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# Optimization on Pretreatment and Enzymatic Hydrolysis of Sugarcane Trash for Ethanol Production

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**Abstract:** The present study was conducted for the optimization of pretreatment and enzymatic hydrolysis of lignocellulosic biomass (sugarcane trash), which is a renewable resource for the production of bioethanol. The pretreatment and enzymatic hydrolysis conditions including alkali (NaOH)/dilute acid ($H_2SO_4$), substrate and chemical concentration for pretreatment, enzyme dosage, pH, temperature and substrate concentration for hydrolysis were varied and evaluated for sugar and ethanol production at the end. The optimum condition was accomplished using 15% w/v DS of 0-2 mm sugarcane trash in size of particle. It was pretreated with two steps of 2% w/v NaOH autoclaving followed by 2% w/v $H_2SO_4$ autoclaving with washing step after pretreatment. An enzymatic hydrolysis was then performed using 15% w/v DS pretreated substrate, hydrolyzed with cellulase 50 filter paper unit (FPU)/g DS at 50 °C and pH 5. After incubating at 160 r for 48 h, 117.16 g/L reducing sugar was obtained. The achieved sugar after enzymatic hydrolysis was finally fermented to ethanol by *Saccharomyces cerevisiae* TISTR 5596, with concentration of 48.17 g/L ethanol or yield 0.509 g/g reducing sugars which was equal to 99.81% of theoretical yield.

**Key words:** Cellulosic biomass, pretreatment and enzymatic hydrolysis, sugarcane trash, ethanol production.

## 1. Introduction

Increasing usage and continuous depletion of non-renewable resource has become a major area of concern for developed and developing countries. Today's world economy and development is totally determined by the non-renewable fuels. Renewable fuels on the other hand are safe, environmentally friendly and reduce the carbon dioxide emission to the atmosphere. Due to this alarming situation, a worldwide search is on to produce renewable fuels which should be viable, sustainable and economically feasible [1, 2].

Ethanol from renewable resources has been of interest in recent decades as an alternative fuel to the current fossil fuels. Lignocelluloses biomass liked wood and agricultural crops residue, e.g., straw [3-5], sugarcane bagasse [6-9] and sugar beet pulp are potential raw materials for producing several high-value products like fuel ethanol and biodiesel [3].

Sugarcane trash (tops and leaves), one of the most abundant biomass in Thailand (approximately 60 million t/year), has a potential alternative renewable feedstock for long-term sustainable production of cellulosic ethanol.

Generally, the conversion of lignocellulosic biomass to bioethanol involves four steps: pretreatment, hydrolysis, fermentation and ethanol recovery [10].

Various pretreatment technologies (alone or in combination) have been proposed in the literature.

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Broadly, pretreatment technologies can be categorized into four types: physical (mechanical), physicochemical, chemical and biological pretreatments. Mechanical pretreatment increases the surface area by reducing the size of biomass [11]. A high control of operation conditions is required in the physicochemical methods because these reactions occur at high temperature and pressure [12]. Chemical methods degrade hemicellulose or remove lignin and thus, loosening the structural of lignin-holocellulose network. Biological pretreatment methods are used for the delignification of lignocellulosic biomass [13].

The main challenge of pretreatment is to remove hemicelluloses and lignin which significantly enhances the hydrolysis of cellulose [10]. Therefore, enzymatic hydrolysis can enhance the yield of fermentable sugars, which are precursors of ethanol production.

This study aims to optimize the sugarcane trash pretreatment and enzymatic hydrolysis for cellulosic ethanol production.

## 2. Materials and Methods

### *2.1 Preparing of Materials and Composition Analysis*

Sugarcane trashes were collected from Khamphang-Phet province, Thailand. They were sundried and milled in Hammer mill, subsequently passed through 2 mm of screen size. The composition was analyzed as described by the technical association of the pulp and paper industry (TAPPI) methods [14]. The milled sugarcane trashes were stored at room temperature and used as substrate in the experiments.

### *2.2 Enzyme*

A commercial blended enzyme, Cellic® CTec2 (Novozymes, Denmark) derived from *Trichoderma reesei*.

The enzyme containing of cellulase, β-glucosidase and hemicellulase, was used in enzymatic hydrolysis step.

### *2.3 Pretreatment*

Milled sugarcane trashes were slurried in 100 mL of pretreatment chemicals in 250 mL duran bottles. They were then pretreated in an autoclave at 121 °C, 15 lb/in$^2$ for 15 min. After the mixtures were cooled down, they were then washed with water and adjusted to pH 5.0. The solid residue was then separated from the liquid fraction by filtering through muslin cloth. The solid residue was subsequently used as substrate for enzymatic hydrolysis.

