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Examination of different pretreatment and saccharification methods for biobutanol production in SSF process

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EXAMINATION OF DIFFERENT PRETREATMENT AND SACCHARIFICATION METHODS FOR BIOBUTANOL PRODUCTION IN SSF PROCESS

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Chumangalah Thirmal

Abstract

Examination of different pretreatment and saccharification methods for biobutanol production in SSF process

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2010

The objective of this thesis was to examine different pretreatment and saccharification processes of the agriculture residue (i.e., wheat straw) for enhanced production of biobutanol. The purpose was to define the best conditions to obtain maximum sugar yield during the saccharification and butanol yield during the simultaneous saccharification and fermentation (SSF).

Three different pretreatment methods for the wheat straws were examined in the present work in comparison with no chemical pretreatment as a reference. This included water, acidic, and alkaline pretreatment. For all cases, physical pretreatment represented by 1 mm size reduction of the straws was applied prior to each pretreatment. Results showed that 16.91 g/L glucose concentration and 100% glucose yield were produced from saccharification with just the physical pretreatment (i.e., no chemical pretreatment). This represented ~5-20 % lower sugar release in saccharification compared to the other three pretreatment processes. Saccharification with acid pretreatment obtained the highest sugar concentrations, which were 18.77 g/L glucose and 12.19 g/L xylose.

Water pretreatment with SSF was compared with SSF alone (i.e., no chemical pretreatment with SSF). Both processes converted more than 10 % of wheat straw into butanol product. This was 2% higher than previous studies. The results illustrated that SSF with no chemical pretreatment obtained 2.61 g/L butanol. Kinetic model was developed for both processes to determine concentration profile of butanol.

The SSF with no chemical pretreatment obtained 1.21% root mean square error in comparison with the kinetic model. Similarly, SSF with water pretreatment obtained 0.83% root mean square error.

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Finally, I would like to thank my parents for providing me with continuous support and guidance to become a better individual.

Dedication

Life is like a trend of water waves with accomplishments and struggles. It can only move forward like a river in a guided pathway. Positive pathways are made possible with good family and good friendship. Therefore, I really would like to dedicate this thesis to my parents, my brother, and my friends particularly Ruston Bedasie and Erlita Mastan. They have made sure that my struggles are fewer burdens on me but my accomplishments are more blessings of my life.

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adj	adjusted
β	β-glucosidase
B	cellobiose
C	cellulase (or cellulose during the study of kinetics)
CMM	cooked meat medium
exp't	experiment
G	glucose
HMF	hydroxymethylfurfural
k_1	Combination of constant including specific rate for r_1 (h^{-1})
k_2	Combination of constant including specific rate for r_2 (g/L·h)
K_m	Michaelis constant (g/L)
K_{1B}	Inhibition constant of cellulase by cellobiose (g/L)
K_{1G}	Inhibition constant of cellulase by glucose (g/L)
K_{2G}	Inhibition constant of β-glucosidase by glucose (g/L)
K_p	Inhibition constant of cellulase by butanol (L/g)
K_3	monod constant of glucose for bacteria growth
K_i	constant of cell growth inhibition by glucose
k_d	specific rate of cell death
m	specific rate of glucose consumption for maintenance energy
mM	milimolar solution
MEA	monoethanolamine
ODE	Ordinary differential equation
P	butanol
SSF	simultaneous saccharification and fermentation
NaOH	sodium hydroxide
sol'n	solution
H_2SO_4	sulfuric acid
H_2O	water
r_1	Volumetric rate of cellulose utilization (g/L·h)
r_2	Volumetric rate of cellobiose utilization (g/L·h)
r_3	glucose consumption rate by the bacteria during SSF
r_4	Volumetric rate of glucose utilization (g/L·h)
with/out	with or without xylanase
with	with xylanase
without	without xylanase
X	xylanase
Y_x	average yield coefficient of cell mass on glucose

I HAVE A DREAM THAT WE WILL ACHIEVE A GREENER ENVIRONMENT IN THE NEAR FUTURE!

1 Introduction

This thesis examines different pretreatment, saccharification, and fermentation processes for producing biobutanol from agriculture residue. The focus on biologically produced butanol has increased due to the growing demand for replacing fossil fuels with biofuels. Butanol can be used alone or can be mixed with fossil fuels (Lee et al., 2008b). Bioutanol is an ideal fuel replacement because abundant supply of agricultural residue is applied. Also butanol is less volatile compared to gasoline and ethanol, which in result emits less pollutant.

1.1 Problem statement

Attaining butanol from biomass in an inexpensive process without generating air pollution or inhibitors is a complex chemical engineering problem because optimizing the yield is difficult. Typical processes for developing butanol from biomass such as acid pretreatment with enzymatic hydrolysis is expensive and generates air pollution. Pretreatment processes develop inhibitors that suppress butanol fermentation with 33% of the total cost for butanol production (Perez et al., 2008). During large scale butanol production, pretreatment catalysts such as sulfuric acid are hazardous and cause air pollution that lead to acid rain (Hill, 2010). Inhibitors such as hydroxymethylfurfural (HMF) and furfural are promoted by sulfuric acid through acid degradation reactions (Kootstra et al., 2009). Agricultural waste is used as a low cost biomass, but it has a limited availability of polysaccharides.

1.2 Objective

The objective of this thesis was to examine different pretreatment, saccharification, and fermentation processes to increase the yield. Sugar yield from the conversion of all polysaccharides available in the biomass into monosaccharides. Biobutanol yield from the conversion of biomass into the fuel product during fermentation. The different pretreatment methods examined will be economically and environmentally friendly. Kinetic model will be derived in order to predict the production profile of butanol. This model will consider several parameters that include the effect of biomass concentration, type of pretreatment, and saccharification temperature during simultaneous saccharification and fermentation (SSF) process.

1.3 Benefits of bioenergy

Recently, the British Petroleum's offshore oil spill has increased the need to replace oil with other abundant resources due to loss of natural resource (i.e. gasoline). Biobutanol has been demonstrated to effectively replace petroleum in automobile engines without any necessary modification (Lee et al., 2008b). It can also be mixed in gasoline easily because it is less hydroscopic compared to ethanol (Durre, 2007). This will be a resolution for great oil spills in water. Butanol replacement of gasoline also does not require any modification of the existing gasoline pipelines.

Butanol production generates less air pollution than petroleum generation. Emission of sulfur and nitrogen oxide pollutants is reduced in the mixture of gasoline and butanol compared to gasoline alone (Bruno et al., 2009). Butanol is more advantageous to use in gasoline, compared to ethanol since its energy content is higher, and it has a lower miscibility with water, higher octane number, and a lower volatility (Durre, 2007). Fuel additives require high octane numbers such as in butanol. Table 1 illustrates that butanol has better characteristics than ethanol for bioenergy.

Previously, butanol was only produced through chemical synthesis. Now, scientists have isolated butanol producing bacterial strains (Ezeji et al., 2007a). These bacterial strains depend on a carbon source for the production of butanol. Canada and United States have abundant sources of biomass that provides a high source of carbon. Biomass such as agricultural waste could be used as the carbon source for butanol fermentation. This carbon source is applied during butanol fermentation.

Agricultural waste consumed for butanol production will reduce build up of wastes as well. More people could use automobiles without worrying about its air pollution which requires more manufacturing of vehicles. Economic benefits from higher vehicle sales, larger travel distances, and more frequent trips will generate more jobs.

Table 1
Comparison of butanol and ethanol

Butanol	Ethanol
Replace gasoline	Only mix with gasoline
No modifications of engines	Require some modifications
No modifications of pipelines	Require some modifications
More carbons, covalent bonds	Less carbon, less covalent bonds
Low vapour pressure	High vapour pressure
Less volatile	More volatile
Less pollution	More pollution
Do not mix in water	Slightly hydrophilic
Nitrogen is used	Oxygen is used
Anaerobic fermentation	Aerobic fermentation

1.4 What is butanol?

Butanol is a colorless liquid that is miscible with organic solvents (Lee et al., 2008). Molecular formula for butanol is C_4H_9OH and the molecular weight is 74.12 g/mol. This chemical can cause irritant effect on mucous membranes and narcotic effect at high concentrations. Butanol is less corrosive and less hydroscopic.

Butanol can be used as diluent for brake fluid, as solvent for certain pharmaceutical products, as direct replacement of gasoline, fuel additive, etc. Half of butanol production is used as butyl acrylate and methacrylate esters. Ten to twelve billion pounds of butanol is produced per year with the cost of 7.0-8.4 billion dollars. The butanol market is expected to increase 3% per year.

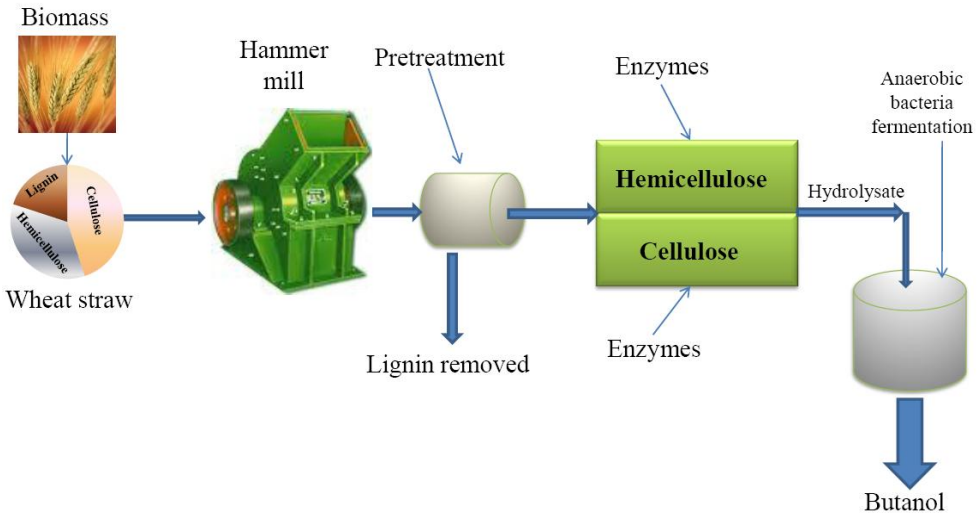
Butanol can be synthesized chemically through oxo synthesis, reppe synthesis, or crotonaldehyde hydrogenation synthesis (Lee et al., 2008b). It can also be produced biochemically with the aid of microorganisms. Fermentation process is discussed later on. There are three different methods to produce butanol through chemical methods. The first is oxo synthesis, which requires hydroformylation of carbon

monoxide and hydrogen to carbon-carbon double bond. Cobalt, rhodium, and rhuthenium are examples of catalysts used in oxo synthesis. It requires two steps where butanol is produced in second step using two aldehydes from first step. The second, reppe synthesis, is where propylene, carbon monoxide, and water react with the application of a catalyst. This requires only one step to produce butanol at low temperature and pressure. However, it is not cost effective. The final chemical synthesis method is crotonaldehyde hydrogenation, which requires three steps: aldol condensation is the first step, dehydration is the second step, and hydrogenation is the third step. All of these methods use petroleum or chemicals which will be harmful to environment and expensive. Hence, it is better to synthesize butanol using biological methods as follows.

1.5 Biological production of butanol

An overview of butanol production through pretreatment, saccharification, and fermentation is illustrated in Figure 1. Here wheat straw is applied as the biomass. First the wheat straw is grinded through hammer mill using a sieve screen. Then pretreatment is applied to remove lignin. During saccharification, enzymes are applied to break down hemicellulose and cellulose into simple sugars. These simple sugars are applied in anaerobic butanol fermentation to produce butanol.

Figure 1
Butanol production through biomass pretreatment, saccharification, and bacteria fermentation



1.5.1 Wheat straw as the biomass

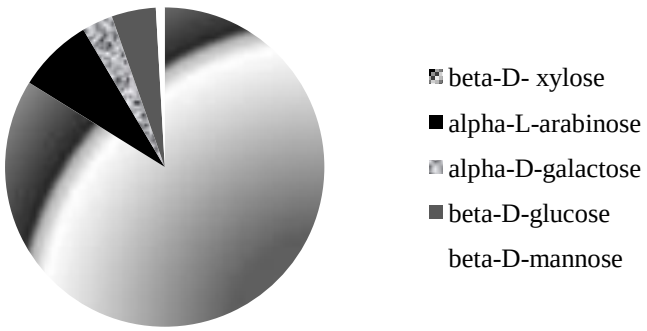
Wheat straw from agricultural waste is used to examine butanol fermentation in this thesis because of its abundance in Canada. Table 2 lists the polysaccharide composition of wheat straw. Wheat straw contains a high source of polysaccharides. Wheat straw is composed of lignin, hemicellulose, and cellulose. Different studies provide different compositions of wheat straw because each wheat straw grows in a unique way at different locations.

Table 2
Polysaccharide composition of wheat straw

Cellulose %	Hemicellulose %	Lignin %	Reference
35-40	20-30	20	Sun et al. (1996)
35-40	30-35	<20	Qureshi et al. (2007)
30.2	22.3	17	National Renewable Energy Laboratory
50	30	15	Zugenmaier (2008)

Cellulose is mainly composed of linear chain of 1,4-β-glycosidic bonds or glucose monomers which is (illustrated in Figure 9, see Literature Review). However, hemicellulose is a complex polysaccharide and composed of mainly xylose molecules (O'Sullivan, 1997). However, small section of hemicellulose is composed of arabinose, galactose, mannose, and glucose (Girio et al., 2010). Composition of hemicellulose is illustrated in Figure 2. The complexity of hemicelluloses creates difficulty for enzymes to hydrolyse simple sugars during saccharification.

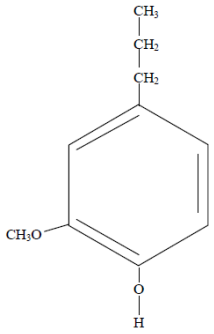
Figure 2
Components of hemicellulose



1.5.2 Pretreatment process

Figure 3 illustrates the structure of lignin. Pretreatment is required to remove the lignin and some of the hemicellulose (Jones & Woods, 1986). Lignin is not important for butanol production because it is not composed of any sugar molecules. Polysaccharides are surrounded by lignin which is why lignin is removed or separated from the polysaccharides. Removal of water molecules from sugar molecules within the lignin will create an irreversible aromatic structure of lignin. This hydrophobic molecule protects the hydrophilic polysaccharide components in the wheat straw from water. The polysaccharides, hemicellulose and celluloses are hydrophilic. There are also other types of pretreatment processes with the application of chemical catalysts such as sulphuric acid. This catalyst is dissolved in water. Polysaccharides separated through pretreatment process will be dissolved in the water. Physical pretreatment such as cutting the wheat straw can also remove lignin away from biomass (Talebnia et al., 2010).

Figure 3
Structure of lignin monomer



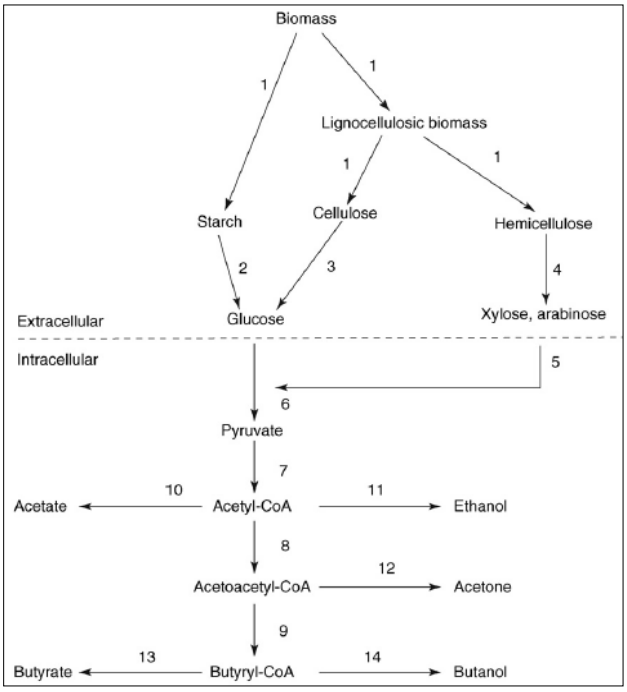
1.5.3 Saccharification process

Today, the competitive market provides several choices of enzymes. Enzymes are used as catalysts for saccharification of polysaccharides into sugar monomers. More than one enzyme is required to break down one type of polysaccharide such as cellulose. Enzymes are composed of protein molecules and they depend on temperature, pH, and time. Best temperature, pH, and time conditions of saccharification will be determined in this thesis. Effect of different enzymes will also be demonstrated in this thesis.

1.5.4 Fermentation process

Glycolic pathway of butanol producing bacteria is illustrated in Figure 4. Here biomass is converted to simple sugars using pretreatment and saccharification processes before being applied to fermentation. Then the simple sugars are converted into energy and products by the bacteria during anaerobic fermentation. Acetone is the highest product and butanol is the second highest. This thesis study will mainly focus on butanol production. There are also several choices of butanol producing bacteria and one of the best strain will be examined in this thesis.

Figure 4
Glycolic pathway of butanol producing bacteria(from Ezeji et al., 2007)



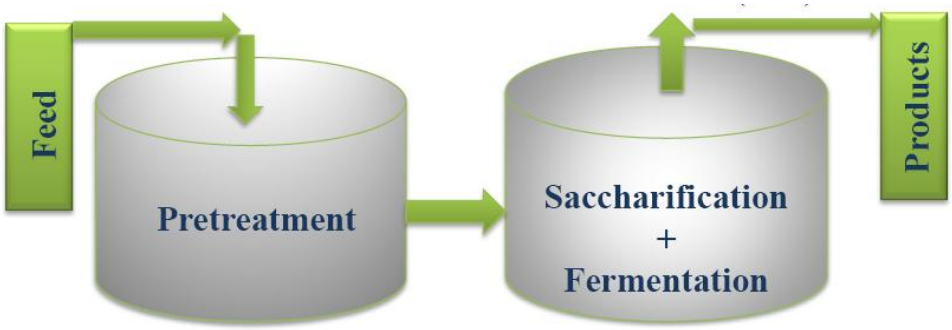
1.5.5 Simultaneous saccharification and fermetnation

Butanol fermentation can be performed in two different ways. The first method is called separate hydrolysis and fermentation (SHF), which is illustrated in Figure 5. Here pretreatment, saccharification, and fermentation are performed separately in series. This requires three different reactors. During simultaneous saccharification and fermentation (SSF), pretreatment is performed separately but saccharification and fermentation are performed in the same reactor. This requires two reactors as

illustrated in Figure 6. The second method is called simultaneous saccharification and fermentation (SSF). SSF reduced the number of reactors, space, time, energy, and costs for butanol production. Here, the feed is composed of wheat straw, enzymes, and bacteria. The products are composed of butanol and other fermentative products.

Figure 5
Separate hydrolysis and fermentation (SHF)

Figure 6
Simultaneous saccharification and fermentation (SSF)



2 Literature Review

In biological synthesis, sugars extracted from biomass such as agricultural waste, can be fermented to produce butanol. Agricultural waste must be pretreated before it is suitable for sugar extraction. Following this stage, enzymatic hydrolysis is employed, before performing fermentation using microorganisms such as *Clostridium* bacteria. There are several methods available to produce butanol which will be discussed briefly in this chapter.

2.1 Pretreatment of wheat straw

Wheat straw was commonly used as the carbon source for butanol fermentation in several studies. This was also referred to as the substrate or biomass. The lignin in wheat straw must be removed because cellulose and hemicellulose were trapped underneath the lignin. Chemical or physical pretreatment methods were used to remove lignin. Some of the effective chemical methods were discussed in this section. Most effective pretreatment methods will also break down hemicellulose polysaccharide into sugar monomers. However, enzymes were still necessary to break down most of the cellulose and hemicellulose into sugar monomers. Sugar monomers such as glucose, xylose, arabinose, and galactose were commonly derived from biomass such as wheat straw. Some sugars are more effective substrate than others to produce butanol, which will be elaborated next.

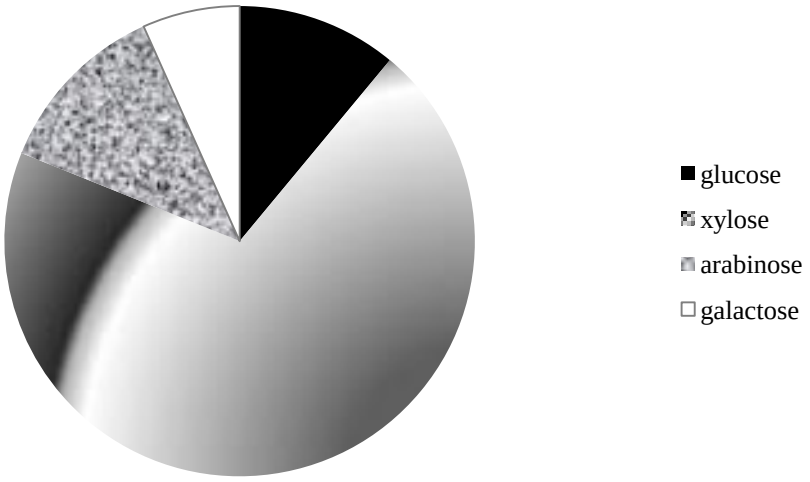
Dilute sulfuric acid pretreatment removed lignin and broke down hemicelluloses into pentose sugars without any application of enzymes (Qureshi, Saha, & Cotta, 2008). Alkali pretreatment on the hand could not break down the hemicelluloses into monosaccharides. In fact, alkali pretreatment solubilised hemicellulose (Ezeji, Dien, Cotta, & Blaschek, 2007). Residues from pretreated corn fibre with acid or alkaline solution developed inhibitors against fermentation which required resins to remove these inhibitors. This was one good reason to avoid corn fibre. Wheat straw did not require intensive removal process of inhibitors as compared to the removal process in corn fiber (Ebener, Qureshi, & Blaschek, 2003).

2.1.1 Acid pretreatment

Qureshi, Saha, & Cotta (2008) Part I used dilute sulfuric acid to pretreat wheat straw. They grounded wheat straw into small particles using a hammer mill before pretreatment. They used 1.0% (v/v) dilute

sulfuric acid mixed in distilled water to pretreat 8.6% (w/v) biomass concentration. Pretreatment was processed by autoclaving the wheat straw mixture at 121^{o}C for 1 hour. The pH was adjusted to 5.0 to provide optimum conditions for butanol fermentation. These samples from clear liquid were used to measure sugar concentrations in HPLC. One advantage of using acid pretreatment was that it did not require any enzymes to hydrolyse most of hemicellulose. These results after acidic pretreatment were summarized in Figure 7**.** Here 25.4 g/L of total sugars were achieved. This was a surprise because enzymes were important to extract sugar monomers. These sugars were mostly composed of xylose. This implied that saccharification of xylose did not depend on enzymes. However, hydrolysis of cellulose definitely depended on enzymes.

Figure 7
Sugar concentrations after acidic pretreatment: results obtained from Qureshi, Saha, & Cotta (2008 Part I)



The wheat straw hydrolysate after pretreatment was applied in butanol fermentation. This successfully produced more than 6.05 g/L of butanol at the completion of fermentation. The concentration of the butanol was reduced compared to fermentation with the application of wheat straw from pretreatment and saccharification. Hence, these authors proved that some butanol concentration could be achieved without any application of enzymes.

2.1.2 Acid and water pretreatment

Balleseros et al. (2006) examined sulfuric acid pretreatment by applying wheat straw as the substrate. They fractionated biomass successfully to improve saccharification. Acid catalysts such as SO_2 or H_2SO_4 were demonstrated to reduce temperature and residence time required for fractionation and saccharification (Ramos, 2003). They used 20% biomass concentration with 0.9% (w/v) sulfuric acid. The temperature and residence time of this acidic pretreatment were summarized in Table 3. Steam explosion was applied to pretreat wheat straw at very high temperatures. Here, direct saturated steam was used at 4.12 MPa (Negro, Manzanares, Ballesteros, Oliva, Cabanas, & Ballesteros, 2003). They also examined pretreatment without any catalysts, which could also be called water pretreatment. The temperature and residence time for water pretreatment was also summarized in Table 3.

Wet material from the steam explosion was recovered in a cyclone and then cooled to required temperature for saccharification. The wet material was filtered to separate water insoluble solids and filtrate. About 42-60% of solids were recovered after steam explosion. More solids were recovered at lower temperature and residence time of steam explosion. Acid treatment further reduced solid recovery compared to water pretreatment. Maximum amount of cellulose content was recovered after treating with acid catalyst and steam explosion for 10 minutes at 180ºC. These results were illustrated in Table 3. Steam explosion above 180ºC produced less cellulose because extreme temperature harmed the cellulose fibre which lost linear chain of glucose molecules (Alfani, Gallifuoco, Saporosi, Spera & Cantarella, 2000). Acid catalyst with steam explosion for 10 minutes at 180ºC extracted highest amount of polysaccharides. However, sulfuric acid pretreatment at 160ºC provided the maximum amount of hemicellulose. High content of hemicellulose was also recovered by water pretreatment with the application of steam explosion.

Although acid pretreatment removed more cellulose compared to water pretreatment, water pretreatment was the best pretreatment. High content of cellulose was achieved through acid pretreatment. However, water pretreatment produced very small amount of inhibitors. The inhibitors, furfural and hydroxymethylfurfural (HMF), were formed during the pretreatment. Table 3 illustrated that higher the temperature the more HMF and furfural were formed. Steam explosion using water pretreatment at all temperatures produced very low HMF and furfural. Acid pretreatment at higher temperature increased the amount of inhibitors produced. Hence, acidic pretreatment always produced higher inhibitors compared to water pretreatment.

Table 3
Reduction of inhibitors from steam explosion: results obtained from Balleseros, Negro, Oliva, Cabanas, Manzanares, & Ballesteros (2006)

Pretreatment method	T(°C)	Time (min)	Lignin %	Cellulose %	Hemicellulose %	Glucose recovery %	pH	HMF	Furfural
0.9 % (w/w) H_2SO_4	160	20	25.4	50.3	15.0	9.5	1.8	0.03	0.03
	170	5	25.5	54.1	8.6	12.3	1.8	0.04	0.02
		10	27.0	58.5	5.9	10.9	1.7	0.05	0.02
	180	5	27.5	62.7	5.2	14.5	1.9	0.25	0.07
		10	32.6	63.5	1.3	25.1	2.0	0.32	0.07
	190	5	28.3	54.8	1.8	23.4	1.9	0.51	0.08
		10	33.6	55.8	1.0	29.2	1.8	0.75	0.14
	200	5	33.1	55.2	1.2	25.1	2.0	1.51	0.24
Water	170	10	20.8	50	13.6	12.1	3.8	0.01	0.01
	180	10	25.9	60.2	7.5	7.2	3.8	0.03	0.01
	190	10	23.5	59.6	7.6	5.0	3.8	0.06	0.02
	200	10	27.9	61.9	4.5	6.3	3.5	0.04	0.16
No chemical pretreatment and saccharification			15.3	30.2	22.3				

2.1.3 Alkaline pretreatment

Monoethanolamine (MEA) was illustrated to be an excellent catalyst during alkaline pretreatment. Shah et al. (1991) applied different concentrations of MEA at 186°C for 3 hours to pretreat hardwood chips. The pH was adjusted using 0.01M citrate buffer solution prior to saccharification. The results from MEA pretreatment and saccharification were summarized in Table 4. This illustrated that pretreatment with higher composition of MEA removed most lignin without affecting carbohydrates present in the hardwood. High concentrations of MEA did not improve extraction of polysaccharides. MEA was the best pretreatment to remove lignin especially compared to supercritical CO_2-SO_2 pretreatment. During supercritical CO_2-SO_2 pretreatment higher content of polysaccharides was achieved at low temperatures. However, higher lignin was removed at higher temperature. Unfortunately, Table 4 illustrated that MEA pretreatment produced better results compared to supercritical CO_2-SO_2 pretreatment.

2.1.4 Comparison of results from different pretreatment processes

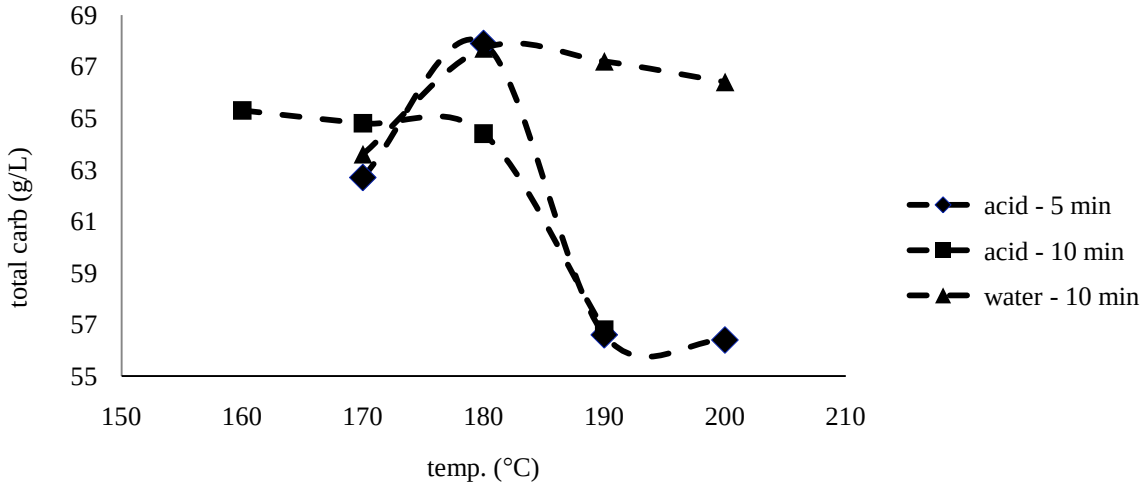
Ballesteros et al. (2006) illustrated that acid pretreatment at very high temperatures successfully separated most of the polysaccharides from lignin. Similarly, water pretreatment at very high temperatures also extracted high content of polysaccharides. Total carbohydrates extracted from both pretreatment processes were compared in Figure 8. Here, the total carbohydrates extracted at different temperatures during pretreatment processes were examined. Total carbohydrates did not exhibit any correlations with temperature of the pretreatment process. The optimum pretreatment temperature was 180°C. The amount of carbohydrates decreased if the pretreatment was processed at temperatures above 180°C. Also acid pretreatment with less time produced best results. However, water pretreatment and acid pretreatment extracted similar amount of total carbohydrates. This implied that water pretreatment was also a very successful procedure to extract carbohydrates away from the lignin in the biomass.

Shah, Song, Lee, & Torget (1991) published higher carbohydrates yields through MEA pretreatment. These yields were achieved through division of total carbohydrates after pretreatment by total carbohydrates available in the biomass prior to pretreatment. High yields were achieved even during pretreatment with dilute MEA. However, this study used hardwood chips as the substrate. In the latter cases, wheat straw was used as the substrate. Supercritical SO_2-CO_2 pretreatment at lower temperatures also removed high amount of polysaccharides. Unfortunately, they were lower compared to MEA pretreatment.

Table 4
Monoethanolamine (MEA) pretreatment: results obtained from Shah, Song, Lee, & Torget (1991)

Pretreatment	Solids recovered %	Lignin removed %	Hexose %	Pentose %	Total Carbohydrates %	ABE solvents	
MEA (%V/V)						g/100g pretreated aspen	g/100g dry aspen
5	66.8	49.8	43.2	14.1	82.3	22.6	15.1
10	64.9	61.5	44	15.4	85.3	23.1	15
20	63.8	76.5	42.3	15.6	83.2	23.2	14.8
30	63.2	79.6	42.8	15.5	83.8	23.7	15
40	62.7	87.9	43.1	16.2	85.2	23.4	14.7
50	61.4	91.2	41.1	17.1	83.6	24	14.7
Supercritical SO_2-CO_2							
130°C, 4h	66	60.8	43.5	14.3	83	23.7	15.6
140°C, 4h	58	84	42.9	11.6	78.3	22.7	13.2

Figure 8
Total carbohydrates removed: results obtained from Ballesteros et al. (2006)



2.1.5 Advantages and disadvantages of pretreatment processes

Pretreatment will remove most of lignin with the suitable catalyst and temperature quickly. Sulfuric acid is an excellent catalyst which successfully removed lignin and some hemicellulose. Polysaccharides are completely hydrolysed into sugar with higher sulphuric acid concentrations. Furfural and HMF are caused by degradation reactions in acidic pretreatment (Kootstra, Beeftink, Scott, & Sanders, 2009). They inhibit butanol production (Baltz, Davies, & Demain, 2010). Furfural is derived from pentose sugars in hemicellulose and HMF are derived from hexose sugars in hemicellulose. These inhibitors must be removed because they suppress fermentation (Nardi et al., 1967). Dilute sulfuric acid pretreatment will cause acid rain in a large scale production (Hill, 2010). Acidic pretreatment is also an expensive procedure. More than 33% of total cost of fermentation is required for acidic pretreatment (Perez et al., 2008). This thesis will examine different parameters of saccharification with acid pretreatment to determine feasible methods.

Water pretreatment on the other hand removes lignin and hemicellulose without any application of catalysts. This reduces costs of catalysts. Enzymes can still access polysaccharides more efficiently after water pretreatment (Schell et al., 1989). Furfural and HMF are eliminated because water pretreatment does not cause any acidic degradation. However, water pretreatment is still expensive because, heat or steam must be provided during pretreatment at high temperatures. Heat costs energy and money. Then cooling process must be applied prior to SHF or SSF which will require additional costs.

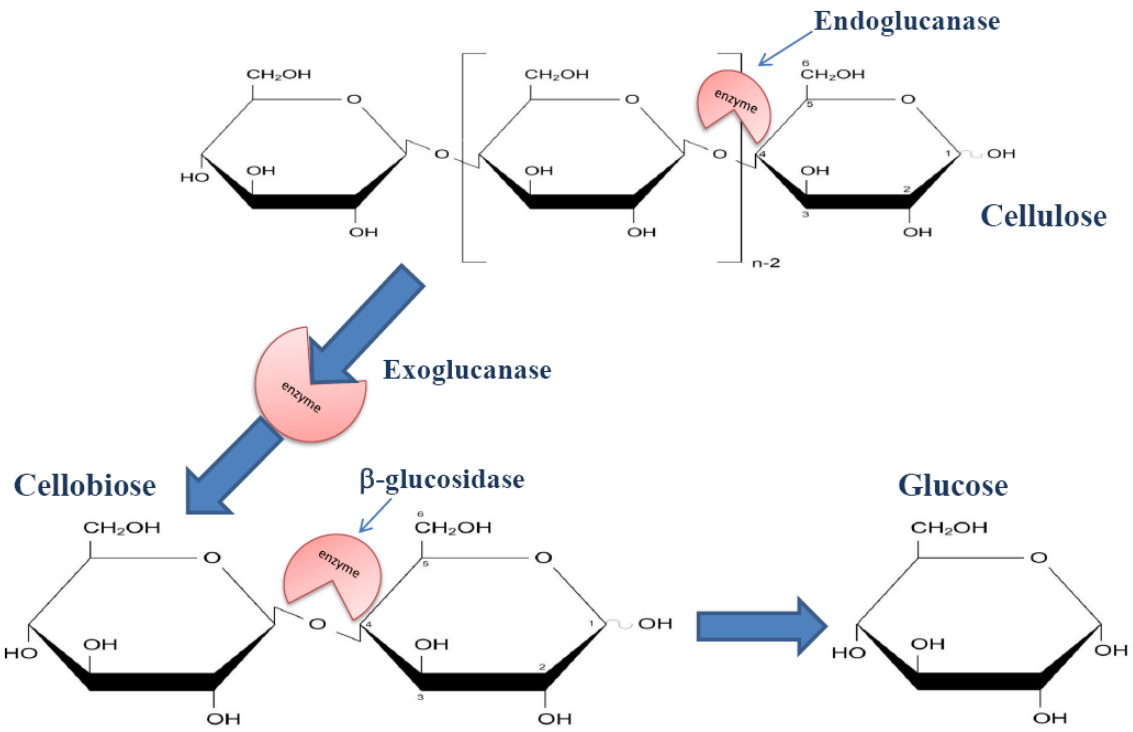
No chemical pretreatment resolves several issues. Here, physical pretreatment alone with saccharification can be effective to produce high amount of sugars (Talebnia, Karakashev, & Angelidaki, 2010). Air pollution was eliminated because there were no hazardous chemicals used. There was no heat applied, which will reduce costs. There will be no requirements for pH adjustments or removal of inhibitors. This process will be the ultimate goal to achieve a greener environment in the near future.

