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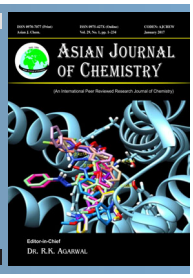

Production of Bioethanol from Corn Stover

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Bioethanol is biodegradable, low toxicity and colourless liquid biofuel that can be produced through fermentation process from biomass feedstock and had become one of the most popular alternative source of energy to reduce the consumption of fossil fuel based energy. The aim of this research is to produce bioethanol from agriculture waste, corn stover through simultaneous saccharification and fermentation (SSF) process with the help of different pretreatment. Corn stover is a promising bioethanol producing raw material as it contain high level of lignocellulose, which can convert into glucose molecules through pretreatment process (size reduction, acid and alkaline) to increase the total surface area and decrease the biomass crystallinity for better enzymatic hydrolysis. In simultaneous saccharification and fermentation process, enzyme cellulase from *Aspergillus niger* is used in enzymatic hydrolysis while *Saccharomyces cerevisae* and *Escherichia coli* are used in fermentation process. After high performance liquid chromatography, *Saccharomyces cerevisae* show the highest concentration of bioethanol produced which is 33.45 % in alkaline condition.

Keywords: Bioethanol, Corn stover, Simultaneous saccharificationand fermentation process, *Saccharomyces cerevisae*.

INTRODUCTION

Corn stover is a lignocellulosic byproduct in the corn grain production. Generally, corn stover include all parts of the maize plant except the roots and the kernels. The parts that included are husks, cobs, leaves and stalks which will be remain in the field most of the time after harvest of corn [1]. In United States, corn stover acts as the primary lignocellulosic biomass source used to produce bioethanol [2]. From the study, it indicates that the farmers can harvest the corn stover for lignocellulosic sugars which act as feedstock to be fermented into bioethanol and the cost of production is low [3]. Besides, the attention and consequences about global warming and greenhouse gases emission had prompted the great interest on using corn stover as an alternative renewable energy producer. However, some crucial problems had obstructed the opportunity to harvest the corn residues from farm such as lack of high commercial conversion technologies, lack of markets and less concerns about soil sustainability [4].

Pretreatment method is one of the crucial techniques used for the conversion and alteration of the composition of biomass which include hemicellulose, lignin and cellulose into bioethanol [5]. The pretreatment method helps to break down these lignocellulosic complex matrixes in plant to minimize the cellulose degree of crystallinity and thus, change into the finest structure to make it more accessible for the enzyme [6].

Simultaneous saccharification and fermentation (SSF) is a process where saccharification and fermentation process was carried out simultaneously. The lignocellolusic biomass was used to produce reducing sugars such as glucose through the process of enzymatic hydrolysis with the help of fermenting microorganisms in the culture to convert these fermentable sugar into bioethanol [7]. Previous research showed that this process is cost effective and reduced the chances of contamination [8].

In this research, the main objectives were to study the efficiency of producing bioethanol from corn stover by using simultaneous saccharification and fermentation (SSF) technique with the help of different pretreatment. Besides to identify the effect of using different pretreatment process to the amount of bioethanol produce and to determine the effect of using different microorganisms for the production of bioethanol.

EXPERIMENTAL

Size reduction: The corn stover which obtained from Jeli, Kelantan corn farm was washed by using tap water to remove dirt and other microorganisms. Initially, the corn stover was grounded by using grinder machine into small fragment form and then sieved into powder form. These corn stover powder were then dried in a drying oven at 75 °C to remove the water content and stored for further use.

Acid pretreatment: 2 % dilute sulphuric acid (0.303 cm^3/mL) was prepared in 2 L beaker by mixing 1 L of distilled water with 11.32 mL of concentrated sulphuric acid. Then 100 g of corn stover was added into the beaker. The mixture was then autoclaved at 121 °C with the sterilization process for 30 min. After that, the pretreated corn stover was separated through filtration process for two times. First time was filtered by using gauze to remove large particle residues and second times by using filter paper to ensure complete removal of corn stover residues. Then the corn stover residues was neutralized by using sodium hydroxide before it was disposed.

