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Different physical and chemical pretreatments of wheat straw for enhanced biobutanol production in simultaneous saccharification and fermentation

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Abstract
The objective of this study is to increase butanol product yields using wheat straw as the biomass. First this study examined different pretreatment and saccharification processes to obtain the maximum sugar concentration. Three different physical and chemical pretreatment methods for the wheat straws were examined in the present work in comparison with physical pretreatment alone 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 13.91 g/L glucose concentration was 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. Second this study produced butanol from simultaneous saccharification and fermentation (SSF) using wheat straw hydrolysate and *Clostridium beijerinckii* BA101. Water pretreatment was applied to separate lignin and polysaccharides from the wheat straw. Physical pretreatment was applied prior to water pretreatment where, wheat straw was grounded into fine particles less than 1 mm size. Another experiment was conducted where physical pretreatment was applied alone prior to SSF (i.e. no chemical pretreatment was applied). 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 physical pretreatment alone obtained 2.61 g/L butanol.

Keywords: Biobutanol; Biofuel; Saccharification; Pretreatment; Wheat straw; Biomass.

1. Introduction

Today research in biofuel production from agricultural residues became in most demand due to high price of gasoline. Biobutanol can replace petroleum in automobile engines without any necessary modification [1]. Butanol replacement of gasoline also does not require any modification of the existing gasoline pipelines [2].

Wheat straw from agricultural waste is used to examine butanol fermentation in this study because of its abundance in Canada. Wheat straw contains a high source of polysaccharides. Wheat straw is composed of lignin, hemicellulose, and cellulose. Cellulose is mainly composed of linear chain of 1,4-β-glycosidic bonds or glucose monomers. However, hemicellulose is a complex polysaccharide and composed of mainly xylose molecules [3].

Chemical pretreatment is applied to remove the lignin. Physical pretreatment which is to reduce the size of wheat straw particles will also remove lignin [4]. This study will examine three different physical and chemical pretreatment processes in comparison with physical pretreatment alone. The chemical pretreatments are acidic, alkaline, and water pretreatment. Sulfuric acid and monoethanolamine (MEA) are applied as catalysts during acid and alkaline pretreatment. No catalysts are applied during water pretreatment. Heat is applied during all three chemical pretreatment processes. For example, wheat straw is mixed in dilute sulphuric acid and autoclaved at 121°C for 1 h. This study will examine acid pretreatment without applying heat to eliminate the costs of heat [5]. This process is called soaking process where, wheat straw will be soaked in sulphuric acid for few hours.

Saccharification will be applied after all four types of pretreatment processes. During saccharification, enzymes are applied to break down hemicellulose and cellulose into simple sugars. This study will examine the effect of cellulose, β-glucosidase, and xylanase at different conditions during saccharification. The complexity of hemicelluloses within the wheat straw creates difficulty for enzymes to hydrolyse simple sugars during saccharification. Optimum conditions for enzymes are very important to hydrolyse hemicelluloses into monomers, which will be analysed in this study.

Simple sugars derived from saccharification will be consumed by anaerobic bacteria such as *Clostridium beijerinckii* BA101. These bacteria will produce butanol during metabolism. Since several bacteria are grown in a reactor to produce high concentration of butanol, this process can also is called fermentation. *Clostridium beijerinckii* BA101 grows best at 35°C and pH 5.0 [6]. These bacteria also require vitamins and minerals to maintain an optimum growth [7]. A strong buffer will be required because these bacteria produce acidic products such as butyric acid and acetic acid [8]. Butyric acid will be effectively converted into butanol product at optimum pH [9]. These bacteria are anaerobic. This study will apply resazurin to indicate the presence of oxygen molecules [10]. L-cysteine will also be applied during fermentation to reduce any oxygen molecules and produce water [11].

Fermentation can be processed separately from saccharification which is called separate hydrolysis and fermentation (SHF). It can also be processed simultaneously with saccharification which is called simultaneous saccharification and fermentation (SSF). During SSF, less number of reactors, time, and cost will be required compared to SHF. This study will examine SSF in batch process. The best chemical pretreatment and saccharification with most suitable parameters will be applied in SSF to determine butanol production and yields. The butanol yield will be determined through the ratio of biomass and butanol concentrations.