MEA is a weak base which is previously applied in the study by Shah et al. (1991). This is the least hazardous catalyst used in pretreatment processes because it is composed of nitrogen. Nitrogen is an essential part of any life system. MEA is also an organic compound, which causes less harm to living organisms. This provides an excellent opportunity to remove lignin and hemicellulose in a safe manner.

2.2 Saccharification of wheat straw polysaccharides

Cellulose was a linear polymer composed of cellobiose and 1-4 β-D-glucopyranose (Zugenmaier, 2008). This polysaccharide was hydrolysed by non-aggregating and aggregating enzymes. Generally, aggregating enzymes were required for saccharification of crystalline cellulose. Plant cellulose was usually amorphous. Crystalline cellulose was only a synthetic polymer which would not be discussed here. Non-aggregating enzymes were β-1,4-D-glucan glucanohydrolase, 1,4-β-D-glucan cellobiohydrolase, and β-D-glucoside glucohydrolase. These enzymes were commonly known as endoglucanase, exoglucanase, and β-glucosidase (Philippidis, Smith, & Wyman, 1992). Endoglucanase would break the β-1,4-glycosidic bonds in the cellulose polysaccharide. This was called cellobiose which would be released by exoglucanase. Finally, β-glucosidase would break down cellobiose into glucose. These reactions were illustrated in Figure 9. All three reactions would occur synergistically. Sometimes, glucose was produced in first step by endoglucanase while breaking β-1,4-glycosidic bonds into cellobiose.

Figure 9
Saccharification of cellulose into glucose molecules



Hemicellulose was a heterogeneous macromolecule which was a component of plant cell wall. This polysaccharide was composed of pentoses, hexoses, and uronic acids. Examples of pentoses in hemicellulose were β-D-xylose and α-L-arabinose. Hemicellulose was also composed of hexoses such as, β-D-mannose, β-D-glucose, and α-D-galactose. Xylan was the major component of hemicellulose, which was composed of xylose linked with b-1,4-glycosidic bonds. Hence, xylan was well abundant in agricultural biomass (Girio et al., 2010).

Several enzymes were required to hydrolyse xylan. The enzyme β-1,4-endoxylanases would split glycosidic bonds in xylan backbone. Arabinofuranosidase attached to arabinose side-chains and α-glucuronidase separated glucorinic acid side-chains from xylose molecules. Xylosidase hydrolysed xylobiose into xylose. All of these enzymatic activities occurred simultaneously (Gilbert & Hazlewood, 1993).

Each enzyme had optimum temperature and environmental conditions. Different enzymes were most active at slightly different pH conditions. Most of the enzymes required pH of 5.0, which was slightly acidic. This pH was similar to pH found in distilled water (from Ryerson University). Also enzymes were

most active at certain temperatures. Most of the following literatures applied saccharification at 45°C. This was also time-dependant. Completion of enzymatic hydrolysis took several hours and sometimes days. Hydrolysis and pretreatment must be performed separately due to adverse reactions (Qureshi, Saha, & Cotta, 2007; Wade, 1999).

Qureshi, Saha, & Cotta (2008) examined saccharification after acidic pretreatment. The pH was adjusted to 6.5 prior to saccharification. The temperature was maintained at 45°C throughout saccharification. Sufficient amount of sugars were extracted after 72 h of continuous hydrolysis. Another saccharification was examined after acid pretreatment at different pH. Here, the pH was adjusted to 5.0. The results from both saccharification processes were compared in Figure 10. Saccharification at pH 5.0 provided better results than pH of 6.5. Hence pH 5.0 was optimum for saccharification with the same enzymes applied in their study.

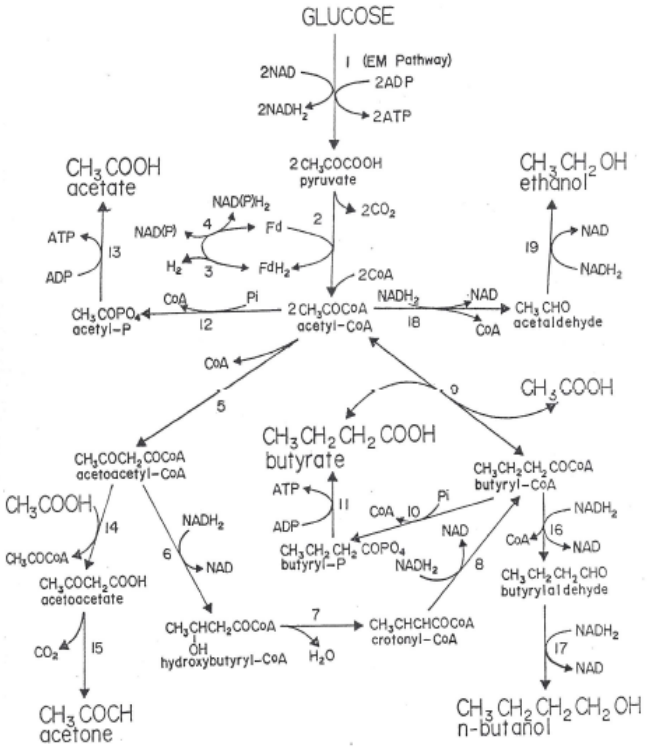
Figure 10
Sugar recovery from WSH at different pH: results obtained from Qureshi, Saha, & Cotta (2008)

Saccharification after acid and water pretreatment at very high temperatures were also examined by (Ballesteros et al., 2006). Here the pH was adjusted to 4.8 but the saccharification was performed at 50°C for 72 h. Highest glucose concentration was recovered after acid pretreatment and saccharification. Glucose recovery after saccharification was illustrated in Table 3.

2.3 Fermentation of butanol

Butanol producing bacteria was applied in fermentation process to produce butanol. *C.beijerinckii* was the best available strain to produce high composition of butanol, which would be elaborated in the following sections. *C.beijerinckii* could only grow under optimum temperature and pH. Figure 11 illustrated that *Clostridia* consume simple sugars such as glucose for their metabolism. Energy was produced in the form of adenosine triphosphate (ATP). The addition of phosphate from adenosine diphosphate (ADP) to ATP required energy. But when the phosphate was released from ATP to ADP, energy was also released. Intermediate products such as butyric acid and acetate were produced. These acid components may decrease the pH. Hence, a strong buffer was required to maintain a constant pH. If the optimum conditions were maintained then final products of acetone, butanol, and ethanol would be produced. Acetone would be the highest concentration and butanol would be the second highest concentration. This thesis would focus on improving butanol production.

Figure 11
Metabolism of glucose by butanol producing bacteria: figure obtained from Huang (1985)



2.3.1 Butanol producing bacteria

There were several species found within the genus *Clostridium*. These bacteria had the properties of gram-positive. They were rod-shaped and capable of creating their own spores. *Clostridium* bacteria were anaerobic, because they could not survive under oxygen. One unique property of *Clostridia* was that they can produce chiral products that were difficult to synthesize through chemical reactions. *Clostridia* were an excellent choice to produce butanol through biochemical reactions. Most common *Clostridia* that were applied in butanol fermentation were *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharobutylicum,* and *Clostridium scharoperbutylacetonicum*. *Clostridium* could be abbreviated as *C.*, for example *Clostridium beijerinckii* could also be called *C.beijerinckii*.

Clostridium commonly produced butanol in two different phases. The first phase was called acidogenic. During this phase, *Clostridia* produced acetate, butyrate, hydrogen, and carbon dioxide. Acidogenic phase usually occured during exponential growth phase of the bacteria. Second phase was called solventogenic. During this phase acetone, butanol, and ethanol were produced. This was commonly known as ABE solvent production. *C.beijerinckii* was unique because, isopropanol could also be produced instead of acetone during solventogenic phase. The two phase changes were manipulated by different types of genes. However, similar genes were used to control solventogenic phase and sporulation.

Clostridium beijerinckii NCIMB 8052 was the most common solventogenic clostridia used in butanol fermentation (Wang, Kashket, & Kashket, 2005). *Clostridium beijerinckii* BA101 was the hyper-solvent producing strain derived from NCIMB 8052 (Chen & Blaschek, 1999). NCIMB 8052 was the parental strain. Similarly, *C.acetobutylicum* ATCC 824 was the mutated strain of ATCC 4259, which was composed of 210 kb plasmid Psol1 (Lee, Cho, Park, Chung, Kim, Sang, & Um, 2008). This plasmid encoded genes for solventogenic phase. Loss of this plasmid would cause degeneration. Fortunately, *C.beijerinckii* NCIMB 8052 had a single circular chromosome without any plasmids. Presence of chromosomes would never cause degeneration unless gene coding was altered through genetic engineering methods. Scientists reported that *C.beijerinckii* BA101 was inhibited at higher butanol concentration compared to *C.beijerinckii* NCIMB 8052 (Qureshi, Blaschek, 2001). Therefore, *Clostridium beijerinckii* BA101 was the most recommended strain for butanol fermentation.

2.3.2 Optimum conditions for optimum growth of *C.beijerinckii* BA101

The P2 medium was commonly used to grow the bacterial culture. P2 medium was composed of three stock solutions: minerals, buffer, and vitamins. Preparations of these solutions were demonstrated in the study by Qureshi & Blaschek, (1999). In another study by Qureshi, Karcher, Cotta, & Blaschek (2004), corn steep liquor was used as the growth medium. The authors concluded that CSL was more economical to use but P2 medium was most effective in butanol fermentation.

Qureshi, Lai, & Blaschek (2004) performed experiments by limiting nutrients for *C.beijerinckii* BA101 strain. This illustrated that all nutrients such as mineral solution, vitamins, yeast extract, and buffer were essential for better growth and maximum solvent production. It was not possible to limit these nutrients during fermentation even during an excessive cell growth. Excessive cell growth caused blockage in the reactor. However, high solvent production required excessive growth of solventogenic cells in the reactor. Butanol concentration above 25 g/L on the other hand was toxic to *C.beijerinckii* BA101 (Qureshi & Blaschek, 2000).

Each bacteria had different requirements of temperature, pH, and nutrients. *Clostridi*a required high concentration of carbon, which was provided by biomass. Agricultural waste such as corn fibre, wheat straw, and soy provided an excellent source of carbon. *Clostridi*a also required iron, phosphate, and sodium acetate for an optimum growth. Iron was used to convert pyruvate to acetyl-CoA during metabolism. Sodium acetate prevented degeneration of plasmids. *Clostridia* required nitrogen, high redox potential, and mesophillic temperature. Nitrogen could also be provided by yeast extract. Mesophillic temperature was between 25^{o}C to 37^{o}C. Optimum growth of *Clostridium beijerinckii* BA101 occurred at 35°C. *Clostridia* preferred slightly acidic environment such as, 4.5 to 5.0 pH. Maintenance of optimum pH throughout fermentation process improved the rate of growth.

Each strain required different amounts of sugars and substrate for an optimum growth. However, they were commonly grown in growth medium before transferring them to fermentation medium (Lienhardt, Schripsema, Qureshi, & Blaschek, 2002). The exponential phase must be reached at the growth medium. This would allow an active growth in fermentation medium (Ennis & Maddox, 1985). Each strain reached exponential phase at different time intervals. When the culture was inoculated into the fermentation medium with the required stock solutions and substrate, the exponential phase would be reached before any butanol production was initiated.

Degeneration in bacterial strain was a risk factor, which decreases productivity and forms more spores (Lee, Cho, Park, Chung, Kim, Sang, & Um, 2008). Butanol productivity and bacterial growth depended on pH, acetate, butyrate, and phosphate concentrations. Degeneration could be caused by repetition of batch culture. The study by Chen & Blaschek (1999) cultivated *C.beijerinckii* repetitively with the addition of sodium butyrate. This proved to prevent degeneration. Maximum butanol concentration without repetitive culturing was lower. Repetitive culturing allowed higher butanol production for longer period of time. This reduced the negative effect of metabolic shift between acidogenesis and solventogenesis, which was normally reduced optimum butanol production. Thus, degeneration can be avoided by repetitive culturing of bacteria.

2.3.3 Comparison of different strains of bacteria

Table 5 illustrated comparisons of different types of substrates and bacteria applied in fermentation of butanol. The highest solvent concentration was obtained by Qureshi, Saha, & Cotta, (2007) and lowest solvent concentration was obtained by Qureshi, Karcher, Cotta, & Blaschek, (2004). Wheat straw hydrolysate with glucose supplement achieved the highest solvent production, when *C.beijerinckii* P260 was used. Corn steep liquor provided better results when *C.beijerinckii* BA101 was applied. However, compared to all processes *C.beijerinckii* P260 achieved the highest solvent concentration. Perhaps, if wheat straw substrate was used in the fermentation with *C.beijerinckii* BA101, then this strain would have achieved higher solvent concentration. However, highest butanol production was achieved with the application of *C.beijerinckii* P260.

Table 5
Comparison of substrate, reactors, and bacteria

Substrate	ABE (g/L)	Bacteria	References
CFAX + 5g/L xylose	24.67	*C.acetobutylicum* P260	Qureshi, Li, Hughes, Saha, & Cotta, (2006)
WSH	21.42	*C.beijerinckii* P260	Qureshi, Saha, & Cotta, (2008)
100 g/L Glucose	23.50	*C.beijerinckii* P260	Qureshi, Saha, & Cotta, (2007)
WSH + 35g/L glucose	47.6	*C.beijerinckii* P260	Qureshi, Saha, & Cotta, (2007)
Corn steep liquor	6.29	*C.beijerinckii* BA101	Qureshi, Karcher, Cotta, & Blaschek, (2004)
Butyrate	5.70	*C.beijerinckii* BA101	Qureshi, Karcher, Cotta, & Blaschek, (2004)

2.4 Simultaneous saccharification and fermentation (SSF)

SSF was most preferred by the industries. Saccharification required several hours to break down all the polysaccharides into sugar monomers, and this extra time can be reduced by SSF. Qureshi, Saha, & Cotta (2008) examined SSF after dilute acid pretreatment. The pH was adjusted to 6.5. This pH was slightly higher than optimum pH. Results from saccharification at the initial stage of fermentation were illustrated in Figure 12 and Table 6. Here total sugar concentrations of 25.6 g/L were achieved, which was mostly composed of xylose monosaccharides.

Table 6 compared sugar concentrations obtained through different saccharification processes. All of these saccharification processes were performed after dilute acidic pretreatment. These results demonstrated that highest sugar concentrations were achieved through SHF. The wheat straw hydrolysate from SHF was mostly composed of glucose concentrations. During SSF, higher xylose concentration was achieved compared to glucose concentration. These studies demonstrated that saccharification of xylose was not negatively influenced during SSF. Enzymes were not required to extract xylose as illustrated in Figure 8**.** Acidic pretreatment alone also extracted high concentration of xylose. This implied that acid pretreatment already produced some sugars which were available prior to SSF.

Figure 12
Wheat straw hydrolysate at the initial stage of SSF: reults obtained from Qureshi, Saha, & Cotta (2008 Part I)

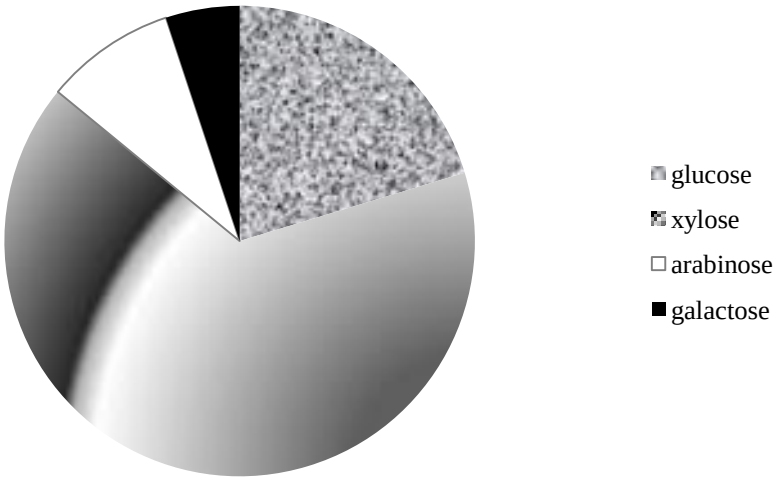


Table 6
Comparison of simultaneous saccharification and fermentation with separate hydrolysis and fermentation followed by acidic pretreatment

Method	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Galactose (g/L)	Mannose (g/L)	Butanol (g/L)	References
SHF	27.5	19.5	4.8	3.3	2.8	12.0	Qureshi et al. (2007)
SHF	19.1	17.1	2.6	3.1	0.0	8.1	Qureshi et al. (2008)Part I
SSF	5.2	16.8	1.3	2.3	0.0	7.4	Qureshi et al. (2008)PartI

3 Material and Methods

Procedures for different pretreatment, saccharification, and SSF processes were illustrated in two different sections. Analysis of each product is summarized in the third section. Table 7 summarized all the chemicals applied during these processes. The purity of these chemicals was in general above 97% and was used as received from the supplier unless explained otherwise in the methods section.

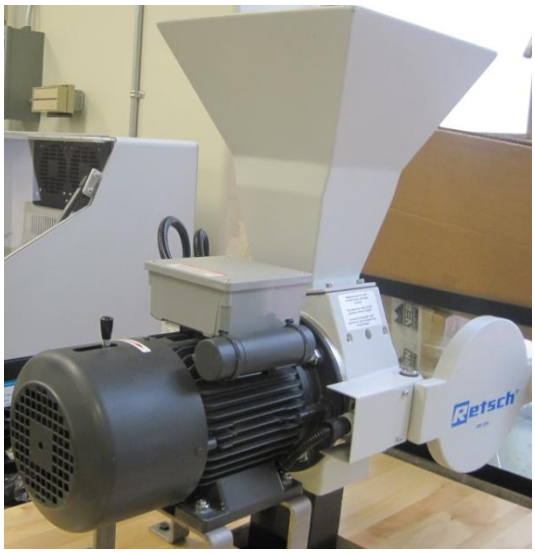
Table 7
List of chemicals and enzymes applied during experiments

Product	**Company**	**Catalogue #**
Sulfuric acid	Sigma-Aldrich	339741
Monoethanolamine	VWR	CAJT9339-1
Xylanase	Sigma-Aldrich	X2753
Celluclast 1.5L	Sigma-Aldrich	C2730
Novozym 188	Sigma-Aldrich	C6105
Clostridium beijerinckii BA101	Cedarlane Labs	ATTC # PTA-1550
Cooked meat medium	Oxoid	MT0350
L-cysteine	Sigma-Aldrich	W326305
Resazurin	Sigma-Aldrich	199303
KH_2PO_4	Sigma-Aldrich	322431
K_2HPO_4	Sigma-Aldrich	G0139
Ammonium acetate	Sigma-Aldrich	A1542
PABA	Sigma-Aldrich	6930
Thiamine	Sigma-Aldrich	T4625
Biotin	Sigma-Aldrich	B4501
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich	63138
$MnSO_4 \cdot 7H_2O$	Sigma-Aldrich	M7634
$FeSO_4 \cdot 7H_2O$	Sigma-Aldrich	F8048
NaCl	Sigma-Aldrich	S7653

3.1 Pretreatment and Saccharification

Wheat straw was obtained from a farmer located in Barrie, Ontario. This wheat straw was grounded to fine particles using 1.00 mm sieve screen in a hammer mill (model # 12930143D and manufactured by Retsch GmbH Inc. in USA). Hammer mill was illustrated in Figure 13. This was considered as the physical pretreatment that was applied to all straws used in the present work. Three different pretreatment methods were applied in comparison to the untreated wheat straws (i.e. no chemical pretreatment). These methods include water pretreatment, acid pretreatment, and alkaline pretreatment.

Figure 13
Hammer mill used to ground wheat straw into fine particles:
model # 12930143D; manufactured by Retsch GmbH Inc., U.S.A.



3.1.1 No chemical pretreatment

Nine different shake flasks were cleansed thoroughly to conduct nine experiments. Different conditions were illustrated in Table 8. After physical pretreatment, required amount of wheat straw was added to each flask. Then 100 ml of cold distilled water was added to each shake flasks. The distilled water was autoclaved at 121°C for 15 min before adding to each flask for sterilization. Here, pH was not required to be adjusted prior to saccharification.

Table 8
Summary of parameters examined during saccharification with no chemical pretreatment

Exp't	Biomass %(w/w)	Cellulose (g/L)	Hemicellulose (g/L)	pH	Enzymes			Hydrolysis (°C)
					cellulase	β-glucosidase	xylanase	
1	8.60	38.70	30.10	5.8	✓	✓		37
2	5.00	22.50	17.50	5.14	✓	✓		40
3	2.50	11.25	8.75	5.23	✓	✓		40
4	5.00	22.50	17.50	5.14	✓	✓		45
5	2.50	11.25	8.75	5.23	✓	✓		45
6	5.00	22.50	17.50	5.14	✓	✓		35
7	2.50	11.25	8.75	5.23	✓	✓		35
8	5.00	22.50	17.50	5.14	✓	✓	✓	45
9	2.50	11.25	8.75	5.23	✓	✓	✓	45

3.1.2 Water pretreatment

Following physical pretreatment of wheat straw, required amount of wheat straw was weighed in a dry glass beaker and then poured into a 500ml shaker flask using a glass funnel. All flasks contained 100 ml of water. Then all flasks were autoclaved at 135°C for 1 h. No pH adjustments were required prior to saccharification. Here, pretreatment was analysed at high temperatures to examine if temperature improves separation of lignin and polysaccharides. Saccharification was processed under different temperatures and enzymes as illustrated in Table 9.

3.1.3 Acidic pretreatment

These conditions were summarized in Table 10. Following physical pretreatment of wheat straw required amount was added to each shaker flask. Again, 100 ml of sterilized distilled water was added to all flasks. Required amount of sulfuric acid was added, while maintaining the total volume to 100 ml. Then, most of the shaker flasks were covered with aluminum foil and autoclaved at 135°C or 121°C for 1 h. One of the flasks was left at room temperature for 2.25 h. This method was called "soaking" process. Another shaker flask was left in room temperature for 3.5 h but this was also autoclaved at 121°C for 1 h.

Some of the shaker flasks were pH adjusted prior to saccharification using small drops of 10 M NaOH. The pH was approximately adjusted to 5.0. Some of the pretreated solution were not pH adjusted prior to saccharification. Saccharification was processed under different temperature, enzymes, and pH.

3.1.4 Alkaline pretreatment

Six experiments were performed to examine alklaine pretreatments. Following physical pretreatment of wheat straw, required amount was added to each shaker flask. The biomass concentration at 3.33%(w/w) was applied throughout all six experiments. All flasks contained 100 ml of sterilized distilled water and required amount of monoethanolamine. The following weak base concentrations were observed: 0.10, 0.50, 1.00, 2.00, 3.33, and 6.00% (v/v). All of these six shaker flasks were covered with aluminum foil and autoclaved at 135°C for 1 h.

After pretreatment, wheat straw sludge was soaked in 2.00% NaOH for 24 h. Then the pH was adjusted to approximately 4.5 using 0.01 M citrate buffer. These mixtures were covered with aluminum foil and autoclaved again at 121°C for 15 min as recommended by Shah et al. (1991). When the mixtures were cooled, saccharification was conducted at 35°C, 80 rpm.

3.1.5 Saccharification

During saccharificaiton, 0.375 ml of each Celluclast 1.5L and Novozyme 188 were applied in all of the experiments prior to saccharification. Celluclast 1.5L was composed of cellulase from *Trichoderma reesei* with the enzyme activity of 700 IU/g, and Novozyme 188 is composed of Cellobiase from *Aspergillus niger* with the enzyme activity of 250 IU/g. Some enzymatic hydrolysis included the application of the third enzyme called Xylanase. This was derived from Thermomyces lanuginosus with the activity of 2500 IU/g. Here, 0.375 ml of xylanase was applied. The incubator shaker was adjusted to 80 rpm and temperature was adjusted to 35°C, 37°C, 40°C, or 45°C. Enzymatic hydrolysis was observed from 35°C to 45°C because this temperature range was optimum for saccharification or butanol fermentation. Saccharification was optimum at 45°C and butanol fermentation was optimum at 35°C.

Table 9
Summary of parameters examined during water pretreatment and saccharification (C: cellulase; β: β-glucosidase; X: xylanase)

Exp't	Biomass %(w/w)	Cellulose (g/L)	Hemicellulose (g/L)	pH	Enzymes			Hydrolysis (°C)
					C	β	X	
1	8.00	36.00	28.00	5.21	✓	✓	✓	37
2	6.67	30.00	23.33	5.26	✓	✓	✓	37
3	7.14	32.14	25.00	5.22	✓	✓	✓	40
4	2.50	11.25	8.75	5.25	✓	✓	✓	40
5	4.00	18.00	14.00	5.26	✓	✓	✓	40
6	3.33	15.00	11.67	5.24	✓	✓	✓	40
7	7.14	32.14	25.00	5.22	✓	✓		45
8	2.50	11.25	8.75	5.25	✓	✓		45
9	4.00	18.00	14.00	5.26	✓	✓		45
10	3.33	15.00	11.67	5.24	✓	✓		45
11	7.14	32.14	25.00	5.22	✓	✓	✓	35
12	2.50	11.25	8.75	5.25	✓	✓	✓	35
13	4.00	18.00	14.00	5.26	✓	✓	✓	35
14	3.33	15.00	11.67	5.24	✓	✓	✓	35
15	7.14	32.14	25.00	5.22	✓	✓	✓	45
16	2.50	11.25	8.75	5.25	✓	✓	✓	45
17	4.00	18.00	14.00	5.26	✓	✓	✓	45
18	3.33	15.00	11.67	5.24	✓	✓	✓	45

Table 10
Summary of parameters examined during acidic pretreatment and hydrolysis (C: cellulase; β: β-glucosidase; X: xylanase)

Exp't	Biomass %(w/w)	Cellulose (g/L)	Hemicellulose (g/L)	Acid %(v/v)	Soak (h)	Pretreat (°C)	pH Adj		pH	Enzymes			Hydrolysis (°C)
							Yes	No		C	β	X	
1	8.60	38.70	30.10	1.00	-	121	✓		5.07	✓	✓		45
2	8.60	38.70	30.10	1.00	2.25	121	✓		5.07	✓	✓		45
3	8.00	36.00	28.00	10.00	3.50	20		✓	5.14	✓	✓		37
4	8.00	36.00	28.00	10.00	-	135	✓		5.39	✓	✓		37
5	8.00	36.00	28.00	10.00	-	135		✓	0.10	✓	✓	✓	35
6	4.71	21.18	16.47	10.00	-	135		✓	0.12	✓	✓	✓	35
7	3.81	17.14	13.33	10.00	-	135		✓	0.14	✓	✓	✓	35
8	3.20	14.40	11.20	10.00	-	135		✓	0.19	✓	✓	✓	35
9	3.33	15.00	11.67	1.00	-	135		✓	0.78	✓	✓	✓	35
10	3.33	15.00	11.67	2.00	-	135		✓	0.62	✓	✓	✓	35
11	3.33	15.00	11.67	6.00	-	135		✓	0.23	✓	✓	✓	35
12	3.33	15.00	11.67	0.10	-	135		✓	0.93	✓	✓	✓	35
13	3.33	15.00	11.67	0.50	-	135		✓	0.90	✓	✓	✓	35
14	3.33	15.00	11.67	0.01	-	135		✓	2.79	✓	✓	✓	35
15	3.33	15.00	11.67	0.50	-	135	✓		5.06	✓	✓	✓	35
16	3.33	15.00	11.67	0.01	-	135	✓		5.25	✓	✓	✓	35

3.2 Simultaneous saccharification and fermentation

3.2.1 Culture and cell propagation

Clostridium beijerinckii BA101 was stored in distilled water at -80°C. One tube or 10 ml of cooked meat medium (CMM) was transferred to a small 20 ml glass vial. Then 0.25 ml of 0.025% resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) was added to this glass vial (Mills & McGrady, 2008). Resazurin was used to indicate low redox potentials which were commonly used in anaerobic growth tests (Visser et al., 1990). The CMM vial was closed with a blue neoprene solid stopper and crimped.

The vial was vacuumed inside the glove box (model # 1681-29C-EX-001; series # 100; manufactured by Terra Universal). This was illustrated in Figure 14. Then nitrogen was surged through the vial at 150 ml/min for 10 to 20 min. Then, 0.5 to 1.0 ml of *C.beijerinckii* BA101 was inoculated into the tightly closed vial. The bacterial culture was inoculated using a sterilized syringe needle under anaerobic conditions. The syringe needle could be sterilized further with ethanol flame or ethanol (Madigan et al., 2000). Approximately, 0.1 g of L-cysteine was added to the oxygen-free vial by using a syringe needle under anaerobic conditions. Water was produced during the reaction with L-cysteine and oxygen (Sevilla et al., 1987).

Figure 14
Glove box used to create an anaerobic environment:
model # 1681-29C-EX-001; series # 100; manufactured by Terra Universal

After the inoculation, CMM was stored inside an incubator at 35°C (incubator from VWR). Bacteria reached exponential phase during 16 to18 h of incubation. Then 5.0 to 6.0 ml *C.beijerinckii* BA101 were inoculated into a second CMM vial. Second CMM vial was incubated for 16 to 18 h at 35°C. *C.beijerinckii* BA101 was inoculated several times to avoid degeneration (Lee, et al., 2008).

There were three types of stock solutions used here, and they were buffer, vitamins, and mineral solution. Buffer solution was composed of 5.00 g/L KH_2PO_4, 5.00 g/L K_2HPO_4, and 22 g/L ammonium acetate. Vitamins were composed of 0.01 g/L PABA, 0.01 g/L thiamine, 0.0001 g/L biotin, 2.00 g/L $MgSO_4 \cdot 7H_2O$, and 0.10 g/L $MnSO_4 \cdot 7H_2O$. Mineral solution was composed of 0.10 g/L $FeSO_4 \cdot 7H_2O$, and 0.10 g/L NaCl. Stock solution was used in P2 medium and fermentation medium as follows.

P2 medium was created in a 250 ml glass vial as follows. Approximately, 3.00 g glucose and 0.10 g of yeast extract was added to distilled water to produce 100 ml solution. Then, 1.00 ml of resazurin solution was added. This vial was also closed with a blue neoprene solid stopper and crimped. Then this was sterilized at 121°C for 15 min. Approximately 1.00 ml of each filter sterilized stock solutions were added. This vial was also vacuumed for 15 to 20 min. Then nitrogen was surged through the liquid phase at 150 ml/min for 40 to 45 min, until the oxygen indicator became clear. Approximately, 1.0 ml of 0.01% (w/v) L-cysteine was added. Then P2 medium was inoculated with 6.0 to 7.0 ml of *C.beijerinckii* BA101 during exponential growth phase. This was left to incubate again for 16 to 18 h at 35°C.

3.2.2 Pretreatment and batch SSF

Four types of pretreatment and SSF were examined in this thesis and they were illustrated in Table 11. Physical pretreatment was conducted in all four experiments. All four vials were composed of 2.50% (w/w) biomass concentration. One ml of 0.025% resazurin was added to each vial prior to chemical pretreatment or saccharification. All vials were closed with blue neoprene stopper and crimped.

Oxygen free nitrogen gas was surged through the gaseous and liquid phase of each vial at 150 ml/min for 40 to 45 min, until the indicator turned clear. The latter step was performed with inlet and outlet needle. The outlet needle was released 1 to 2 min prior to releasing inlet needle. At least 1.00 ml of 0.01% (w/v) L-cysteine was added to each vial to reduce any oxygen molecules. Saccharification was conducted under anaerobic conditions as illustrated in Table 11. During fermentation, 4 to 5 ml of actively growing *C.beijerinckii* BA101 from P2 medium was inoculated into each vial. Agitation was neglected during these batch fermentation processes to avoid any bacteria lysis.

Table 11
Description of four types of pretreatment and SSF

Experiment	Pretreatment	Saccharification
S10	physical (1mm sieve) + water (135°C for 1 h)	0.12 ml of cellulase + β-glucosidase inserted through syringe 20 h prior to fermentation
S11	physical (1mm sieve) + no chemical	0.12 ml of cellulase + β-glucosidase inserted through syringe 20 h prior to fermentation
A10	physical (1mm sieve) + water (135°C for 1 h)	0.12 ml of cellulase + β-glucosidase inserted through syringe with fermentation
A11	physical (1mm sieve) + no chemical	0.12 ml of cellulase + β-glucosidase inserted through syringe with fermentation

3.2.3 Control batch fermentation

Control fermentation experiments were conducted to compare the butanol production. Here, anhydrous sugars were applied and the compositions were derived from the study by Qureshi, Saha, & Cotta (2007), which was illustrated in Table 6 obtained during separate hydrolysis and fermentation. The following sugar composition was applied: 28.0 g/L glucose, 19.0 g/L xylose, 5.0 g/L arabinose, 3.3 g/L galactose, and 2.7 g/L mannose. *C.beijerinckii* BA101 was inoculated during fermentation.

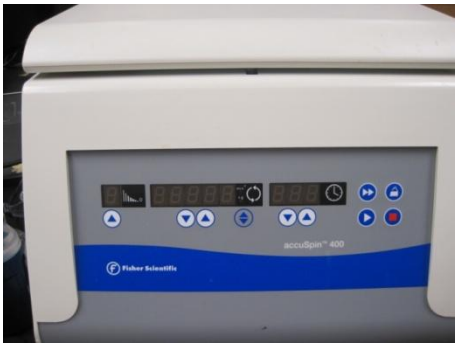
3.3 Analysis

3.3.1 Sugars, butyric acid, and butanol during SSF

Sample size of 1 ml was taken after each time interval for sugar analysis. These samples were centrifuged at 15000g for 15 min and filtered through 0.2μm syringe filters. Centrifuge was illustrated in Figure 15. They were stored at -80°C before sugar analysis. Sugar concentrations were measured using high performance liquid chromatography (HPLC) equipped with an automatic sample injector which was illustrated in Figure 16. Two HPLC columns were purchased from Shodex and they were called KC811 and SP0810. These two columns were used in series to measure sugars and inhibitors. Double distilled water was used as the solvent. The solvent was filter sterilized and autoclaved at 121°C for 15 min. Then

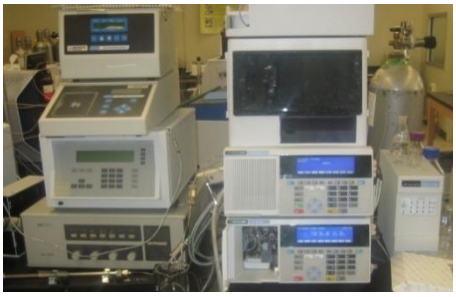
this was degassed using helium gas pipeline attached to HPLC equipment. A blank sample with only double distilled water was applied in the first sample vial tray of HPLC. This blank was used to increase the flow rate of the solvent from 0.0 ml/min to 0.6 ml/min. The flow rate was maintained at 0.6 ml/min for 1 h while, increasing the temperature of the HPLC column from 20°C to 60°C. This also cancels some noise created during the analysis. Then each sample vials were injected with 0.1 µl in sequence.

Figure 15
Centrifuge applied to separate wheat straw solids from liquid:
model # accuSpin 400; manufactured by Fisher Scientific



Sugar, butanol, and butyric acid concentrations were measured using HPLC equipped with an automatic sample injector. BioRad Aminex Resin-based HPX-87H HPLC column was used. Similar procedures were applied as previous section, except the parameters were modified. Samples were measured at 0.6 ml/min and at 60°C with 0.05 mM sulfuric acid as the solvent. Each sample was analysed through the HPLC for 1 h.

