hr. g biomass) was determined. A xylulose uptake rate of 0.701 ± 0.214 (mmol/hr. g of biomass) was determined. The output rates of products are listed in the Table 3.1. Experimentally determined uptake rates were used as constraints in the model and solved for maximizing the biomass formation reaction as the objective function. The specific growth rate was determined to be 0.048 g/g. hr experimentally. The specific growth rate from the model was 0.048 g/g.hr when a non-growth associated ATP requirement of 6.58 mmole ATP/hr. g biomass was used. The model outputs are comparable to the experimentally determined data (Fig.3.1). The model suggests an increase of 1.036 mmol/hr. g biomass of ethanol due to the consumption of xylulose under the given conditions. This was calculated by simulation using experimentally determined glucose uptake rate considering the xylulose uptake was zero from the same experimental data.

Table 3.1. Experimentally determined fluxes

Flux type	Metabolite	Experimental flux value (mmol/hr. g biomass)	Model flux value (mmol/hr. g biomass)
Input	Glucose Xylulose	5.256±0.172 0.701±0.214	- -
Output	Ethanol CO_2 Glycerol Biomass	13.302±0.4798 11.15±0.071 0.89±0.172 0.048 g/hr. g	10.818 11.134 0.328 0.048 g/hr. g

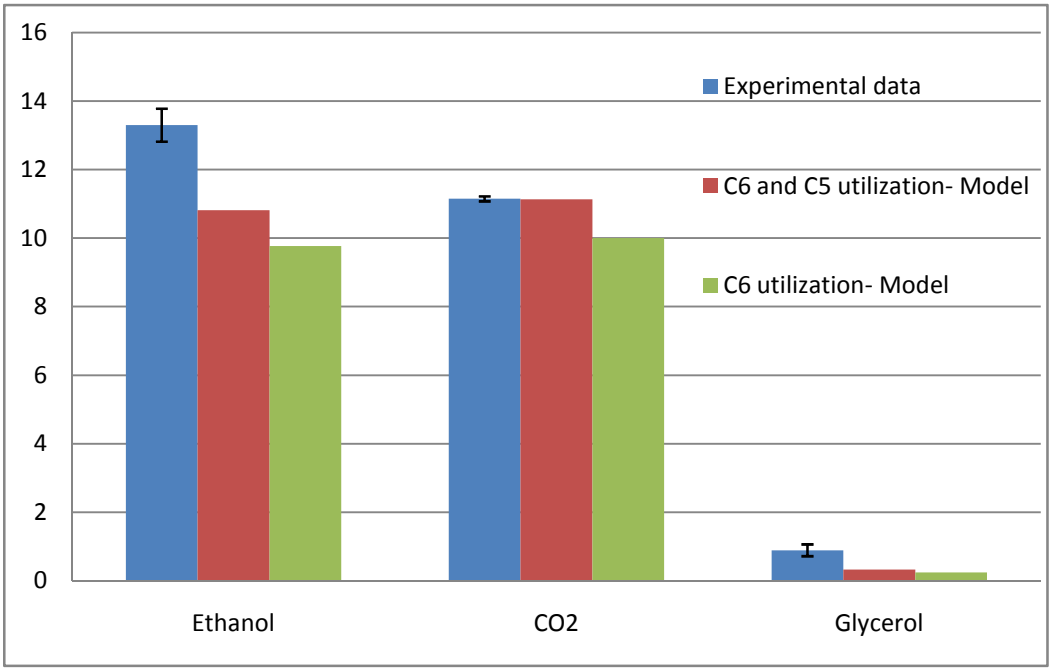

Figure 3.1. Comparison of experimental and model output flux values

Discussion

Anaerobic conditions were simulated by considering oxygen to be a non-available nutrient by fixing its exchange flux to be zero. Additionally, protons, water,

phosphate, sulphate, sodium and potassium were unconstrained allowing them to freely enter/leave the system. Urea was used as the nitrogen source in the experiments. Urea exchange was unconstrained considering it to be a non limiting nutrient. Ergosterol and fatty acids such as oleic acid are not synthesized by *Saccharomyces cerevisiae* in absence of oxygen (de Kock et al., 2001, you et al., 2003). Anaerobic simulations also required ergosterol, zymosterol, palmitoleate (C16:1), stearate (C18:20), oleate (C18:1), and linoleate (C18:2) to be freely exchanged in the *Saccharomyces cerevisiae* iND750 model. Marginal values in the GAMS output file suggested a requirement of succinate output to be unconstrained. A reduced costs analysis was done to determine the fluxes of other products which needed to be unconstrained under these conditions. Reduced cost analysis is performed to determine the necessary changes in the objective function coefficients needed so that a variable, flux such as succinate, which is currently zero in the optimal solution would be non-zero in the new optimal solution. Reduced cost values can be obtained from the marginal values of the reaction fluxes in the GAMS output file. Based on such reduced cost analysis, a minimum of 0.010 mmol/hr. g biomass of succinate needed to be produced to improve the objective function values from 0 to 0.048 g/hr. g biomass.

Experimentally determined biomass specific growth rates were used to determine the maintenance ATP requirement under the experimental conditions. A maintenance ATP requirement of 6.58 mmol ATP/hr. g biomass was determined and fixed for simulating the experiment in the flux balance based model. The experimental flux values of ethanol and glycerol were higher than the simulated flux values. The

medium used was a complex medium containing supplements in the form of yeast extract, consisting of amino acids, and carbohydrates and hence the exact composition information was not available. Therefore, presence of supplements in the media was not simulated in the analysis and could be one of the reasons for lower ethanol and glycerol production in the model simulations. There was 81.32%, 99.8% and 36.8% agreement between the model outputs and these experimental results for ethanol, CO_2 and glycerol fluxes respectively. Comparison of the elemental composition of biomass to the biomass forming metabolites in the model shows that experimental C: N ratio of 0.1808 deviates 13.9 % from the model biomass composition C: N ratio of 0.1557. It is to be noted that, Duarte et al (2004) in their gene deletion study using the iND750 model reported an 82.6 % (positive phenotypes) agreement between model phenotype predictions and experimental observed phenotypes. Also, biomass formation reaction has been identified as the important source of the false predictions as only the most important components constituting the biomass have been included in the biomass reaction and other trace metabolites have not been included (Duarte et al, 2004). This along with minor experimental errors can be the reason for the observed lower C: N ratio of the model biomass composition than the experimentally determined ratio.

Xylulose is utilized through the pentose phosphate pathway. It is then channeled into the glycolysis pathway. Xylulose is initially phosphorylated to xylulose-5-phospahte. It then combines with ribose-5-phosphate and is catalytically converted to sedoheptulose-7-phospahte and glyceraldehyde-3-phosphate by transketolase. Transaldolase enzyme converts sedoheptulose-7-phospahte and glyceraldehyde-3-

phosphate to erythrose-4-phosphate and fructose-6-phosphate. Fructose-6-phosphate enters the glycolysis. Another molecule of xylulose-5-phosphate combines with erythrose-4-phosphate to form glyceraldehyde-3-phosphate and fructose-6-phosphate. These products enter the glycolysis. The utilization of glyceraldehyde-3-phosphate for the reaction catalyzed by transketolase is from glycolysis and is competitively not preferred over glycolysis (Senac et al., 1990). This is the reason for the experimentally observed low utilization rates of xylulose when compared to glucose.