2. Experimental section

2.1 Materials

Clostridium beijerinckii BA101 (ATTC # PTA-1550) was purchased from the American Type Culture Collection (ATCC), Manassas, VA 20108, USA. Chemicals; Sulfuric acid, Xylanase, Celluclast 1.5L, Novozym 188, L-cysteine, Resazurin, KH_2PO_4, K_2HPO_4, Ammonium acetate, PABA, Thiamine, Biotin, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl, NaOH; were purchased from Sigma-Aldrich and were used as received. Monoethanolamine was purchased from VWR. Cooked meat medium was purchased from Oxoid.

2.2 Methods

2.2.1 Physical pretreatment of wheat straw

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). This was considered as the physical pretreatment that was employed prior to all experiments conducted in this study.

2.2.2 Physical pretreatment alone (no chemical) with saccharification

Required amount of wheat straw was added to 100 ml of sterilized distilled water in a 200 ml shaker flask. The effect of biomass concentration was examined here. Biomass concentration was varied from 2.5 to 5.0% where, 2.5 g and 5.0 g of wheat straw were added. The effect of temperature and the effect of combination of xylanase, cellulase, and β-glucosidase were examined during saccharification.

2.2.3 Physical and water pretreatment with saccharification

Here, required amount of physically pretreated wheat straw was added to 100 ml of sterilized distilled water in a 200 ml shaker flask. The biomass concentration of 2.5, 3.3, 4.0, and 7.0% were examined by adding 2.5, 3.3, 4.0, and 7.0 g of wheat straw to each shaker flask. Then each flask was autoclaved at 135°C for 1 h which was called water pretreatment. After pretreatment, the effect of temperature and the effect of combination of xylanase, cellulase, and β-glucosidase were examined during saccharification.

2.2.4 Physical and acid pretreatment with saccharification

First, 3.3 g of wheat straw was added to 100 ml of dilute sulphuric acid solution in a 200 ml shaker flask. The effect of different sulphuric acid concentration was examined by adding 0.01, 0.1, 0.5, 1.0, 2.0, and 6.0 of sulphuric acid in each flask. Then each flask was autoclaved at 135°C for 1 h. After the acidic pretreatment, saccharification was conducted at 35°C without adjusting the pH. During the repeated experiments, some flasks were pH adjusted to 5.0 by applying 10M sodium hydroxide. Only cellulase and β-glucosidase were employed to all experiments during saccharification. During this soaking process, 8.0 g of wheat straw was mixed in 10 ml of sulphuric acid and 90 ml of sterilized distilled water for 3.5 h. During no soaking process, the wheat straw in dilute sulphuric acid solution was autoclaved for 1 h at 135°C. Then saccharification was employed at 37°C, 80 rpm.

2.2.5 Physical and alkaline pretreatment with saccharification

Alkaline pretreatment and saccharification was examined by adding 3.3 g of wheat straw in 100 ml monoethanolamine solution in a 200 ml shaker flask. The monoethanolamine concentration was varied from 0.1, 0.5, 1.0, 2.0, 3.3, and 6.0% (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 2.0% 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 [12]. When the mixtures were cooled, saccharification was conducted at 35°C and 80 rpm.

2.2.6 Saccharification of wheat straw

During all experiments, 0.375 ml of Celluclast 1.5L and Novozyme 188 (Sigma Aldrich) were employed in 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. The effect of temperature was examined where the saccharification was conducted at 35°C, 40°C, and 45°C. Also the effect of xylanase enzyme in combination with cellulase and β-glucosidase were examined in some experiments by adding 0.375 ml of each enzyme. Xyalanase was derived from Thermomyces lanuginosus with the activity of 2500 IU/g (Sigma Aldrich). All experiments during saccharification were conducted in a water shaker bath at 80 rpm. Samples were taken every 10 h until steady state of saccharification was obtained. Sugar concentration was measured using analytical methods from each sample.

2.2.7 Culture and cell propagation for SSF

First, 10 ml of cooked meat medium (CMM) was transferred to a 20 ml glass vial. Then 0.25 ml of 0.025% resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) was added. The vial was crimped and vacuumed inside the glove box (model # 1681-29C-EX-001; series # 100; manufactured by Terra Universal). Then nitrogen was surged through the vial at 150 ml/min for 10 to 20 min.

About 0.5 to 1.0 ml of *C.beijerinckii* BA101 was inoculated into the crimped CMM vial [6]. Approximately, 0.1 g of L-cysteine was added to the oxygen-free vial by using a syringe needle under anaerobic conditions. CMM was incubated at 35°C for 16 to18 h. Then 5.0 to 6.0 ml *C.beijerinckii* BA101 was inoculated into a second CMM vial and incubated for 16 to 18 h at 35°C.