Figure 16
HPLC used to measure sugars, butyric acid, and butanol
Refractive index: model # HP 1047A; manufactured by Hewlett Packard
HPLC: model # 600; manufactured by Perklin Elmer



3.3.2 Sugar yield

Glucose yield was a ratio of glucose concentration and cellulose concentration. Xylose yield is a ratio of xylose concentration and hemicellulose concentration. Other sugars such as arabinose and galactose yield ratio of each sugar concentration and hemicellulose concentration. Cellulose concentration was calculated with the assumption of 45% of wheat straw was composed of cellulose (Zugenmaier, 2008). Another assumption was made here which was that 35% of wheat straw was composed of hemicellulose (Qureshi, Saha, & Cotta, 2007).

3.3.3 Cell concentration

Bacteria cell concentration was measured in Guava flow cytometry (illustrated in Figure 17). Sample tube of 1.5 ml size was inserted with 950 µl Guava check diluents fluid with 50 µl Guava check beads. Guava check beads code, expiry date, and expected particles concentration were filled out in the Guava settings. Then the diluents were inserted inside the flow cytometry with the cap open. Three replicates were examined until the green light was turned on. Then new data set with new file was created. This would now allow measurements of cell concentrations.

Figure 17
Flow cytometer applied to measure cell concentration:
model # GTI_2800080228; manufactured by Guava Technologies

4 Results and Discussion

This chapter was divided into three main sections. First, three types of pretreatment and saccharification processes were examined in comparison with saccharification alone (i.e. physical pretreatment only). The parameters summarized in Table 17 were elaborated in the first section. Second, all of these parameters were compared. Finally different pretreatment and SSF processes were examined.

4.1 Pretreatment and saccharification

Concentrations of arabinose, galactose, and mannose were not examined here because the total concentrations of all these three sugars were very small. Also they were negligible during fermentation according to previous studies. These sugars were only applied when glucose and xylose were not available. However, maximum concentrations of these sugars would be illustrated in section 0

4.1.1 Saccharification with no chemical pretreatment

Saccharification with no chemical pretreatment was examined at few different conditions. These conditions include the biomass concentration (2.5%, 5.0%, and 8.7%) and enzymatic hydrolysis temperatures (35°C, 37°C, 40°C, and 45°C).

Figure 18 illustrated glucose released from 5.00% (w/v) biomass concentration at different saccharification temperature and in the absence of xylanase enzyme. The concentrations at different time to estimate the time required to reach equilibrium. Equilibrium of xylose production was approached quickly. Approximately, 80% of glucose concentrations were achieved at 50 h. The optimum temperature was 45°C because highest glucose concentrations were obtained. Enzymes were most active at optimum temperature. The concentrations were almost similar at 40°C and 35°C but slightly increasing at 40°C. This implied that increase in temperature increased glucose production towards optimum temperature. Similarly, xylose concentrations increased during saccharification at increasing temperature from 35°C to 45°C, which was illustrated in Figure 19.

Figure 18
Glucose concentrations from 5.00% biomass concentrations at different saccharification temperatures in the absence of xylanase during saccharification with no chemical pretreatment

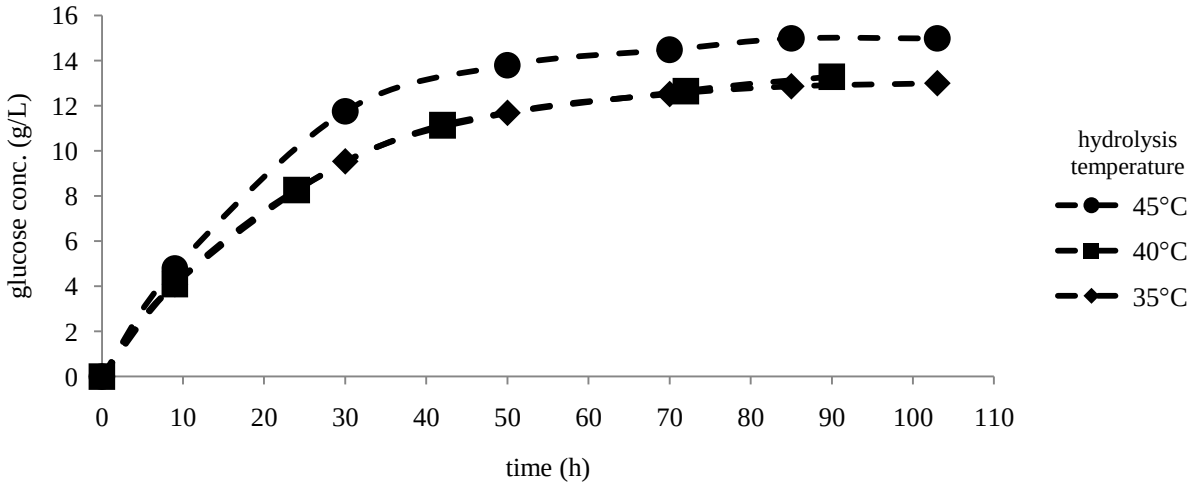


Figure 19
Comparison of sugar concentrations at different saccharification temperatures in the absence of xylanase during saccharificaiton with no chemical pretreatment:
1 (beside glucose and xylose) represented 5.00% biomass concentration and
2 (beside glucose and xylose) represented 2.50% biomass concentration

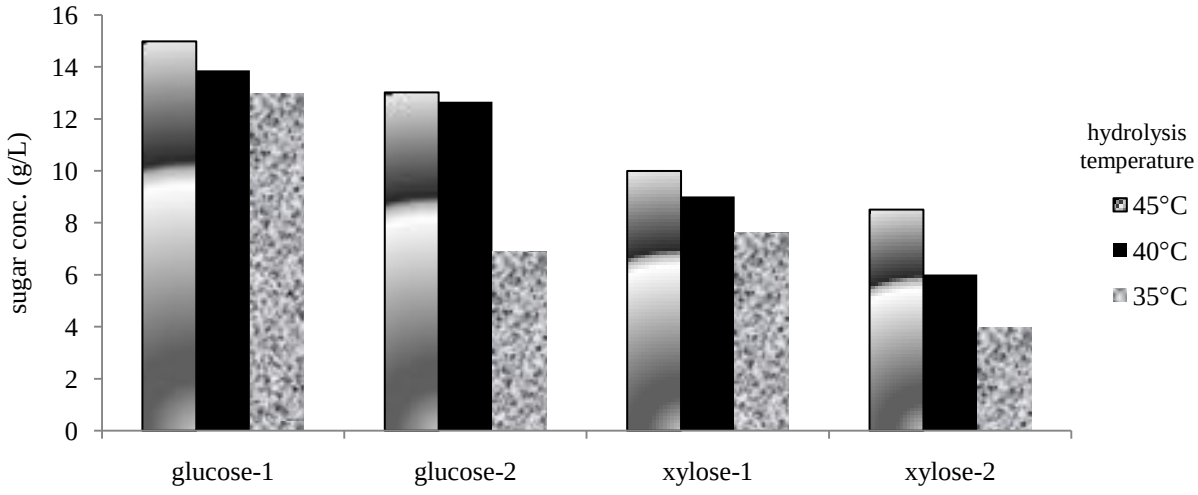


Figure 20 illustrated glucose and xylose concentrations extracted from 5.00% and 2.50% biomass concentrations in the presence and absence of xylanase. Glucose concentrations increased in the presence of xylanase. Surprisingly, xylose concentrations were reduced in the presence of xylanase. Xylose concentrations in the presence of xylanase at 45°C were lower than in the absence of xylanase at 35°C

(illustrated in Appendix A). It is common that xylanase was used to break down xylan from hemicellulose into xylose. Here, the results illustrated that xylose was produced without the application of xylanase. Cellulase alone was sufficient enough to produce a high concentration of xylose (Girio et al., 2010). This occurred because hemicellulose could be attacked by cellulase and result in xylose production (Gilbert & Hazlewood, 1993). However, glucose concentrations slightly increased in the presence of xylanase, indicating that the xylanase may have interacted with cellulase. This interaction between xylanase and cellulase decreased hydrolysis of hemicellulose into xylose. Also, when biomass concentration was doubled, sugar concentrations were not doubled. This may had occurred because the enzyme molecules were trapped underneath the lignin of the wheat straw fibre.

Figure 20
Comparison of sugar concentrations in the presence and absence of xylanase during saccharification with no chemical pretreatment:
1 (beside glucose and xylose) represented 5.00% biomass concentration and
2 (beside glucose and xylose) represented 2.50% biomass concentration

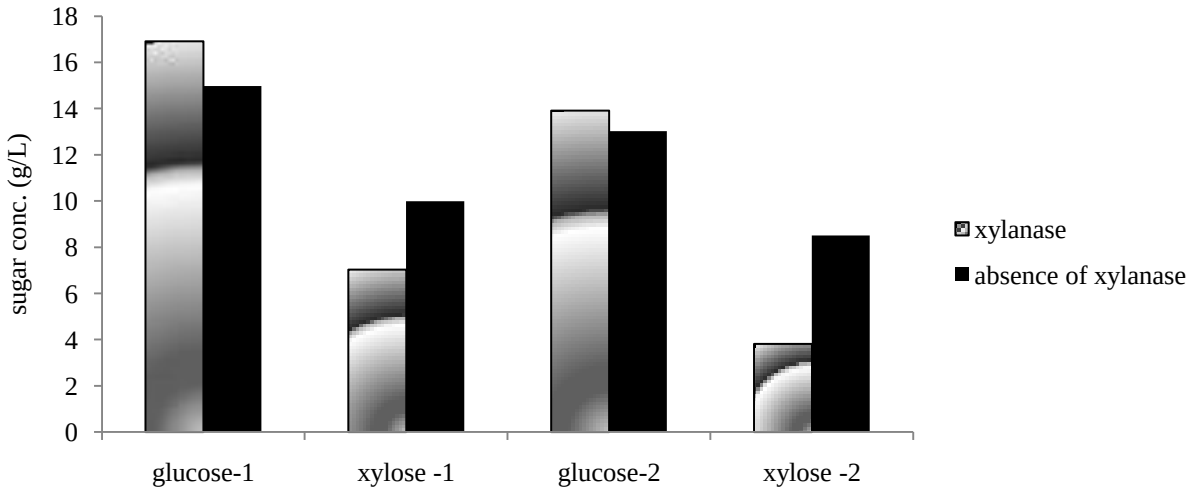


Table 12 illustrated sugar yields in the presence and absence of xylanase. Examining results in Table 12 reveals that maximum yield obtained was 100%. This can be explained by two reasons. Probably, small concentration of wheat straw utilized in the present work promotes higher exposure during the saccharification stage, which enhances the yield of the sugar at the end of the process. Some glucose molecules are derived from hemicellulose. These calculations did not encounter the conversion of hemicellulose into glucose because this conversion was very low. Glucose yields from 2.50% biomass concentration were significantly higher than glucose yields from 5.00% biomass concentration during saccharification with all three enzymes. However, this was not the case in xylose yields because they

were almost same in both biomass concentrations during saccharification with all three enzymes. This occurred because only minimum amount of xylose was produced during saccharification with xylanase. In fact, xylanase reduced xylose yields during saccharification with cellulase and β-glucosidase because this mixture of enzymes suppressed hydrolysis of hemicellulose. The same interaction between three enzymes improved glucose yields. Perhaps, cellulase from Celluclast 1.5L alone hydrolysed hemicellulose into xylose production.

Table 12
Comparison of sugar yields during saccharification with no chemical pretreatment in the presence and absence of xylanase: glucose yield was obtained through cellulose concentration and xylose yield was obtained through hemicellulose concentration

	xylanase		absence of xylanase	
Biomass %(g/g)	glucose yield %(g/g)	xylose yield %(g/g)	glucose yield %(g/g)	xylose yield %(g/g)
5.00%	0.75	0.40	0.67	0.57
2.50%	1.02	0.44	1.00	0.97

Table 13 illustrated glucose and xylose yields in the absence of xylanase and during saccharification at different temperatures. Here, also higher yields were achieved from 2.50% biomass concentration compared to 5.00% biomass concentration. The same explanation for Table 12 could be applied here. Only 97% xylose yield was achieved because 3% of hemicellulose was composed of other sugars such as glucose, arabinose, and galactose. Maximum sugar yields were achieved at optimum temperature of 45°C. However, 100% glucose yield was still achieved at 40°C even though lower xylose yield was obtained. This may have occurred because the enzymes were capable of hydrolysing cellulose quickly at 40°C but not with hemicellulose. This implied that conversion of hemicellulose into xylose definitely required optimum temperature. Hemicelluloses were more difficult to be removed without any pretreatment methods. The pretreatment catalysts were used to complete reaction quickly.

Table 13
Comparison of sugar yields during saccharification at different temperatures and in the absence of xylanase with no chemical pretreatment: glucose yield was obtained through cellulose concentration and xylose yield was obtained through hemicellulose concentration

Biomass %(g/g)	temp	glucose yield %(g/g)	xylose yield %(g/g)
5.00%	45°C	0.67	0.57
	40°C	0.62	0.51
	35°C	0.58	0.44
2.50%	45°C	1.01	0.97
	40°C	1.00	0.69
	35°C	0.61	0.46

4.1.2 Saccharification with water pretreatment

No pH adjustment was implemented since all water pretreatment experiments were examined at a maximum temperature of 135°C. In previous studies (Table 3), water pretreatment including steam explosion was performed at temperatures above 160°C which in result decreased the pH. Steam explosion produced small amounts of inhibitors due to these acidic conditions. The different parameters that were examined in this section include biomass concentration, temperatures, and type of enzymes used.

Figure 21 and Figure 22 represents the change in glucose and xylose concentrations obtained at different saccharification temperatures. Enzymatic hydrolysis was examined at 35°C, 40°C, and 45°C in the presence of xylanase. Also biomass concentrations from 2.50% to 7.14% were examined here. The results delineated that the increase in enzymatic hydrolysis temperature increased sugar production. Maximum sugar concentrations were obtained at 45°C. Glucose production was linearly proportional to hydrolytic temperature in the presence of xylanase. Similarly, xylose concentration increased when the saccharification temperature was increased from 35°C to 45°C. This phenomena was similar to what was obtained through saccharification with physical pretreatment only (i.e. no chemical pretreatment) but during saccharification without xylanase. This implied that sugar production depended on saccharification temperature more than the addition of xylanase during saccharification.

Figure 21
Comparison of glucose concentrations during saccharification with water pretreatment at different biomass concentrations: saccharification in the presence of xylanase; water pretreatment at 135°C

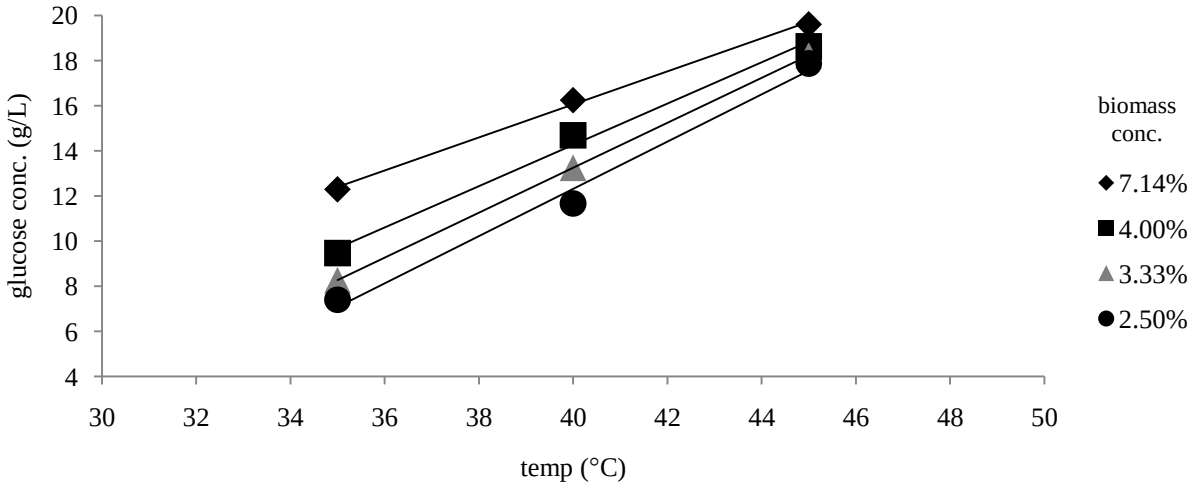


Figure 22
Comparison of xylose concentrations during saccharification with water pretreatment at different temperatures and biomass concentrations: saccharification in the presence of xylanase; water pretreatment at 135°C

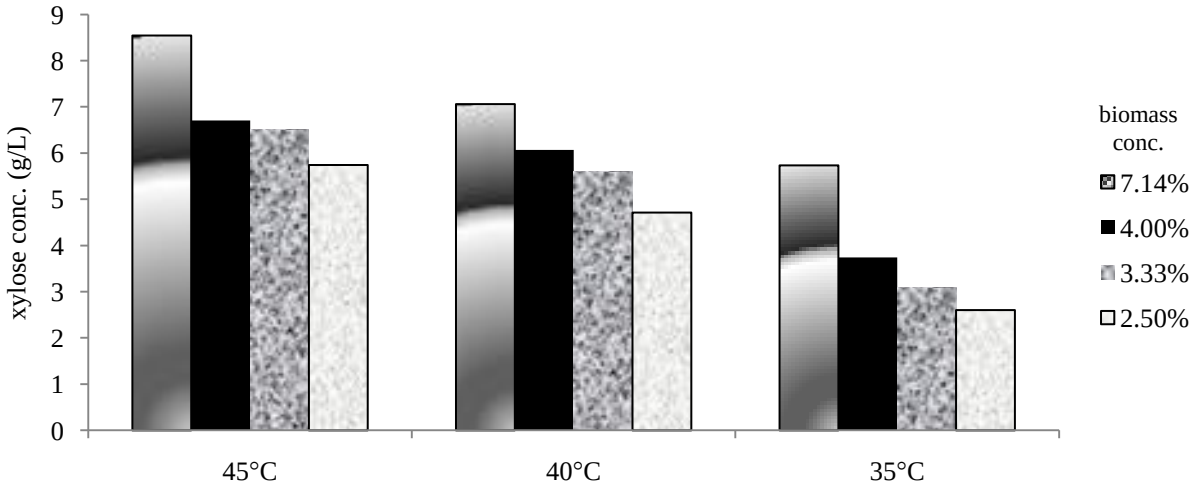


Figure 23
Comparison of glucose concentrations during saccharification with water pretreatment at different biomass concentrations: saccharification in the absence and presence of xylanase; water pretreatment at 135°C

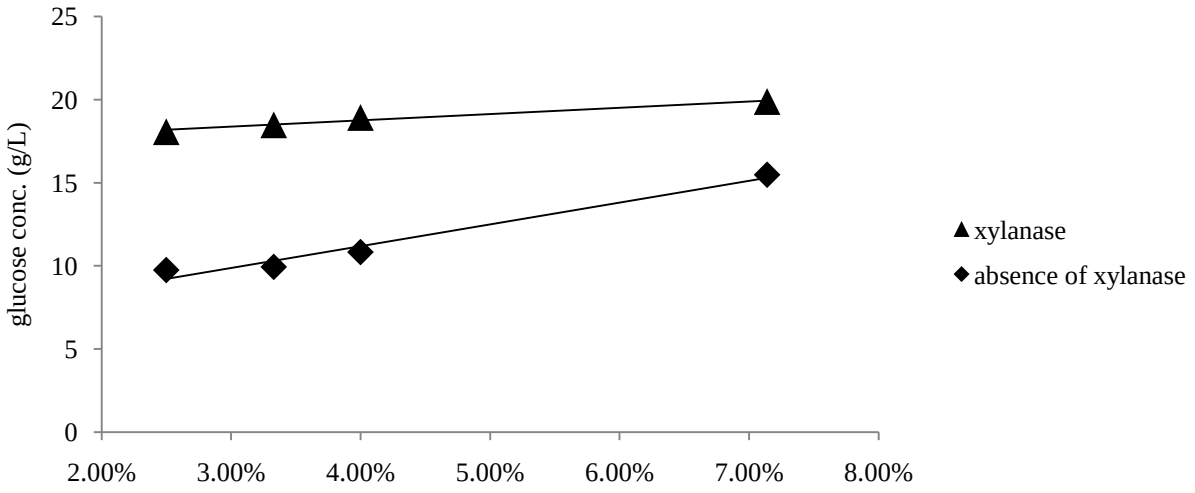


Figure 23 illustrated glucose concentrations in the absence and presence of xylanase during saccharification after water pretreatment at different biomass concentrations. Biomass from 4.00% through 2.50% produced similar glucose concentrations in the absence of xylanase. Except 7.14% biomass concentration which produced slightly higher glucose concentration. However, glucose concentrations increased when the biomass concentration was increased during saccharification with cellulase and β-glucosidase only (i.e. absence of xylanase). This implied that xylanase interacted with cellulase and β-glucosidase and allowed similar glucose concentrations at all biomass concentrations in the range of 2.50% to 7.14%. The same phenomena occurred during xylose production which was illustrated in Figure 24. However, glucose concentrations were higher during the presence of xylanase compared to absence of xylanase. This was again due to interaction with xylanase and cellulase during saccharification with all three enzymes. The same interaction suppressed hydrolysis of hemicellulose into xylose.

Figure 24
Comparison of xylose concentrations during saccharification with water pretreatment at different biomass concentrations: saccharification in the absence and presence of xylanase; water pretreatment at 135°C

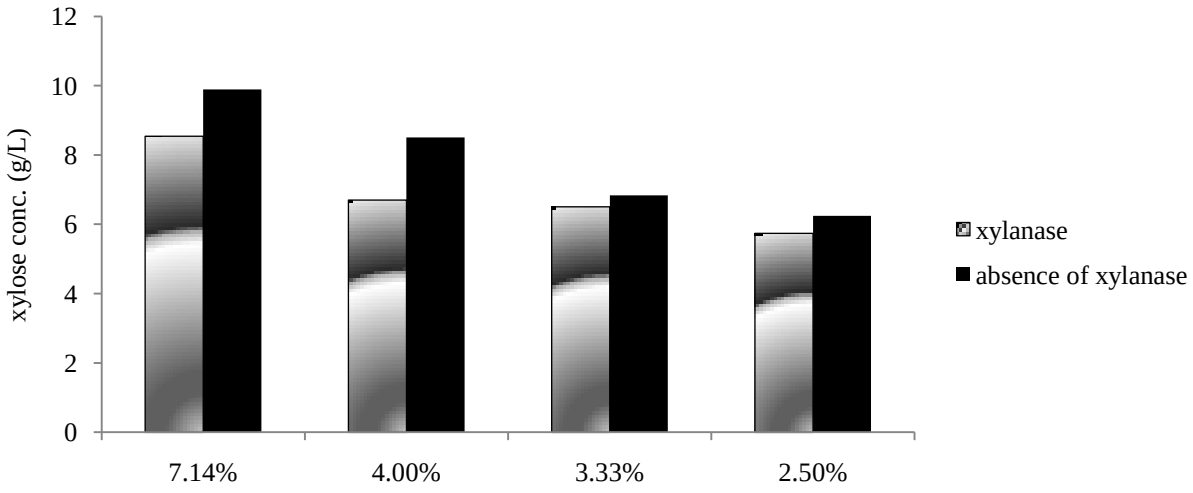


Table 14 illustrated sugar yields in the presence and absence of xylanase at different biomass concentrations. Xylose yields were higher when no xylanase was added compared to saccharification with all three enzymes. This was due to suppression of hydrolysis of hemicellulose in the presence of all three enzymes. The same explanation for Figure 23 and Figure 24 could be applied here. Except the low biomass concentrations produced higher glucose and xylose yields. Here, small amount of wheat straw allowed more exposure to each particle during pretreatment stage and enzymes during saccharification stage. This results in producing higher glucose and xylose yields during saccharification after water pretreatment with low biomass concentration.

Table 15 shows results for the glucose and xylose yields at different saccharification temperatures and biomass concentrations. Here, 100% glucose yield was achieved at 45°C and 40°C with 2.50% biomass concentration. Similar explanation for Figure 22 could be applied here. Except decrease in biomass concentration increased glucose and xylose yields. The same phenomena occurred during saccharification with no chemical pretreatment. Low concentration of wheat straw allowed more exposure for enzymes during saccharification. Also this allowed more exposure to heat and water to remove more lignin and some polysaccharides and may even hydrolyse some of them.

Table 14
Comparison of sugar yields during saccharification with water pretreatment at different biomass concentrations: saccharification in the absence and presence of xylanase; water pretreatment at 135°C; glucose yield was obtained through cellulose concentration; xylose yield was obtained through hemicellulose concentration

	xylanase		absence of xylanase	
Biomass %(g/g)	glucose yield %(g/g)	xylose yield %(g/g)	glucose yield %(g/g)	xylose yield %(g/g)
7.14%	0.61	0.34	0.48	0.40
4.00%	1.00	0.51	0.60	0.54
3.33%	1.00	0.56	0.66	0.59
2.50%	1.01	0.66	0.87	0.71

Table 15
Comparison of sugar yields during saccharification with water pretreatment at different biomass concentrations and saccharification temperatures: saccharification in the presence of xylanase; water pretreatment at 135°C: glucose yield was obtained through cellulose concentration and xylose yield was obtained through hemicellulose concentration

Biomass %(g/g)	temp	glucose yield %(g/g)	xylose yield %(g/g)
7.14%	45°C	0.61	0.34
	40°C	0.52	0.28
	35°C	0.39	0.23
4.00%	45°C	1.01	0.51
	40°C	0.86	0.43
	35°C	0.56	0.27
3.33%	45°C	1.01	0.56
	40°C	0.93	0.48
	35°C	0.58	0.26
2.50%	45°C	1.02	0.66
	40°C	1.01	0.54
	35°C	0.69	0.30

4.1.3 Saccharification with acid pretreatment

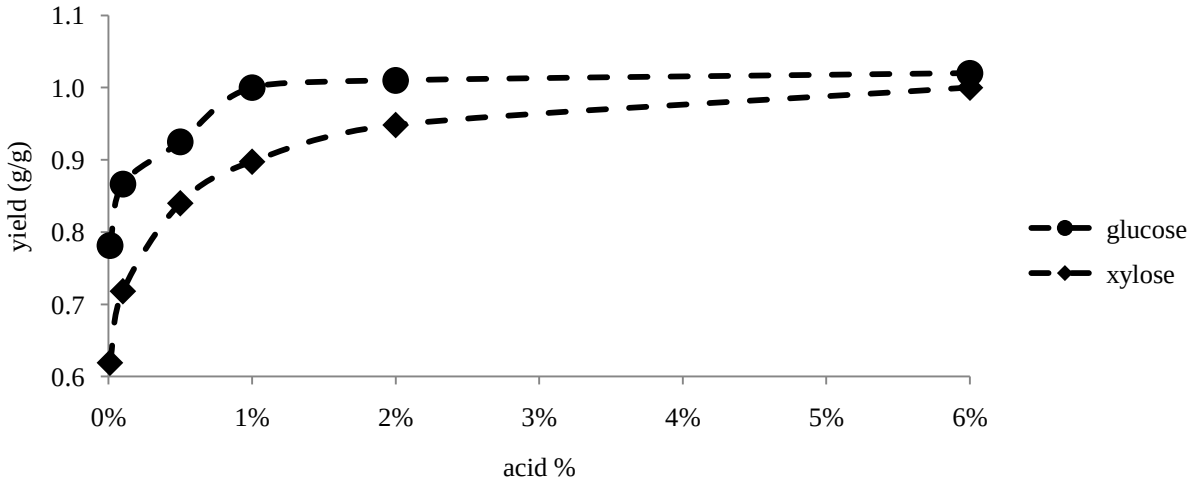
Saccharification with acid pretreatment was examined by varying biomass concentration, pH, acidic concentration, pretreatment temperature, hydrolysis temperature, and time. The biomass concentration was varied from 3.33% through 8.60%. Maximum biomass concentration was 8.60% because; higher

biomass concentration would affect the SSF. Bacteria cells required liquid space to grow and produce end products (Cinar et al., 2003). Hence, better fermentation would be achieved by low biomass concentration and high liquid volume. The pH was recommended to be 5.0 for best enzymatic hydrolysis. Here some pretreated hydrolysate was pH adjusted and some were not. This was performed to determine the effect of pH on enzymatic hydrolysis.

Sulfuric acid concentrations between 0.01% through 10.00% were observed. High concentrations of sulfuric acid would destroy healthy bacterial growth during fermentation (Zoysa & Morecroft, 2007). Effects of pretreatment temperature were observed at 0°C, 121°C, and 135°C. Enzymatic hydrolysis was performed at 35°C, 37°C, and 45°C. Most of the enzymatic hydrolysis experiments were performed at 35°C because this was the optimum temperature for the SSF. In addition, the effect of soaking wheat straw with sulfuric acid before autoclaving was examined. Most of the sugar concentrations obtained from acidic pretreatment and enzymatic hydrolysis were observed at different time intervals. However, this section mostly examined final concentrations obtained at each condition. Also not all of the parameters were analysed here but, all results were illustrated in Appendix A.

Figure 25 examined glucose and xylose yields extracted from 3.33% biomass concentration during saccharification at 35°C. Here, saccharification with acid pretreatment was examined from 0.01% to 6.00% (v/v) sulfuric acid concentrations. Glucose and xylose yields increased at high acidic concentration. Similarly, glucose and xylose concentrations increased with increase in acid concentrations (illustrated in Appendix A). In fact, sulfuric acidic concentrations from 1.00% through 6.00% achieved approximately 100% glucose yield. Slightly more than 100% glucose yields was achieved when high acidic concentrations were applied because, some glucose molecules were derived from hemicellulose as well. Similarly, 100% xylose yields were achieved with 6.00% acid concentration.

Figure 25
Comparison of sugar yields during saccharification with acid pretreatment at different concentrations of sulfuric acid: 3.33% biomass concentration; pretreatment at 135°C; saccharification at 35°C



These results implied that increase in sulphuric acid concentration removed more lignin and hemicellulose. In some cases, acidic pretreatment alone hydrolysed hemicellulose into xylose (Qureshi, Saha, & Cotta, 2008, Part I). This was due to presence of COOH groups located on the outside region of lignin and hemicellulose. H+ ions in acidic molecules were attracted to the negative charges present in lignin and hemicellulose. Unfortunately, these forces and reactions created inhibitors such as HMF and furfural especially when pretreatment was performed at very high temperatures.

Figure 26 illustrated glucose and xylose yields from pH adjusted and no pH adjusted saccharification. Here pH adjusted represented that pH was adjusted prior to saccharification. Acid concentrations of 0.01% and 0.5% were examined here. Enzymes were most active at pH 5.0 (Shuler & Kargi, 2002). Saccharification with pH adjustments improved glucose and xylose yields. Similarly sugar concentrations were increased. Xylose yields were approximately equal after pH adjustments at 0.5% and 0.01% sulfuric acidic concentrations. This implied that acid concentrations were negligible during pH adjusted saccharification of xylose. Also the pH was an important parameter during saccharification of hemicelluloses. However, cellulose did not depend on pH because cellulose was composed of linear bonds of glucose molecules which provided easy access for enzymes to break away each bond. Hemicelluloses on the other hand, were complex, which required optimum conditions of enzymes to successfully break away each bond between two sugar monomers.

Figure 26
Comparison of sugar yields during saccharification with acid pretreatment:
1 represented pH adjusted; 2 represented no pH adjustments; 3.33% biomass concentration; pretreatment at 135°C; saccharification at 35°C

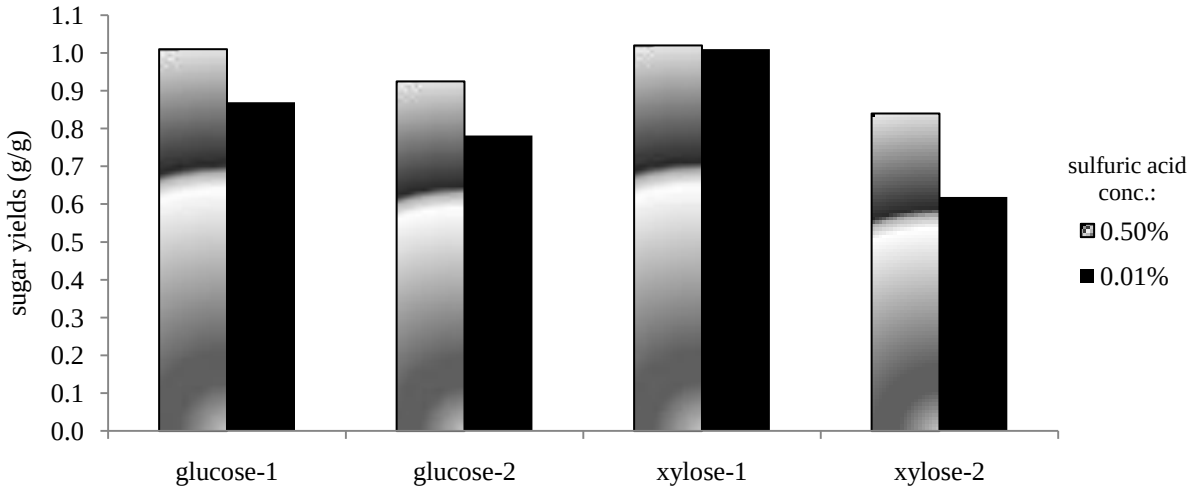


Figure 27
Comparison of sugar concentrations from soaking and no soaking process:
1 (beside soaking and no soaking) represented glucose concentration and 2 (beside soaking and no soaking) represented xylose concentration

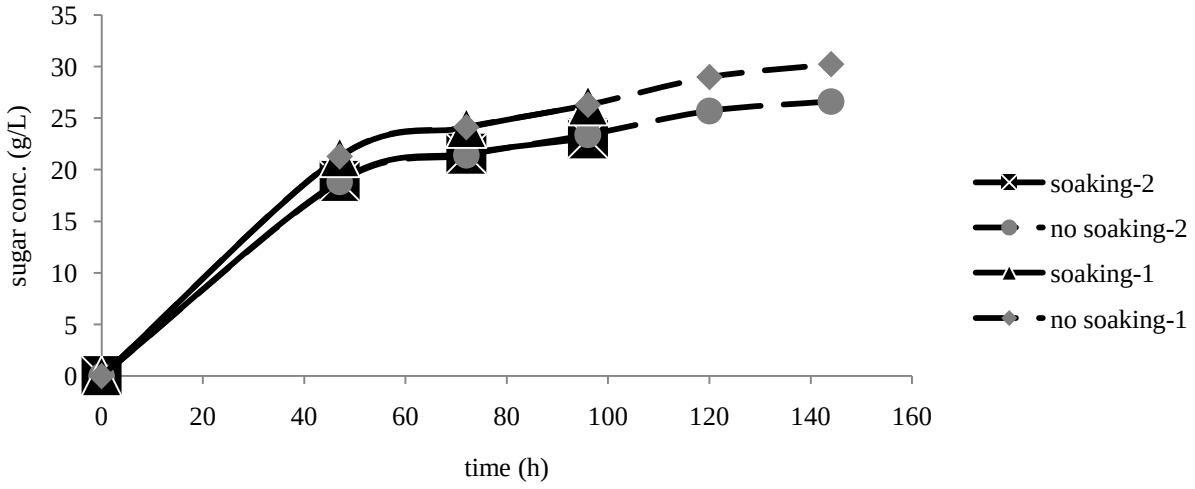


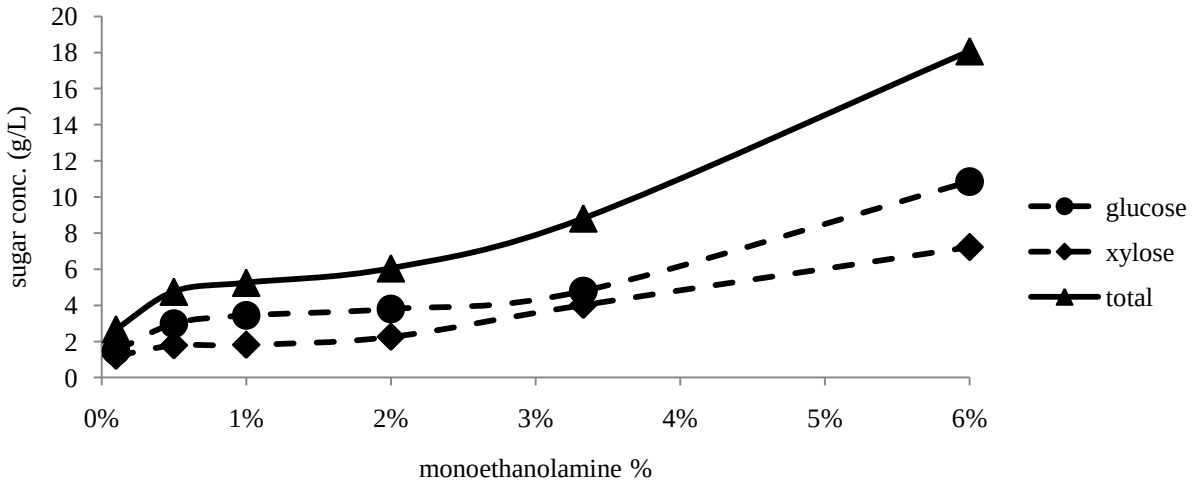
Figure 27 illustrated glucose and xylose concentration during soaking and no soaking process. Soaking process produced the same glucose and xylose concentrations as the no soaking process at all time intervals. The yields were also same which was illustrated in Appendix A. This proved that heat was not required during acid pretreatment. Acid pretreatment only depended on exposure time to absorb acid. This

was highly advantageous to industries because costs of supplying heat and energy during pretreatment was eliminated. Pretreatment at room temperature for a few hours reduced the production of inhibitors such as HMF and furfural. The study by Ballesteros et al. (2006) illustrated that exposure to acid for a longer period of time at low temperatures reduced the latter two inhibitors.