### *2.4 Enzymatic Hydrolysis*

Enzymatic hydrolysis was done by using wet pretreated solid residue, washed with water and adjusted to pH 5.0. Cellic® CTec2 (50 FPU/g DS) was then added to the pretreated solid residue fraction and incubated at 50 °C, 160 rpm for 48 h. Samples were withdrawn periodically, centrifuged at 9,000 rpm for 10 min. The supernatants were analyzed for reducing sugar by dinitrosalicylic acid (DNS) method [15].

### *2.5 Ethanol Fermentation*

Sugar hydrolysate from enzymatic hydrolysis of pretreated sugarcane trash was used as substrate for ethanol production. The inoculum of *S. cerevisiae* TISTR 5596 ($10^7$ cells/mL) grown in MGYP medium, was inoculated into the conical flask containing sugar hydrolysate. The ethanol fermentation was conducted on orbital shaking incubator at 30 °C, 160 rpm. Aliquots (2-3 mL) were withdrawn aseptically and assayed for ethanol concentration, reducing sugar and cell number [16].

The variables considered to be important in enzymatic hydrolysis and ethanol production were pretreatment method (NaOH + autoclaving, $H_2SO_4$ + autoclaving and NaOH + autoclaving followed by $H_2SO_4$ + autoclaving) [16], substrate concentration for pretreatment (12.5%, 15% and 17.5% w/v) chemical concentration (1, 2% w/v NaOH and 1, 2% w/v $H_2SO_4$), enzyme dosage (40, 45, 50 and 55 FPU/g substrate), substrate concentration for hydrolysis (15% and 17.5% w/v), pH (4, 5, 6 and 7), and temperature (40, 50 and 60 °C) were varied and evaluated for sugar and ethanol production at the end.

*2.6 Analytical Methods*

The contents of cellulose, hemicellulose and lignin were determined according to the TAPPI standard test method [14]. Cell number was determined by haemacytometer and reducing sugars were estimated by DNS method [15]. Ethanol concentration was analyzed by gas chromatography, Agilent 6890 series (Agilent GC system, USA) using 19091N-133 Innowax column and flame ionization detector [16]. The activity of cellulase was measured according to the reference of Ghose [17].

## 3. Results and Discussion

*3.1 Composition of Raw Materials*

The dried sugarcane trash, prior to pretreatment, contain 35.2% cellulose, 23.4% hemicellulose, 12.6% lignin and 6.59% ash (Table 1). Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment mainly because the lignin in plant cell walls forms a barrier against enzyme attack [18]. An ideal pretreatment would reduce the lignin content and crystallinity of the cellulose and increase the surface area.

*3.2 Effect of Pretreatment Method on Ethanol Production*

In the present study, the reducing sugar concentration obtained after enzymatic hydrolysis of NaOH, $H_2SO_4$ and two steps of NaOH followed by $H_2SO_4$ pretreatment were 106.04, 53.44 and 90.84 g/L, respectively. However, the two steps (NaOH followed by $H_2SO_4$ pretreatment) could give the maximum concentration 30.40 g/L of ethanol, followed by NaOH pretreatment (25.91 g/L), and $H_2SO_4$ pretreatment (19.34 g/L), respectively (Fig. 1).

One of the main challenges of pretreatment is to remove most of the non-cellulose structure, preserving more cellulose accessible to enzymatic hydrolysis and producing reducing sugar for ethanol production [10].

**Table 1 The compositions of sugarcane trash.**

| Composition | Percentage (DW) |
|---|---|
| Holocellulose | 58.6 |
| Cellulose | 35.2 |
| Hemicellulose | 23.4 |
| Lignin | 12.6 |
| Ash | 6.59 |

The values were the mean of two independent samples.