Conclusion

A flux balance model of *Saccharomyces cerevisiae* was set up using the metabolic network from the fully compartmentalized genome scale metabolic model of *Saccharomyces cerevisiae* iND750. Additional reactions involved in xylulose utilization were added to the model. Experimentally determined data were used and the model outputs were compared to the experimental outputs. The results of the experiment and the model were comparable with an increase of 1.047 mmol/hr. g biomass of ethanol production contributed by xylulose utilization under the given conditions and there was 81.32%, 99.8% and 36.8% agreement between the model outputs and these experimental results for ethanol, CO_2 and glycerol fluxes respectively.

Acknowledgements

We acknowledge the Systems biology research group of UCSD and Genencor, Danisco Inc.

Chapter 4

Effect of furfural and higher yeast inoculum concentration on the utilization of hemicellulose by yeast

Abstract

Furfural is an important inhibitor formed in the pretreatment process of lignocellulosic ethanol production. Furfural, mainly formed from xylose in the hemicellulose is inhibitory to the fermentation by yeasts. Effect of furfural concentration on the fermentation of hemicellulose hydrolyzate was studied in a synthetic media using the strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* which were adapted to the hemicellulose like environment. Three different concentrations of furfural (0, 0.5 and 2 g/L) were used in the medium. A constraint based metabolic model of *Saccharomyces cerevisiae* metabolism was used to perform a flux balance based analysis of furfural metabolism. Also the effect of high inoculum on the fermentation of xylulose was verified by batch experiments with high initial inoculums of the original *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* strains. Performances of different strains under different conditions were compared in terms of xylulose utilization in batch growth. In *Saccharomyces cerevisiae* fermentations, an intermediate concentration of 0.5 g/L of furfural had highest ethanol yield of 0.375Cmoles/Cmoles of total sugar and lowest glycerol yield of 0.041Cmoles/Cmoles of total sugar. In *Schizosaccharomyces pombe*, absence of any furfural in the medium had highest xylulose utilization of 51.84% over 120 hrs. Higher

inoculum concentrations did not improve batch xylulose utilization in both the yeast strains but the utilization rates were improved.

Introduction

Pretreatment of lignocellulosic biomass is an important process step in the production of ethanol from lignocellulosic ethanol. Acid hydrolysis is one of the preferred methods of pretreatment. One of the drawbacks of acid pretreatment is the formation of inhibitory compounds (Palmqvist et al., 2000a, 2000b). Furfural and hydroxy methyl furfural are formed due to further conversion of xylose and glucose in the hydrolyzate due to browning reaction (Larsson et al., 1990, Palmqvist et al., 2000b). Furfural formation is a greater concern in utilization of hemicellulose because hemicellulose is mainly composed of xylose (up to 60%). Furfural has been found to be an inhibitor of fermentation by yeasts (Horvath et al., 2003) even at very low levels. The yeasts detoxify the furfural to lesser inhibitory compounds furoic acid and furfurol under aerobic and anaerobic conditions respectively (Taherzadeh et al., 1999, 2000).

In this research, batch fermentation experiments of the strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were conducted with different concentrations of furfural under anaerobic conditions in the hemicellulose media. The aim was to understand the effect of furfural concentration on the fermentation of xylose/xylulose. A flux balance based model was set up using the reaction set of the *Saccharomyces cerevisiae* iND750 model (Natalie et al., 2003) in GAMS (General

Algebraic modeling system). Additional reactions for the metabolism of furfural and xylulose were included in the model (Table 4.1). Our aim was to use the experimentally determined data in the model and do a flux balance based analysis.

Table 4.1 Additional reactions included in the model

Reaction	Stoichiometry
Xylulose exchange and transport	Xylulose_extracellular ⟶ Xylulose_intracellular
Furfural exchange and transport	Furfural_extracellular ⟶ Furfural_intracellular
Aerobic furfural metabolism	Furfural + NADH ⟶ Furoic acid + NAD + H^+
Anaerobic furfural metabolism	Furfural + NADH ⟶ Furfurol + NAD + H^+
Furoic acid exchange and transport	Furoic acid_intracellular ⟶ Furoic acid_extracellular
Furfurol exchange and transport	Furfurol_intracellular ⟶ Furfurol_extracellular

We investigated the effect of a higher inoculum concentration compared to previous batch experiments. If higher xylulose utilization was achieved by higher cell loading, this strategy can be used to better utilize the pentose fraction of lignocellulosic feedstock. In the previous experiments, a ratio of 1:45 of inoculum to sugar ratio was used. Increasing the inoculum to sugar ratio to 1:9 was tried to study the effect of high inoculum on the utilization of xylulose. Also, the xylulose utilization during batch growth of the strains *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* adapted, under different furfural and inoculum concentrations were compared.

Materials and Methods

Batch growth with furfural

The adapted strains of *Saccharomyces cerevisiae and Schizosaccharomyces pombe* were grown in an isomerized hemicellulose medium (Table 2.2) with high sugar concentrations (glucose 45g/L and xylose 45g/L). Initial concentration of 0.5 and 2 g/L of furfural were used in the medium. The medium was bubbled with nitrogen gas to remove the dissolved oxygen. The experiments were performed in batch mode in shake flasks of 225 mL volume. An initial inoculum level of 0.5 g was used. Fermentation was carried out at 30°C and pH 5 with an agitation of 100 rpm for 120 hrs. Samples and weight loss measurements were taken at 0, 4, 6, 9, 12, 24, 48, 72, 96 and 120 hrs. Weight loss measurements were considered as CO_2 measurements (Smits et al., 1996). Samples were filtered to remove any cells to prevent growth during storage and handling. The samples were stored at 4°C until used for HPLC analysis. All treatments were conducted in triplicates.

Batch growth with higher inoculum

The original strains *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were grown in the isomerized hemicellulose medium with high sugar concentrations (with respect to usual sugar concentrations in hydrolyzate). A high inoculum to sugar ratio of 1:9 (inoculums: total sugar) was used in the batch experiments done in 125 mL shake flasks. An initial inoculum level of 1.25g was used. Fermentation was carried out at 30°C and pH 5 with an agitation of 100 rpm for 120 hrs. Samples and weight

loss measurements were taken at 0, 4, 6, 9, 12, 24, 48, 72, 96 and 120 hrs. Weight loss measurements were considered as CO_2 measurements (Smits et al., 1996). Optical density was measured at 600 nm in a spectrophotometer (Spectronic-Genesys 10 Bio) and the optical density was correlated to the concentration of cells in (g/L). Samples were filtered to remove any cells to prevent growth during storage and handling. The samples were stored at 4°C until used for HPLC analysis. All treatments were conducted in triplicates.

HPLC Analysis of samples

HPLC analyses were done using the Agilent 1200 series HPLC system. Aminex HPX-87H (Bio-Rad, Hercules, CA) column was used for analyzing the samples. Glucose, xylose, xylulose, glycerol, xylitol, furfural and ethanol were detected using the refractive index detector (Model G1362A). Each sample was filtered using 0.2µm filters and an injection volume of 20µl was used per sample. The column was maintained at 65°C and each sample was run for 30 minutes using 0.005 M H_2SO_4 as the mobile phase. The data was processed using the Chemstation software package.

Results and Discussion

Batch fermentation with furfural

Batch fermentations were done in hemicellulose media isomerized using xylose isomerase. 0, 0.5 and 2 g/L of furfural were used in the media. Batch fermentation results show consumption of glucose, xylulose and furfural, production of ethanol,

glycerol and xylitol over a period of 120hrs. Xylose levels remain constant over the period of time indicating that xylose was not consumed during fermentation. Weight loss measurements account for the release of CO_2 during fermentation. Batch fermentation profiles show that the furfural is metabolized immediately within four hrs (Fig.4.1and Fig.4.2).