4.1.4 Saccharification with alkaline pretreatment

Figure 28 illustrated glucose and xylose concentration from saccharification with different concentrations of alkaline pretreatment. Glucose and xylose concentrations increased when monoethanolamine (MEA) concentrations increased. Similar sugar concentrations were produced with 0.50% to 2.00% alkaline concentrations. These results approved with Shah et al. (1991). Similar results occured in sugar yields as well which were illustrated in Appendix A.

Figure 28
Comparison of sugar concentrations during saccharificaiton with alkaline pretreatment at different concentrations of monoethanolamine: at 3.33% biomass, pretreatment at 135°C, and saccharification at 35°C



MEA was illustrated to be a poor choice of catalyst to pretreat wheat straw which was also illustrated in Figure 34 (see next section). Although, MEA was a poor catalyst, 100% glucose and xylose yields were achieved through MEA pretreatment (illustrated in Table 16). Yields also increased with increase in alkaline concentration. These results exceeded yields obtained by Shah et al. (1991). Hence, using small surface particle size of wheat straw improved glucose and xylose yield through pretreatment with MEA and enzymatic hydrolysis. Here only 6.00% of MEA was required to achieve 100% sugar yields.

Table 16
Comparison sugar yields during saccharification with alkaline pretreatment at different concentrations of monoethanolamine: at 3.33% biomass, pretreatment at 135°C, and saccharification at 35°C

MEA% (v/v)	glucose yield% (w/w)	xylose yield% (w/w)
0.10%	0.10	0.10
0.50%	0.20	0.15
1.00%	0.23	0.16
2.00%	0.25	0.19
3.33%	0.68	0.63
6.00%	1.01	1.00

These results implied that negative charge ions (especially OH^-) present in MEA attracted positive charge groups present in the outer region of lignin and hemicelluloses. This may had caused some increase in sugar production when MEA concentration above 3.33% was applied. Unfortunately nitrogen molecules present in the MEA compound reacted with enzymes. Enzymes were also composed of proteins, which were mainly composed of nitrogen molecules. This in result suppressed enzymatic hydrolysis. If enzyme molecules were restructured with new nitrogen or any molecules, then the function of enzymes would be dormant or modified. These phenomena may have occurred when MEA concentration below 2.00% was applied.

4.2 Comparisons of all four pretreatment and saccharification

Acid, alkaline, water pretreatment followed by physical pretreatment were examined in comparison with physical pretreatment alone (i.e. no chemical pretreatment). All of these processes were followed by saccharification. All of the parameters examined were illustrated in Table 17. Each parameter was analysed in this section.

Table 17
Summary of parameters examined during all four pretreatment and saccharification processes

	Parameters	Pretreatment methods			
		Acidic	Alkaline	Water	None
Pretreatment	Temperature of pretreatment	✓		✓	
	Catalyst conc.	✓	✓		
	Solid conc.	✓		✓	✓
	Length of pretreatment	✓			
Saccharification	Effect of pH	✓			
	Different enzymes	✓		✓	✓
	Temperature of enzymatic hydrolysis	✓		✓	✓
	Time	✓	✓	✓	✓

Figure 29
Maximum glucose concentrations from saccharification with no chemical or water pretreatment:
No (beside each biomass concentration) represented no chemical pretreatment;
H2O (beside each biomass concentration) water pretreatment

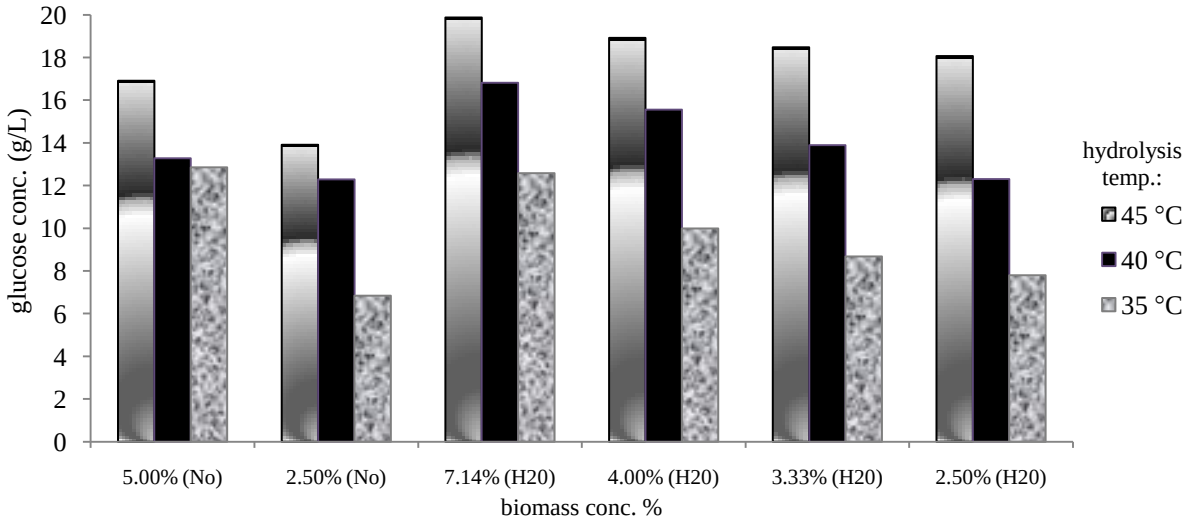
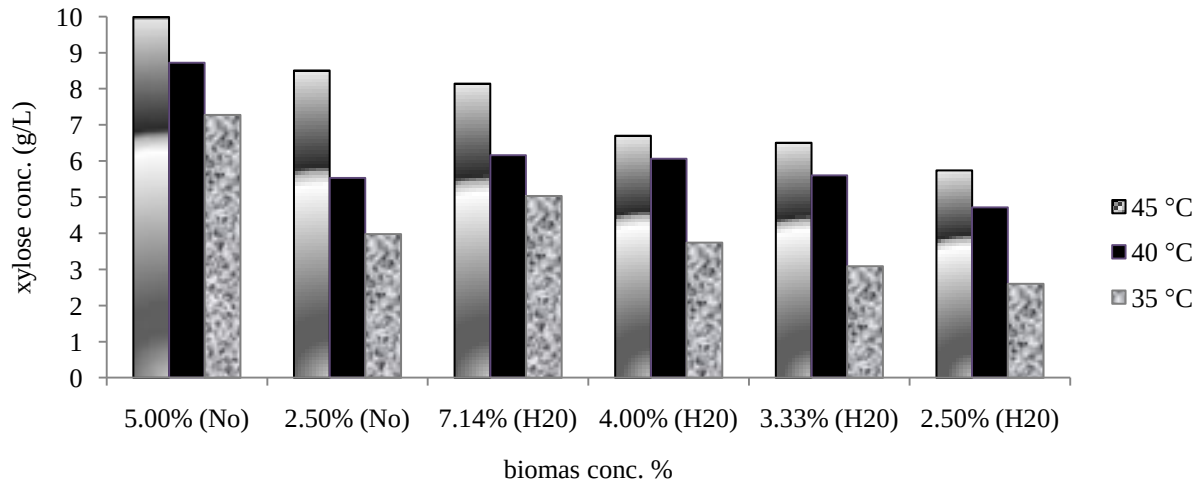


Figure 29 illustrated maximum glucose concentrations from saccharification at different temperatures with no chemical and water pretreatment. Here, saccharification with water pretreatment was examined in the absence of xylanase. However, saccharification with no chemical pretreatment was examined in the presence of xylanase. Highest glucose concentration was obtained through saccharification with water pretreatment when 7.14% biomass concentration was used and enzymatic hydrolysis was performed at

45°C with xylanase. These results implied that similar explanation could be applied for saccharification with water pretreatment and saccharification with no chemical pretreatment. Xylanase slightly improved glucose concentrations due to positive interaction between cellulose, β-glucosidase and xylanase towards hydrolysis of cellulose into glucose. Also increase in biomass concentration allowed more hydrolysis of cellulose into glucose. Increase in saccharification temperature increased hydrolysis of cellulose into glucose as well. The latter two statements could be applied to explain the effect of saccharification temperature and biomass concentration on hydrolysis of hemicelluloses into xylose.

Figure 30
Maximum xylose concentrations during saccharificaiton with no chemical or water pretreatment:
No (beside each biomass concentration) represented no chemical pretreatment;
H2O (beside each biomass concentration) water pretreatment



Xylose concentrations from saccharification with water pretreatment in comparison with saccharification with no chemical pretreatment was illustrated in Figure 30. Surprisingly, highest xylose concentration was obtained from 5.00% biomass concentration during saccharification at 45°C with no chemical pretreatment. The highest xylose concentration was achieved at lower biomass concentration than 7.14%. In fact, 2.50% biomass concentration allowed similar or slightly more hydrolysis of hemicelluloses into xylose after no chemical pretreatment when compared to 7.14% biomass concentration from saccharification with water pretreatment. Higher xylose concentrations from saccharification with no chemical pretreatment were obtained because xylanase was not applied here. Due to negative interaction between xylanase and cellulose, hydrolysis of hemicelluloses into xylose was suppressed. Also these results implied that water pretreatment did not hydrolyse hemicelluloses into xylose prior to

saccharification. Hence, chemical pretreatment could be avoided. This contradicted with phenomena stated in the study by Perez et al. (2008) because some hemicelluloses were already hydrolysed during water pretreatment prior to saccharification.

Figure 31
Comparison of glucose concentrations during saccharificaiton with acid or alkaline pretreatment at different concentrations of acid or alkaline:
3.33% biomass concentration; pretreatment at 135°C; saccharification at 48 h and 35°C

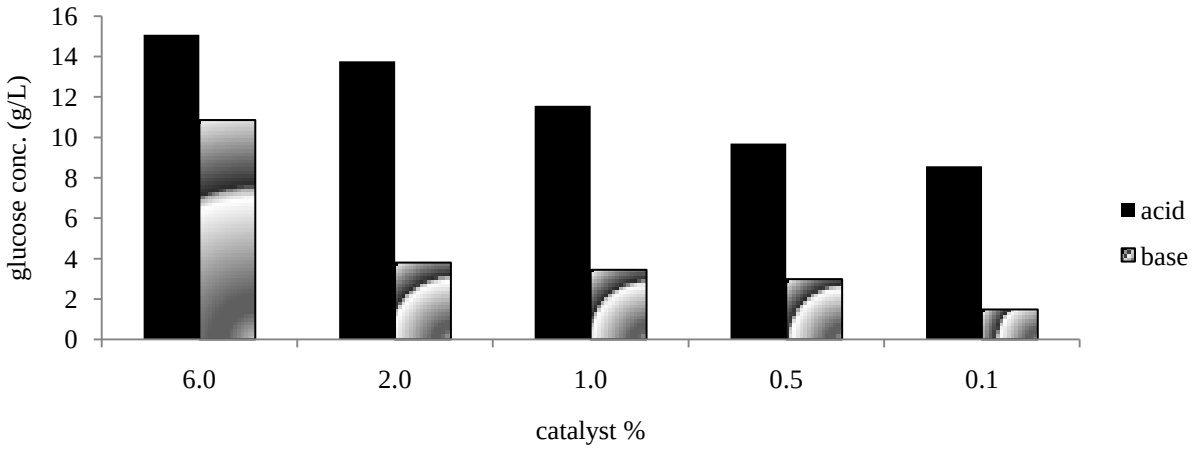


Figure 32
Comparison of xylose concentrations during saccharificaiton with acid or alkaline pretreatment at different concentrations of acid or alkaline:
3.33% biomass concentration; pretreatment at 135°C; saccharification at 48 h and 35°C

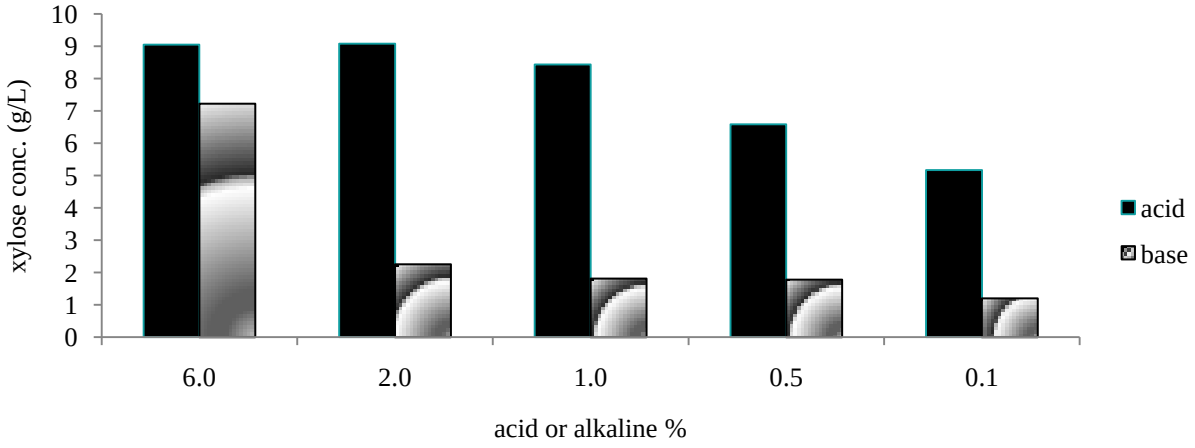
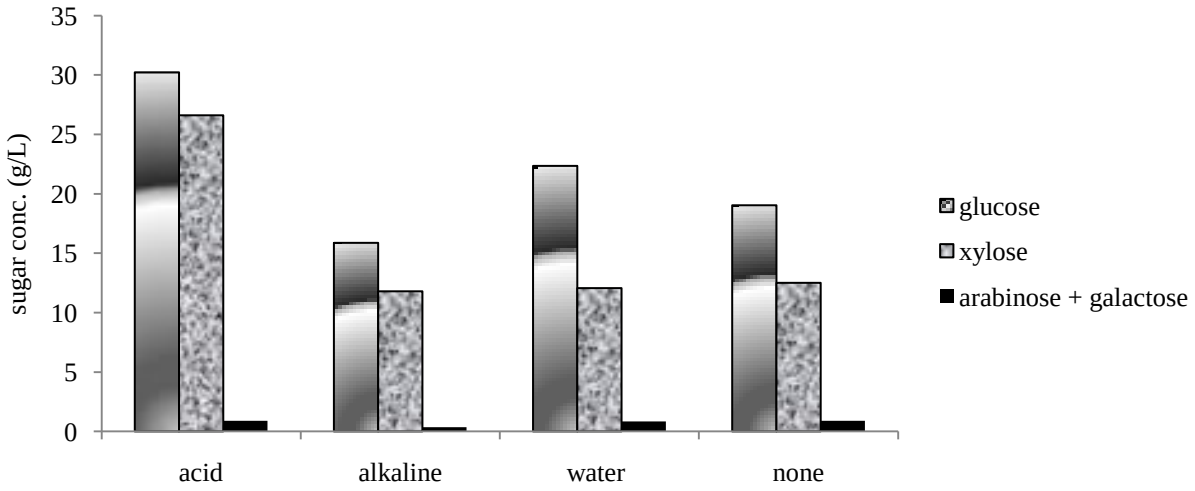


Figure 31 and Figure 32 illustrated glucose and xylose concentrations obtained from 3.33% biomass concentrations during saccharification with acid and alkaline pretreatment. These concentrations were

obtained during hydrolysis at 48 h and saccharification was examined at 35°C. Here, glucose concentration increased when acid concentration was increased because more sulphuric acid allowed more hydrolysis of cellulose into glucose. Glucose concentrations did not increase with increase in MEA concentration below 3.33%. However, glucose concentrations from saccharification with alkaline pretreatment were always lower than glucose obtained through saccharification with acid pretreatment. These results implied that H^+ ions were more effective to hydrolyse cellulose into glucose compared to OH^- ions.

Sulfuric acid was a very strong acid and MEA was a weak base. This implied that it was difficult for a weak base to compete against a strong acid. This may be another factor which caused a reduction in hydrolysis of cellulose into glucose during saccharification with MEA pretreatment. Xylose concentrations on the other hand did not depend on sulphuric acid or MEA concentrations. This implied that either H^+ or OH^- ions were not effective in hydrolysing hemicelluloses into xylose. Figure 26 illustrated that optimum pH condition during saccharification was very important during hydrolysis of hemicelluloses into xylose.

Figure 33
Maximum sugar concentrations from all four types of pretreatment and hydrolysis:
at 8.00% biomass (acid, water, and no chemical), 3.33% biomass (alkaline);
at 10.00% (acid), 6.00% (alkaline)



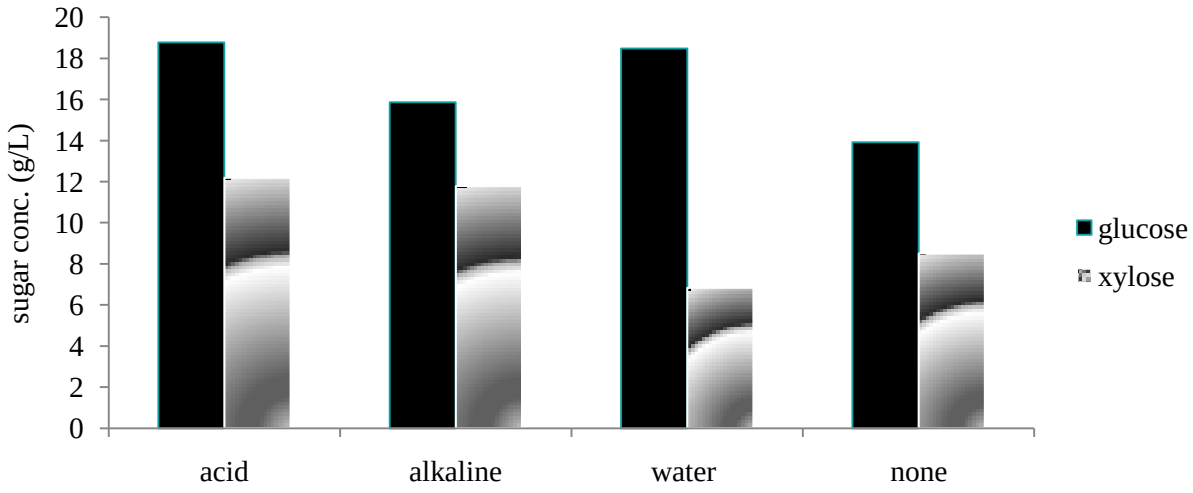
Overall, Figure 33 illustrated that acid pretreatment with 8.00% substrate concentration in 10.00% sulphuric acid and enzymatic hydrolysis at 37°C achieved highest glucose and xylose concentrations.

Hence, acid pretreatment was the best pretreatment to achieve high concentrations of sugars. However, largest biomass concentration was used here along with highest concentration of catalyst, sulphuric acid.

Second highest glucose concentration was obtained through hot water pretreatment with 8.00% biomass concentration and enzymatic hydrolysis with all three types of enzymes at 37°C. There was no catalyst used here but heat was applied during pretreatment at 135°C. The third largest glucose and second highest xylose concentrations were obtained through saccharification with no chemical pretreatment at 37°C with 8.00% biomass concentration. Lowest glucose and xylose concentrations were achieved by alkaline pretreatment with 6.00% MEA, 3.33% biomass concentration and enzymatic hydrolysis at 35°C. Different substrate concentrations were compared here. The latter status would slightly change if same biomass concentration was used.

Figure 34 illustrated maximum sugar concentration from all four pretreatment and saccharification processes with 3.33% biomass concentration. Unfortunately, saccharification with no chemical pretreatment was only conducted with 2.50% biomass concentration. However, Figure 23 illustrated biomass concentrations were linearly proportional to sugar concentrations. So the concentrations from 2.50% biomass concentration could be compared here.

Figure 34
Maximum sugar concentrations:
at 3.33% biomass (acid, alkaline, and water) and 2.50% biomass (no chemical)



Largest glucose concentrations were still obtained by saccharification with acid pretreatment. This was achieved during pretreatment with 6.00% sulfuric acid. Second largest glucose concentration was

achieved by liquid water pretreatment and enzymatic hydrolysis at 45°C with all three enzymes. Third highest glucose concentrations were again achieved by alkaline pretreatment with 6.00% MEA. Lowest glucose concentration was achieved by no chemical pretreatment and saccharification. However, if no chemical pretreatment was performed with 3.33% biomass concentration then, this would have achieved third highest glucose concentration.

In fact, third highest xylose concentrations were achieved saccharification with no chemical pretreatment. Lowest xylose concentrations were achieved by saccharification with water pretreatment. In both hydrolysis without xylanase achieved maximum xylose concentrations. Second highest xylose concentration was achieved by alkaline pretreatment with 6.00% MEA and saccharification.

These results implied that MEA was not a good catalyst. MEA was either dormant during enzymatic hydrolysis or hindered saccharification. This definitely did not improve results compared to water. No catalysts were applied during water pretreatment. Thus, MEA was a poor choice of catalyst to extract sugars from wheat straw. However, this was an excellent choice to remove sugars from hardwood chips (Shah, Song, Lee, & Torget, 1991). This demonstrated that each type of biomass required different type of chemicals and procedures for pretreatment and saccharification.

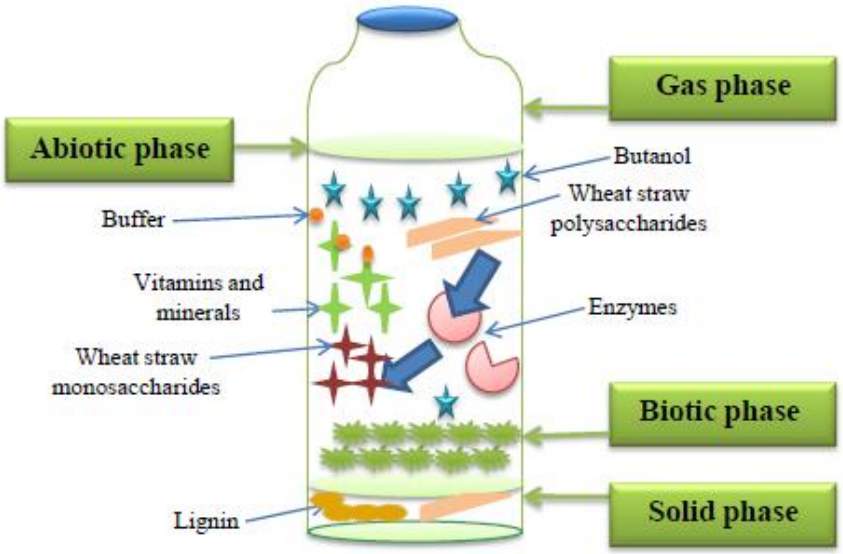
Acidic pretreatment with sulfuric acid extracted highest glucose and xylose concentrations. However, there were several disadvantages involving acidic pretreatment. First, high concentrations of acid catalysts would create inhibitors such as furfural and HMF. Also acidic concentration would destroy bacterial metabolism. Second, it would also be very difficult to adjust pH of a very acidic solution. Third, even dilute sulphuric acid caused acid rain and air pollution (Hill, 2010). This would also burn or irritate human skin.

Several advantages were provided by saccharification with no chemical pretreatment. Even if this process achieved low glucose and xylose concentrations compared to saccharification with acid pretreatment, cost of this procedure was reduced by 33% (Perez et al., 2008). Also there were no inhibitors to be removed which reduced additional time and costs. There was no additional chemical or time required to adjust pH. Fermentation would be completed quickly if chemical pretreatment was eliminated. Less number of reactors was required here. Therefore, saccharification with no chemical pretreatment was the best type of enzymatic hydrolysis for SSF and butanol producing bacteria. Higher xylose concentrations were achieved by saccharification with no chemical pretreatment compared to water pretreatment and saccharification. Water pretreatment and hydrolysis would be second best because it also reduced the costs of catalysts and reduced the amount of inhibitors produced.

4.3 Simultaneous saccharification and fermentation (SSF)

Figure 35 illustrated different activities occurred during batch SSF experiments. Here, pretreatment removed lignin. The lignin settled down in the solid phase. Polysaccharides migrated in to liquid phase. The liquid phase could also be scientifically called abiotic phase (Cinar, Parulekar, & Birol, 2003). These polysaccharides were broken down into monosaccharide during saccharification. Biotic phase is composed of *C.beijerinckii* BA101 cells. These cells were supported by the solid phase. *C.beijerinckii* BA101 consumed vitamins, minerals, and monosaccharide. These bacteria produced energy in the form of adenosine triphosphate (ATP), butanol, and other products through their metabolism. This metabolic pathway was illustrated in Figure 11. The gas phase was mainly composed of nitrogen, which was trapped by neoprene solid stopper. Gas components produced by bacteria dissolved in the gas phase as well. Buffer solution was applied in the abiotic phase to maintain a constant pH. The pH dropped when the butyric acid and acetate were formed during the bacterial metabolism. A strong buffer was required to maintain the optimum pH of the abiotic phase in order to improve the viability of bacterial cells. Again, the optimum pH was 4.5 to 5.5 and optimum temperature was 35°C. More butanol was attained when the viability of biotic phase was increased.

Figure 35
Description of each activity during batch SSF



4.3.1 Control batch fermentation

Figure 36 and Figure 37 illustrated butanol and butyric acid concentration from control batch fermentation. There were no significant pH changes throughout the control batch fermentation. Bacterial cell concentrations were not illustrated here because, bacteria reached stationary phase prior to butanol production. Butyric acid or butanol was not produced until third day of batch fermentation. Butyric acid concentrations were not always increasing.

This would decrease to increase butanol concentration. After most of butyric acid was converted to butanol, more butyric acid was produced to continue butanol fermentation. Butyric acid and butanol were simultaneously produced. This implied that acidogenic phase and solventogenic phase occurred simultaneously.

Figure 36
Butanol concentrations from control batch experiment

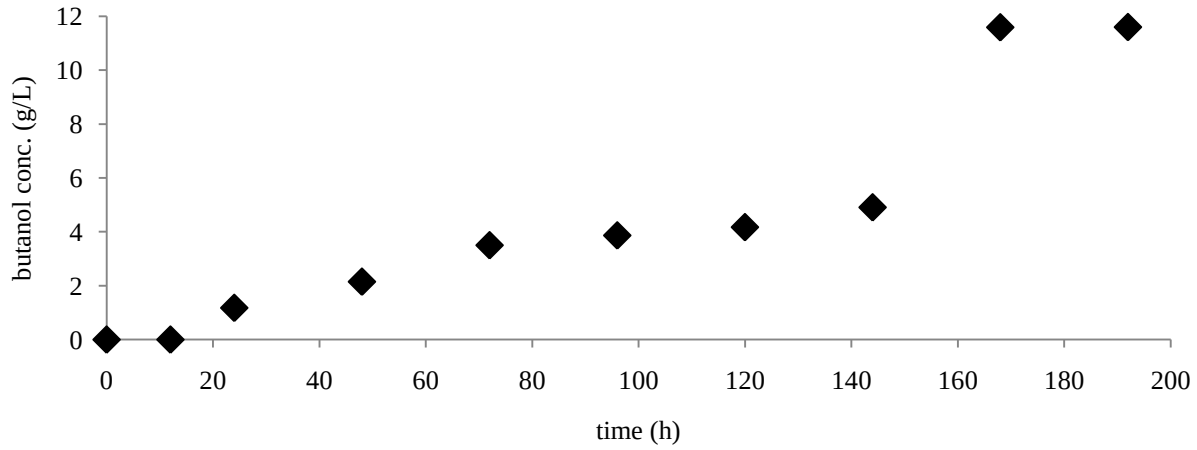
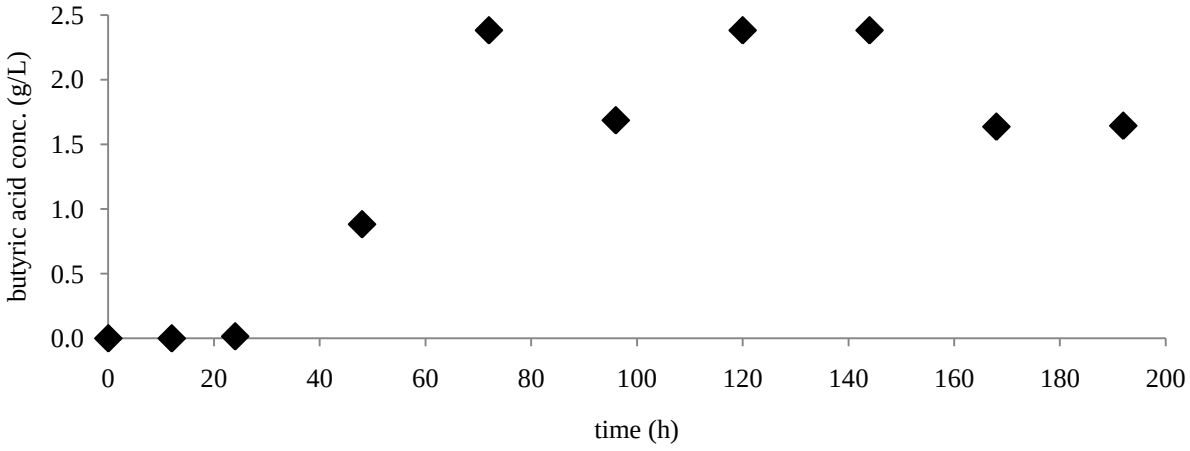


Figure 37
Butyric acid concentrations from control batch experiment

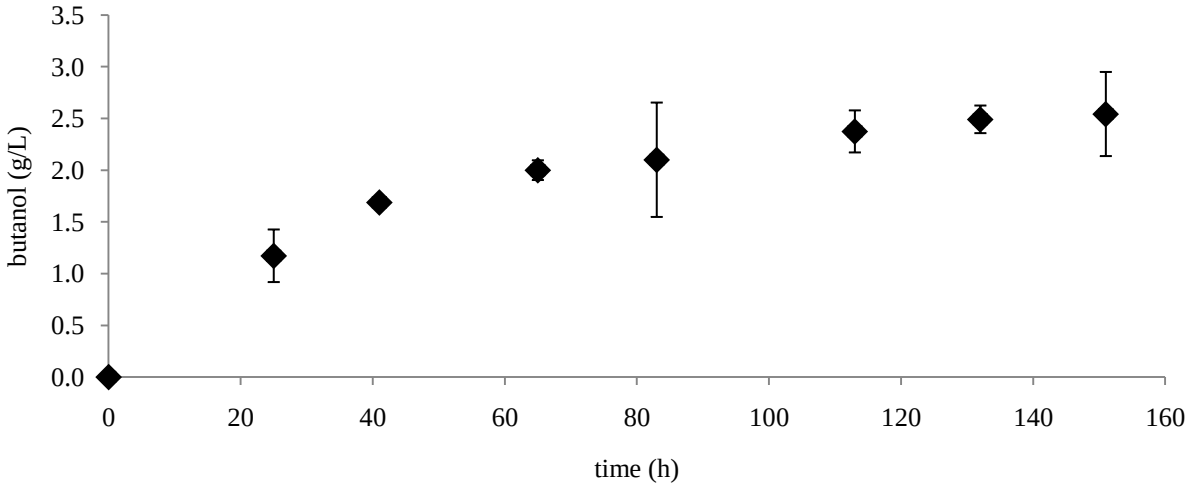


Butanol and butyric acid productions were initiated between 72 and 95 h. Here, sugars were completely consumed before 24 h because, there were no sugar detected throughout the entrire fermentation process. Approximately 12.0 g/L butanol was achieved towards the end of control batch fermentation. This result was similar to the study by Qureshi, Saha, & Cotta, (2007). Sudden increase in butanol concentration from 5.0 g/L to 12.0 g/L occurred in this thesis and in the previous study by Qureshi, Saha, & Cotta, (2007). Here, all of the sugars were available prior to fermentation. Perhaps, bacteria required time to consume all of the sugars before producing high concentration of butanol. This butanol concentration did not increase due to toxicity of butanol towards *C.beijerinckii* BA101.

4.3.2 No chemical pretreatment and SSF

Pretreatment process was completely eliminated in the batch fermentation SSF. During the batch SSF experiment, S11, enzymatic hydrolysis was initiated few hours prior to inoculating *C.beijerinckii* BA101 culture into fermentation medium with biomass. The results obtained through S11 batch fermentation were demonstrated in Figure 38 and Figure 39. Again, bacterial cell concentrations were not illustrated here because, bacteria reached stationary phase prior to butanol production.

Figure 38
Butanol concentrations from S11: no chemical pretreatment and SSF (see Table 11)



No sugar concentrations were detected because *C.beijerinckii* BA101 consumed all the sugar molecules. Butyric acid and butanol production was initiated approximately 24 h after the inoculation. Butanol concentrations were continuously increasing until 132 h, where steady state was reached. Butyric acid concentration decreased at 36 h but this did not influence butanol concentration. There were slight delays in butanol production between 65 and 83 h. This was not due to inhibition because butyric acid concentration did not decrease between these two delays. This implied that bacteria cells continued to metabolize and produce butyric acid but, concentration of butyric acid was not enough to increase butanol concentration a lot. If there were any inhibitors present during butanol fermentation, butyric acid concentration would be decreased. In fact, butyric acid concentrations were decreased when steady state of butanol fermentation was reached. Low concentration of butanol was achieved here because only 2.50% biomass concentration was used. Low biomass concentration provided high sugar yields but low amount of substrate. Hence, less energy was required towards production of sugar monomers.