2.2.8 Stock solutions

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, 2.0 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L $MnSO_4 \cdot 7H_2O$. Mineral solution was composed of 0.1 g/L $FeSO_4 \cdot 7H_2O$, and 0.1 g/L NaCl.

2.2.9 P2 medium

Approximately, 3.0 g of glucose and 0.1 g of yeast extract was added to distilled water to produce 100 ml of P2 medium in a 250 ml glass vial. This vial was sterilized at 121°C for 15 min. Approximately 1.0 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. 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.

2.2.10 Pretreatment and batch SSF

Four experiments were conducted to examine SSF. Each experiment was at least repeated twice. Physical pretreatment was employed in all four experiments. During Experiment 1 and 2, 1.6 g of wheat straw was mixed in 50 ml of sterilized distilled water in 100 ml shaker flask and autoclaved at 135°C for 1 h. During Experiment 3 and 4, 1.6 g of wheat straw in 50 ml of sterilized distilled water in 100 ml shaker flask. All four mixtures were transferred to four different 200 ml vials and crimped. Nitrogen was surged through each vial at 150 ml/min for 45 min. During Experiment 1 and 3, 0.12 ml of each cellulase and β-glucosidase were added to each vial and incubated at 45°C for 20 h. During Experiment 2 and 4, 0.12 ml of each cellulase and β-glucosidase were added during fermentation. Then 5 ml of actively growing *C.beijerinckii* BA101 from P2 medium was inoculated in to all four vials. Also 5 ml of each filter sterilized stock solution was added to all four vials. Then all vials were incubated at 35°C. Samples were taken every 10 h until steady state of butanol production was obtained. Sugar, butanol, and butyric acid concentrations were measured using analytical methods from each sample.

2.2.11 Analytical methods

For the analysis of cell concentrations of samples collected during the BC production, Flowcytometer (Guava EasyCyte Mini System, Guava Technologies, Inc, USA) was used according to the manufacturer's guidelines. The Guava EasyCyte Mini System contained a blue laser (Excitation 488 nm) which emits 20 mW visible laser radiations, three fluorescent detectors, an automated analyzer unit with an internal hard drive connected to a computer, single sample tube format and CytoSoft Software with expandable software modules. Samples were diluted 20-fold using deionized water. The diluted samples were repeatedly vortexed at high speed for 3 min and thereafter, filtered through 0.8 µm membrane filters (Millipore, USA). A volume of 1 mL of each filtered sample was loaded into the sample loader and circulated through the capillary feeder attached to the system. Guava via Count Assay through the built-in Guava® ExpressPluss software Module displayed the direct absolute cell counts as cells/mL in conjunction with subpopulation percentages.

Sugar concentrations were analyzed using pre-calibrated High Performance Liquid Chromatography (HPLC) (Perkin Elmer). This instrument was equipped with an Ion Exchange column (Aminex HPX-87H, Biorad, Hercules, USA), a pump Series 200 (Perkin Elmer), Auto sampler Series 200 (Perkin Elmer) and a Refractive Index Detector (HP1047A, Hewlett Packard). Samples of 50 µL were diluted 20-fold with deionized water and filtered (0.45 µm -Gelman Acrodisc CR PTEF, Millipore). Total of 50 µL from each diluted sample was injected into the column and circulated for 60 min at a flow rate of 0.6 mL/min using filtered (0.2 µm nylon Millipore) and degassed mobile phase of 5 mM H_2SO_4. The column temperature was maintained at 60°C using the column heater CH-30 controlled by an Eppendorff TC 50. Sugar concentrations were quantified from calibration curves that were constructed from standard sugar, butanol, and butyric acid solutions of known concentrations (10-100 µg/mL).

3. Results and discussion

3.1 Saccharification with physical pretreatment alone (no chemical pretreatment)

Figure 1 illustrated glucose released from 5.0 and 2.5% (w/v) biomass concentrations at different saccharification temperature and in the absence of xylanase enzyme. The optimum temperature was 45°C because highest glucose concentrations were obtained. Enzymes were most active at optimum temperature. This implied that increase in temperature increased glucose production towards optimum temperature. Similarly, xylose concentrations increased when saccharification temperature was increased from 35°C to 45°C, which was also illustrated in Figure 1. Also these results were obtained after 100 h of saccharification when steady state was reached.