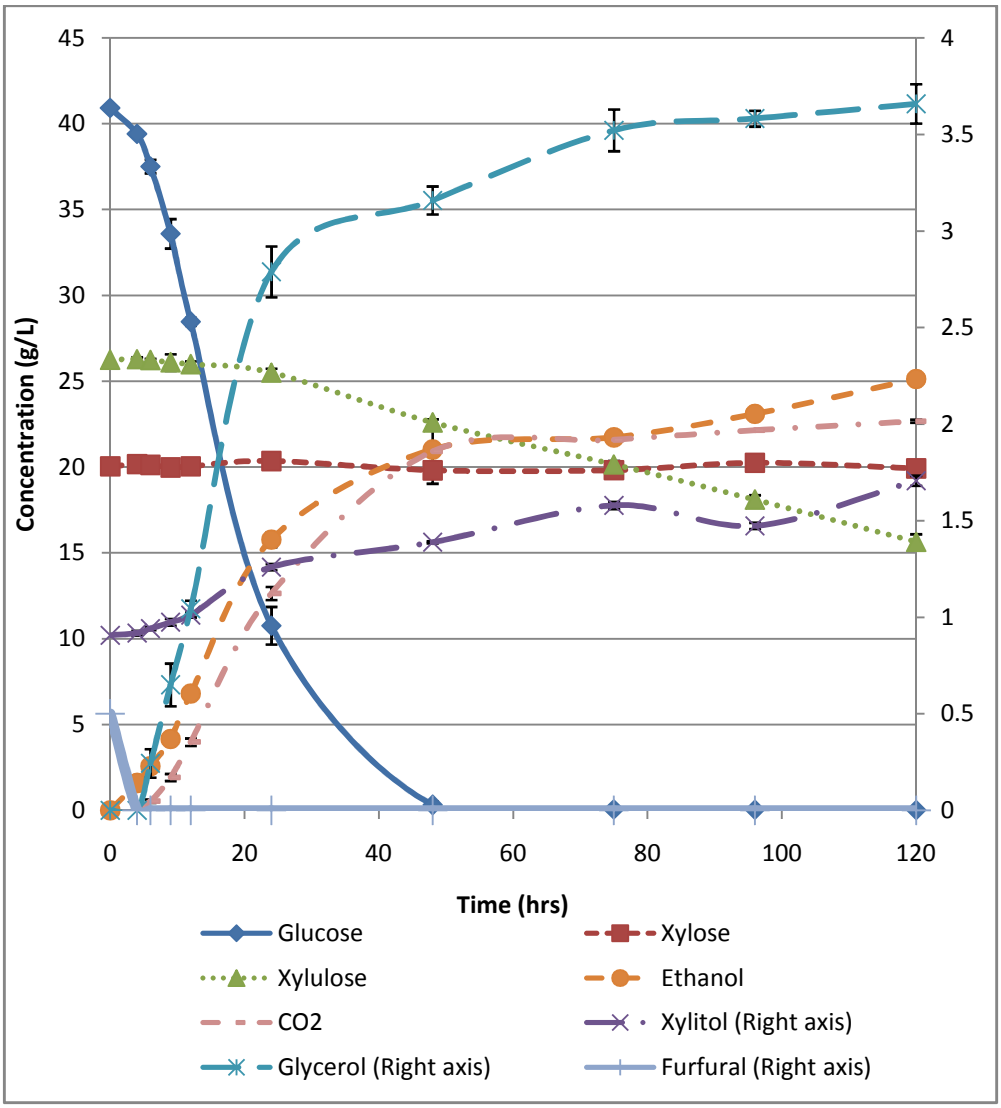

Figure 4.1. Batch fermentation by adapted *Saccharomyces cerevisiae* (0.5 g/L initial furfural concentration)

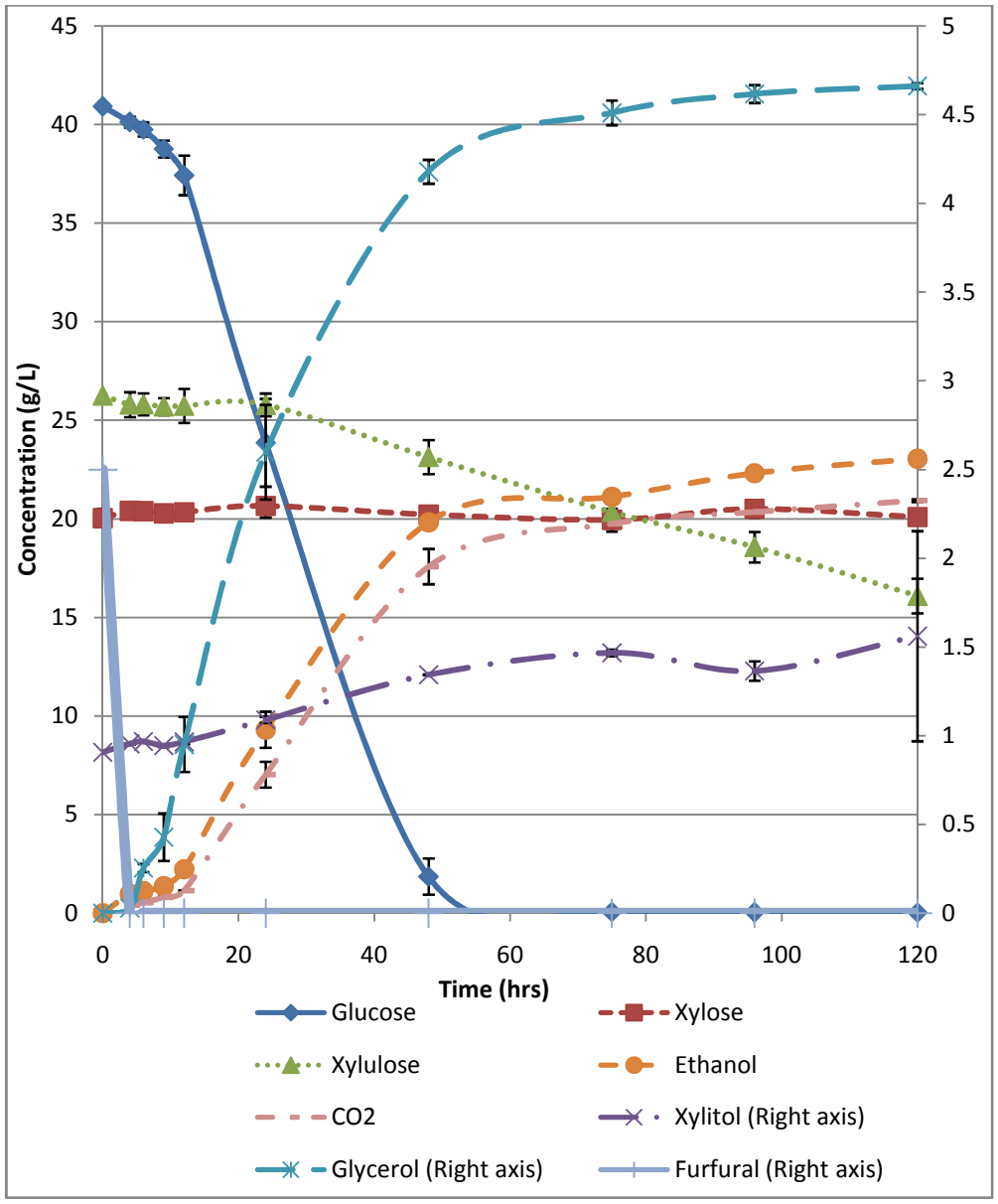

Figure 4.2. Batch fermentation by adapted *Saccharomyces cerevisiae* (2 g/L initial furfural concentration)

In *Saccharomyces cerevisiae*, the presence of furfural delays the utilization of glucose and xylulose whereas once the furfural is detoxified, normal metabolism is seen (Fig.4.1, Fig.4.2 and Fig.4.3).