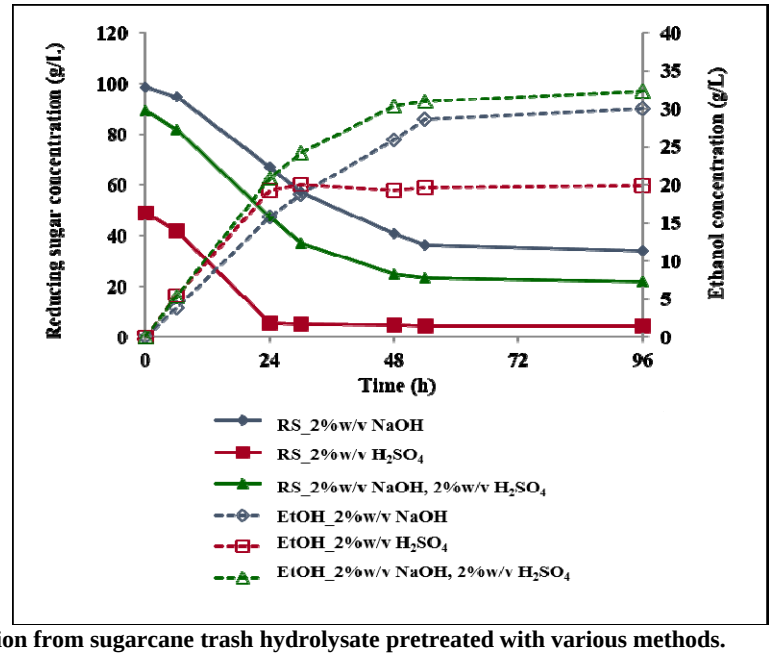


**Fig. 1 Ethanol production from sugarcane trash hydrolysate pretreated with various methods.**

The two steps pretreatment was effective in providing fractionation of the hemicellulose and lignin components, resulting in maximum of glucose, leading to achievement of maximum yield of ethanol [16]. While one step of NaOH pretreatment could produce both glucose and xylose, the yeast used in this study was unable to utilize xylose.

The ethanol concentration obtained was then lower than the two steps pretreatment.

### *3.3 Effect of Pretreatment Chemical Concentration on Ethanol Production*

The optimum pretreatment method was two steps of NaOH followed by $H_2SO_4$ pretreatment. To find the maximum ethanol concentration, the concentrations of NaOH and $H_2SO_4$ of (1% w/v NaOH, 1% w/v $H_2SO_4$), (1% w/v NaOH, 2% w/v $H_2SO_4$), (2% w/v NaOH, 1% w/v $H_2SO_4$) and (2% w/v NaOH, 2% w/v $H_2SO_4$) were varied. The results showed that ethanol concentrations of 30.27, 32.37, 42.18 and 48.17 g/L were obtained after 48 h of fermentation, respectively.

As the results, 2% w/v NaOH followed by 1% or 2% w/v $H_2SO_4$ pretreatment gave more effective result on ethanol yield than 1% w/v NaOH pretreatment. The 1% or 2% w/v $H_2SO_4$ pretreatment has no significant difference on the ethanol yield.

Based on the result of ethanol concentration, 2% w/v NaOH, autoclaving followed by 2% w/v $H_2SO_4$, autoclaving at 121 °C for 15 min pretreatment, was selected for further study (Fig. 2).

### *3.4 Effect of Substrate Concentration for Pretreatment on Ethanol Production*

The pretreatment substrate concentrations of 12.5%, 15% and 17.5% w/v DS were varied.

The results showed that maximum ethanol concentration obtained by using 15% w/v of substrate for pretreatment followed by 12.5% and 17.5% w/v were 48.17%, 42.75% and 36.76 g/L of ethanol obtained after 48 h of fermentation, respectively.

The optimum of substrate concentration for pretreatment was found to be 15% w/v, as the lower (12.5% w/v) and higher (17.5% w/v) substrate concentration gave lower ethanol yield (Fig. 3).

### *3.5 Effect of Enzyme Loading on Ethanol Production*

To compare the ethanol concentration, the enzyme loadings of 40, 45, 50 and 55 FPU/g of substrate were varied. The results showed that ethanol concentrations

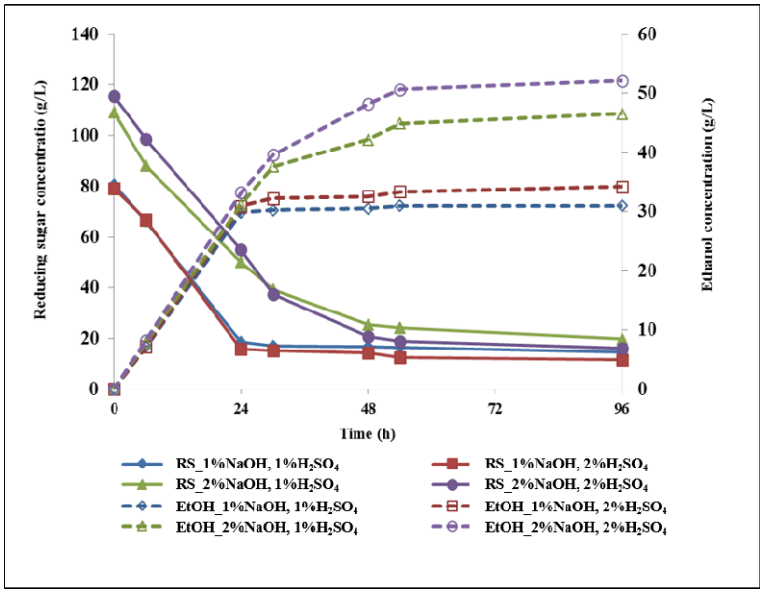


**Fig. 2 Ethanol production from sugarcane trash hydrolysate pretreated with NaOH followed by $H_2SO_4$ at various concentrations.**