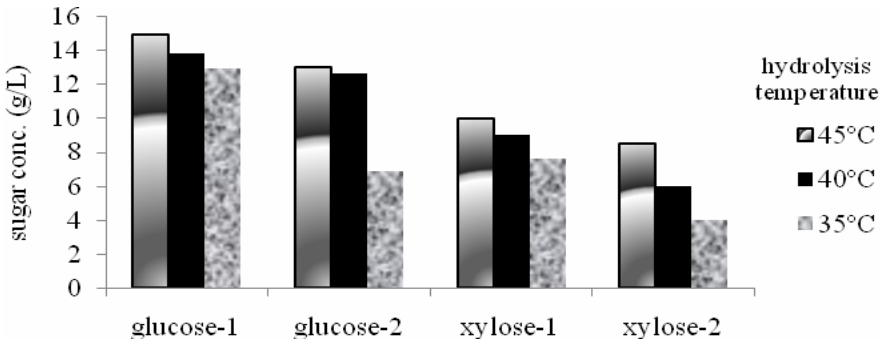

Figure 1. Comparison of sugar concentrations at different saccharification temperatures in the absence of xylanase during saccharificaiton with no chemical pretreatment: 1 (beside glucose and xylose) represented 5.00% biomass concentration and 2 (beside glucose and xylose) represented 2.50% biomass concentration

Figure 2 illustrated glucose and xylose concentrations extracted from 5.0 and 2.5% 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. 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 [13]. This occurred because hemicellulose could be attacked by cellulase and result in xylose production [14]. 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 when more biomass concentration was employed.

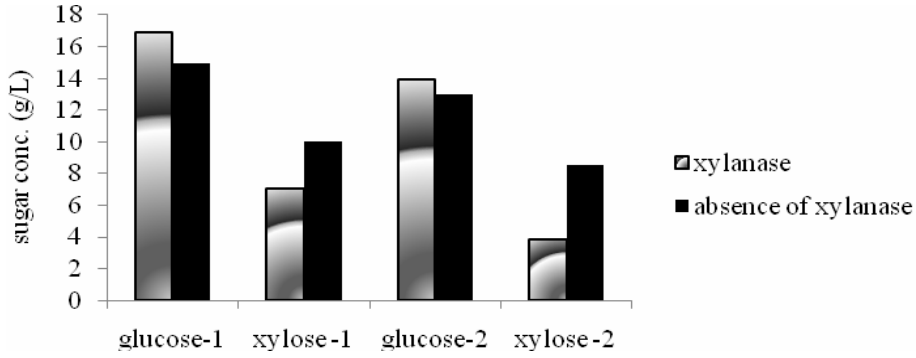

Figure 2. Comparison of sugar concentrations in the presence and absence of xylanase during saccharification with no chemical pretreatment: 1 (beside glucose and xylose) represented 5.00% biomass concentration and 2 (beside glucose and xylose) represented 2.50% biomass concentration

3.2 Saccharification with physical and water pretreatment

No pH adjustment was implemented since all water pretreatment experiments were examined at a maximum temperature of 135°C. In previous studies, water pretreatment including steam explosion was performed at temperatures above 160°C which in result decreased the pH [15]. Steam explosion produced small amounts of inhibitors due to these acidic conditions.

Figure 3 and Figure 4 represented the change in glucose and xylose concentrations obtained at different saccharification temperatures from this study. Enzymatic hydrolysis was examined at 35°C, 40°C, and 45°C in the presence of xylanase. Also biomass concentrations from 2.5, 3.3, 4.0, and 7.0% (w/v) were examined here. These results were obtained after 100 h of saccharification when steady state was reached.

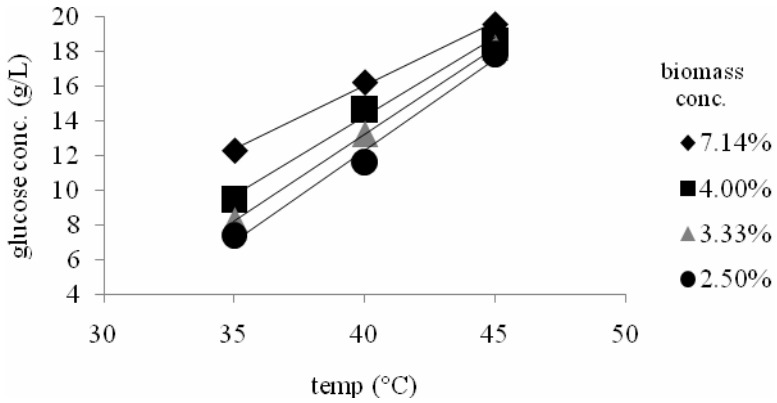

Figure 3. Comparison of glucose concentrations during saccharification with water pretreatment at different biomass concentrations: saccharification in the presence of xylanase; water pretreatment at 135°C