Alkaline pretreatment: 2 % dilute sodium hydroxide (10 g/mL) was prepared in 2 L beaker by mixing with 1 L of distilled water and 10 g of sodium hydroxide powder. Then 100 g of corn stover was added into the beaker. The mixture was then autoclaved at 121 °C with the sterilization process for 2 h. After that the pretreated corn stover was separated through filtration process for two times. First time was filtered by using gauze to remove large particle residues and second times by using filter paper to ensure totally removal of corn stover residues. Then the corn stover residues was neutralized by using sulphuric acid before it was disposed.

Preparation of *Saccharomyces cerevisae* inoculum: 1 g of Baker's yeast *Saccharomyces cerevisae* from the commercial Baker yeast was initially added into 9 mL of distilled water in a test tube. The mixture was shaked well to ensure the yeast was completely dissolved into distilled water. A serial dilution was carried out by using 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Each of the test tube consisted of 9 mL of distilled water. 1 mL of the solution was pipetted out and transferred from falcon tube to 10^{-1} test tube. After that, the mixture was mixed well and 1 mL of the solution was pipetted out and transferred to 10^{-2} test tube. This process was repeated by transferred the solution from 10^{-2} to 10^{-3} and 10^{-3} to 10^{-4} test tube.

An inoculum loop was sterilized by using Bunsen burner and it was allowed to cool down. The inoculum loop was then dipped into 10^{-1} test tube solution and stripped on yeast rich medium (YPD medium) agar plate. This process was repeated by using solution from 10^{-2}, 10^{-3} and 10^{-4}. Then, all the agar plate were incubated for 24 h at 37 °C.

After that, the yeast from YPD medium agar plate was cultured into 50 mL of YPD medium solution for the preparation of inoculum. The mixture was then incubated for 24 h at 37 °C before harvested by using centrifuged machine and centrifuged with the speed of 10000 rpm for 10 min at 4 °C. Then, the pellets after centrifuged was washed repeatedly for about three times by using 0.85 % saline water. The pellets was suspended again with 0.85 % saline water. The inoculum was yield with an optical density of 0.5 by using spectrophotometer with 600 nm and stored in the refrigerator at 4 °C for the next process.

Preparation of *Escherichia coli* inoculum: The bacteria *Escherichia coli* from pure culture plate was initially subcultured on the nutrient agar plate and then incubated for 24 h at 37 °C. After that, the yeast from nutrient agar plate was cultured into 10 mL of tryptic soy broth (TSB) medium for the preparation of inoculum. The mixture was then incubated for 24 h at 37 °C before harvested by using centrifuged machine, centrifuged with the speed of 10000 rpm for 10 min at 4 °C. Then, the pellets after centrifuged was washed repeatedly for three times by using 0.2 % peptone water. The pellets was suspended again with 0.2 % peptone water and stored in the refrigerator at of 4 °C for the next process. Finally, the inoculum was yield with an optical density of roughly 0.5 by using spectrophotometer with 600 nm.

Simultaneous saccharification and fermentation (SSF): 300 mL of acid pretreatment solution, 300 mL of basal medium for yeast, 50 mL of yeast inoculum solution and 2 mL of enzyme cellulase were added into the bioreactor. This step was carried out in the laminar flow. The bioreactor was setting up according to the manual and laptop was connected with the bioreactor to control all the probes during the process. The temperature was set at 37 °C and the fermentation process was run for 24 h. The pH was adjusted with 1 M of hydrochloric acid and 1 M of sodium hydroxide to maintain at pH 5. The agitation speed was set at 250 rpm. The air flow rate in bioreactor was fixed at 1 L/min. Then, the steps were repeated by changing *Saccharomyces cerevisae* inoculum with *Escherichia coli* inoculum in the basal medium Luria Broth.