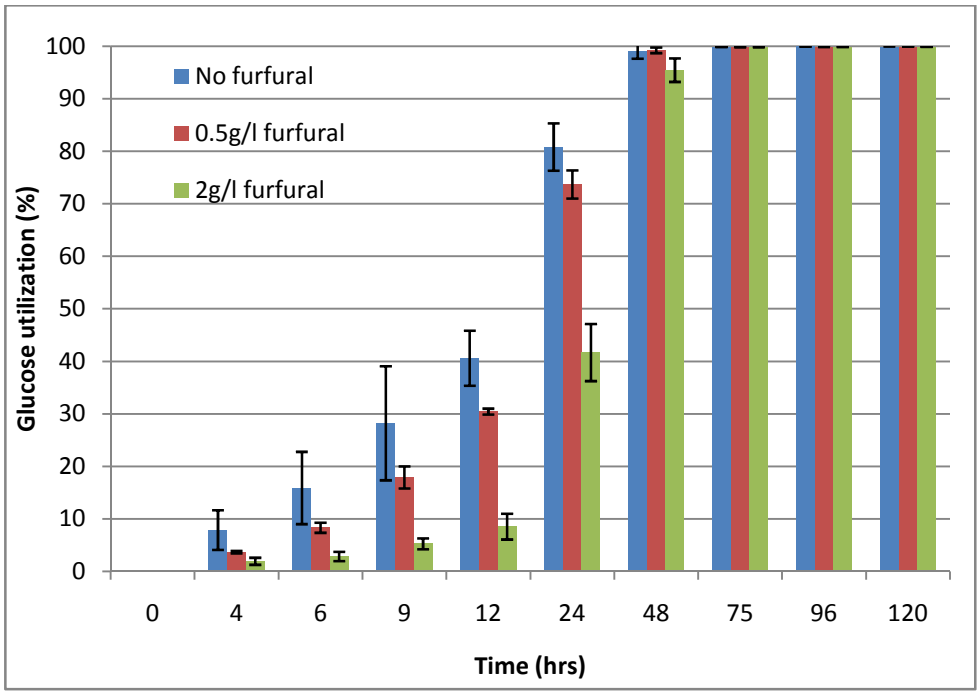

Figure 4.3. Comparison of glucose utilization by adapted *Saccharomyces cerevisiae* in presence of furfural

Furfural has been found to be an inhibitor of fermentation by yeasts (Horvath et al., 2003). Furfural inhibition is mainly due to inhibitory effects on the enzymes alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase (Modig et al., 2002). This inhibition is the reason for the observed poor growth in both aerobic and anaerobic conditions on glucose, also reported by (Horvath et al., 2003). A similar effect is observed in xylulose utilization, which is also slowed in the presence of furfural (Fig.4.4).

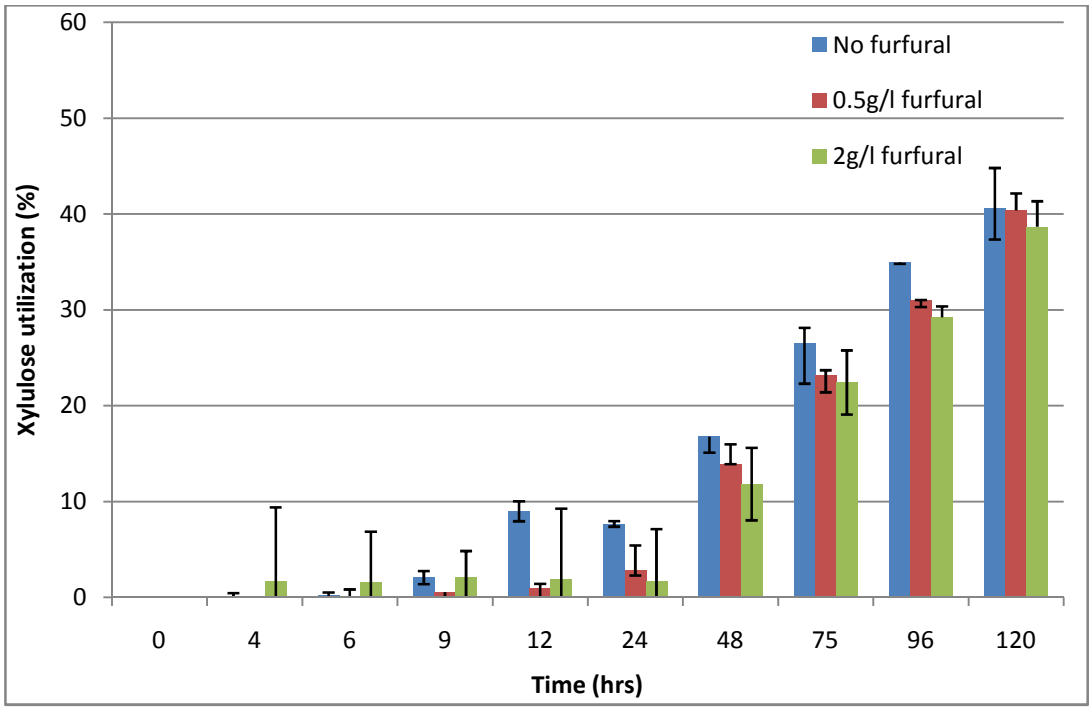

Figure 4.4. Comparison of xylulose utilization by *Saccharomyces cerevisiae* in presence of furfural

This is because utilization of xylulose through the pentose phosphate pathway involves intermediates of glycolysis (Fig. 1.4). Comparison of product yields of *Saccharomyces cerevisiae* suggests that 0.5 g/L furfural when present in the medium gives the highest ethanol yield of 0.375Cmoles/Cmoles of total sugar and lowest glycerol yield of 0.041Cmoles/Cmoles of total sugar (Fig.4.5). The conversion of furfural to furfurol serves as a way of reducing the redox imbalance resulting in reduced glycerol production. Glycerol production is the normal way to reduce the redox imbalance in anaerobic growth and so more glucose is used towards ethanol production instead of glycerol production (Pejo et al., 2008).

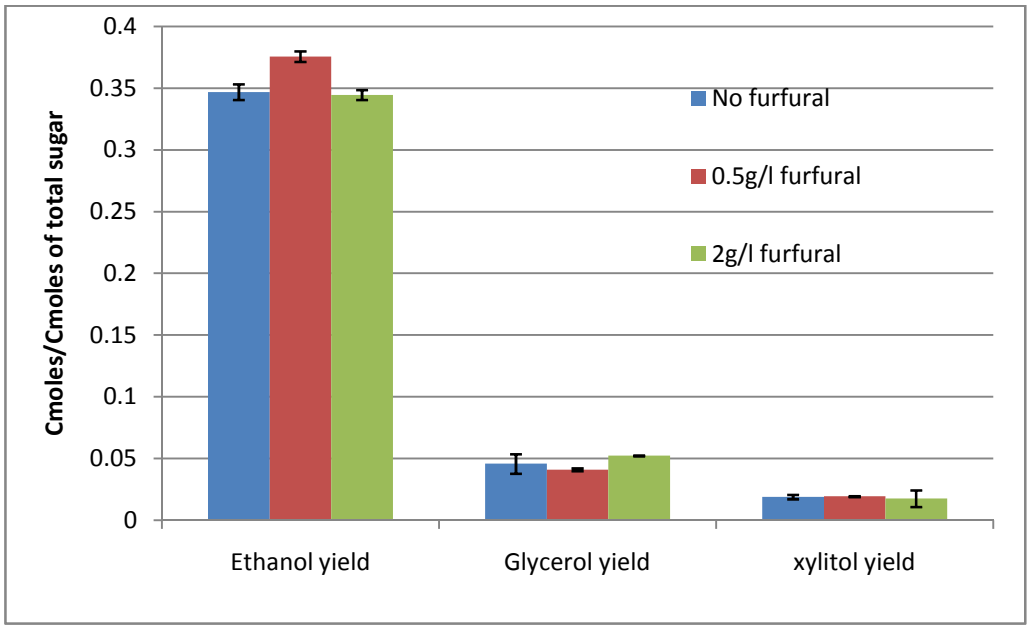

Figure 4.5. Comparison of product yields by adapted *Saccharomyces cerevisiae* in presence of furfural