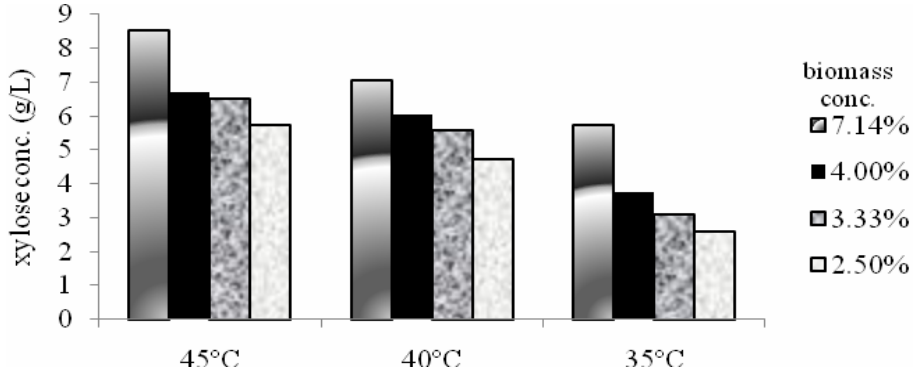

Figure 4. Comparison of xylose concentrations during saccharification with water pretreatment at different temperatures and biomass concentrations: saccharification in the presence of xylanase; water pretreatment at 135°C

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 5 illustrated glucose concentrations in the absence and presence of xylanase during saccharification after water pretreatment at different biomass concentrations. Biomass from 4.0% through 2.5% produced similar glucose concentrations in the absence of xylanase. Except 7.0% 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 2.5 to 7.0%. The same phenomena occurred during xylose production which was illustrated in Figure 6. 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.

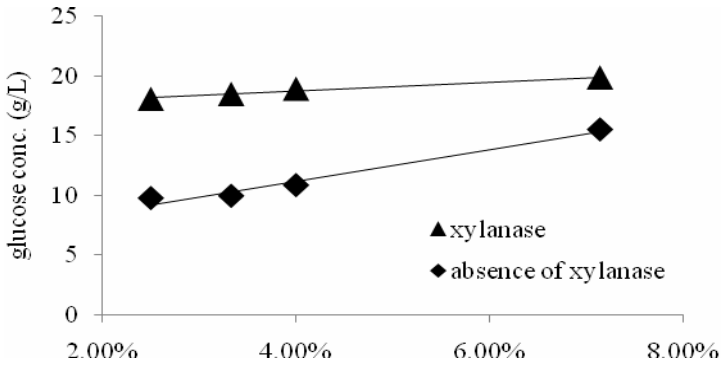

Figure 5. Comparison of glucose concentrations during saccharification with water pretreatment at different biomass concentrations: saccharification in the absence and presence of xylanase; water pretreatment at 135°C

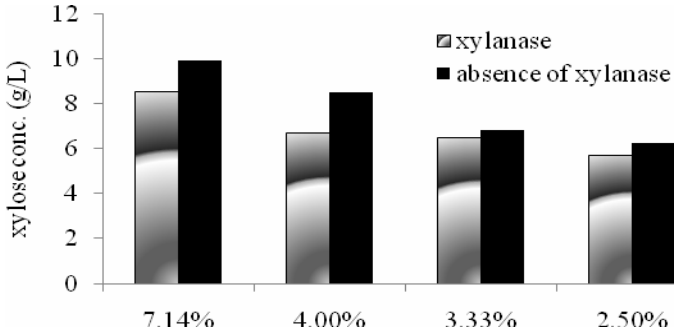

Figure 6. Comparison of xylose concentrations during saccharification with water pretreatment at different biomass concentrations: saccharification in the absence and presence of xylanase; water pretreatment at 135°C.