High performance liquid chromatography (HPLC): The concentration of ethanol was determined by using high performance liquid chromatography (HPLC) after filtered by sterilized string. The conditions of HPLC were set as shown in Table-1.

TABLE-1
CONDITION OF HPLC

Criteria	Condition
Mobile phase	0.05 M *meta*-phosphoric acid diluted with distilled water
Flow rate	1 mL/min
Injected volume	20 µL
Column	Hypersil silica (250 mm × 4.6 mm)
Time	40 min
Temperature	40 °C

3,5-Dinitrosalicylic acid solution (DNS) test: 1 % of 3,5-Dinitrosalicylic acid solution (DNS) was prepared by mixing 10 g of 3,5-dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulphite, 10 g of sodium hydroxide and 1 L of distilled water. 3 mL of 3,5-dinitrosalicylic acid reagent was added into 3 mL of sample in a test tube. The test tube was heated in the water bath for 10 min at 90 °C to develop the red-brown colour. Then, 1 mL of 40 % potassium sodium tartrate solution was added to stabilize the change of colour. After that, the solution was allowed to cool down to room temperature. The absorbance reading was measured and recorded by using spectrophotometer with the wavelength of 575 nm.

A standard glucose curve was prepared to compare with the result of the 3,5-dinitrosalicylic acid test on the samples. 3 mL of glucose solution was mixed with 3 mL of 3,5-dinitrosalicylic acid reagent in a test tube. The test tube was heated in the water bath for 10 min at 90 °C to develop the red-brown colour. Then, 1 mL of 40 % potassium sodium tartrate solution was added to stabilize the change of colour. After that, the solution was allowed to cool down to room temperature. The absorbance reading was measured and

recorded by using spectrophotometer with the wavelength of 575 nm.

RESULTS AND DISCUSSION

Physical pretreatment: The corn stover was obtained from corn farm in Jeli, Kelantan was first undergoes physical pretreatment. The type of physical pretreatment involved was size reduction. The corn stover was grinded into powder form to decrease the particle size and biomass crystallinity. This undeniably increased the total surface area for the enzyme to attack and partially depolymerize cellulose, hemicellulose and lignin which was the main component of the lignocellulosic biomass [9].

Acid pretreatment: The corn stover powder was then undergoes acid pretreatment for further breaking down of the lignocellulose structure. Acid pretreatment able to break down those carbohydrate polymers into monomeric sugar especially hemicellulose as it is easier to be hydrolyze compare with cellulose and lignin [6]. However, acid pretreatment only able to hydrolyze little amount of lignin content due to the reason that in acidic condition, the lignin content was stabilized by the acid through condensation reaction [10].

In this research, sulphuric acid was used for acid pretreatment instead of other conventional acids. This was because sulfuric acid is more economical and act as a powerful hydrolytic agent [5]. Moreover, sulfuric acid able to yield high percentage of fermentable sugar especially from hemicellulose for about 75 to 90 %. These reasons caused sulfuric acid become the primary choice of chemical used in acid pretreatment especially in the industrial sector that produce ethanol [11].

Furthermore, by comparing with sulfuric acid, hydrochloric acid was relatively corrosive and the negative impact to the environment caused its limited application. Besides, phosphoric acid was initially become one of the options to be use in acid pretreatment. This is because after the treatment, the waste that neutralized with sodium hydroxide form sodium phosphate which can act as the nutrient for microorganisms and can be disposed directly to the natural environment without causing any harm. Unfortunately, the lower yield of monomeric sugar content after the treatment caused it removed from the consideration [12].