Figure 39
Butyric acid concentrations from S11: no chemical pretreatment and SSF (see Table 11)

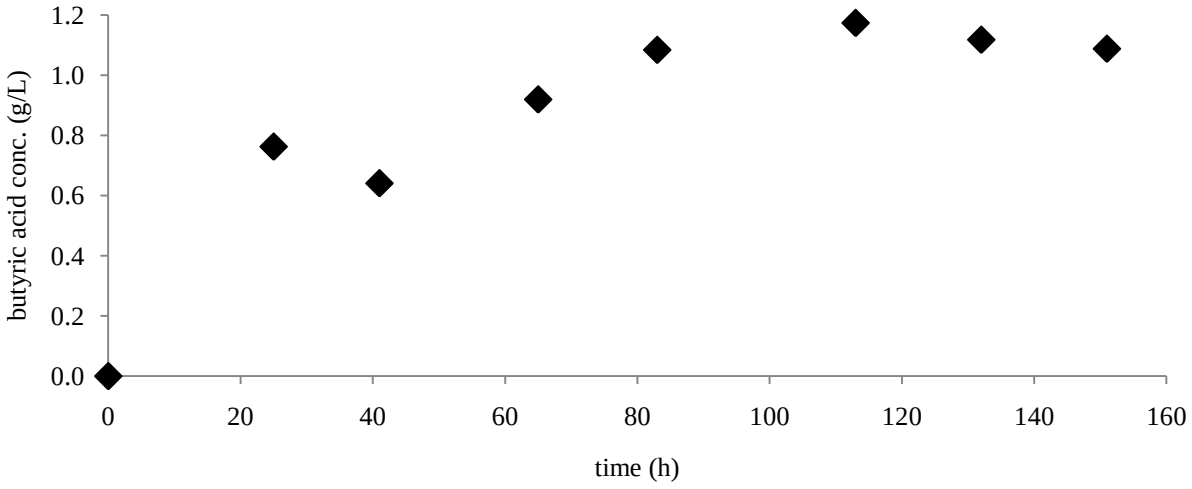


Figure 40
Butanol concentrations from A11: Table 11 no chemical pretreatment and SSF

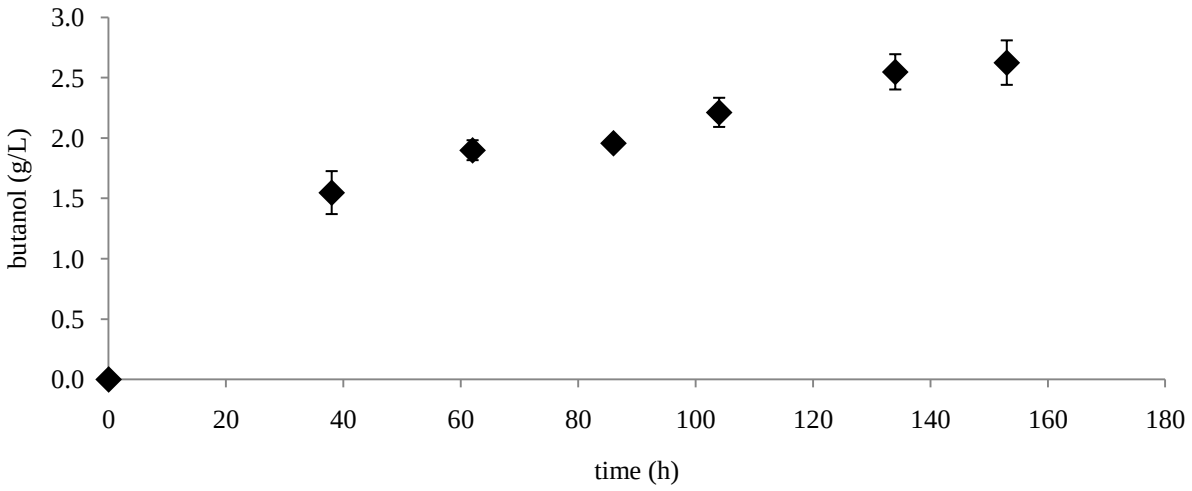
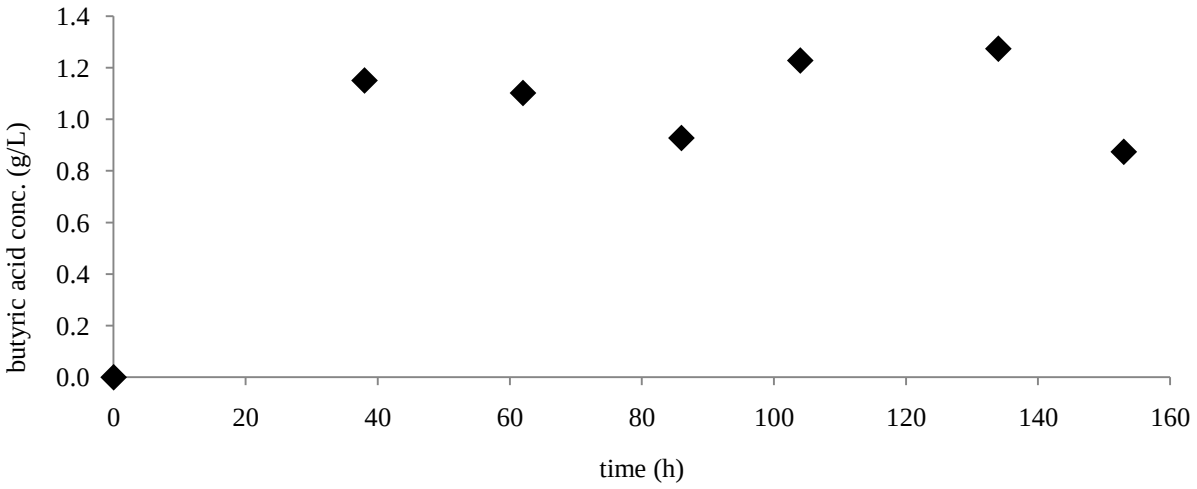


Figure 40 and Figure 41 illustrated the results obtained through batch SSF, A11. There were no chemical pretreatment or saccharification process performed prior to fermentation in A11. Butanol and butyric acid production was initiated within 30 h of fermentation during this experiment as well. This was similar to S11. This implied that butanol production would always begin within 30 h. There was a slight delay between 46 and 60 h. Inhibitors such as, HMF and furfural were not available to cause this delay. This

delay was caused by lack of substrate. Enzyme substrates were competed against several types of molecules including bacteria cells. Bacteria could apply these enzymes for other activities.

Figure 41
Butyric acid concentrations from A11: Table 11 no chemical pretreatment and SSF



Steady state was reached at same time intervals in S11 and A11. This implied that butanol production would be completed quickly whether hydrolysis was initiated prior to fermentation or simultaneously. The final butanol concentrations in A11 were higher than S11. This was more advantageous because procedures in A11 reduced energy, time, and costs of production compared to S11. During experiment A11, saccharification was performed simultaneously during fermentation. During experiment S11, saccharification was performed 20 h prior to fermentation which caused extra time and money.

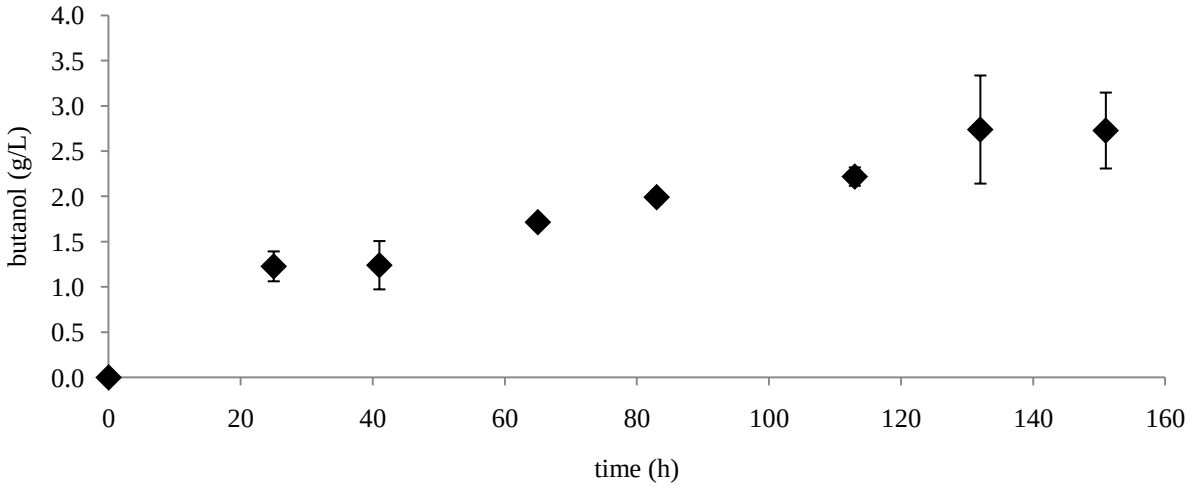
4.3.3 Water pretreatment and SSF

Figure 42 and Figure 43 examined butyric acid and butanol concentrations from experiment S10 in Table 11. Here, enzymatic hydrolysis was initiated 20 h prior to SSF. Concentrations of bacterial strain were not illustrated here because this already approached stationary phase during the production of butanol. There were no significant changes in pH.

Sugars were completely consumed by *C.beijerinckii* BA101 here as well. Butanol concentrations were increasing exponentially, and butanol production was initiated at or before 25 h. There was a small inhibition of butanol fermentation between 25 and 41 h. This inhibition was exceeded by further increase

in butanol production. There could be several reasons for this small inhibition. Butyric acid was reduced at 41 h which may have reduced butanol concentration. Another reason could be that enzyme may have attracted to the wrong substrate. During SSF, there were several types of molecules available. Bacteria may had inhibited the enzyme activity and applied these enzymes for other types of activities. This could have reduced the saccharification process. Another reason could be that bacteria strain paused production of butanol due to lack of metabolism.

Figure 42
Butanol concentrations obtained during SSF in Experiment S10 (Table 11; water pretreatment)



Steady state of butanol production was approached at about 132 h. These results were much lower than control batch experiment because, this was SSF using real biomass as the substrate. Here, only 2.50% biomass concentration was used, which provided very low amount of carbon source for butanol fermentation. High sugar composition was applied in the control experiment compared to experiment S10. Previous sections of this thesis study illustrated that higher sugar yields were achieved with low amounts of biomass. Also all of the sugars produced were completely consumed which did not waste any energy of extracting sugars from biomass. Fermentation was completed faster when biomass was used compared to sugar supplement. Here, butanol and butyric acid production was initiated at 24 h. In the contol experiment, butanol concentrations were not detected until after 72 h.

Figure 43
Butyric acid concentrations obtained during SSF in ExperimentS10 (Table 11; water pretreatment)

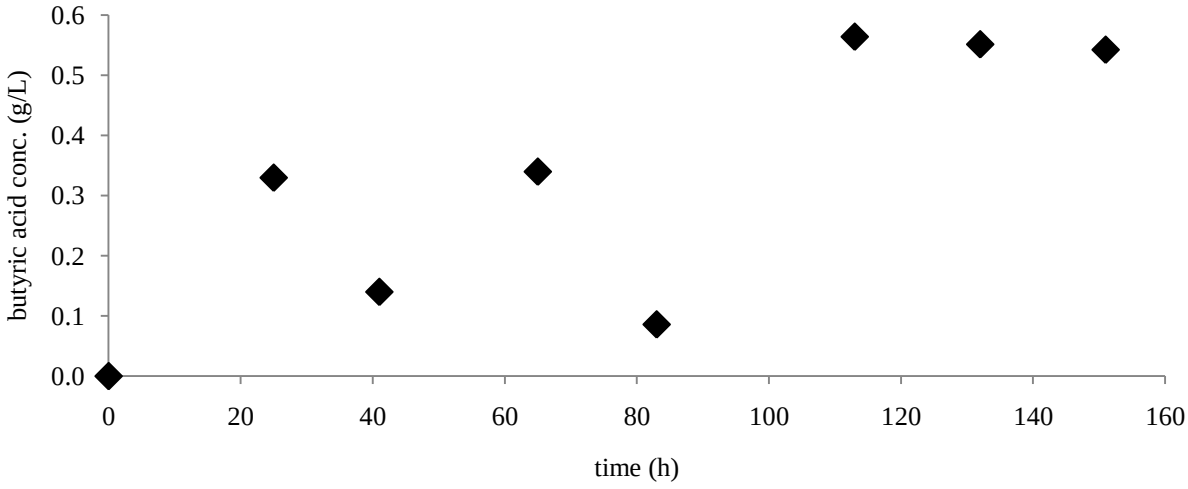


Figure 44
Butanol concentrations obtained during SSF in ExperimentA10 (Table 11; water pretreatment)

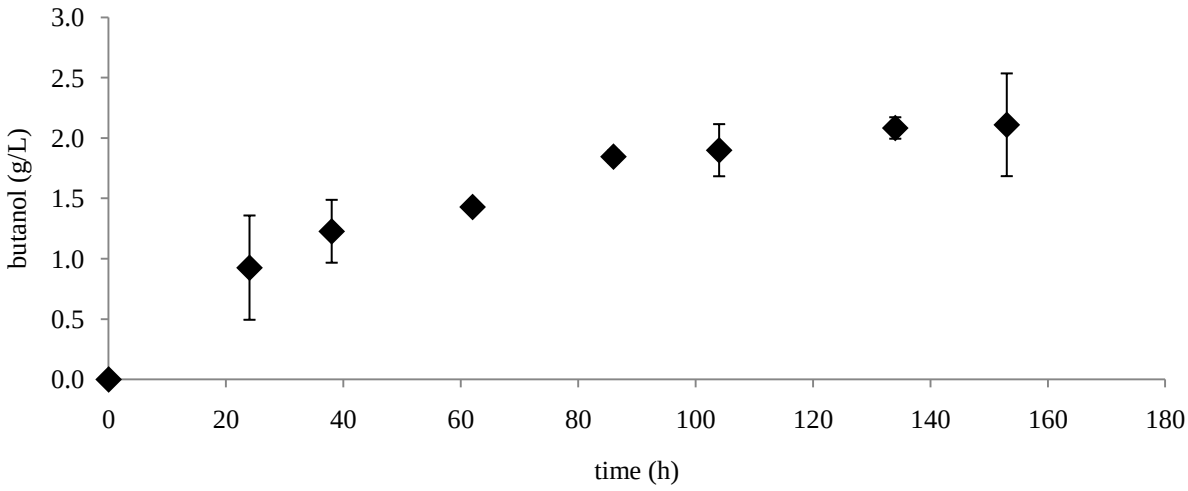
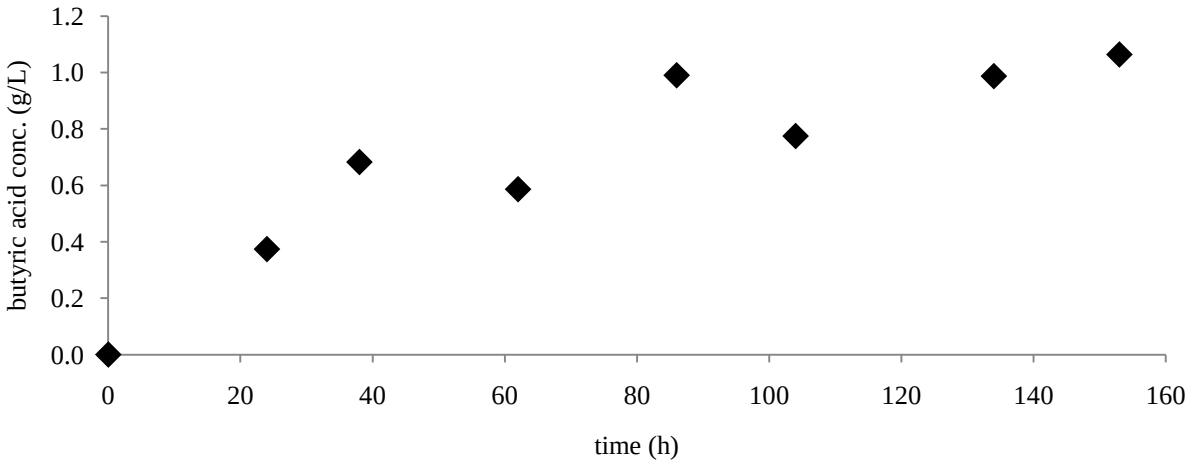


Figure 44 and Figure 45 illustrated butanol and butyric acid concentration from experiment A10 in Table 11. In this experiment, saccharification was conducted simultaneously with fermentation. Here, butanol and butyric acid production was initiated at or before 24 h which was similar to Figure 42 and Figure 43. There were no signs of inhibition in this fermentation. Except there was a slight delay in butanol production after 62 h because butyric acid was decreased at 60 h. Minor reasons such as a small delay in bacteria metabolism or enzyme activity could have caused this small delay. Steady state was reached at

86 h and very low butanol production was achieved. Here very low butanol and butyric acid concentrations were produced.

Figure 45
Butyric acid concentrations obtained during SSF in Experiment A10 (Table 11; water pretreatment)



Several reasons could explain why low butanol and butyric acid concentrations were produced. Hot water pretreatment removed hemicellulose easily but minimized saccharification of cellulose into glucose molecules (Negro et al., 2003). During separate hydrolysis, cellulase and β-glucosidase overcame this inhibition and produced higher glucose concentration by hydrolysing most or all of the cellulose. In the experiment S10 in Table 11, enzymatic hydrolysis was initiated few hours prior to fermentation, which provided enough time to hydrolyse cellulose. Here, the enzymes were confused or tackled by bacteria cells, which minimized the glucose production. It was difficult to determine how much glucose was actually produced because bacteria cells consumed all the glucose molecules as soon as it was produced by the enzymes. There might have been a competition between each bacteria cell to consume the sugar molecules for their metabolism. Since there were not enough glucose molecules available, some bacteria cells could have been dormant.

4.3.4 Acid and alkaline pretreatment with SSF

The above results sections demonstrated that unfortunately, pretreatment with MEA did not remove the highest amount of lignin or hemicelluloses. Also, MEA hindered enzymatic hydrolysis because lower sugar concentrations were achieved compared to hot water pretreatment and hydrolysis. Hence, MEA pretreatment and hydrolysis was not applied in batch SSF. Although, sulfuric acidic pretreatment were applied, this was not illustrated in this chapter due to several reasons. Sulfuric acid pretreatment and SSF failed to produce butanol. The main cause for these failed experiments was lack of bacteria growth. *C.beijerinckii* BA101 required anaerobic conditions. They were very sensitive to harsh chemical such as, sulfuric acid. Batch SSF with 1.00% through 6.00% sulfuric acid pretreatment were observed. Unfortunately, *C.beijerinckii* BA101 failed to grow in any of these pretreated medium whether pH was adjusted or not.

4.3.5 Comparisons of all types of pretreatment and SSF

Table 18 illustrated that highest butanol concentration was achieved in Experiment S10. These values were only slightly higher than butanol production from A11. Surprisingly, butanol production from Experiment A11 was higher than S11. These results illustrated that SSF produced better results than partial SSF process in S11. Lowest products were achieved in A10. Several reasons could have caused this reduction in butanol production. One suitable reason was heat was not reduced to optimum temperature, 35°C, in the entire abiotic phase or solid phase due to lack of heat transfer. Not all particles in the solid and liquid phase had the same temperature. Butanol concentrations obtained in this study were lower than previous study by Qureshi, Saha, & Cotta, (2008 Part I) for several reasons. Different type of bacteria was used here. Low biomass concentration was used here.

Table 18 illustrated the third type of yield examined in this thesis. Here, yield was the ratio of butanol and biomass concentration. These results illustrated that highest yield was achieved in Experiment S10, and the second highest was obtained in Experiment A11. Surprisingly the two highest yields were higher than yield obtained by previous study as illustrated in literature review. In fact, all of the yields obtained by this experiment were higher than previous study. This implied that even if low butanol concentrations were produced, more of the biomass was successfully converted into butanol. Hence, no chemical and water pretreatment with SSF provided excellent choices to achieve maximum butanol concentration with low biomass concentrations even compared to literature values as illustrated in Table 6. Also ratio of

butanol and sugar was calculated to determine the amount of butanol produced per gram of sugar. Here, increase in butanol production illustrated an increase in the ratio of butanol and sugar.

Thus, no chemical pretreatment and SSF successfully produced butanol within a short period of time. There were several advantages towards this process. Emissions of pollutants were eliminated completely. No hazardous chemicals applied or produced here. Even the products butyric acid and butanol were least harmful to human or environment according to Gallagher et al. (2008) and Vernia (2007). Enzymes were the only expensive catalysts used throughout this entire butanol fermentation in Experiment A11, thus approximately 33% of total fermentation costs can be deducted.

Table 18
Comparison of all four pretreatment and SSF

Experiment	Description	biomass (g/L)	butanol (g/L)	total sugar (g/L)	bacteria conc. (cells/L)	butanol yield % (g/g)	ratio of butanol/sugar (g/g)	ratio of cells/butanol (cells/g)
S10	water pretreatment SSF	25.54	2.70	10.4	56621120	10.55%	0.26	4.76E-8
A10	water pretreatment SSF	25.54	2.08	10.4	31141620	8.13%	0.20	6.67E-8
S11	no chemical pretreatment SSF	25.54	2.51	10.8	8493170	9.83%	0.23	2.95E-7
A11	no chemical pretreatment SSF	25.54	2.61	10.8	33972110	10.22%	0.24	7.68E-8
Literature review (Table 6)	acid pretreatment and SSF	86.00	7.00	25.92	-	8.14%	0.27	-

4.3.6 Statistical Analysis

Each experiment was repeated two times to determine the accuracy of results of butanol concentration during simultaneous saccharification and fermentation. The results from both repetitions conducted during Experiment A10 were illustrated in Table 19. Here, 95% confidence intervals were calculated by using t-distribution. The error bars were illustrated in Figure 40. Other error bars from other experiments in SSF were illustrated in Figure 38, Figure 42, and Figure 44. Also 95% confidence intervals for Experiment A11, Experiment S10, and Experiment S11 were illustrated in Table 22, Table 23, and Table 24 (See Appendix F).

The error was also illustrated in Table 19. Here, the maximum error was 7.63% (average error was ~2.5%). This implied that these results were reproducible. There were several sources of errors. For example, butanol concentrations were not measured immediately. Butanol may have not been mixed in equal distribution throughout the entire vial. Other sources of errors include measurements collected from each equipment such as HPLC.

Table 19
Statistical analysis of experimental reproducibility of butanol production from Experiment A10 (See Table 11)

Time (h)	Butanol Exp-1 (g/L)	Butanol Exp-2 (g/L)	Butanol Avg (g/L)	Standard deviation (g/L)	Standard error of the mean (g/L)	95% Confidence interval (g/L)	Error %
24	0.89	0.96	0.92	0.05	0.034	±0.432	7.63%
38	1.21	1.25	1.23	0.03	0.021	±0.260	3.40%
62	1.43	1.43	1.43	0.00	0.003	±0.038	0.42%
86	1.84	1.85	1.84	0.01	0.004	±0.051	0.43%
104	1.88	1.91	1.90	0.02	0.017	±0.216	1.81%
134	2.08	2.09	2.08	0.01	0.007	±0.089	0.67%
153	2.08	2.14	2.11	0.05	0.034	±0.426	3.23%

5 Kinetic analysis

This chapter applied existing kinetic models for saccharification of cellulose. Previous models did not include the effect of different parameters examined in this thesis. Most suitable parameters from different types of pretreatment and saccharification processes were applied to examine the effect of butanol production during batch SSF process in previous sections. The same parameters would be examined in this chapter to improve the existing models to determine the best profile for saccharification process. These models did not encounter the effect of xylanase or hydrolysis of hemicellulose due to lack materials and information. In fact, these models assumed that hydrolysis of cellulose into glucose molecules were most important. Kinetic analysis for butanol production could not be found in pervious literature review. Hence, new model was created in this chapter to determine a long term profile for butanol production during batch SSF process.

5.1 Pretreatment and saccharification process

This section would elaborate on rate of hydrolysis in cellulose polysaccharides. Equation 1 illustrated the rate of hydrolysis of cellulose. Here, cellulose was hydrolyzed by cellulose (Celluclast 1.5L). During this saccharification cellobiose and some glucose were produced. Equation 2 illustrated the rate of hydrolysis of cellobiose. Equation 3 illustrated the glucose consumption rate by the bacteria during SSF (Philippidis, Spindler, & Wyman, 1992). The rate, r_3, was developed through general studies of fermentation in a batch process (Sinclair & Kristiansen, 1987). This was not specific for *Clostridium beijerinckii*. The parameters would be modified in different culture growth.

$$r_1 = \frac{k_1 C}{1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}}$$

Equation 1

$$r_2 = \frac{k_2 B}{K_m\left(1+\frac{G}{K_{2G}}\right)+B}$$

Equation 2

$$r_3 = \frac{1}{Y_x}\frac{dX}{dt} + mX$$

Equation 3

The variable C represented cellulose, B was cellobiose, and G was glucose. Obviously, r_1 and r_2 represented rates of cellulose and cellobiose hydrolysis. The coefficient K_{1B} represented inhibition constant of cellulase caused by cellobiose production. K_{1G} was the inhibition constant of cellulase caused by glucose production. K_{2G} represented inhibition constants of β-glucosidase caused by glucose. There was no significant inhibition caused by cellobiose on the activity of β-glucosidase. Inhibition of enzyme activity could also be found in the study by Beltrame et al. (1984). K_{1B} and K_{1G} were derived from hydrolysis of cellulose while K_{2G} derived from hydrolysis of cellobiose.

K_m represented Michaelis constant for β-glucosidase which depended on the substrate concentration. Small K_m required more substrate but the enzyme activity was performed quickly (Shuler & Kargi, 2002). The parameters k_1 and k_2 represented several factors such as adsorption of cellulase onto cellulose. However, k_1 and k_2 mainly represented specific rate of cellulose and cellobiose.

The parameter Y_x represented the average yield coefficient of cell mass on glucose (Philippidis, Spindler, & Wyman, 1992). Also the paramter, m, in r_3 represented specific rate of glucose consumption for maintenance energy. K_3 was the monod constant of glucose for bacteria growth. Monod constant was correlated to Michaelis constant and Langmuir adsorption isotherm for heterogenous catalysis (Sinclair & Kristiansen, 1987). K_i was the constant of cell growth inhibition by glucose. Finally, k_d was the specific rate of cell death. The variable P represented butanol product.

All of these constants were derived from the study by Philippidis, Smith, & Wyman (1992) and Philippidis, Spindler, & Wyman (1992). These constants were summarized in Table 20. Equation 4 through Equation 7 illustrated the mass balance equations for cellulose, cellobiose, glucose, and bacteria growth. Each of these mass balance depended on r_1 through r_3. The coefficients 1.056 and 1.053

were derived from water molecules during hydrolysis of cellulose and cellobiose. Again, the mass balance for bacteria growth was derived through general assumptions through general studies of bacteria fermentation in a batch reactor, which was not specific to *Clostridium beijerinckii* (Sinclair & Kristiansen, 1987).

$$\frac{dC}{dt} = -r_1$$

Equation 4

$$\frac{dB}{dt} = -1.056\, r_1 - r_2$$

Equation 5

$$\frac{dG}{dt} = 1.053 r_2 - r_3$$

Equation 6

$$\frac{dX}{dt} = \mu_m \left(\frac{G}{K_3} + G + \frac{G^2}{K_i} \right) (1 + P)(X - k_d X)$$

Equation 7

Table 20
Summary of constants applied in kinetic model

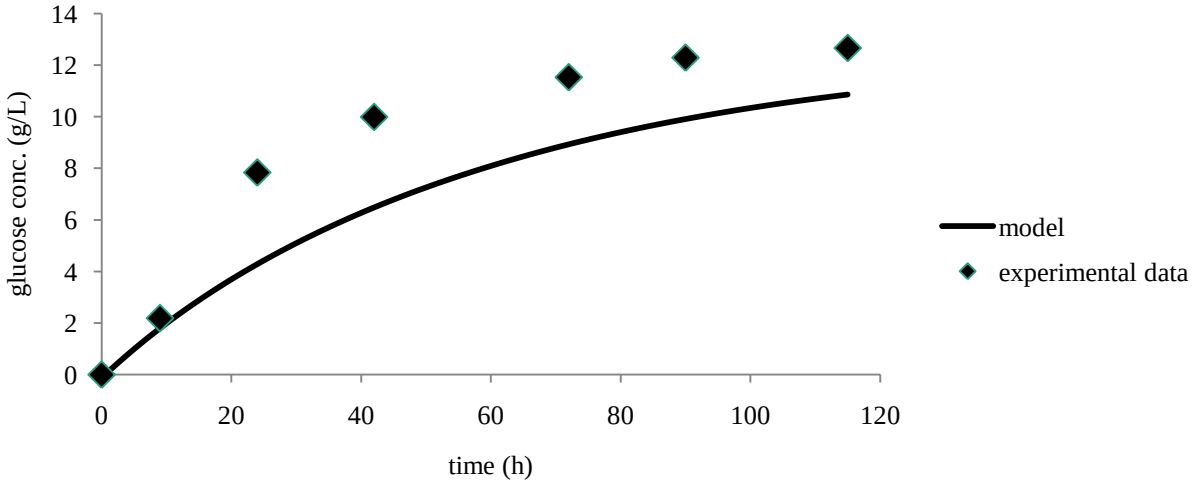
Parameters	r_3	Parameters	r_1	Parameters	r_2
Y_x (g/g)	0.31	k_1 (h^{-1})	0.025	k_2 ($g \cdot L^{-1} \cdot h^{-1}$)	14.22
m (h^{-1})	0.211	K_{1B} (g/L)	5.85	K_2 (g/L)	0.62
μ_m (h^{-1})	0.142	K_{1G} (g/L)	53.16	K_m (g/L)	10.56
K_3 (g/L)	0.171				
K_i (g/L)	33.8				
k_d (h^{-1})	0.0505				

5.1.1 No chemical pretreatment and saccharification

Glucose concentrations were calculated with the application of latter kinetic models and ordinary differential equation (ODE) solver code for ODE 15s in MatLab software program. This program code is illustrated in Appendix C. Hence these kinetic models also provided an excellent tool to estimate the concentrations of cellulose and cellobiose without conducting any expensive experiments. Only initial cellulose concentrations were applied to determine these profiles. Initial concentrations for glucose and cellobiose were zero.

Figure 46 illustrated glucose concentrations with the application of above kinetic models and true experimental values from saccharification with no chemical pretreatment at 2.50% biomass concentration. Glucose profile was obtained from the application of kinetic models. Glucose concentrations were calculated up to 115 h for saccharification at 40°C with 2.50% biomass concentration. Here, the root mean square error was 9.4%.

Figure 46
Glucose concentrations from kinetic models for saccharification with no chemical pretreatment: 2.50% biomass concentration; saccharification at 40°C

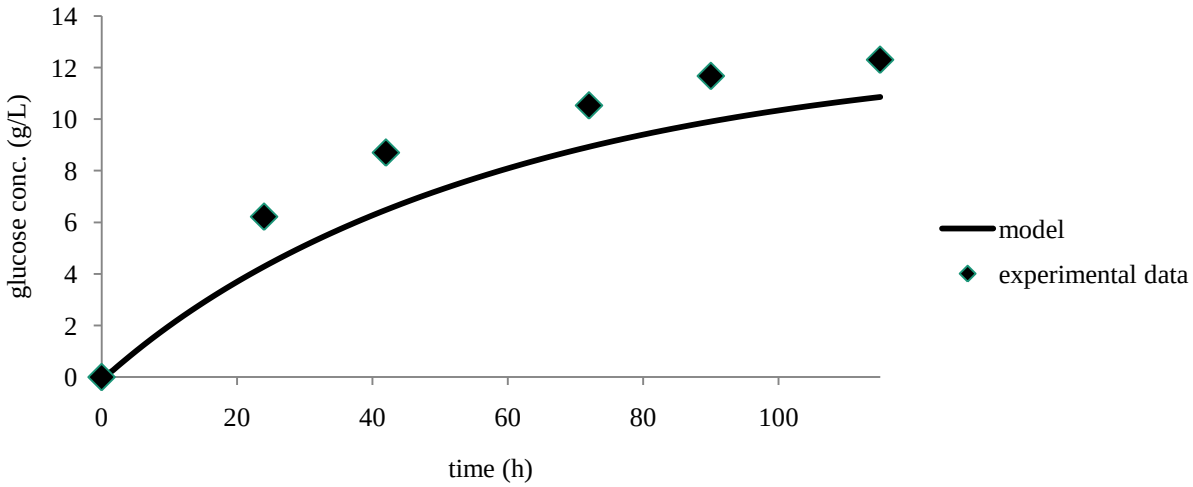


5.1.2 Water pretreatment and saccharification

Similarly, Figure 47 illustrated glucose profile for water pretreatment and saccharification. Here, 2.50% biomass concentration was applied during saccharification at 40°C. Again, saccharification at 40°C was

monitored because this was the optimum saccharification temperature. Here, Equation 4 was applied without any temperature adjustments to determine the glucose profile. The root mean square error was 7.1%. The kinetic model for glucose profile was more suitable for water pretreatment and saccharification.

Figure 47
Glucose concentrations from kinetic models for saccharification with water pretreatment: 2.50% biomass concentration; saccharification at 40°C



5.2 Kinetic models at different saccharification temperature

The previous models were not applicable to different pretreatment and saccharification processes. Here, the models were slightly modified to determine the profile for glucose concentrations obtained through different saccharification temperatures. Only the mass balance of cellulose was modified because, concentration of cellulose determined the glucose production. Other mass balances were not influenced during temperature changes. The new mass balance for cellulose was illustrated in Equation 8. The variable T represented change in saccharification temperature from optimum saccharification temperature. This model assumed that hydrolysis of cellulose was linearly proportional to change in saccharification temperature. The latter statement was proven in Figure 21 during comparisons of glucose concentrations from water pretreatment and saccharification. Same proportionality constant was applied for both no chemical pretreatment and water pretreatment. Parameters of pretreatment and saccharification influenced glucose production in a similar manner during no chemical pretreatment and water pretreatment.

$$\frac{dC}{dt} = -\frac{0.025C}{1 + \frac{B}{5.85} + \frac{G}{53.16} \cdot 2.4 \cdot \Delta T}$$

Equation 8

5.2.1 No chemical pretreatment and saccharification

The new profile for glucose concentrations with temperature adjustments were illustrated in Figure 48 This figure illustrated glucose profile for saccharification with no chemical pretreatmentat 35°C with 2.50% biomass concentration. Here, T was reduced by 5.0°C because optimum saccharification temperature was 40°C. The root mean square error was 5.1%. This error was higher when temperature was not encountered into kinetic models. Also modified kinetic model provided more accurate profile for glucose concentrations compared to profile from old kinetic models. Figure 49 and Figure 50 illustrated profiles for cellulose and cellobiose concentrations. These values were not compared with experimental values due to lack of facilities to measure cellulose and cellobiose concentration. However these profiles were similar to previous studies (Philippidis, Smith, & Wyman, 1992).