In *Schizosaccharomyces pombe*, batch fermentations with 0.5 and 2 g/L concentrations of furfural resulted in similar delayed utilization of glucose (Fig.4.6, 4.7 and 4.8) and xylulose suggesting similar inhibitory effects on glucose and xylulose metabolism as observed in *S.cerevisiae.* Inhibition of enzymes involved in glycolysis should be the reason for the observed delay in utilization of the sugars.

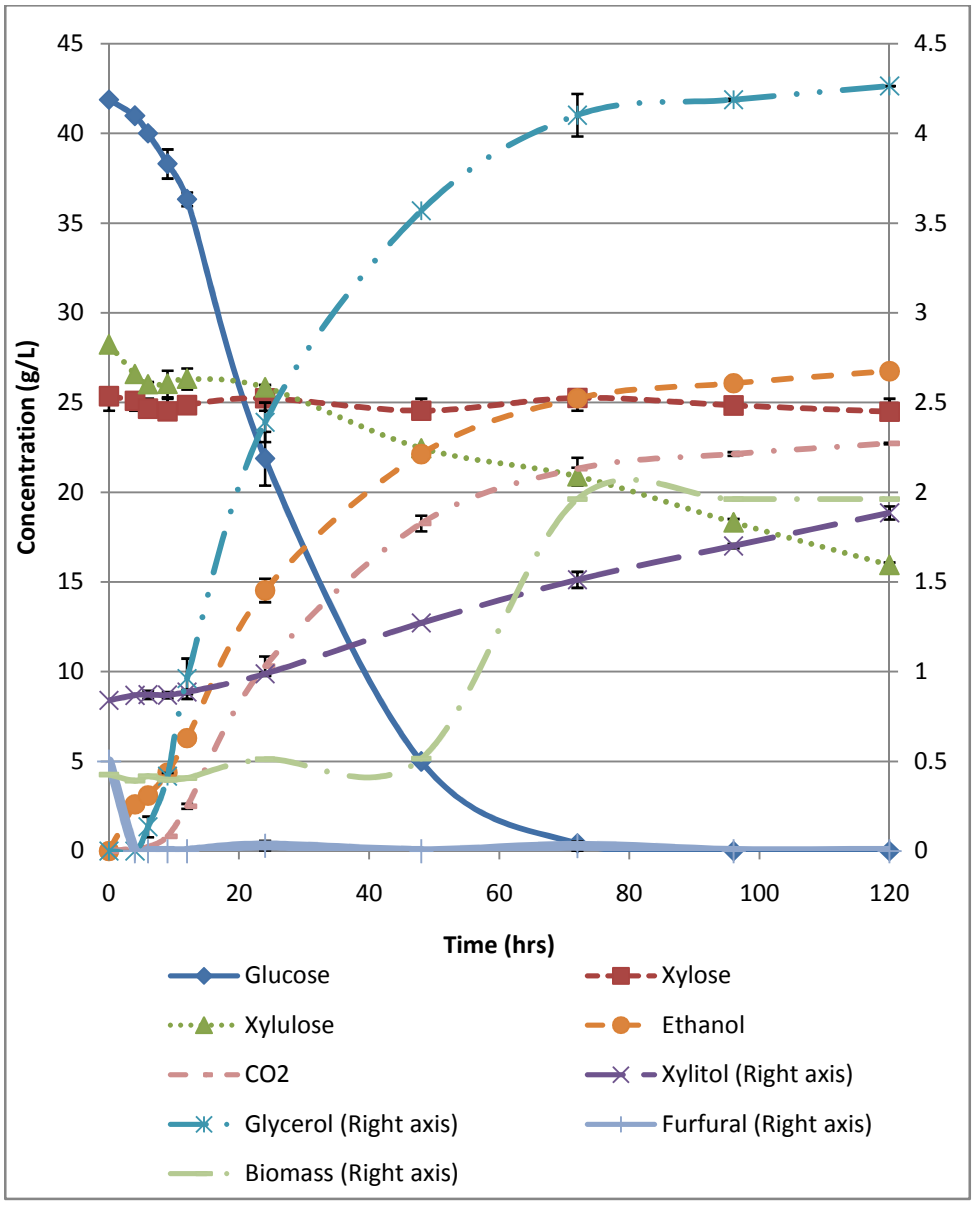

Figure 4.6.Batch fermentation by adapted *Schizosaccharomyces pombe* (0.5 g/L initial furfural concentration)