However, the use of acid pretreatment produce variety of inhibitor by-products such as acetic acid, formic acid, furfural, 5-hydroxymethyl furfural and so forth according to the operation condition [10]. These products act as toxic growth inhibitors for the microorganisms to growth and carried out fermentation [13]. These inhibitors especially furfural and 5-hydromethyl furfural are the strongest inhibitory compounds that will present in acid pretreatment and cause inhibition even in a low concentration. This indirectly will affect the rate of fermentation and led to low concentration of ethanol production [14].

Fig. 1 showed that the optical density values of the samples were decreased when the time of sterilization increased. The optical density values was inversely proportional to the time of sterilization (Table-2). The graph was different with standard glucose curve (Fig. 2) which showing a directly proportional graph.

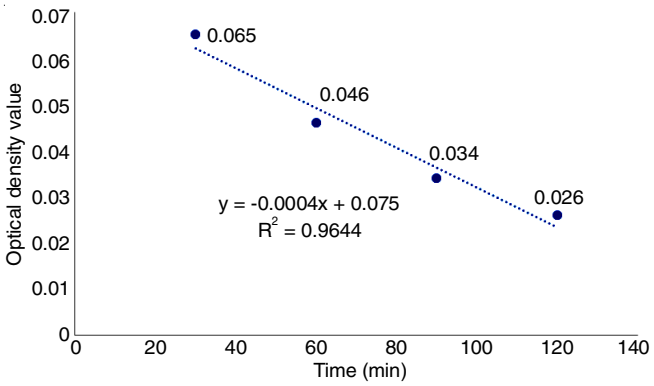

Fig. 1. Optical density curve in acid pretreatment at different sterilization time

TABLE-2
OPTICAL DENSITY VALUES OF ACID PRETREATMENT WITH DIFFERENT STERILIZATION TIME

	Sterilization time (min)			
	30	60	90	120
Replication 1	0.068	0.048	0.034	0.029
Replication 2	0.067	0.046	0.032	0.023
Replication 3	0.060	0.043	0.036	0.027
Average	0.065	0.046	0.034	0.026

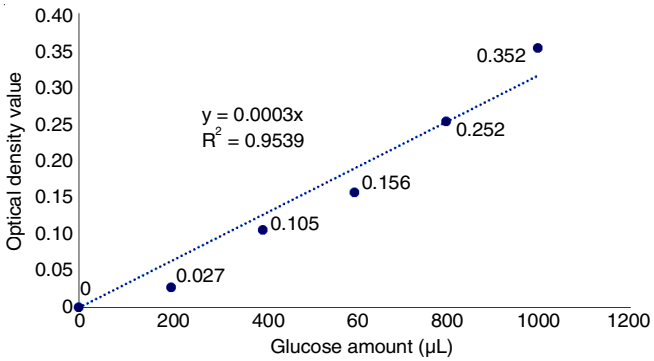

Fig. 2. Standard glucose curve optical density curve

This is because with the longer time of sterilization, the reducing sugar content such as glucose is further decomposed into inhibitory by-product such as furfural and hydroxymethylfurfural. These compounds inhibit the fermentation process carried out by microorganisms [10]. Therefore, in the acid pretreatment, 2 % H_2SO_4 with a sterilization time of 30 min is the best condition to produce high yield of glucose molecules.

Alkaline pretreatment: Beside acid pretreatment, the corn stover powder was also undergoes alkaline pretreatment for further breaking down of the lignocellulose structure. Alkaline is a powerful agent that able to break down lignin and cellulose more efficiently compare with acid [15]. This is because alkaline attacks the intermolecular bonds that cross-linking the molecules in cellulose, hemicellulose and lignin resulting the increased of total surface area and pore size for enzymatic reaction [16]. However, the efficiency of alkaline pretreatment is lesser when utilizing hemicellulose. This is because alkaline reagent can remove acetyl group and various acid substitution on hemicellulose [17].