3.3 Saccharification with physical and acid pretreatment

Saccharification with acid pretreatment was examined by varying pH, acidic concentration, and pretreatment temperature. 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. Also the sulphuric acid concentration was varied from 0.01 to 6.0% to determine the effect of sulphuric acid during pretreatment and saccharification. Finally soaking process was examined to determine the effect of reduced temperature during acid pretreatment and saccharification.

Figure 7 examined glucose and xylose concentrations extracted from 3.3% biomass concentration during saccharification at 35°C. Here, saccharification with acid pretreatment was examined from 0.01 to 6.0% (v/v) sulfuric acid concentrations. These results were obtained after 100 h of saccharification when steady state was reached. Glucose and xylose concentrations increased at high acidic concentration. Sulfuric acidic concentrations from 1.0 to 6.0% achieved above 15 g/L glucose concentrations. Similarly, above 12 g/L xylose concentration was achieved with 6.0% acid concentration.

These results implied that increase in sulphuric acid concentration removed more lignin and hemicellulose. In some cases, acidic pretreatment alone hydrolysed hemicellulose into xylose [6]. 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 hemicelluloses.

Figure 8 illustrated glucose and xylose concentrations 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 [16]. Saccharification with pH adjustments improved glucose and xylose concentrations. Similarly sugar concentrations were increased. Xylose concentration was 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.

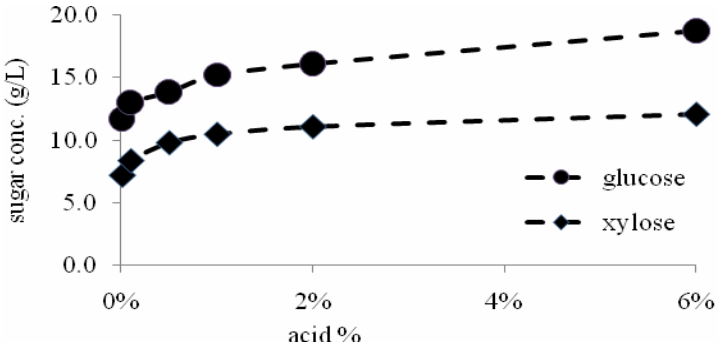

Figure 7. Comparison of sugar concentrations during saccharification with acid pretreatment at different concentrations of sulfuric acid: 3.33% biomass concentration; pretreatment at 135°C; saccharification at 35°C

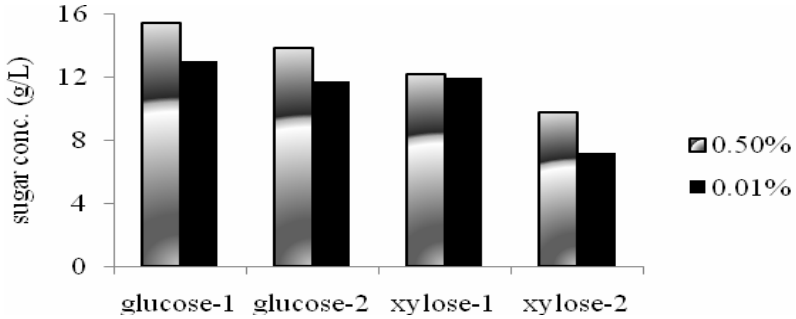

Figure 8. Comparison of sugar yields during saccharification with acid pretreatment: 1 represented pH adjusted; 2 represented no pH adjustments; 3.33% biomass concentration; pretreatment at 135°C; saccharification at 35°C

Figure 9 illustrated glucose and xylose concentrations during soaking and no soaking process. Soaking process produced the same glucose and xylose concentrations as the no soaking process at all time intervals. 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.

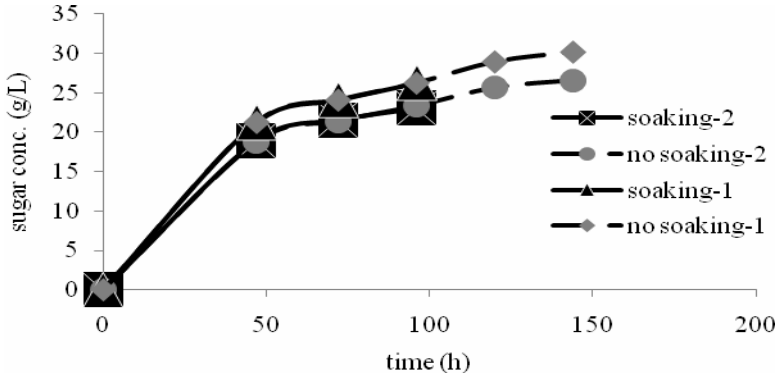

Figure 9. Comparison of sugar concentrations from soaking and no soaking process: 1 (beside soaking and no soaking) represented glucose concentration and 2 (beside soaking and no soaking) represented xylose concentration