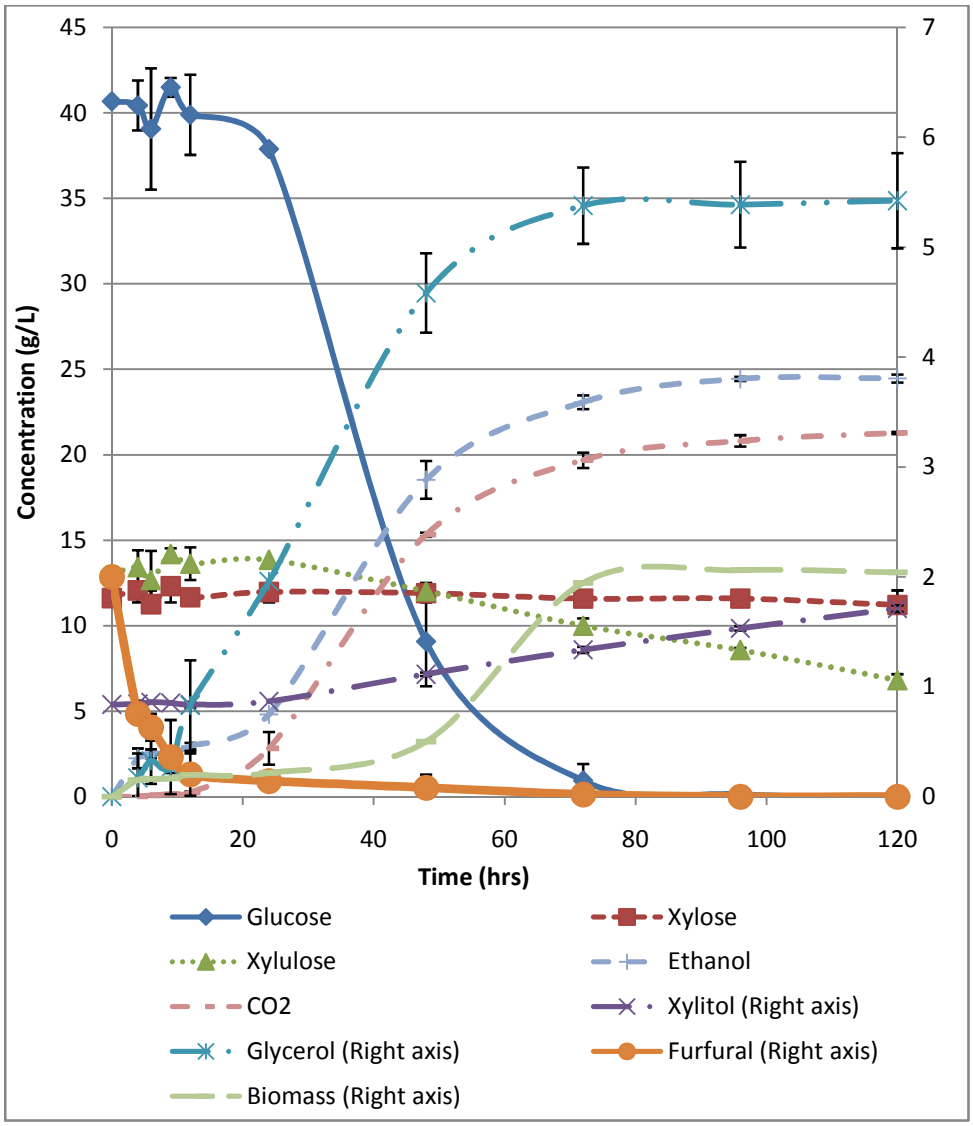

Figure 4.7. Batch fermentation by adapted *Schizosaccharomyces pombe* (2 g/L initial furfural concentration)

Due to the difference in amount of total sugars in the medium, xylulose utilization (% utilized) was used to compare the effect of furfural on fermentation by *S.pombe*. The Fig.4.9 suggests that absence of furfural resulted in highest xylulose utilization in 120 hrs.

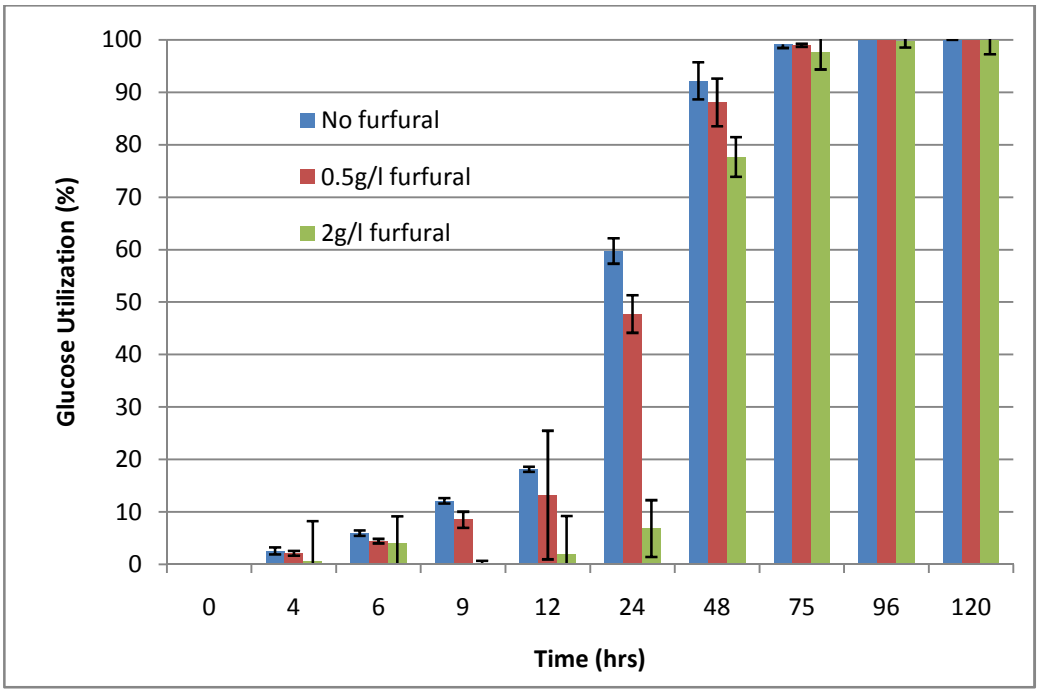

Figure 4.8. Comparison of glucose utilization by adapted *Schizosaccharomyces pombe* in presence of furfural

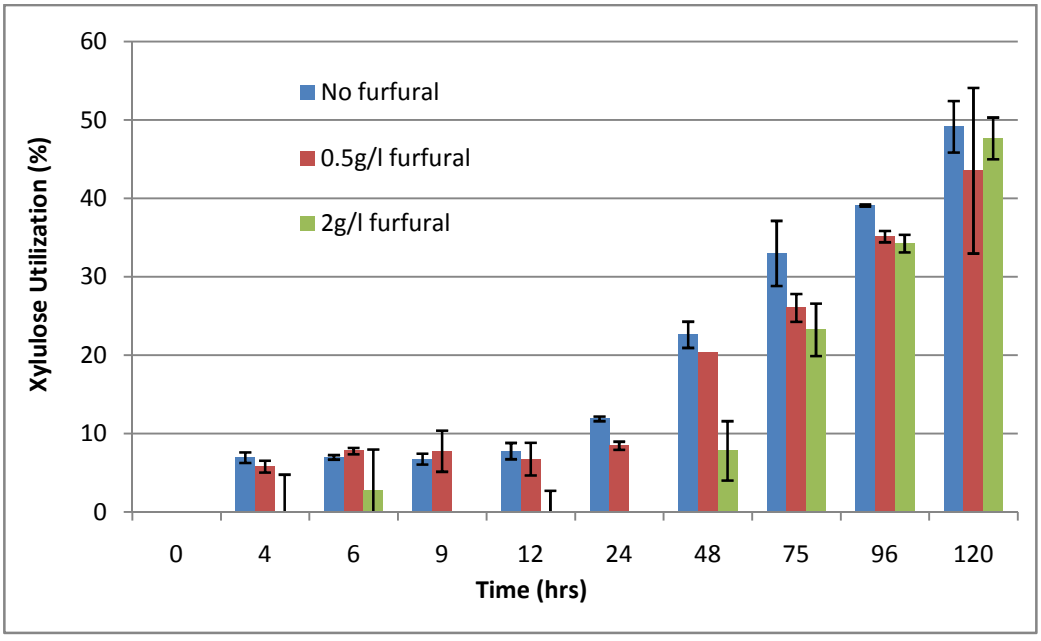

Figure 4.9. Comparison of xylulose utilization by adapted *Schizosaccharomyces pombe* in presence of furfural

Flux balance analysis

The reaction set from the iND750 metabolic model of *Saccharomyces cerevisiae* was used and additional reactions for xylulose and furfural metabolism were added (Table 4.1).In the batch fermentation experiments of *Saccharomyces cerevisiae*, all the furfural was utilized within 4hrs. An exact uptake rate could not be calculated due to insufficient experimental data. A furfural uptake rate of 0.6 g/hr. g of biomass was observed by Taherzadeh et al., 1999 for *Saccharomyces cerevisiae* in anaerobic batch cultures. This uptake rate determined by Taherzadeh et al., 1999 was used in the flux balance model. Data from the exponential stage of growth in batch processes is used to determine uptake rates for use in flux balance models. As the detoxification of furfural was already complete before the start of exponential stage in batch growth, we verified the possibility of detoxification for uptake rates observed under exponential stage in the absence of furfural. The model output rates of products suggested that, to detoxify furfural under normal metabolic functioning would reduce the specific growth rate about 100 folds to 0.00043 g/hr. g of biomass from 0.048 g/hr. g of biomass.