In this research, sodium hydroxide was used instead of other alkalis. This is because it had been proved that delignification and hydrolysis of lignocellulosic biomass by using sodium hydroxide is more effective where released about 60 % of lignin and 80 % of hemicellulose [6]. Moreover, the pretreatment can be carry out in mild condition like low temperature and pressure but with the disadvantage of longer pretreatment time is needed. With the aid of these mild condition, the problems such as condensation of lignin and high lignin solubility can be avoided [5].

Furthermore, alkaline pretreatment produce only minimal amount of inhibitory by-product compare with acid pretreatment. This is because alkaline reagent causes less sugar degradation and the production of inhibitory by-product due to the degradation of sugar content in the solution. Moreover, many of the caustic salt can be recovered or regenerated after the pretreatment process [18].

Fig. 3 showed that the optical density values of the samples were increased when the time of sterilization increased (Table-3). The optical density values was directly proportional with the time of sterilization which was the same as standard glucose curve.

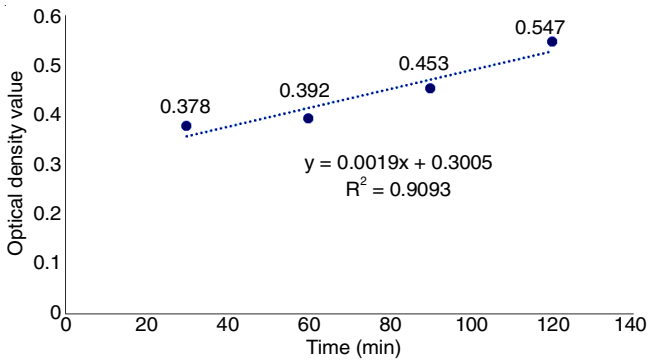

Fig. 3. Optical density curve in alkaline pretreatment at different sterilization time

TABLE-3
OPTICAL DENSITY VALUES OF ALKALINE PRETREATMENT WITH DIFFERENT STERILIZATION TIME

	Sterilization time (min)			
	30	60	90	120
Replication 1	0.382	0.397	0447	0.558
Replication 2	0.379	0.386	0460	0.540
Replication 3	0.374	0.394	0.452	0.543
Average	0.378	0.392	0.453	0.547

This is because sodium hydroxide able to hydrolyze uronic and acetic esters and cause swelling to corn stover lignocellulose more efficiently [12]. Thus, it decreased the recalcitrance of lignocellulosic biomass and improved the enzymatic hydrolysis or saccharification [13]. According to Varga *et al.* [19], high pH able to improve the convertibility of cellulose into high amount of fermentable sugar such as glucose.

Furthermore, according to Taherzadeh and Karimi [15], among sodium hydroxide, hydrogen peroxide, sulfuric acid and ozone pretreatment, sodium hydroxide show the best result where the delignification of lignocellulosic biomass up to 65 % and cellulose conversion up to 60.8 % by using 2 % of NaOH. This is because NaOH able to obtain higher enzymatic conversion compared with H_2SO_4 pretreatment. Alkaline pretreatment is also effective in breaking down ester bond between cellulose, hemicellulose and lignin [6]. This hydrolysis mechanisms is based on the saponification of intermolecular ester bond that crosslinking cellulose, hemicellulose and lignin [5].

In this research, *Saccharomyces cerevisae* and *Escherichia coli* were used in the fermentation process as these two microorganisms are efficient ethanolic fermenters. Their efficiency in carried out fermentation to produce ethanol under oxygen free condition were compared. These two microorganisms carried out fermentation by using the fermentable sugar in the solution as the source of energy [13].

It is important to understand that fermentation process in simultaneous saccharification and fermentation help to reduce the concentration of glucose produced through pretreatment and enzymatic hydrolysis. This is because high concentration of glucose causes inhibition to the enzyme cellulase to carry out its function. Besides, directly conversion of glucose to ethanol in fermentation process reduces the percentage of contamination due to the growth of unwanted microorganisms in the fermentation broth [20].