3.4 Saccharification with physical and alkaline pretreatment

Figure 10 illustrated glucose and xylose concentrations from saccharification with different concentrations of alkaline pretreatment. Glucose and xylose concentrations increased when monoethanolamine (MEA) concentrations increased. Similar sugar concentrations were produced with 0.5% to 2.0% alkaline concentrations. These results were obtained after 100 h of saccharification when steady state was reached.

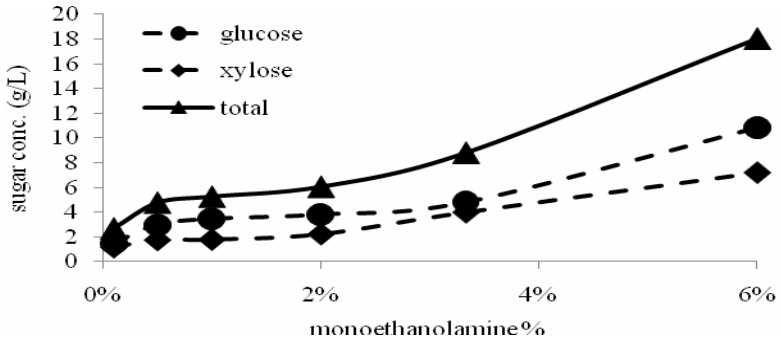

Figure 10. Comparison of sugar concentrations during saccharificaiton with alkaline pretreatment at different concentrations of monoethanolamine: at 3.3% biomass, pretreatment at 135°C, and saccharification at 35°C

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 3.3% was applied. This pattern approved with Shah et al. [12] but, the concentrations were low in this study. MEA was illustrated to be a poor choice of catalyst to pretreat wheat straw. 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.

3.5 Comparisons of all pretreatment and saccharification processes

Similar results occurred during saccharification with water and physical pretreatment in comparison with physical pretreatment only. 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 as well as more hydrolysis of hemicellulose into xylose. Increase in saccharification temperature increased hydrolysis of cellulose and hemicellulose.

Figure 11 illustrated maximum sugar concentration from all four pretreatment and saccharification processes with 3.3% biomass concentration. Unfortunately, saccharification with physical pretreatment was only conducted with 2.5% biomass concentration. However, Figure 3 illustrated biomass concentrations were linearly proportional to sugar concentrations. So the concentrations from 2.5% biomass concentration could be compared here.

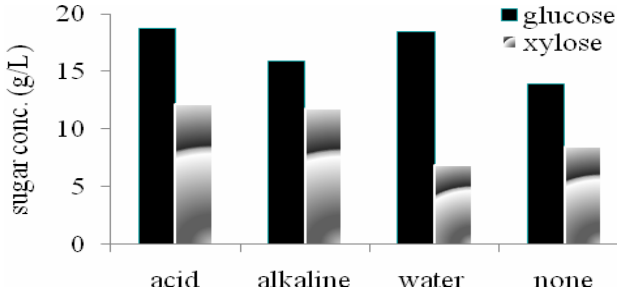

Figure 11. Maximum sugar concentrations: at 3.3% biomass (acid, alkaline, and water) and 2.5% biomass (physical pretreatment only with no chemical pretreatment)

These results implied that MEA was not a good catalyst. MEA pretreatment definitely did not improve results compared to water pretreatment with no catalysts. Acidic pretreatment with sulfuric acid extracted highest glucose and xylose concentrations. However, there were several disadvantages involving acidic pretreatment. Acidic concentration would destroy bacterial metabolism. Second, it would be very difficult to adjust pH of a very acidic solution. Third, even dilute sulphuric acid caused acid rain and air pollution [17]. This would also burn or irritate human skin. Several advantages were provided by saccharification with physical pretreatment without any chemical pretreatment. There was no additional chemical or time required to adjust pH here. Fermentation would be completed quickly with less number of reactors if chemical pretreatment was eliminated.

3.6 Simultaneous saccharification and fermentation (SSF)

The results obtained through all four experiments during batch SSF were demonstrated in Figure 12. 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. The results illustrated in this figure were obtained at 132 h, after the steady state. Low concentration of butanol was achieved here because only 2.5% biomass concentration was used.