Batch fermentations with higher inoculum concentration

The high inoculum dosage (1:9 inoculum to sugar) increased the rate of glucose consumption in *Saccharomyces cerevisiae* with almost all the glucose being consumed by 24 hrs (Fig.4.10)compared to lower inoculum dosage (1:45) where glucose was consumed in 48 hrs (Fig. 2.8). A total of 55.4% of initial xylulose was consumed at the end of 120 hrs. Also the rate of utilization of sugars is higher from 0.072 g of xylulose/hr to 0.124 g of xylulose/hr and 0.68 g of glucose/hr to 1.54 g of glucose/hr.

Higher inoculum concentration did not increase xylulose utilization in *S.pombe* (Fig.4.11). Comparison of different strains under different conditions shows that the original *S.cerevisiae* has the highest xylulose utilization of 53.1% and 55.4% when a higher inoculum concentration was used (Fig.4.12).

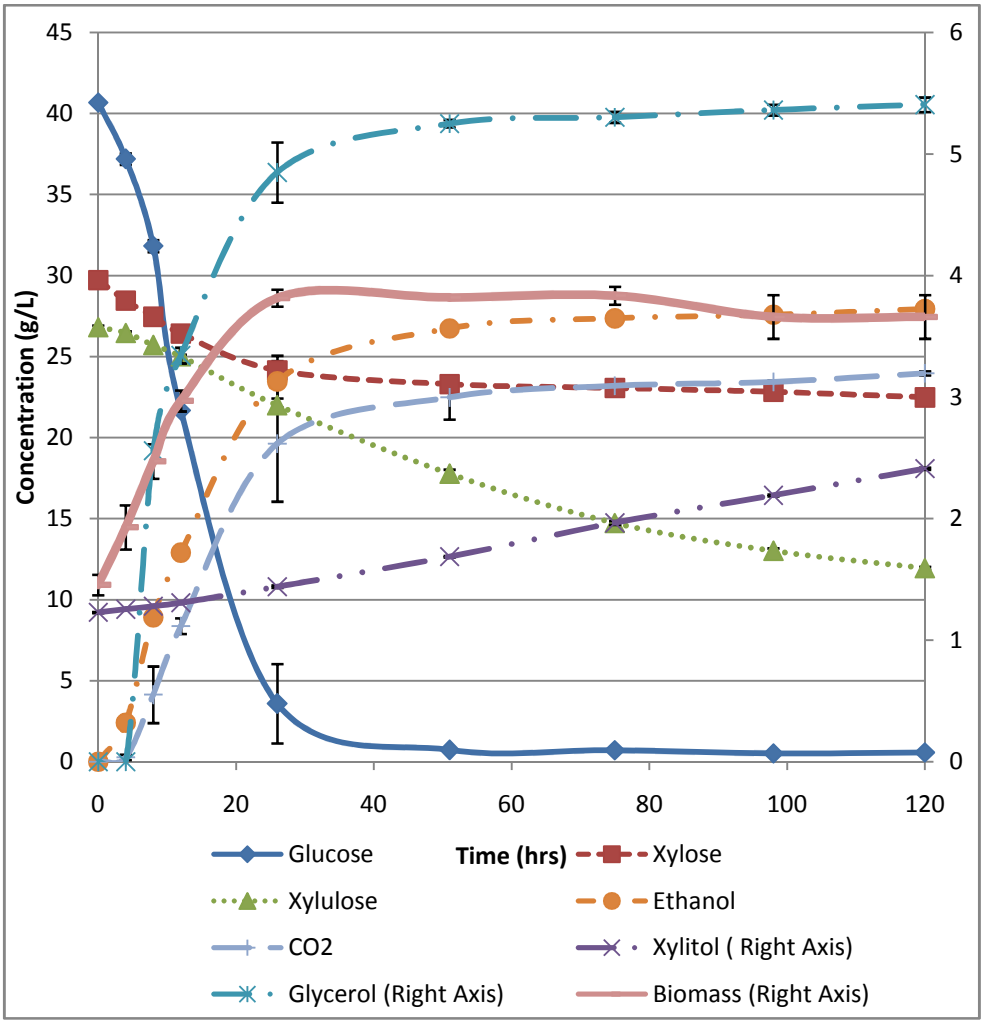

Figure 4.10. High inoculum batch fermentation by *Saccharomyces cerevisiae* in hemicellulose medium

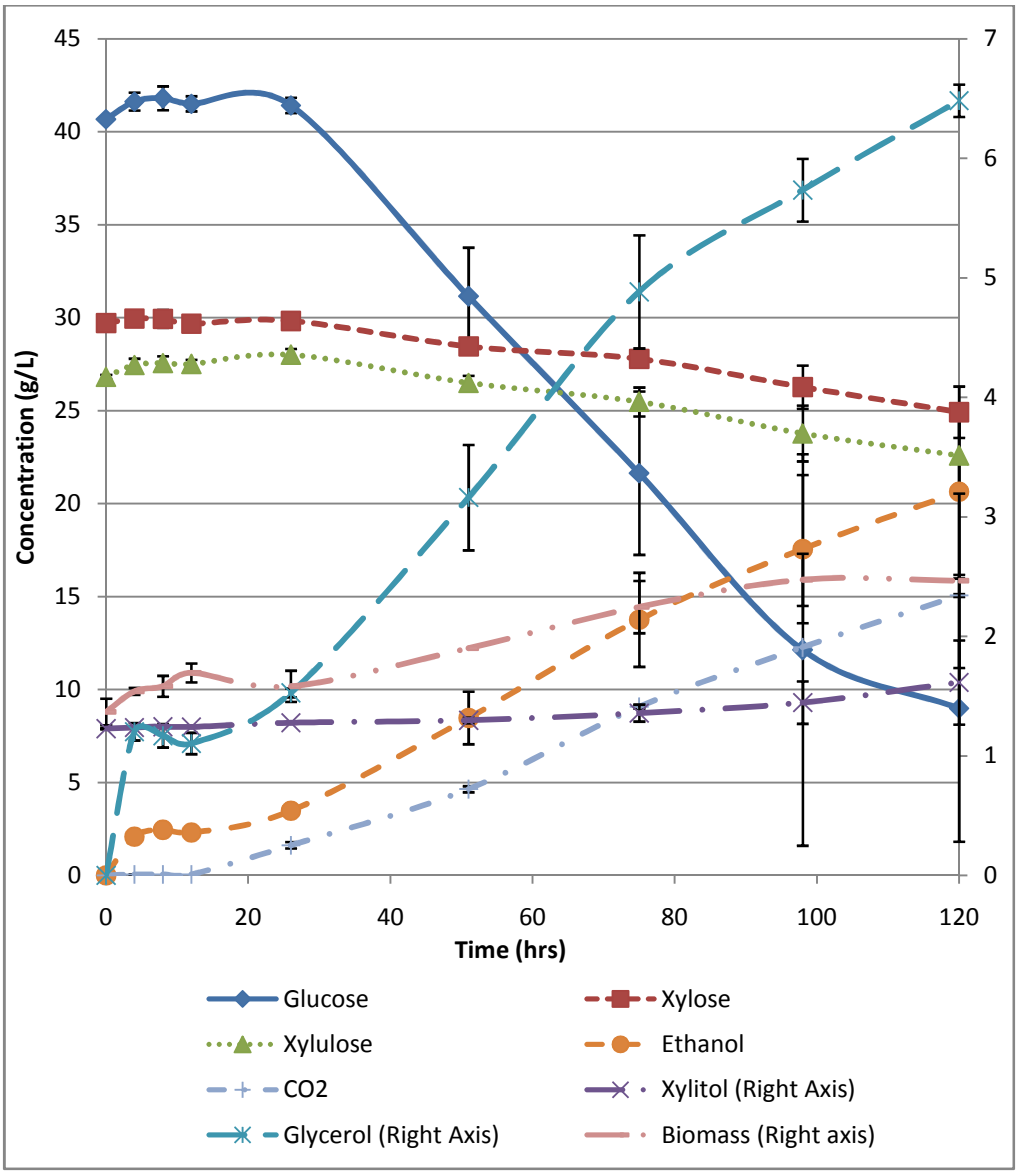

Figure 4.11. High inoculum batch fermentation by *Schizosaccharomyces pombe* in hemicellulose medium