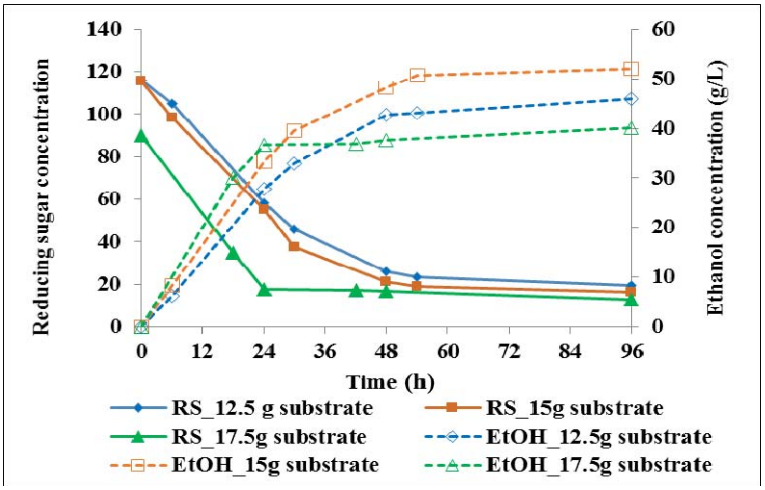


**Fig. 3 Ethanol production from sugarcane trash hydrolysate pretreated with various substrate concentrations.**

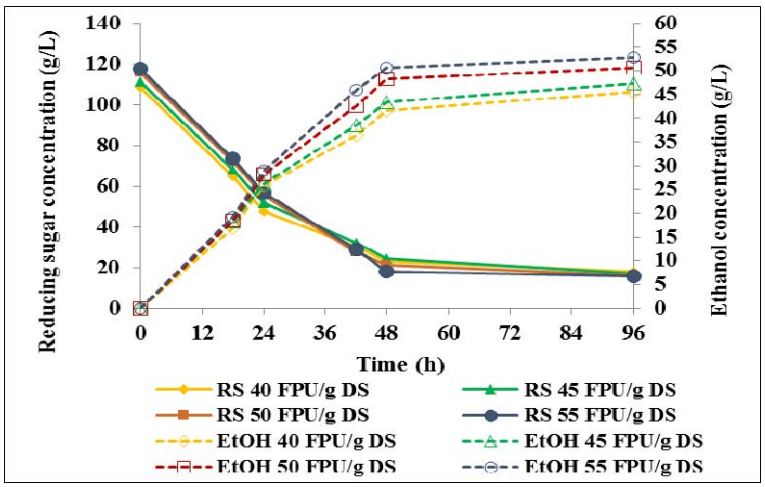


**Fig. 4 Ethanol production from sugarcane trash hydrolysate hydrolyzed with various enzyme concentrations.**

of 41.66, 43.44, 48.17 and 50.62 g/L were obtained after 48 h of fermentation, respectively.

A cellulase concentration of 50 FPU/g of substrate was found to be optimum, as increase in cellulase concentration (55 FPU/g of substrate) was not economically feasible. It was also observed that the increase in enzyme loading from 50 FPU/g to 55 FPU/g of substrate, the ethanol concentration increased only 5.09% (Fig. 4).

### *3.6 Effect of Substrate Concentration for Hydrolysis on Ethanol Production*

To find the optimum substrate concentration for hydrolysis, substrate concentrations of 15% and 17.5% w/v DS were compared. The results showed that ethanol concentration 48.17 g/L from substrate concentration 15% w/v and 38.47 g/L of ethanol from substrate concentration 17.5% w/v were obtained after 48 h of fermentation (Fig. 5).

As a result, substrate concentration of 15% w/v was found to be optimum for enzymatic hydrolysis and ethanol production. An increase in substrate concentration of 17.5% w/v DS limited the saccharification and ethanol yield, due to difficulties in shaking and enzyme inhibition exerted by saccharification products [19, 20].