Figure 48
Glucose concentrations from temperature adjusted kinetic models for saccharification with no chemical pretreatment: 2.50% biomass concentration; saccharification at 35°C

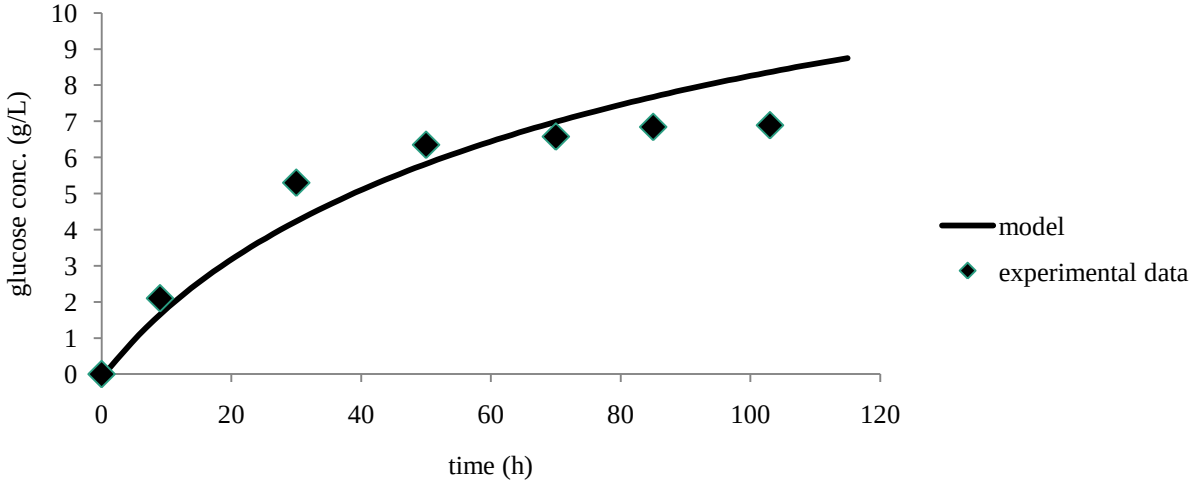


Figure 49
Cellulose concentrations from temperature adjusted kinetic models for saccharification with no chemical pretreatment: 2.50% biomass concentration; saccharification at 35°C

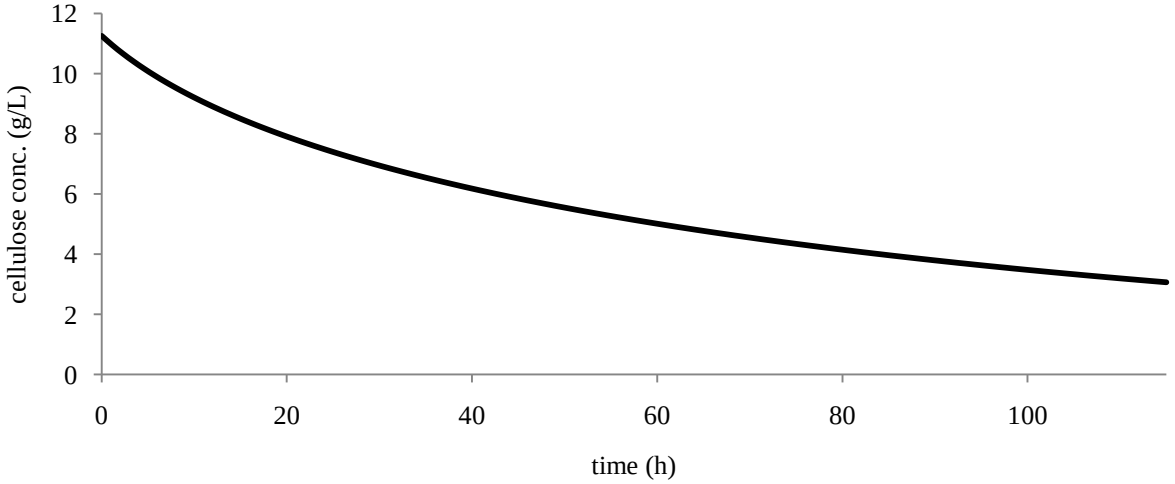
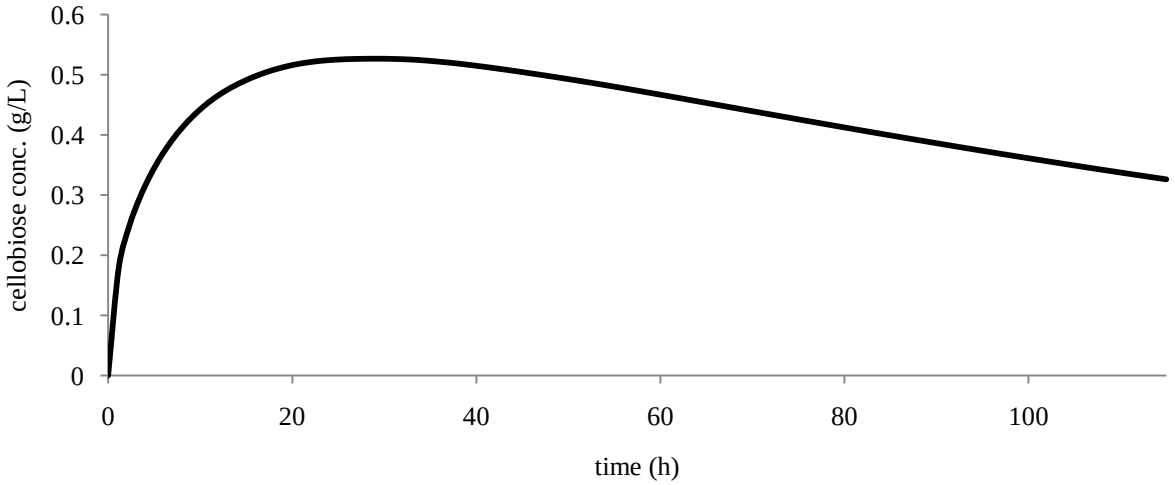


Figure 50
Cellobiose concentrations from temperature adjusted kinetic models for saccharification with no chemical pretreatment: 2.50% biomass concentration; saccharification at 35°C



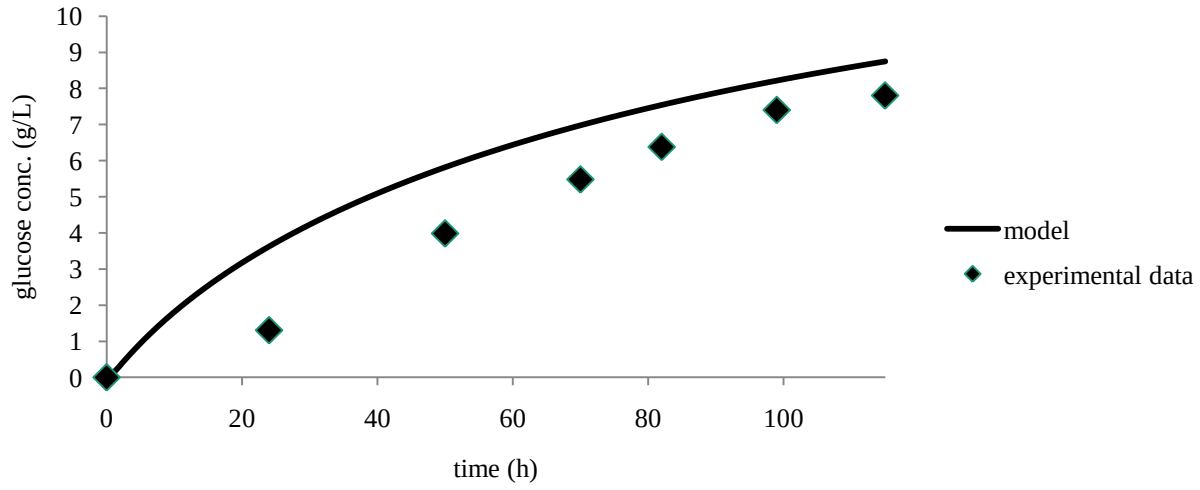
5.2.2 Water pretreatment and saccharification

Although previous kinetic models provided better fit here, temperature changes were not implemented into previous kinetic models. Equation 8 was developed with an assumption that glucose concentrations

were linearly proportional to saccharification temperature. Figure 21 approved the latter proportionality. Final glucose concentrations from saccharification with water pretreatment at all biomass concentrations and saccharification temperature were applied in Figure 21. The linear pattern was followed in all four biomass concentrations. The residual sum of squares was approximately 1.0.

Figure 51 illustrated glucose profile with the application of new model. This profile determined glucose concentrations for 2.50% biomass concentration with saccharification with water pretreatment at 35°C. Here, the root mean square error was 4.5%. This error was lower when compared with previous model without the temperature parameter.

Figure 51
Glucose concentrations from temperature adjusted kinetic models for saccharification with water pretreatment: 2.50% biomass concentration; saccharification at 35°C



5.3 Development of kinetic model for butanol production

In order to find a kinetic model to determine a long time profile for butanol production in batch SSF process, some calculations must be performed. First third rate of reaction, r_4, must be developed because butanol was produced after the production of glucose molecules. Equation 9 illustrated a model for r_4. The variable P represented butanol product. Coefficient K_p must be determined through some calculations. Here, unit of P was g/L then units of K_p must be L/g.

$$r_4 = \frac{dG}{dt}\left(\frac{1}{1+K_p P}\right)$$

Equation 9

In order to find coefficient K_p, r_4 must be set to zero which was illustrated in Equation 10. The effect of other two rates was assumed to be constant because this was SSF process and there was no change at time zero. The units of constant, c, must be (g/L·h).

$$(r_4)_{t=0} = \frac{c}{1+K_p P}$$

Equation 10

At time zero, r_4 was determined through calculations of r_1 at different time intervals. Here, rate of cellulose was applied because cellulose hydrolysis must begin at time zero. Other hydrolysis and products did not exist at time zero. These rates were normalized through conversion of percentages. Then these values were compared with true values of butanol concentrations produced during experiments A10 and A11. These latter comparisons were created in a graphical form to determine the coefficient K_p.

Finally, r_4 was applied in Equation 11 to determine the kinetic model for butanol production. Hence, Equation 11 provided a new model to determine the long term profile of butanol concentrations without conducting any expensive or tedious experiments. Here, mass balance of butanol, $\frac{dP}{dt}$ was determined by multiplying mass balance of glucose by r_4. Mass balance of glucose was applied here because butanol production strictly depended on the production of glucose.

$$\frac{dP}{dt} = r_4$$

Equation 11

5.3.1 No chemical pretreatment and SSF in batch process

Concentrations of cellulose were crucial to determine initial rate of r_4. Only initial concentrations of cellulose were applied to obtain the concentrations of cellulose, cellobiose, and glucose. Equation 8 was applied to determine the rate of cellulose during saccharification at 35°C. The rate, r_1, was calculated at different time intervals using the ODE solver in Mat Lab software program. These rates were converted to percentages which could be called normalized hydrolysis rate.

Concentrations of butanol were compared with the normalized rates of cellulose in Figure 52. The correlations between hydrolysis rate and butanol production could be determined by the application of Equation 10. Coefficient K_p was 1.951 which was determined through applications of non linear regression methods. Here, the sum of residual squares was 0.94. This coefficient was applied to determine r_4.

Then, r_4 was applied to determine the long term profile for butanol production. This profile for butanol concentrations were also obtained through the application of ODE solver in Mat Lab software program. Figure 53 illustrated this butanol profile. These values were compared with experimental values from A11. The root mean square error was only 1.2%. Hence, the error was very low, which approved that this model was excellent fit to determine butanol profile from no chemical pretreatment and SSF.

Figure 52
Comparison of normalized hydrolysis rate at different butanol concentration obtained during no chemical and SSF (A11)

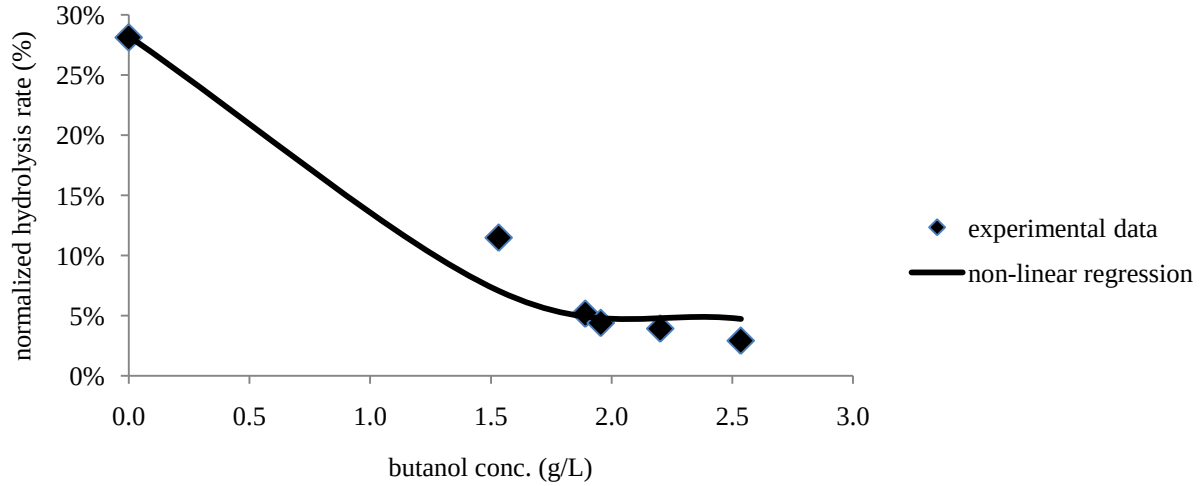
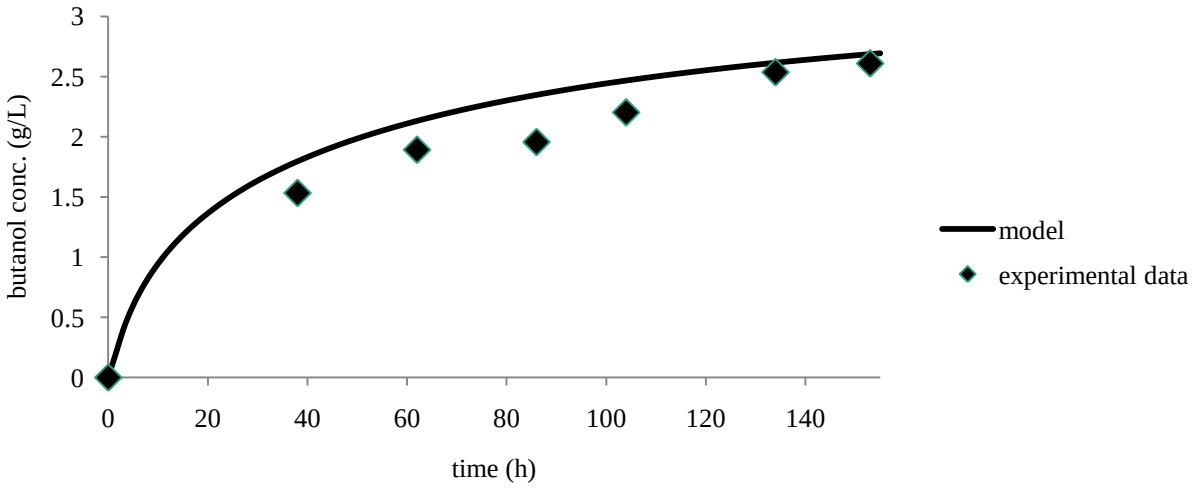


Figure 53
Butanol profile for no chemical pretreatment and SSF (A11):
2.50% biomass concentration; SSF at 35°C; batch process



5.3.2 Water pretreatment and SSF in batch process

Similarly models for butanol production could be developed for water pretreatment and SSF in a batch process. Different K_p was determined here because butanol production depended on different glucose concentrations. The parameter, K_p implemented the effect of different type of pretreatment processes.

Figure 54
Comparison of normalized hydrolysis rate at different butanol concentration obtained during no chemical and SSF (A11)

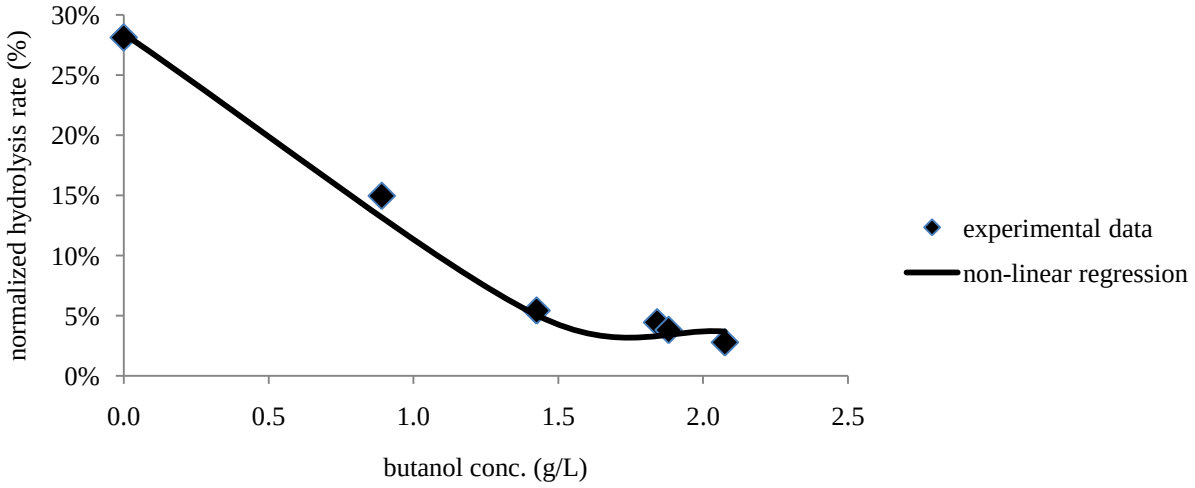
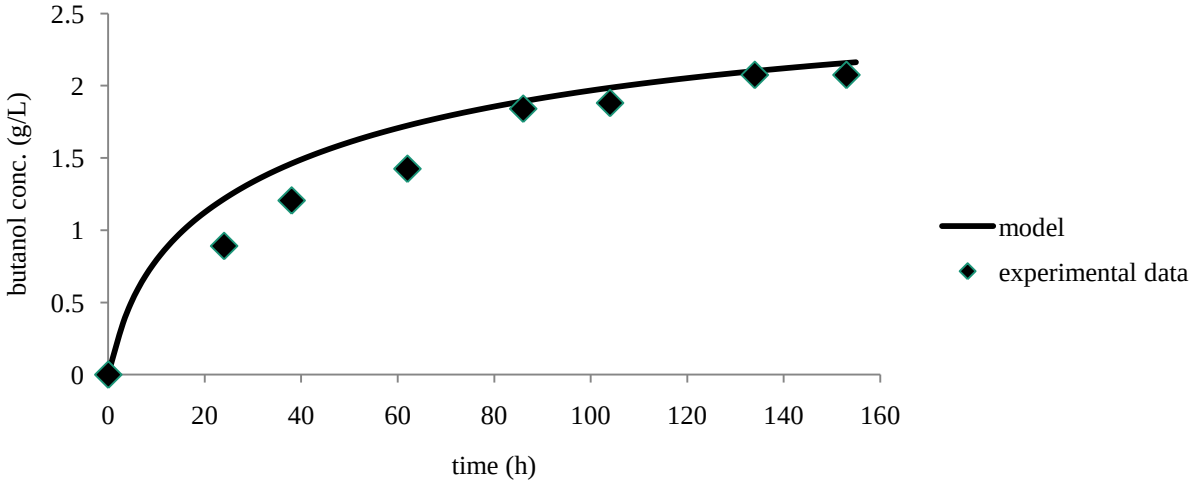


Figure 54 illustrated normalized hydrolysis rate of cellulose and butanol concentrations from experiment A10. Here, K_p was 3.245, which was again determined through non-linear regression method. Then, profile for butanol concentrations was determined through the application of kinetic models. This was illustrated in Figure 55. The root mean square error was only 0.83%. Hence, this model with different K_p provided excellent prediction for butanol profile for water pretreatment and SSF.

Figure 55
Butanol profile for water pretreatment and SSF (A10):
2.50% biomass concentration; SSF at 35°C; batch process



6 Conclusions

This thesis study achieved its objective of producing butanol from biomass in an inexpensive process without generating air pollution or inhibitors at optimum sugar yields. Several methods for butanol production existed but they had several factors, which were unfavourable to massive production in industries.

No chemical pretreatment and SSF methods provided results that achieved the objectives of this thesis. Butanol yield was more than 10% during strict SSF conditions. Table 18 illustrated that this value was higher than previous literature values. This reduced the costs of fermentation by approximately 30%. Butanol production was guaranteed at all times because there were no inhibitors. Fermentation was completed successfully and quickly. Low biomass concentration after saccharification with no chemical pretreatment achieved 100% glucose yields.

Water pretreatment and SSF also provided results that achieved the objectives of this thesis. Highest butanol conversion by the biomass was achieved when partial saccharification was conducted few hours prior to fermentation. Higher glucose concentrations were achieved compared to saccharification with no chemical pretreatment. However, 100% glucose yields were achieved in both cases. Xylose concentrations were lower than results from saccharification with no chemical pretreatment. Inhibitors were present at small amount. This caused delays in SSF. More expensive compared to saccharification with no chemical pretreatment. Hazardous environment is provided at massive butanol production in industries because pretreatment at high temperatures above 121°C were applied. Sulfuric acid pretreatment inhibited SSF. There were several disadvantages, which should be avoided. Inhibitors were produced which did not always guarantee a successful butanol fermentation. Experiments in this thesis demonstrated inhibition of SSF caused by dilute sulfuric acid. This was most expensive pretreatment because it recovered highest glucose and xylose concentrations. Soaking process reduced some costs because heat was eliminated during pretreatment.

Saccharification with MEA pretreatment provided the least best choice for SSF. Here, 100% glucose and xylose yields were achieved when 6.0% MEA was applied. Sugar concentrations were lower than hot water pretreatment. This process only increased costs of fermentation.

Kinetic models provided excellent profiles for cellulose, cellobiose, glucose, and butanol. These profiles could be advantages as follows. These profiles were determined for two types of pretreatment process

prior to batch SSF: no chemical pretreatment and water pretreatment. Previous models did not consider the effect of different types of pretreatment. Models developed in this thesis considered the effect of different biomass concentration, saccharification temperature, and pretreatment process. Expenses of unnecessary experiments were eliminated. These models only require initial concentration of cellulose, saccharification temperature, and the type of pretreatment to determine each profile. Root mean square error for the models was 1.21% for SSF with no chemical pretreatment and 0.83% for SSF with water pretreatment.

Therefore, no chemical pretreatment and SSF provided the best solutions to resolve several issues. Most or all of the cellulose in the biomass was converted into glucose molecules. All of these glucose molecules and other sugars were completely consumed for an effective butanol production. In result, butanol concentration continuously increased without any inhibition and fermentation was completed quickly.

7 Recommendations

No chemical pretreatment and saccharification SSF methods of batch fermentation are recommended to be applied in large scale production. This process could be further improved in continuous butanol fermentation. Immobilized reactors are recommended for high achievement of butanol fermentation. Product removal would also be recommended for continuous production of butanol.

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Appendix A **Figures from experimental results**

A.1 No chemical pretreatment and saccharification

Figure 56
Xylose concentrations from 5.00% biomass concentrations at different saccharification temperatures

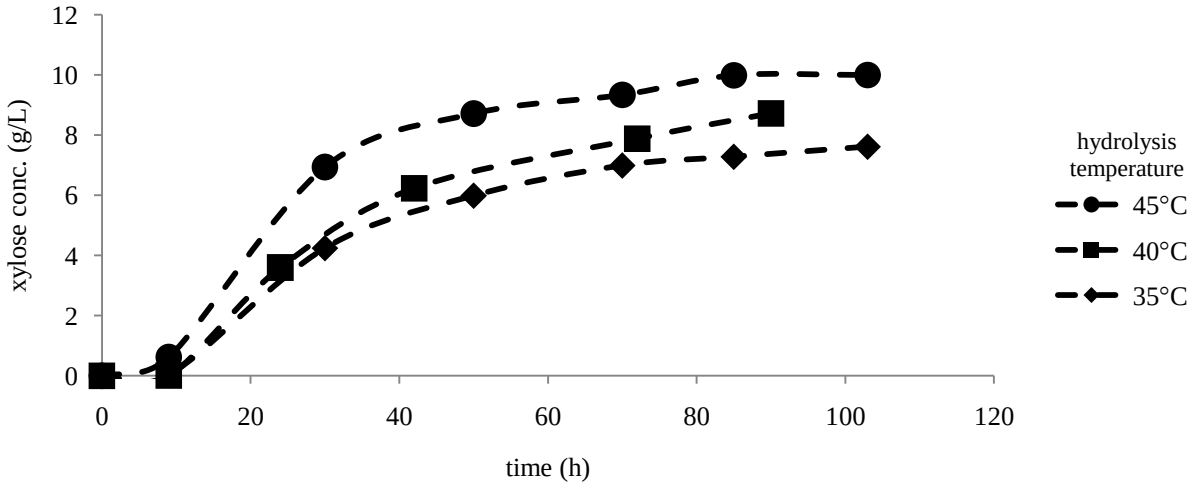


Figure 57
Sugar concentrations from 5.00% biomass concentrations during saccharification with cellulase, β-glucosidase, and xylanase

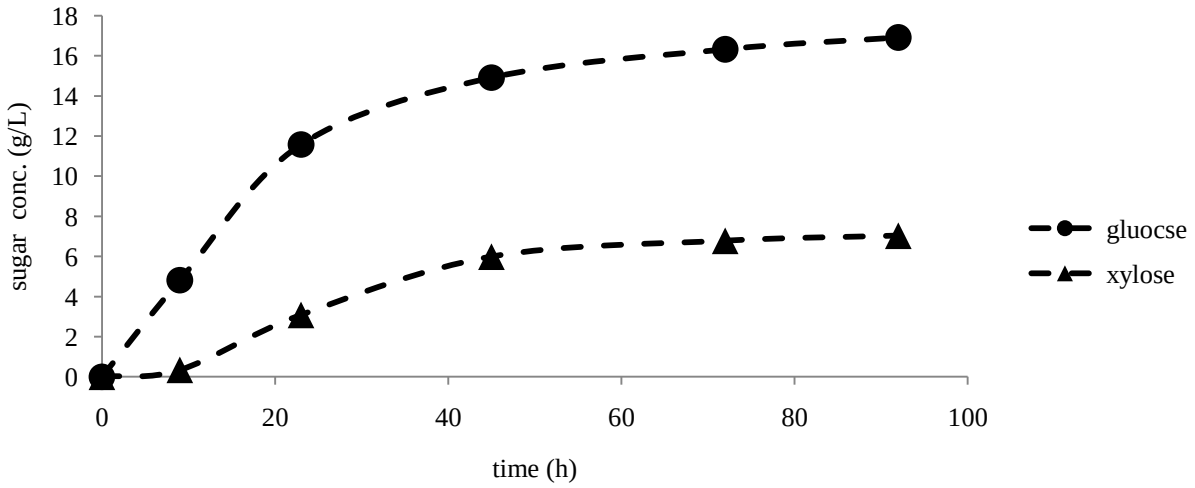


Figure 58
Glucose concentrations from 2.50% biomass concentrations at different saccharification temperatures

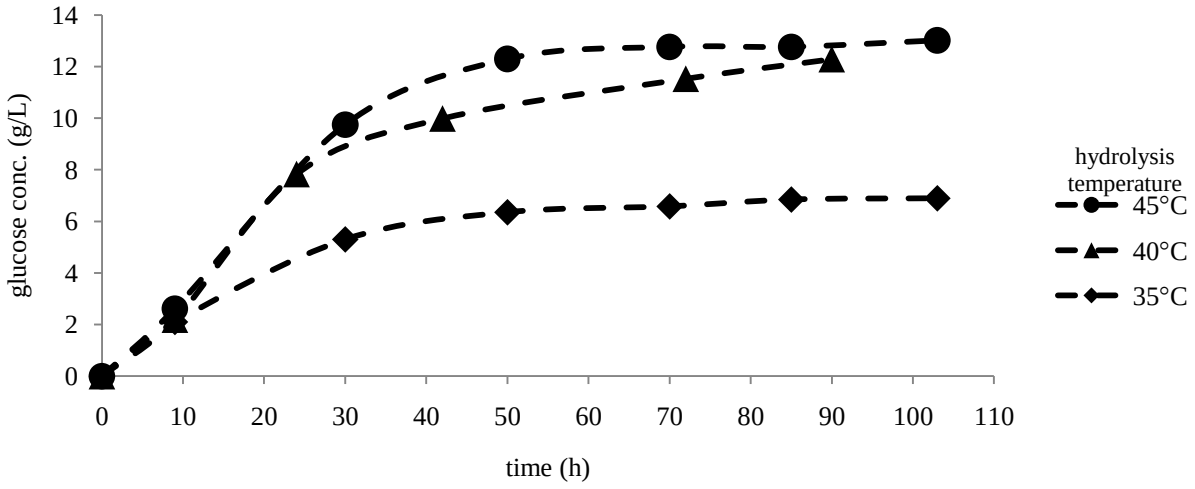


Figure 59
Xylose concentrations from 2.50% biomass concentrations at different saccharification temperatures

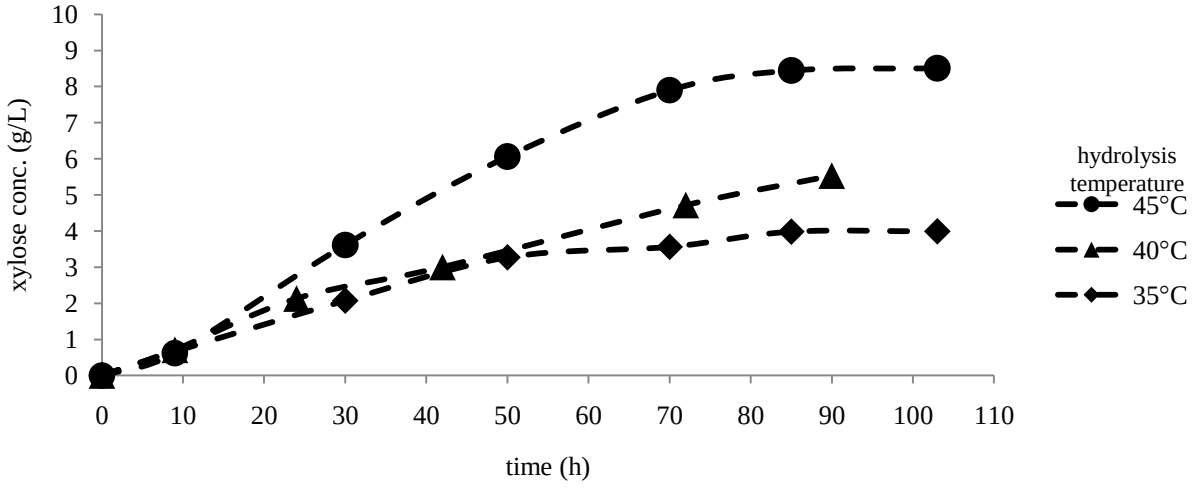


Figure 60
Sugar concentrations from 2.50% biomass concentrations during saccharification with cellulase, β-glucosidase, and xylanase

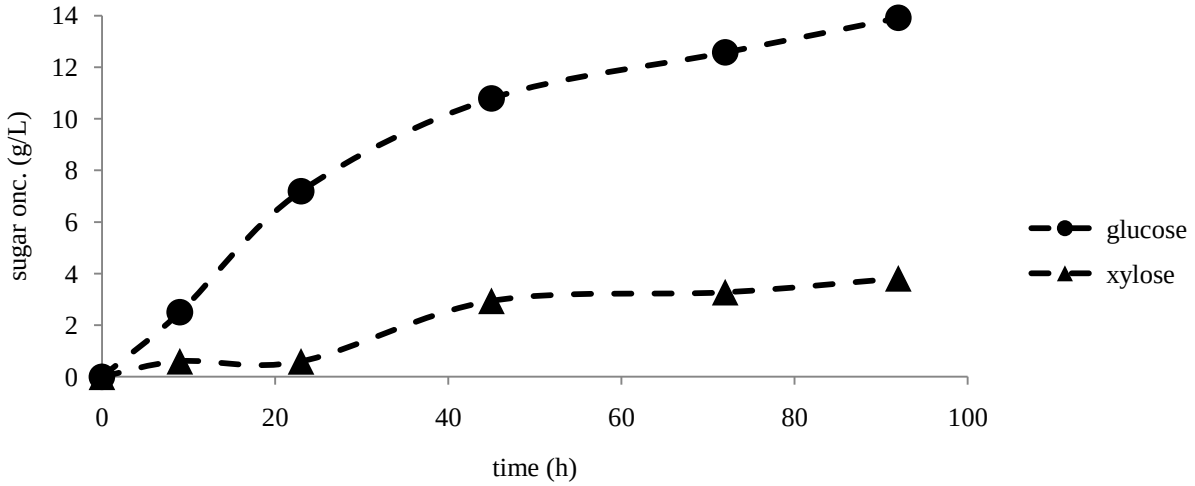
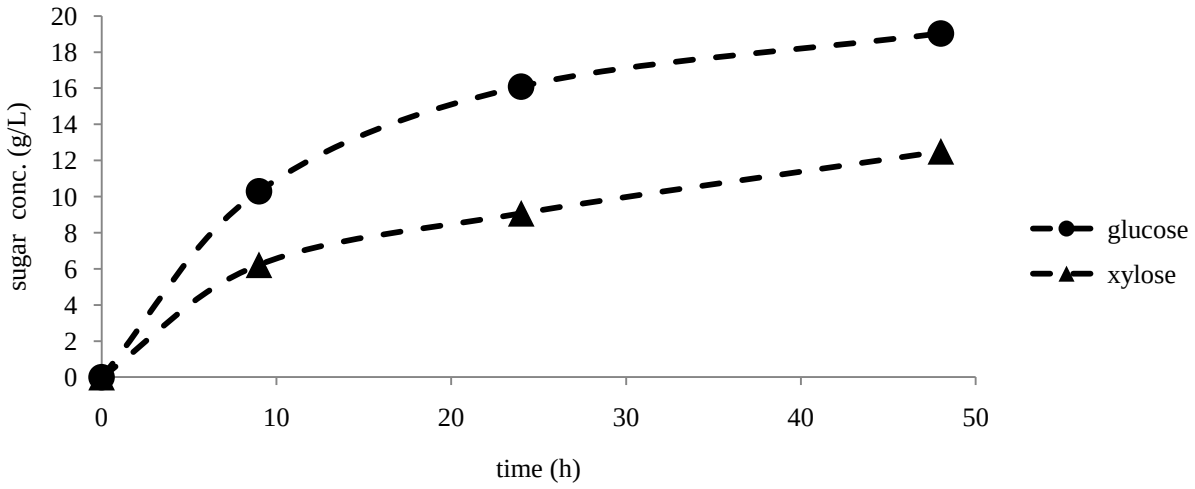


Figure 61
Sugar concentrations from 8.60% biomass concentrations and saccharification



A.2 Water pretreatment and saccharification

Figure 62
Glucose concentrations from 7.14% biomass concentration at different saccharification temperatures

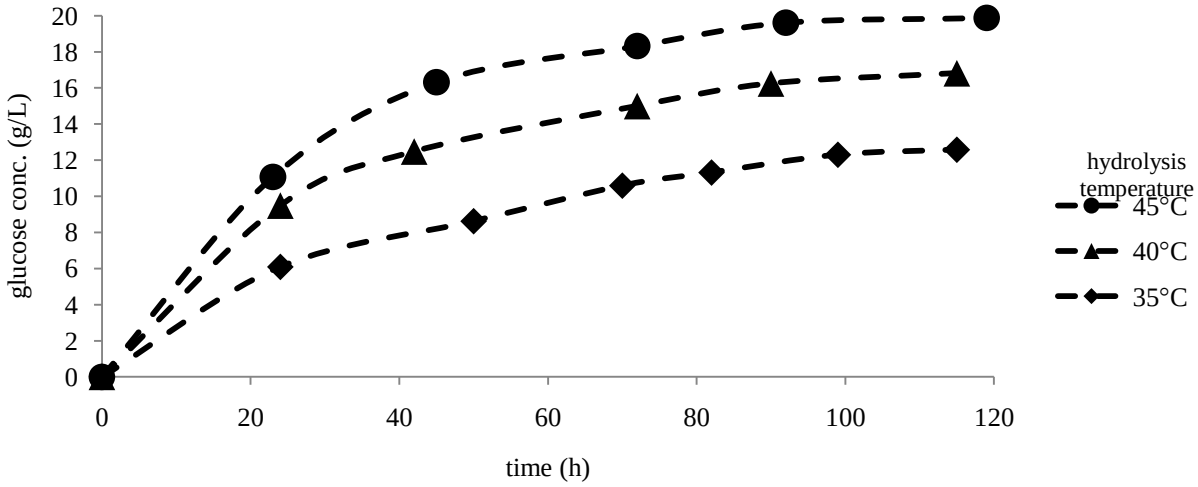


Figure 63
Xylose concentrations from 7.14% biomass concentration at different saccharification temperatures