It is concluded that the alkaline pretreatment is better than acid pretreatment as both ethanol concentration by using different microorganisms are higher than that of acid pretreatment. While Baker's yeast *S. cerevisae* appeared to be a better fermenting microbe than bacteria *Escherichia coli* as the ethanol concentration is relatively higher in alkaline condition. However, the ethanol concentration in acid pretreatment by using bacteria *Escherichia coli* is relatively lower than that of using Baker's yeast *S. cerevisae* (Figs. 4-7) This may be due to the unflavourable living condition which inhibited *Escherichia coli* to carry out fermentation process.

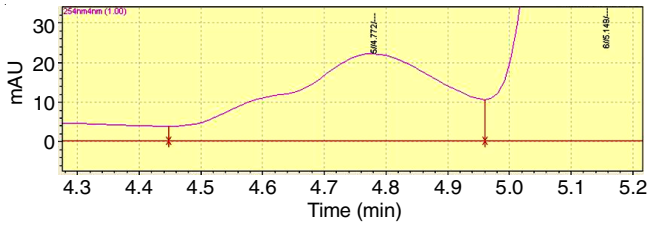

Fig. 4. Ethanol peak area of acid pretreatment by using *S. cerevisae*

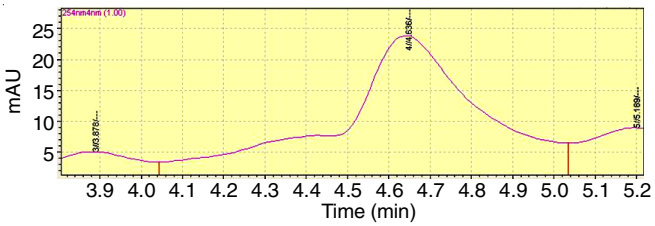

Fig. 5. Ethanol peak area of alkaline pretreatment by using *S. cerevisae*

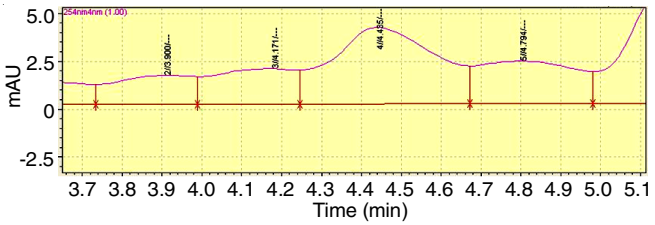

Fig. 6. Ethanol peak area of acid pretreatment by using *E. coli*

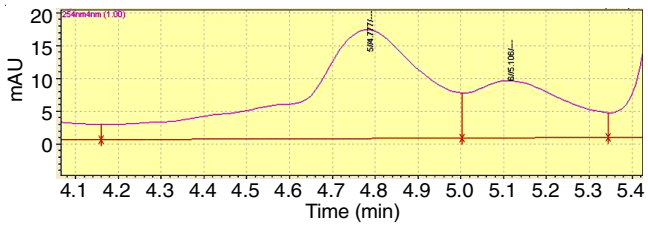

Fig. 7. Ethanol peak area of alkaline pretreatment by using *E. coli*

Conclusion

In conclusion, in acid pretreatment, the best condition to produce highest amount of glucose was by using 2 % sulphuric acid, 30 min of sterilization time at 121 °C. In alkaline pretreatment, the best condition to produce highest amount of glucose was by using 2 % sodium hydroxide, 120 min of sterilization time at 121 °C. The samples were then undergoes simultaneous saccharification and fermentation process to generate bioethanol by using Baker's yeast *Saccharomyces cerevisae* and bacteria *Escherichia coli* in the bioreactor for 24 h at 37 °C. Enzyme cellulase was added to enhance the hydrolysis of cellulose inside the corn stover. The Baker's yeast *Saccharomyces cerevisae* shows the highest concentration of bioethanol produced which was 33.45 % in alkaline condition. While the bacteria *Escherichia coli*, produced only 19.8 % of bioethanol in alkaline condition.

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