### *3.7 Effect of pH on Enzymatic Hydrolysis*

To find the maximum concentration of reducing sugar after 48 h of hydrolysis, the pH of hydrolysis 4, 5, 6 and 7 were varied. The results showed that reducing sugar concentrations obtaining from hydrolysis at pH 4,

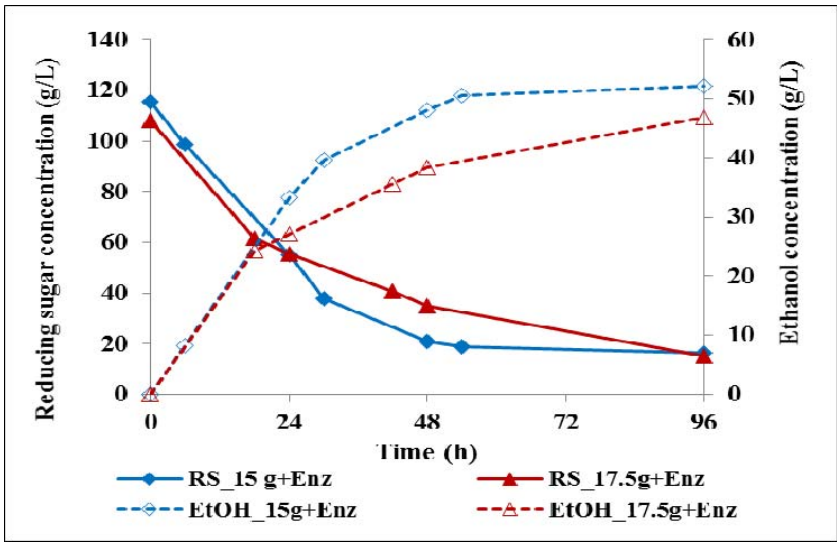


**Fig. 5 Ethanol production from sugarcane trash hydrolysate hydrolyzed with 15% w/v and 17.5% w/v of pretreated substrate.**

5, 6 and 7 were 111.74, 117.16, 111.22 and 104.60 g/L, respectively (Table 2). Maximum yield was reachable at pH 5, although pH in the range (4-6) did not affect the sugar yield. The slight decrease in the saccharification at lower pH values is favourable to the simultaneous saccharification and fermentation (SSF) process. The optimal pH for yeast growth was between 5 and 5.5. A decrease in below 4 was however taken place during fermentation. These results show that such a change in pH during fermentation does not bring about a great effect on the total efficiency of enzymatic hydrolysis [19, 20].

### *3.8 Effect of Temperature on Enzymatic Hydrolysis*

To compare the maximum reducing sugar concentration after enzymatic hydrolysis, the temperature of hydrolysis at 40, 50 and 60 °C was varied. The results showed that reducing sugar concentrations of 113.03, 117.16 and 99.50 g/L were obtained using temperature 40, 50 and 60 °C, respectively (Table 2). A temperature of 50 °C was found to be optimum temperature for hydrolysis. At temperature 40 °C, only a negligible decrease in sugar yield was however observed [21].

**Table 2 Reducing sugar concentration from enzymatic hydrolysis of pretreated sugarcane trash at various pH values and temperatures.**

| Parameter | Level | Reducing sugar concentration (g/L) |
|---|---|---|
| pH | 4 | 111.74 |
| | 5 | 117.16 |
| | 6 | 111.22 |
| | 7 | 104.60 |
| Temperature (°C) | 40 | 113.03 |
| | 50 | 117.16 |
| | 60 | 99.50 |

This result is useful for the SSF process, as higher temperature (60 °C) is limiting factor for yeast growth as well as ethanol yield [19, 20].

Enzymatic hydrolysis is an ideal approach for degrading cellulose into reducing sugars because mild reaction conditions (pH between 4.8-5.0 and temperatures between 45-50 °C) can be used, it does not present corrosion problems in the reactors and result in negligible by-products formation with high sugar yields [22].

## 4. Conclusions

The optimal conditions for pretreatment of sugarcane trash was dried substrate of 15% w/v pretreated with 2% w/v of NaOH, autoclave at 121 °C, 15 lb/in$^2$ for 15 min followed by 2% w/v of $H_2SO_4$, autoclave at 121 °C, 15 lb/in$^2$ for 15 min.

The optimal conditions for enzymatic hydrolysis of cellulose to obtain high glucose and ethanol

concentration was determined as substrate concentration 15% w/v, enzyme loading 50 FPU/g substrate at 50 °C and pH 5 for 48 h. At this condition reducing sugar concentration of 117.16 g/L and ethanol concentration of 48.17 g/L or yield 0.509 g ethanol/g sugar which was equal to 99.81% of theoretical yield were obtained by using $10^7$ cells/mL of *S. cerevisiae* TISTR 5596 as inoculum.

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