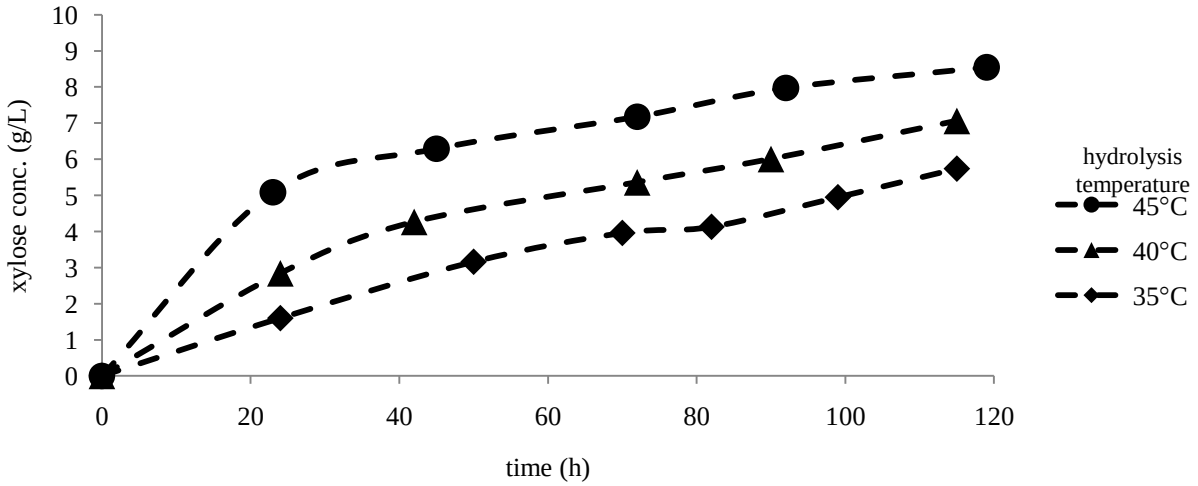


Figure 64
Sugar concentrations from 7.14% biomass concentration during saccharification with water pretreatment with cellulase and β-glucosidase

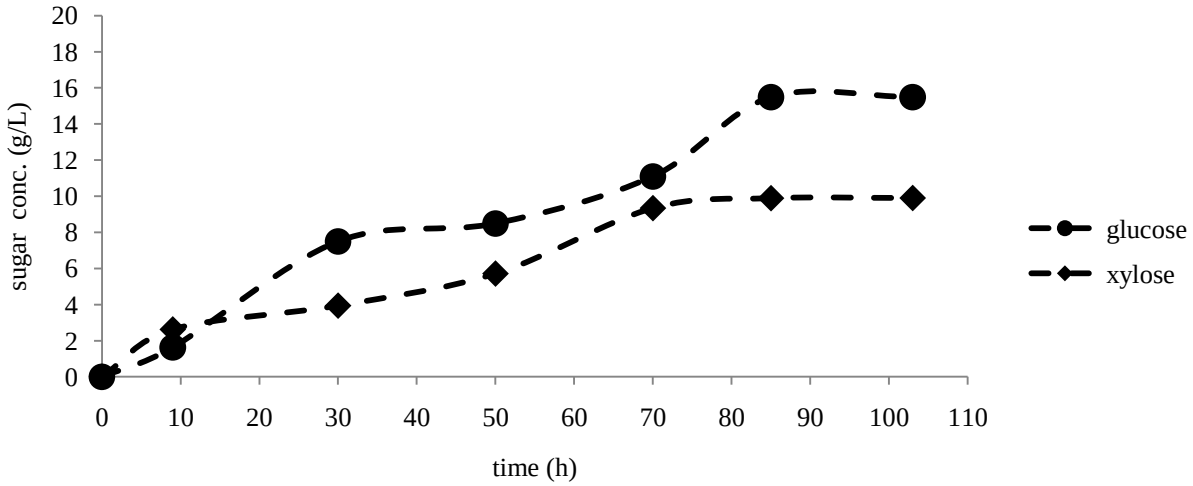


Figure 65
Glucose concentrations from 4.00% biomass concentration at different saccharification temperatures

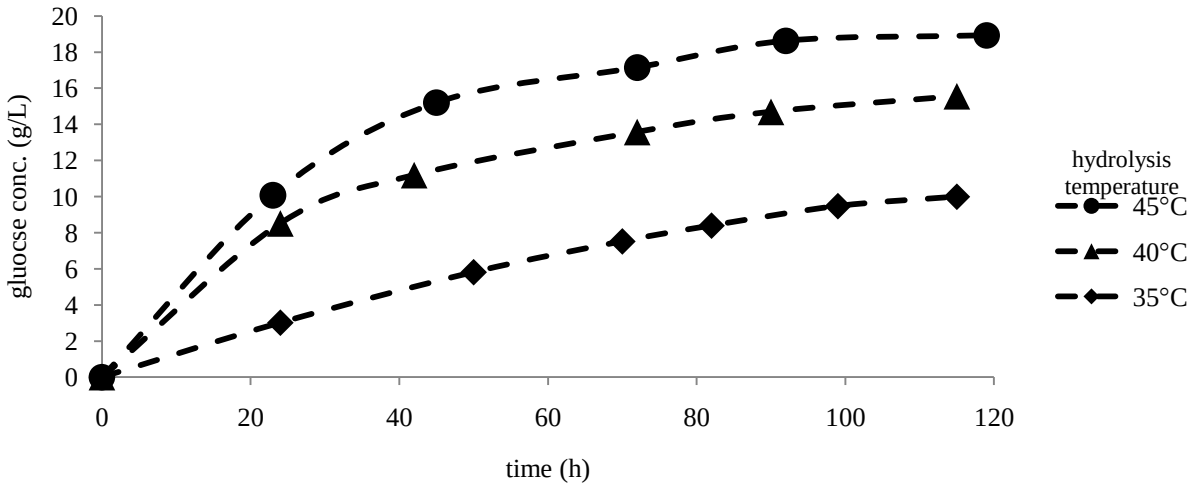


Figure 66
Xylose concentrations from 4.00% biomass concentration at different saccharification temperature

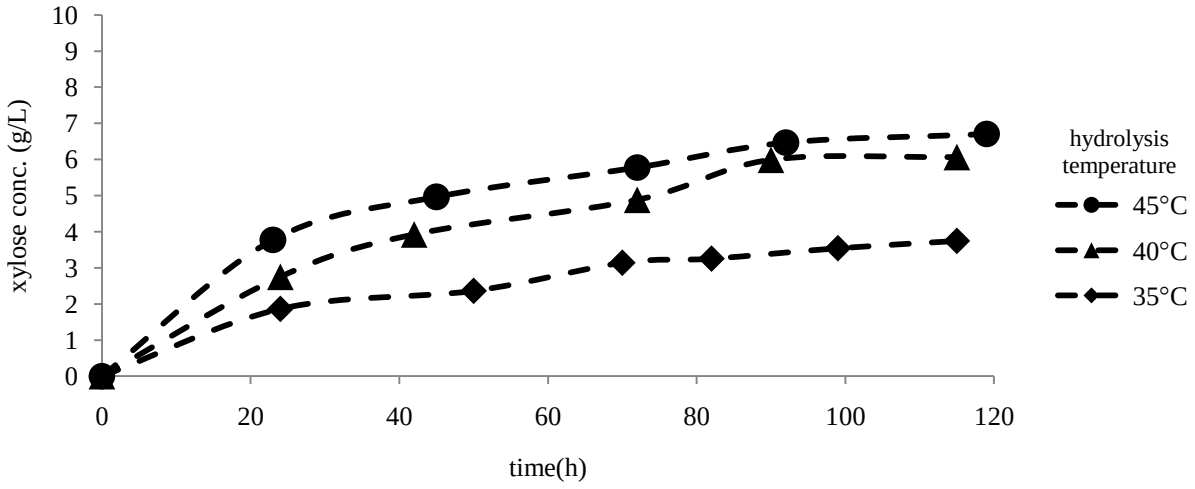


Figure 67
Sugar concentrations from 4.00% biomass concentration during saccharification with water pretreatment with cellulase and β-glucosidase

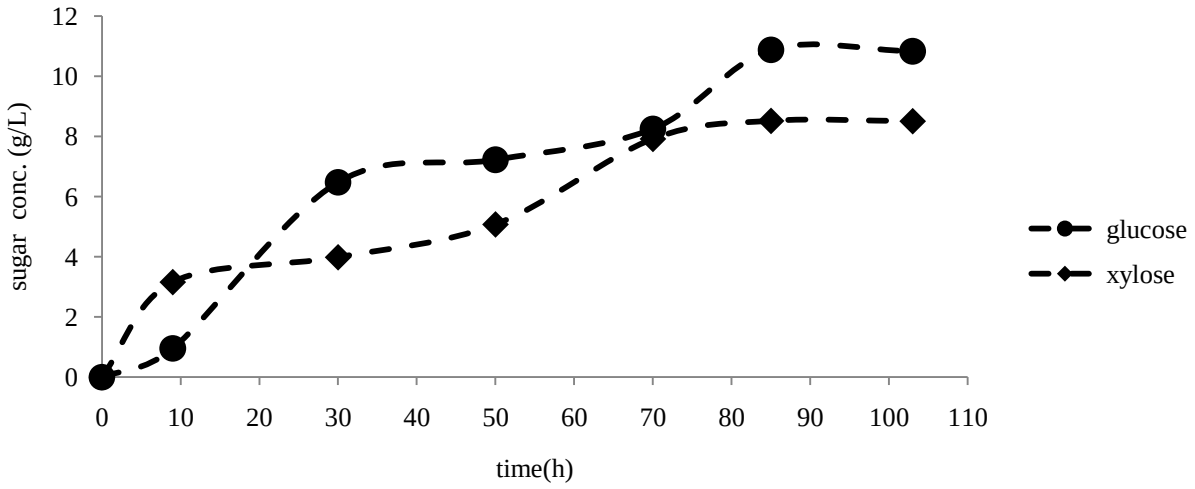


Figure 68
Glucose concentrations from 3.33% biomass concentration at different saccharification temperatures

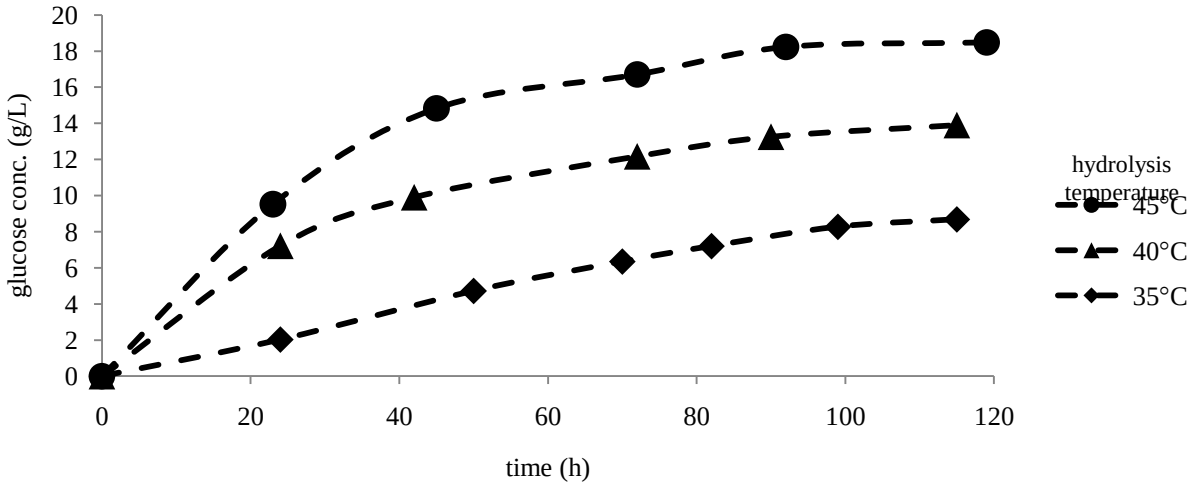


Figure 69
Xylose concentrations from 3.33% biomass concentration at different saccharification temperatures

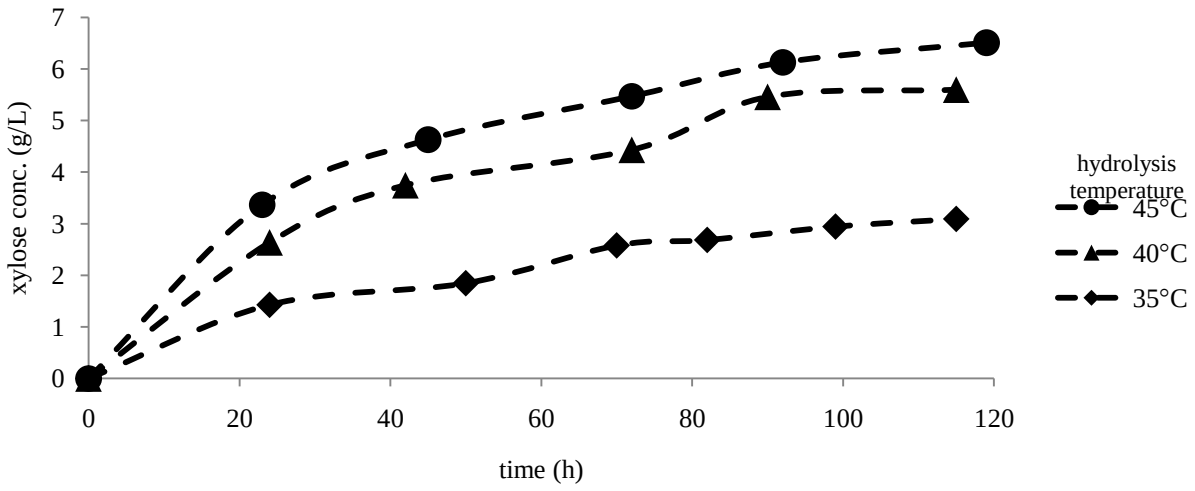


Figure 70
Sugar concentrations from 3.33% biomass concentration during saccharification with water pretreatment with cellulase and b-glucosidase

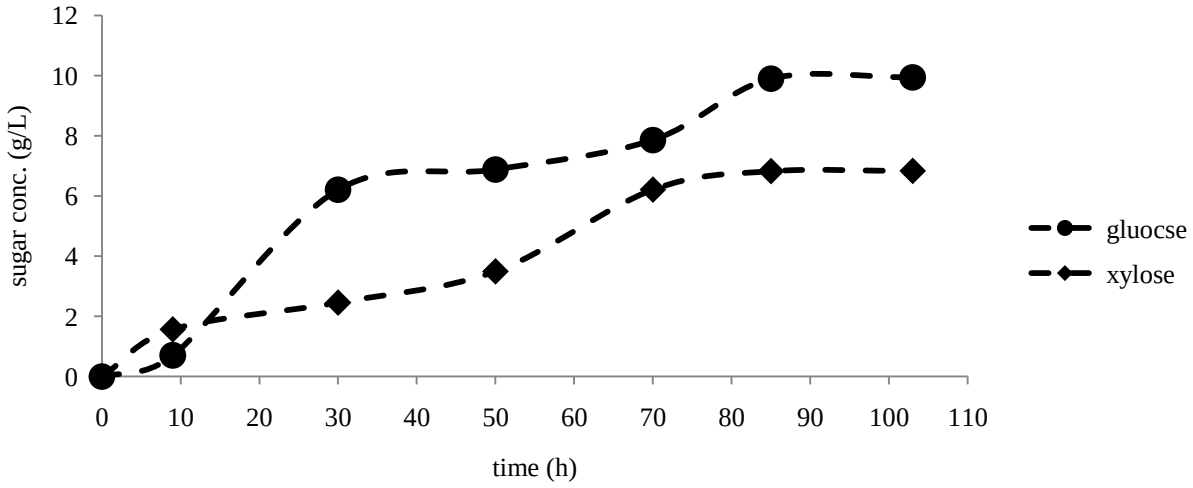


Figure 71
Glucose concentrations from 2.50% biomass concentration at different saccharification temperatures

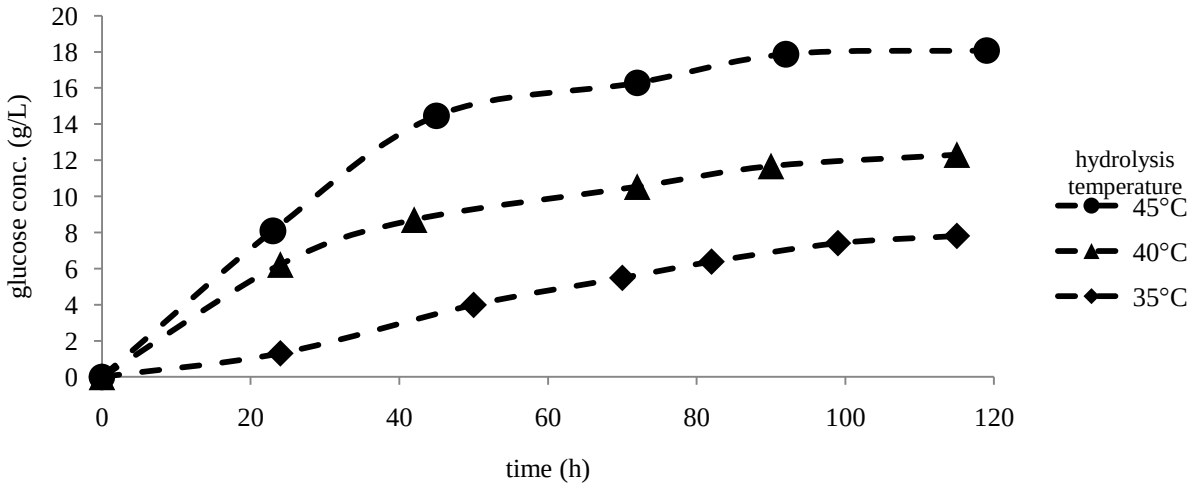


Figure 72
Xylose concentrations from 2.50% biomass concentration at different saccharification temperatures

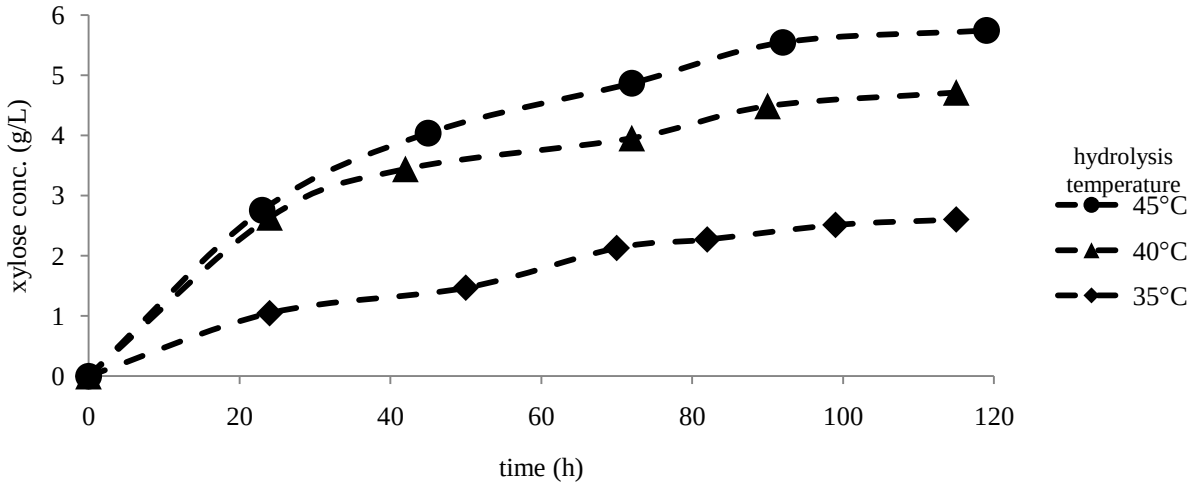
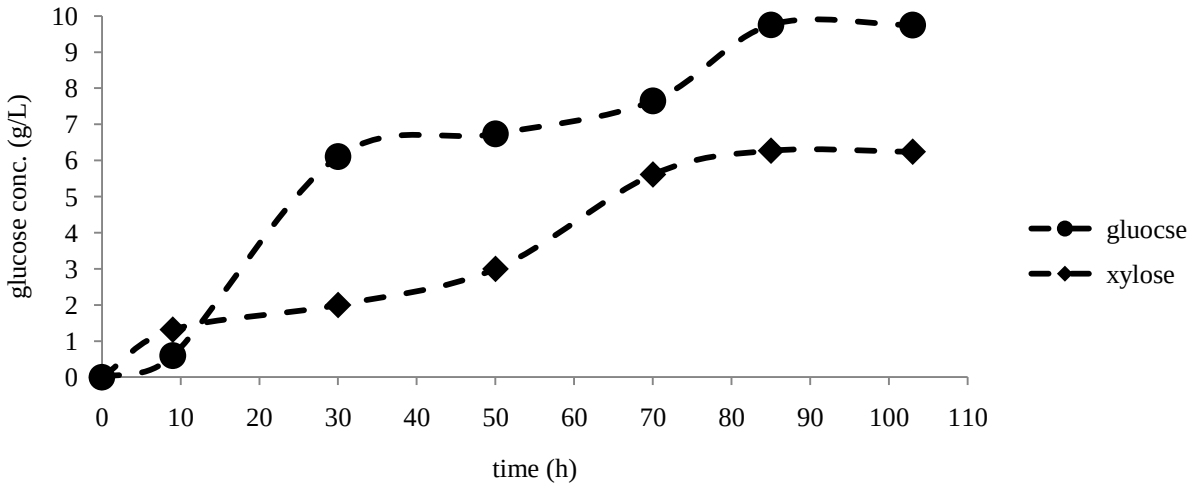


Figure 73
Sugar concentrations from 2.50% biomass concentration during saccharification with water pretreatment with cellulase and β-glucosidase



A.3 Acid pretreatment and saccharification

Figure 74
Sugar concentrations obtained from 0.50% acidic pretreatment and pH adjusted saccharification

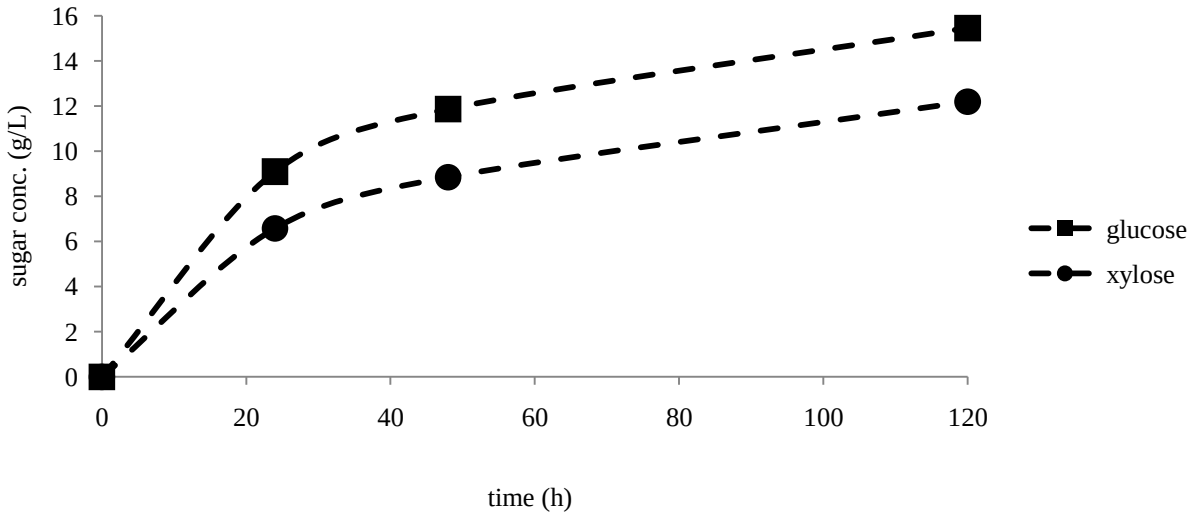


Figure 75
Sugar concentrations obtained from 0.01% acidic pretreatment and pH adjusted saccharification

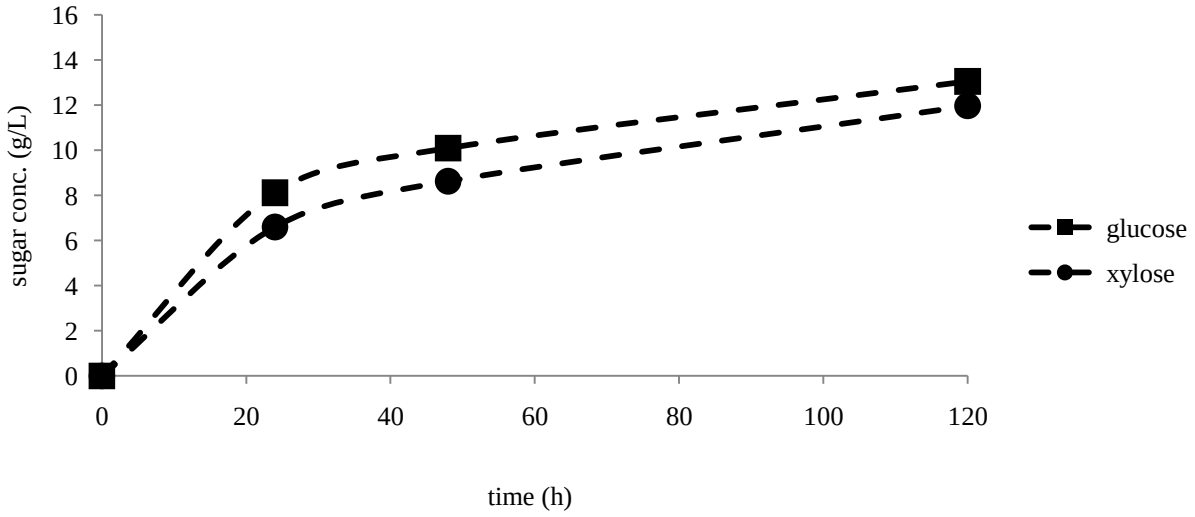


Figure 76
Sugar yields obtained from 0.50% acidic pretreatment and pH adjusted saccharification

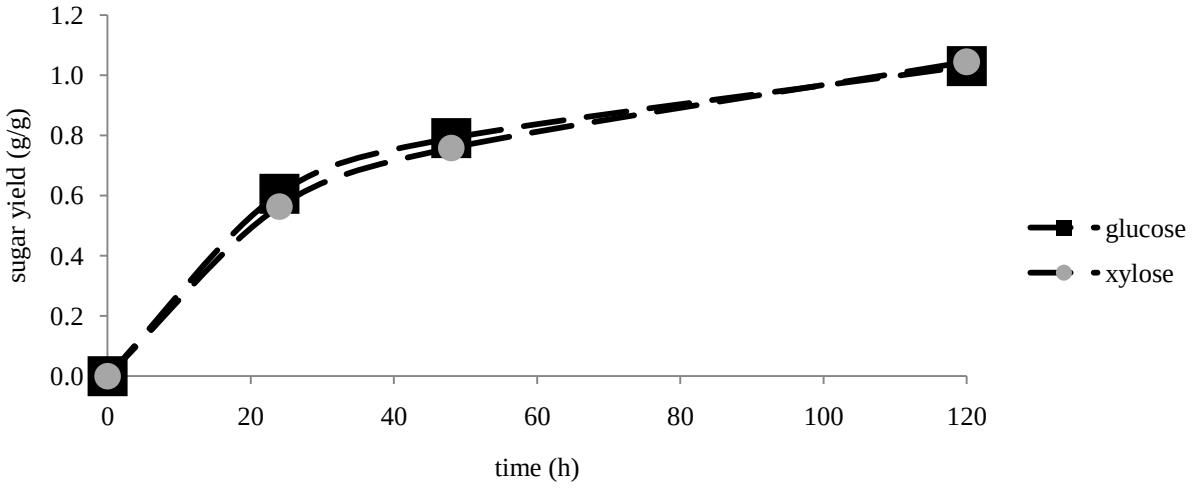


Figure 77
Sugar yields obtained from 0.01% acidic pretreatment and pH adjusted saccharification

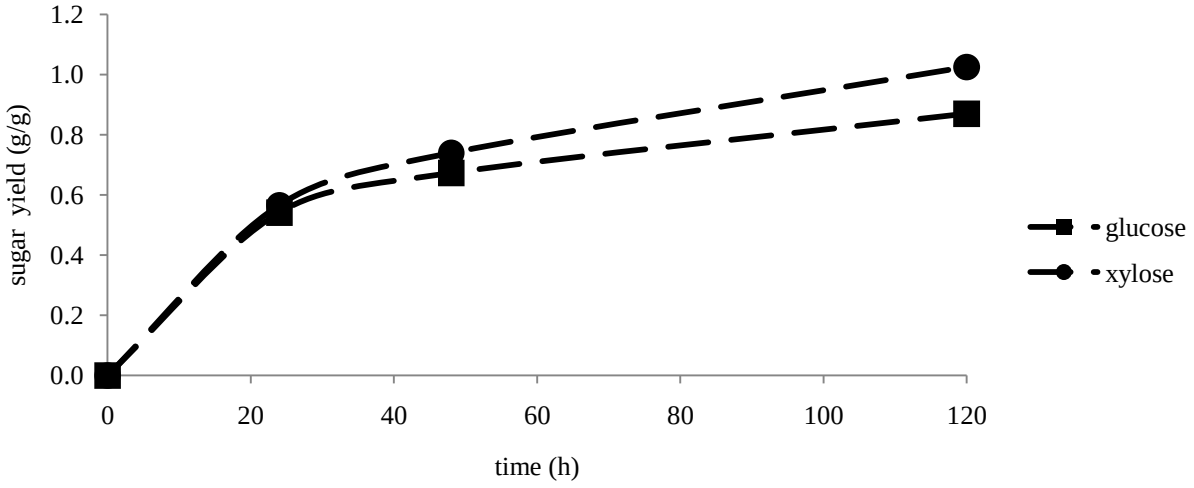


Figure 78
Sugars from acidic pretreatment at different biomass concentrations

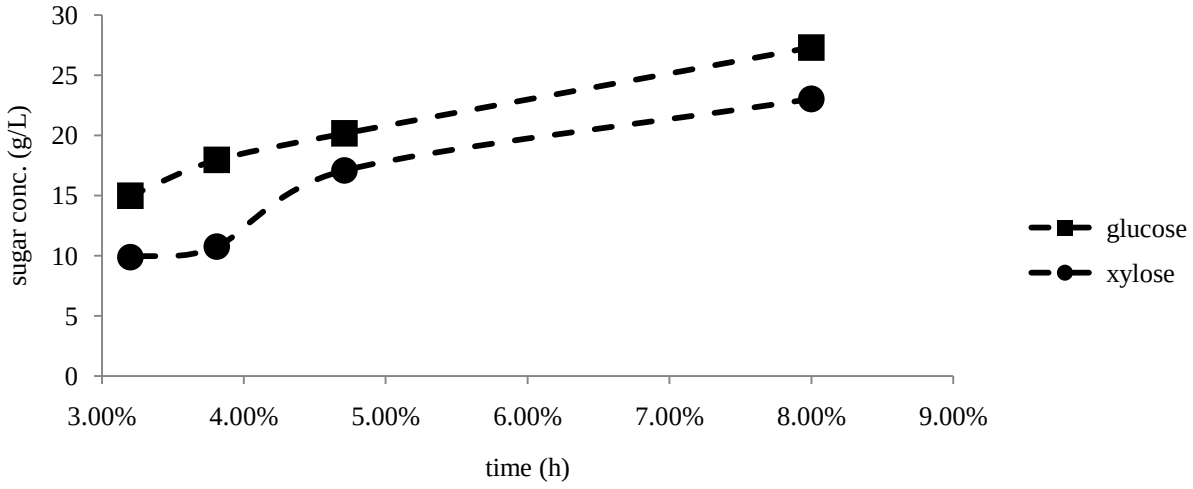


Figure 79
Glucose yields from soaking versus no soaking pretreatment and saccharification