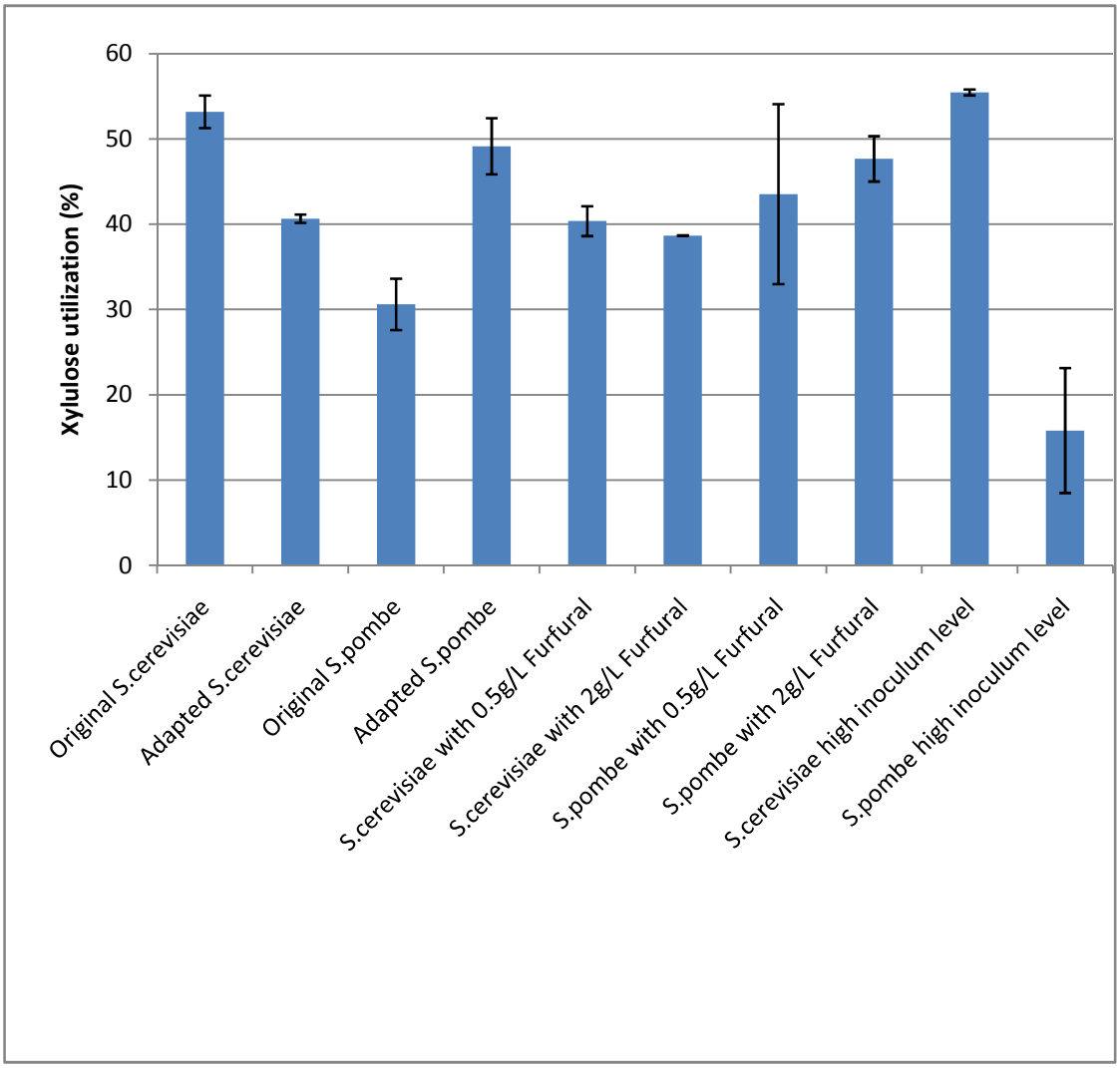

Figure 4.12. Comparison of xylulose utilization

Conclusion

Effect of furfural, an inhibitor formed from xylose due to pretreatment was studied on the fermentation of *S.cerevisiae* and *S.pombe* of the hemicellulose media. Three different concentrations of furfural were used in the initial medium. It was determined

that 0.5 g/L of furfural gave the highest ethanol yield of 0.375Cmoles/Cmoles of total sugar and lowest glycerol yield of 0.041Cmoles/Cmoles of total sugar. In *Schizosaccharomyces pombe*, absence of any furfural in the medium had highest xylulose utilization of 51.84% over 120 hrs. Higher inoculum concentrations did not improve xylulose utilization in both the yeast strains. A flux balance model was used to verify if the detoxification of furfural by *Saccharomyces cerevisiae* can be done under normal metabolic activity. The model output rates of products suggested that, detoxification of furfural under normal metabolic functioning would reduce the specific growth rate to 0.00043 g/hr. g of biomass from 0.048 g/hr. g of biomass. The effect of high inoculum on the fermentation of hemicellulose was studied using high inoculum to sugar ratio. Comparison of different strains under different conditions shows that the original *S.cerevisiae* has the highest xylulose utilization of 53.1% and 55.4% when a higher inoculum concentration was used.

Chapter 5

Overall Conclusions

Findings and Contributions of this research

Adaptation resulted in marginally better utilization of xylulose by the strains, isomerization and utilization of xylose as xylulose was found to be a feasible solution for fermentation of xylan fraction present predominantly in the hemicellulose hydrolyzate. In the experiments conducted using a synthetic hydrolyzate like media, xylose utilization as such was prevented and as a result xylitol production was very low. Although xylitol may have useful application as an artificial sweetener, xylitol production during lignocellulosic ethanol production results in loss of valuable carbon which can in turn be directed towards ethanol production. Yields of up to 0.41 Carbon mole of ethanol per Carbon mole of total sugars was obtained from the co-fermentation of the glucose and xylulose fraction from the batch fermentations performed over 120hrs. This is comparable to the yields obtained by Chandrakant and Bisaria (2000). Xylulose utilization and furfural metabolism were added to the iND750 model of *Saccharomyces cerevisiae* metabolism. Experimentally observed uptake rates were used as constraints in the model and the output rates simulated in the model were comparable to the experimentally observed output rates. Batch fermentations with furfural in the medium resulted in delayed utilization of sugars as observed by other researchers (Horvath et al., 2003). Higher inoculum to sugar ratio resulted in higher rates of utilization.

Future directions

Future research is required in many areas for hemicellulose fermentation to be successful at industrial scale. This includes developing better isomerization technology, with improved systems for efficient isomerization of xylose to xylulose with high xylulose: xylose ratio at low enzyme loading to reduce the cost of isomerization. Genetically engineered strains can be employed for better utilization of hemicelluloses. Research especially directed towards improved co-metabolism of xylulose and glucose by strategies such as increased expression of enzymes in pentose phosphate pathway. Flux balance models can be used to analyze the effects of gene additions/deletions that can improve the co-metabolism of xylulose along with glucose and these strategies can be experimentally studied. Also, better strategies to detoxify inhibitors or to remove the inhibitors formed during pretreatment of biomass are needed. Further, research on identifying consolidated bio-processing capable microbial strains can lead to technologies that are both cost effective and will have higher efficiency.

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