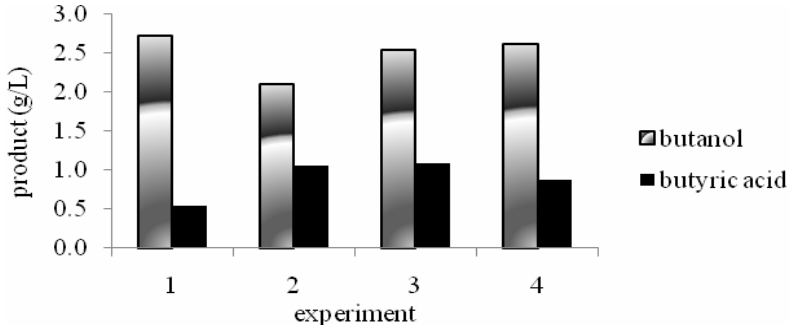

Figure 12. Comparison of products from all four experiments during SSF: Experiment 1 and 2 examined physical and water pretreatment prior to SSF. Water pretreatment was conducted at 135°C for 1 h. Experiment 3 and 4 examined physical pretreatment prior to SSF. *Clostridium beijerinckii* BA101, cellulase, and β-glucosidase were employed during SSF.

Several reasons could explain why low butanol and butyric acid concentrations were produced in Experiment 2. Water pretreatment removed hemicellulose easily but minimized saccharification of cellulose into glucose molecules [18]. 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 1, enzymatic hydrolysis had sufficient time to hydrolyse cellulose.

It was impossible to compare butanol concentrations with previous studies because different studies used different biomass concentrations. However, product yields determined through ratio of biomass concentration and butanol concentration would provide a better comparison between different studies. Table 1 summarized the values of yields obtained in this study. These results illustrated that highest yield was achieved in Experiment 1, and the second highest was obtained in Experiment 4. Surprisingly these product yields were higher than yield obtained by previous study [6]. This implied that even if low butanol concentrations were produced, more of the biomass was successfully converted into butanol. Hence, these SSF processes in this study provided excellent choices to achieve maximum butanol concentration with low biomass concentrations.

Thus, physical pretreatment and SSF (i.e. no chemical pretreatment) 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 [19, 20]. Enzymes were the only expensive catalysts used throughout this entire butanol fermentation in Experiment 4, thus approximately 33% of total fermentation costs can be deducted [5].

Table 1. Examining SSF during all four experiments and comparing the results with previous study of SSF

Experiment	Description	biomass (g/L)	butanol (g/L)	total sugar (g/L)	bacteria conc. (cells/L)	butanol yield % (g/g)
1	water pretreatment SSF	25.54	2.70	10.4	56621120	10.55%
2	water pretreatment SSF	25.54	2.08	10.4	31141620	8.13%
3	no chemical pretreatment SSF	25.54	2.51	10.8	8493170	9.83%
4	no chemical pretreatment SSF	25.54	2.61	10.8	33972110	10.22%
Qureshi et al., (6)	acid pretreatment and SSF	86.00	7.00	25.92	-	8.14%

4. Conclusions

This study achieved its objective of producing optimum sugar concentrations without generating excess amount of pollutants. Physical pretreatment and saccharification provided results that achieved the objectives of this study. Low biomass concentration after saccharification with physical pretreatment (no chemical) achieved 13.91 g/L glucose and 8.51 g/L xylose concentrations. Water and physical pretreatment with saccharification also provided results that achieved the objectives of this study. Higher glucose concentrations were achieved compared to saccharification with physical pretreatment alone. However, xylose concentrations were lower than results from saccharification with physical pretreatment only.

Sulfuric acid and physical pretreatment recovered highest glucose and xylose concentrations. Soaking process reduced some costs because heat was eliminated during pretreatment. Saccharification with MEA and physical pretreatment provided the least best choice for SSF. Here, sugar concentrations were lower than water and physical pretreatment with saccharification.

This study also obtained optimum butanol yields above 10% which was higher than previous study of SSF. Butanol production from physical pretreatment alone or with water pretreatment prior to SSF provided several advantages. These two processes emitted less pollutant. Application of catalyst during pretreatment was eliminated. Difficulty of adjusting pH prior to SSF was eliminated. These two processes may had produced low butanol concentration and required low biomass concentration. However, these processes would continuously produce butanol in a continuous free cell reactor during SSF.

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