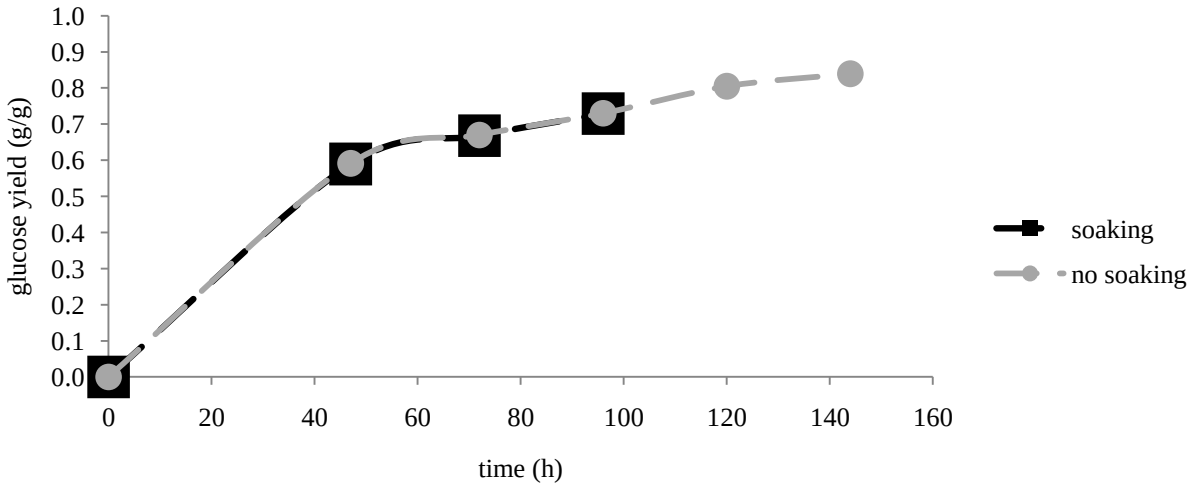


Figure 80

Xylose yields from soaking versus no soaking pretreatment and saccharification

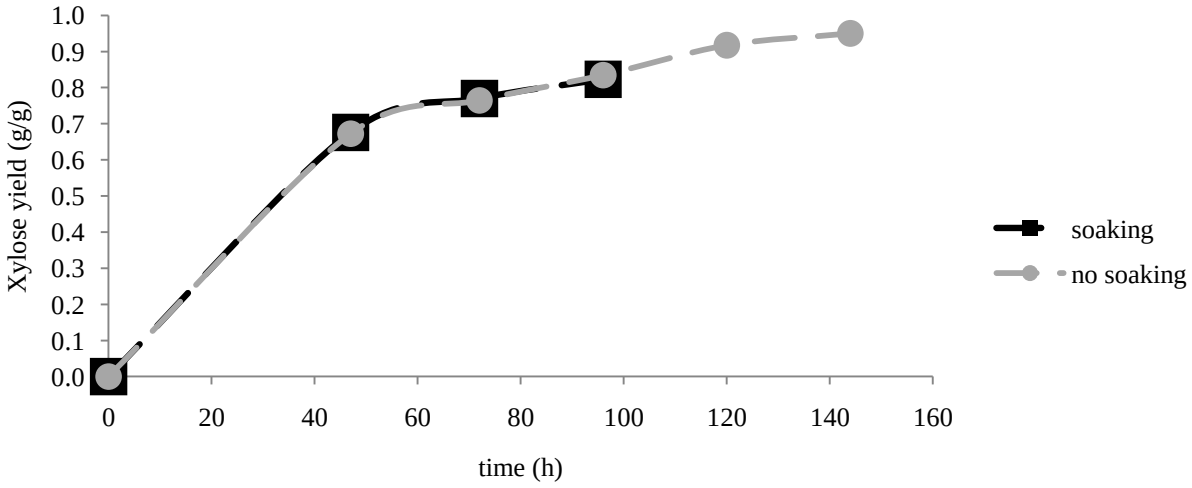


Figure 81

Glucose concentrations at different saccharification temperatures

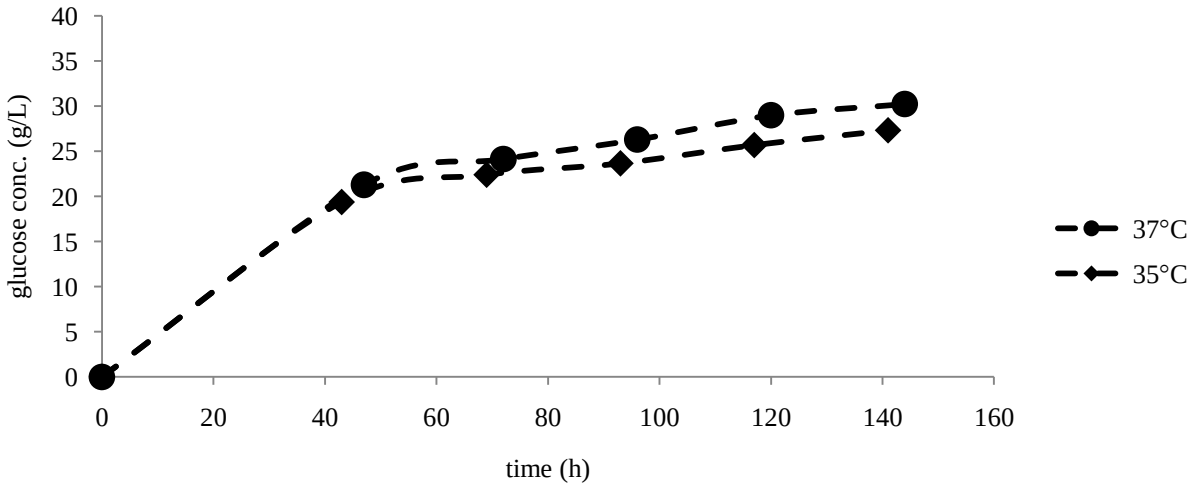
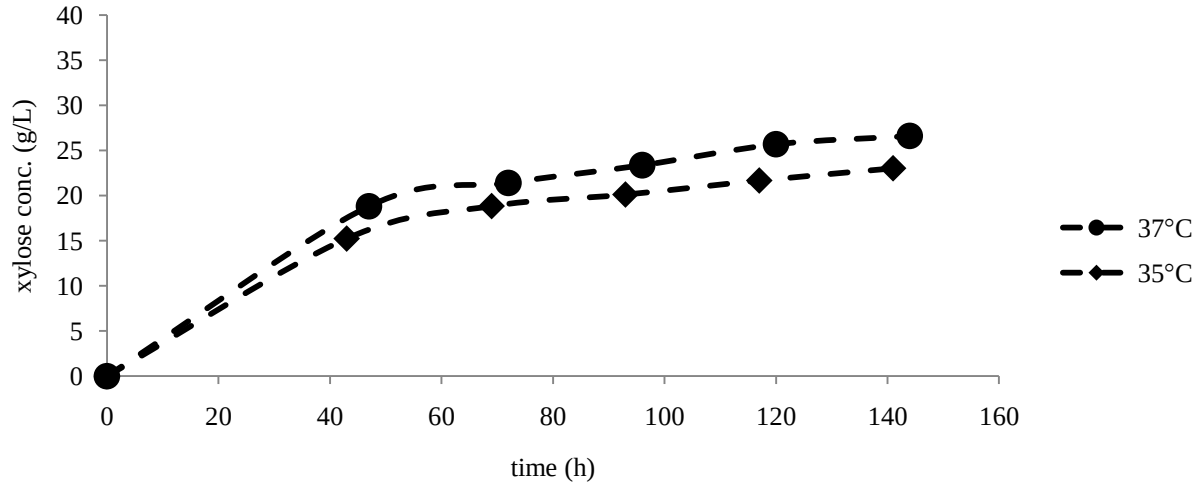


Figure 82
Xylose concentrations at different saccharification temperatures



A.4 Saccharification with alkaline pretreatment

Figure 83
Sugar yields at different concentrations of monoethanolamine

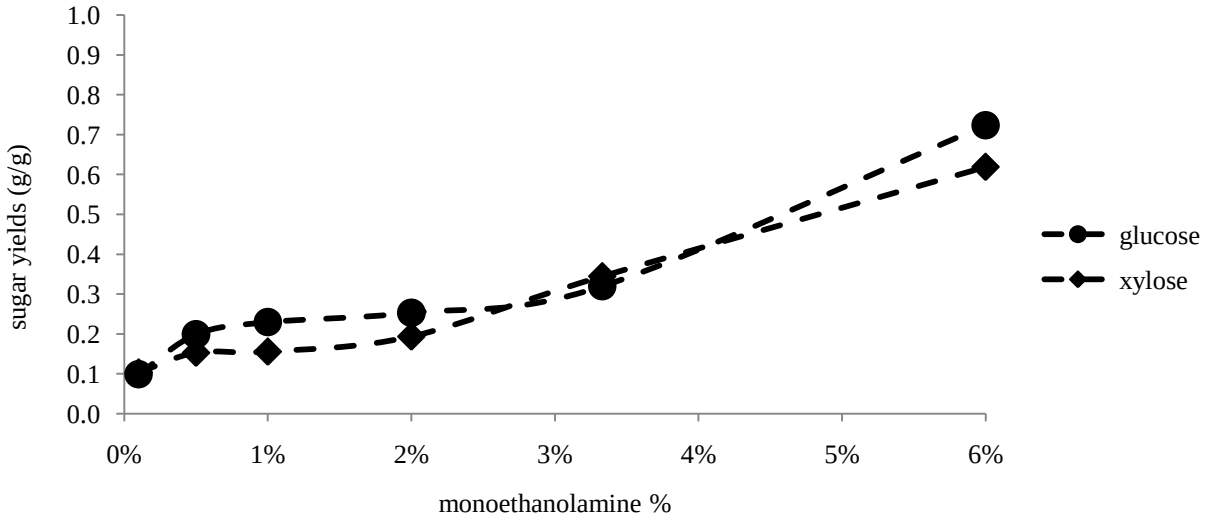


Figure 84
Xylose concentrations versus time from alkaline pretreatment and hydrolysis

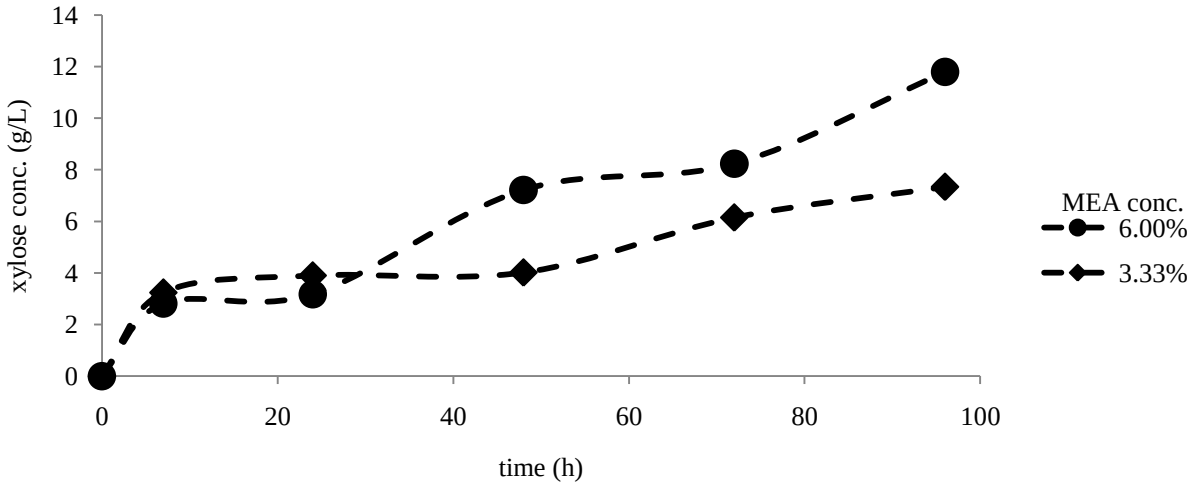


Figure 85
Glucose yields versus time from alkaline pretreatment and hydrolysis

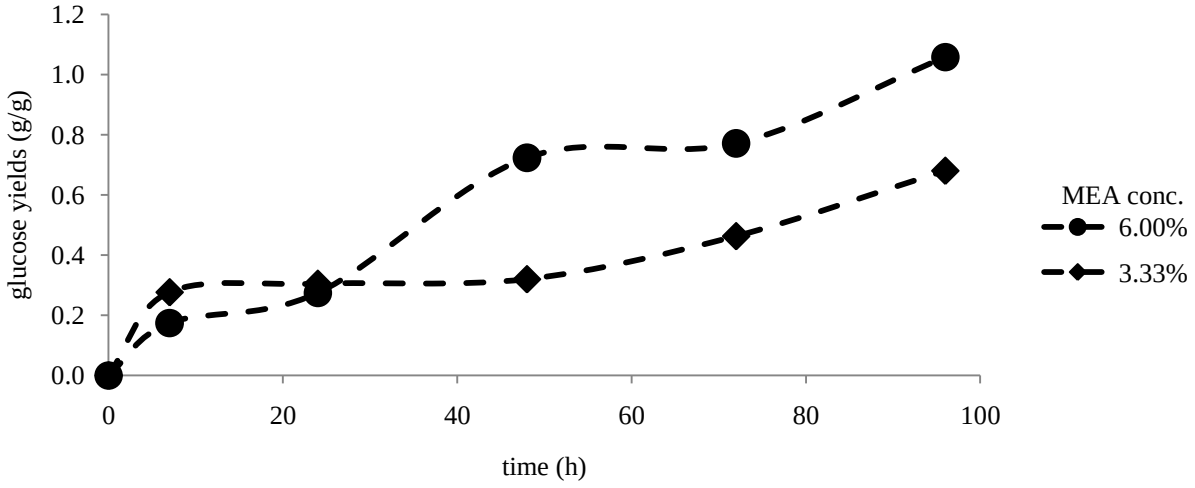
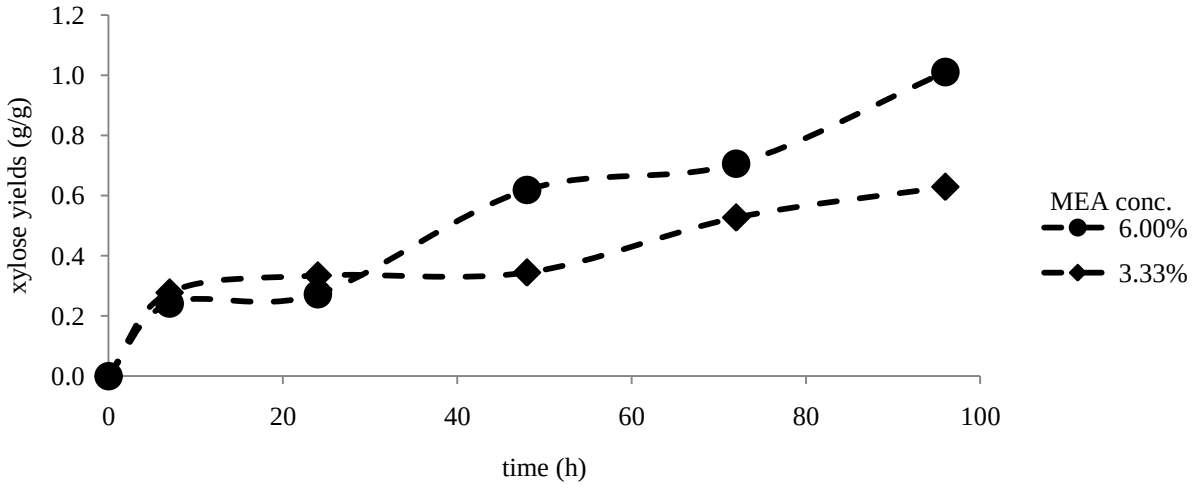


Figure 86
Xylose yields versus time from alkaline pretreatment and hydrolysis



A.5 Comparison of all four pretreatment and saccharification

Figure 87
Maximum xylose concentrations from acid and alkaline pretreatment followed by saccharification

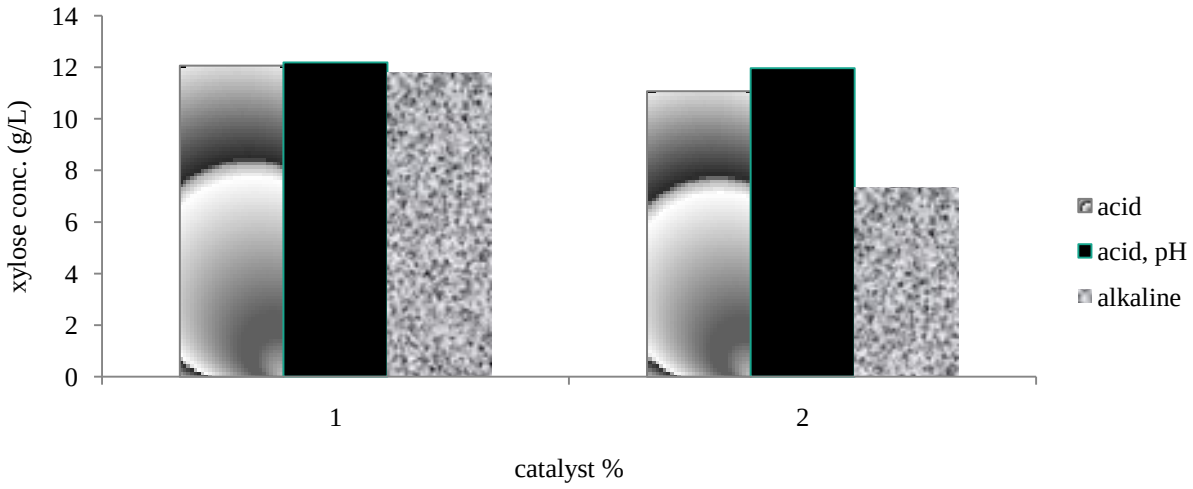


Figure 88
Maximum glucose concentrations from acid and alkaline pretreatment followed by saccharification

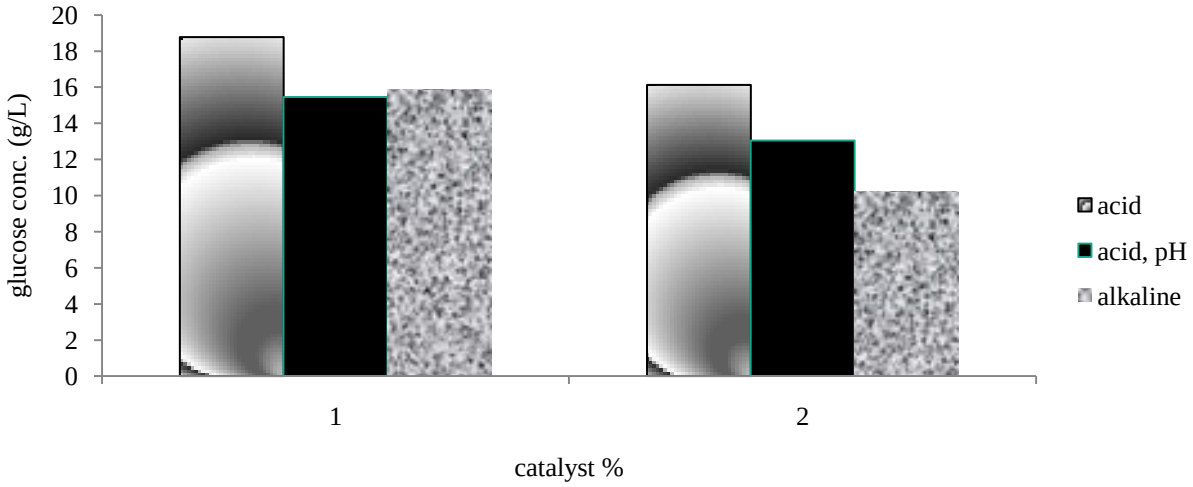


Figure 89
Glucose yields from acid and alkaline pretreatment followed by saccharification

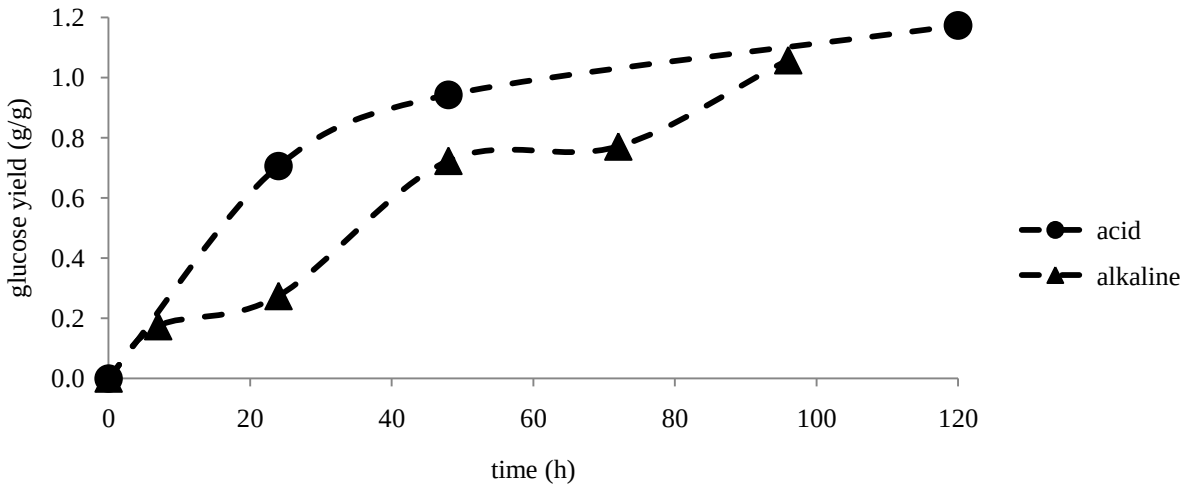
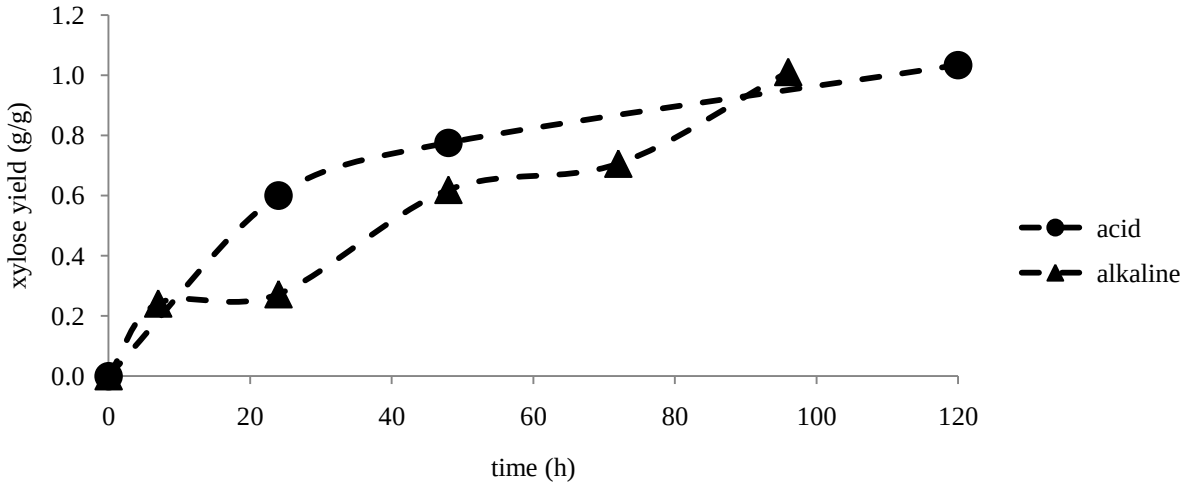


Figure 90
Xylose yields from acid and alkaline pretreatment followed by saccharification



Appendix B **Correlations between butanol production and xylose consumption**

There was no sugar molecules detected through the HPLC analysis for experiments A10, S10, A11, and S11. This implied that the bacteria consumed all of the available sugar molecules. Previous sections in 4 illustrated rate of glucose and xylose production. Correlations between sugar consumption and butanol production was illustrated in Figure 91 and Figure 92. The data from no chemical pretreatment and saccharification for 2.50% biomass concentration at 35°C were applied here. These data were compared with the data from experiment A10.

Figure 91 demonstrated that butanol production depended on xylose production. Hence, butanol concentrations were linearly proportional to xylose concentrations. The sum of residual squares was approximately 0.98. The slope here was 1.29. This proved that butanol was produced as soon as the xylose was consumed. In other words, 1.0 g of butanol was produced when 1.29 g of xylose was consumed in 1 L batch SSF medium.

Figure 91
Correlations between xylose consumption and butanol production

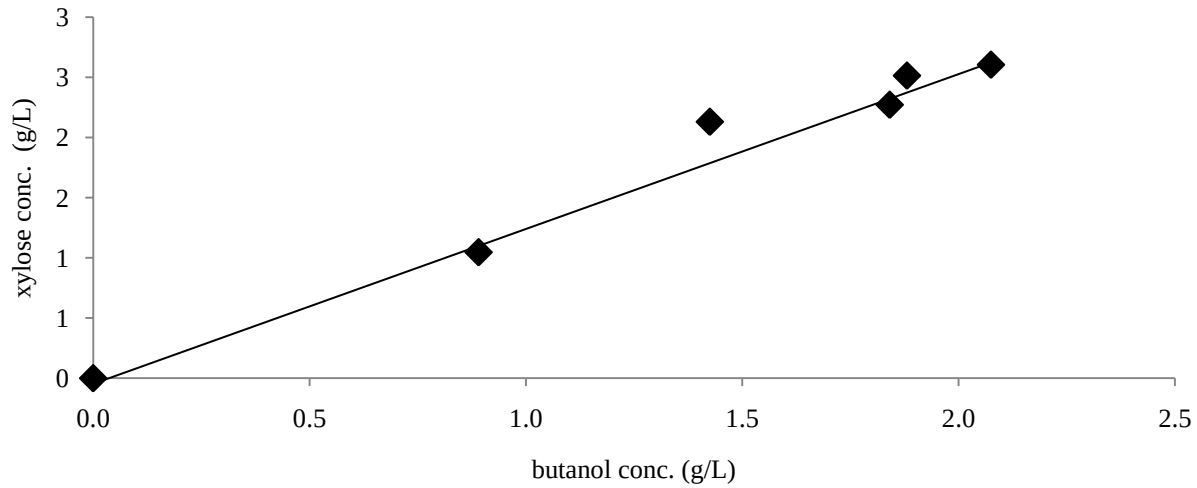
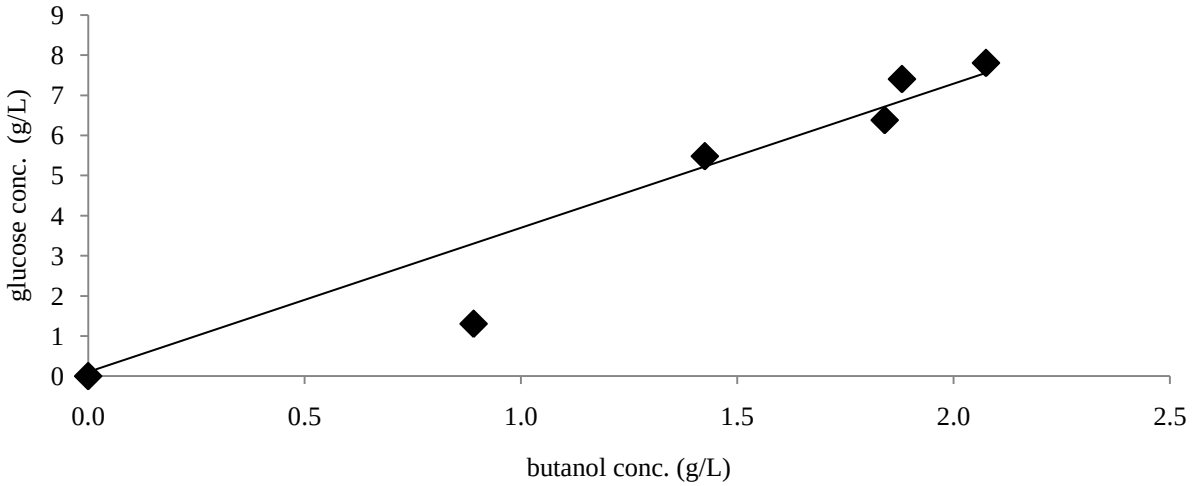


Figure 92
Correlations between glucose consumption and butanol production



Similar correlations between glucose and butanol were illustrated in Figure 92. Here also the sum of residual squares was approximately 0.96. This implied that *C.beijerinckii* BA101 produced butanol while consuming all of the glucose molecules available in the batch system. Here the slope was 2.94 which implied approximately 2.94 g of glucose was consumed for each g of butanol produced in 1 L batch SSF medium.

Appendix C **Matlab code**

This section illustrated the pseudocode applied to determine the profiles of cellulose, cellobiose, glucose, and butanol. Here, ODE solver from Mat Lab library was applied to solve four different ordinary differential equations. Two different Mat Lab files were created. First file designed the models applied to determine the profile. Second file provided initial conditions and codes to determine each profiles in a graphical format. These two files were illustrated below.

```
%MAIN FUNCTION
% model parameters
data.pa.K1B=5.85; % g/L
data.pa.K1G=53.16; % g/L
data.pa.Km=10.56; % g/L
data.pa.K2G=0.62; % g/L
data.pa.k1=0.025; % 1/hr
data.pa.k2=14.22; % g/L*hr
data.pa.temp = 5.0; %(°C)
data.pa.Um = 0.142; %1/hr
data.pa.K3 = 0.171; %g/L
data.pa.Ki = 33.8; %g/L
data.pa.kd = 0.0505; %1/hr
data.pa.Yxg=0.310; %g/g
data.pa.m = 0.211; %1/hr
data.pa.a = 0.00269;
data.pa.b = 0.198;
data.pa.K4 = 0.0;


% initial values
C0=11.25; % g/L
B0=0; % g/L
M0=0; %g/L
G0=0; % g/L
E0=0; %g/L
x0=[C0; B0; M0; G0; E0];

% call ODE solver
t0=0; % initial time, hr
tf=80; % end time, hr

tspan=t0:tf/100:tf;
[t,x]=ode15s(@(t,x)model(t,x,data),tspan,x0);

% plot data
figure(1); plot(t,x(:,1)); xlabel('time (h)'); ylabel('C (g/L)');
figure(2); plot(t,x(:,2)); xlabel('time (h)'); ylabel('B (g/L)')
figure(3); plot(t,x(:,3)); xlabel('time (h)'); ylabel('M (g/L)')
figure(4); plot(t,x(:,4)); xlabel('time (h)'); ylabel('G (g/L)')
figure(5); plot(t,x(:,5)); xlabel('time (h)'); ylabel('P (g/L)')
```

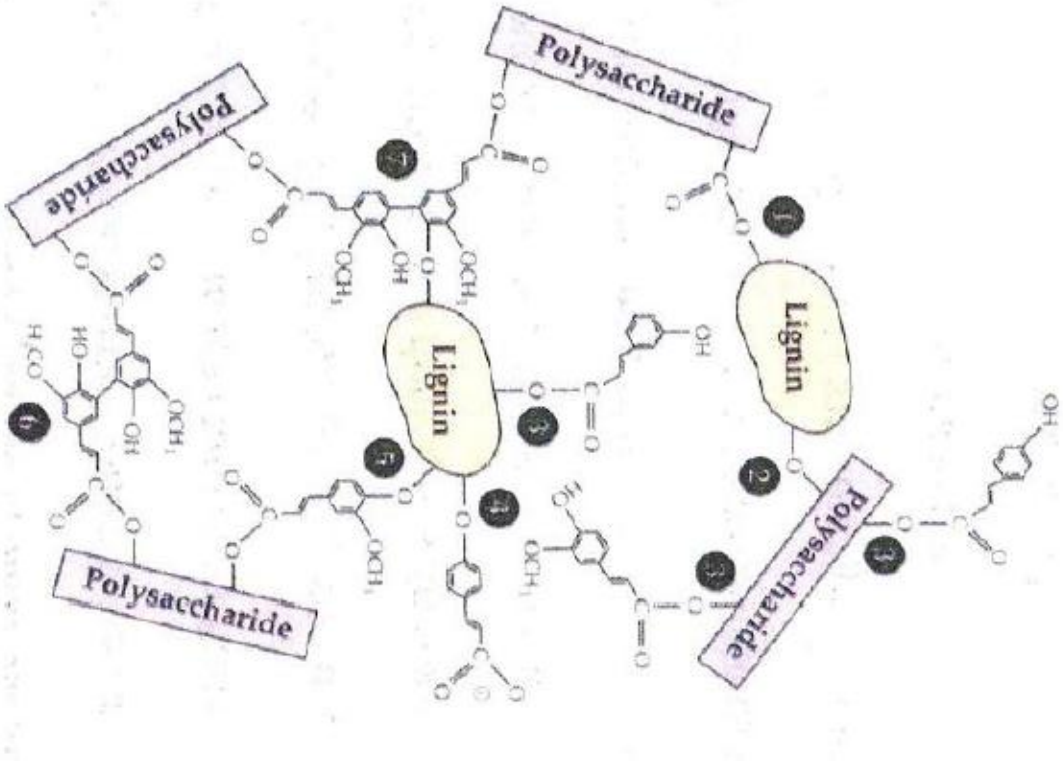
```
% MODEL FUNCTION
function dx = model(t, x, data)
% MODEL describes the enzymatic hydrolysis of cellulose.

% obtain state variables
C=x(1);
B=x(2);
M=x(3);
G=x(4);
E=x(5);

% obtain parameters
k1=data.pa.k1;
k2=data.pa.k2;
K1B=data.pa.K1B;
K1G=data.pa.K1G;
K2G=data.pa.K2G;
Km=data.pa.Km;
temp=data.pa.temp;
Um=data.pa.Um;
K3=data.pa.K3;
Ki=data.pa.Ki;
kd=data.pa.kd;
Yxg=data.pa.Yxg;
m=data.pa.m;
a=data.pa.a;
b=data.pa.b;
K4=data.pa.K4;


% model equations
dC=-k1*C/(1+B/K1B+G/K1G * 2.4*temp);
dB=-1.056*dC-k2*B/(Km*(1+G/K2G)+B);
dM=Um*(G/K3 + G + (G*G)/Ki)*(M)-kd*M;
dG=1.053*(1.056*dC+dB)- (1/Yxg)*dM + m*M;

dE=(a*dM+b*M)*(G/K4 + G);
%dP=dG*(1/(1 + 3.2451*P));
dx=[dC; dB; dM; dG; dE];
end
```

Appendix D **Structure of wheat straw**

Structure of wheat straw is illustrated in Figure 93, next page. This illustrates that polysaccharides are surrounded by lignin. The lignin is attacked during pretreatment. The polysaccharides are attacked during saccharification. Polysaccharides and lignin are connected with different linkages as illustrated in the figure with different numbers. Each chemical bonds will be broken during different stages or simultaneous during saccharification. Bonds between lignin and polysaccharides will be broken during pretreatment process. Most of these linkages are listed below according to each number listen in the figure.

1. Direct ester linkage
2. Direct ester linkage
3. Hydroxycinnamic acid ester
4. Hydroxycinnamic acid ester
5. Ferulic acid bridge
6. Dehydrodiferulic acid diester bridge
7. Dehydrodiferulic acid diester-ether bridge

Figure 93
Structure of lignin and polysaccharides (Peterson, 2009)



Appendix E **Comparisons of all products**

Table 21 illustrated ratio of all products. First, the total sugar yield was calculated through ratio of total sugars obtained and total polysaccharides available. Second, butanol yield was calculated through ratio of butanol and total biomass available. Third yield illustrated a ratio of cells per total sugars. This determined how many cells were created per gram of sugar monomers. Fourth ratio of cells per butanol was calculated. This determined how many cells were utilized during fermentation to produce one gram of butanol. Here the number of bacteria cells was not proportional to butanol production. This cell count included all types of cells available within the sample. Hence, not all cells were active to produce butanol. Finally, ratio of butanol and sugar was calculated to determine the amount of butanol produced per gram of sugar. Here, increase in butanol production illustrated an increase in the final ratio.

Table 21
Ratio of all products

Experiment	biomass (g/L)	total sugars (g/L)	butanol (g/L)	cells/L	total sugar yield %(w/w)	butanol yield %(w/w)	ratio of cells/sugars (cells/g)	ratio of cells/butanol (cells/g)	ratio of butanol/sugar %(g/g)
S10	25.54	10.40	2.70	56621120	99%	10.55%	5444338.0	20970785	0.26
A10	25.54	10.40	2.08	31141620	99%	8.13%	2994387.0	14971933	0.20
S11	25.54	10.88	2.51	8493170	100%	9.83%	780622.2	3383733	0.23
A11	25.54	10.88	2.61	33972110	100%	10.22%	3122426.0	13016092	0.24

Appendix F **Statistical Analysis**

Table 22
Statistical analysis of experimental reproducibility of butanol production from Experiment A11 (See Table 11)

Time (h)	Butanol Exp-1 (g/L)	Butanol Exp-2 (g/L)	Butanol Avg (g/L)	Standard deviation (g/L)	Standard error of the mean (g/L)	95% Confidence interval (g/L)	Error %
0	0.00	0.00	0.00	0.00	0.000	0.000	0.00%
38	1.53	1.56	1.55	0.02	0.014	0.178	1.83%
62	1.89	1.90	1.90	0.01	0.006	0.083	0.69%
86	1.96	1.96	1.96	0.00	0.001	0.013	0.10%
104	2.20	2.22	2.21	0.01	0.010	0.121	0.86%
134	2.54	2.56	2.55	0.02	0.012	0.146	0.91%
153	2.61	2.64	2.62	0.02	0.015	0.184	1.11%

Table 23
Statistical analysis of experimental reproducibility of butanol production from Experiment S10 (See Table 11)

Time (h)	Butanol Exp-1 (g/L)	Butanol Exp-2 (g/L)	Butanol Avg (g/L)	Standard deviation (g/L)	Standard error of the mean (g/L)	95% Confidence interval (g/L)	Error %
0	0.00	0.00	0.00	0.00	0.000	0.000	0.00%
25	1.21	1.24	1.23	0.02	0.013	0.165	2.14%
41	1.22	1.26	1.24	0.03	0.021	0.267	3.44%
65	1.72	1.72	1.72	0.00	0.000	0.006	0.06%
83	1.99	2.00	1.99	0.01	0.005	0.070	0.55%
113	2.21	2.23	2.22	0.01	0.008	0.102	0.72%
132	2.69	2.79	2.74	0.07	0.047	0.597	3.49%
151	2.70	2.76	2.73	0.05	0.033	0.419	2.45%

Table 24
Statistical analysis of experimental reproducibility of butanol production from Experiment S11 (See Table 11)

Time (h)	Butanol Exp-1 (g/L)	Butanol Exp-2 (g/L)	Butanol Avg (g/L)	Standard deviation (g/L)	Standard error of the mean (g/L)	95% Confidence interval (g/L)	Error %
0	0.00	0.00	0.00	0.00	0.000	0.000	0.00%
25	1.15	1.19	1.17	0.03	0.020	0.254	3.47%
41	1.68	1.69	1.69	0.00	0.003	0.038	0.36%
65	1.99	2.01	2.00	0.01	0.007	0.095	0.75%
83	2.06	2.14	2.10	0.06	0.044	0.553	4.23%
113	2.36	2.39	2.37	0.02	0.016	0.203	1.36%
132	2.48	2.50	2.49	0.01	0.011	0.133	0.85%
151	2.51	2.57	2.54	0.05	0.032	0.407	2.55%