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# Evaluation of agricultural wastes for the use in ethanol production by *Candida shehatae* TISTR 5843

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## Abstract

This study aimed to evaluate four agricultural wastes: rice straw, oil palm empty fruit bunch (oil palm EFB), sugarcane bagasse and corncob, for their potential when used as feedstocks in ethanol production. The waste materials were subjected to acid pretreatment by autoclaving at 121°C followed by enzyme (Accellerase 1500®) hydrolysis prior to ethanol fermentation. By varying the times in acid pretreatment, 10 minutes was shown to be sufficient based on the test of susceptibility to cellulose hydrolysis. The enzyme dosage study resulted in the use of 60 FPU/g dry solid (DS) for all acid-treated materials except for oil palm EFB which required the dosage of 110 FPU/g DS. Different forms of substrates used in enzyme hydrolysis (the acid-treated solids versus the acid-treated slurries) did not affect the amount of ethanol produced from resulting oil palm EFB and sugarcane bagasse hydrolysates, but they did affect in the case of rice straw and corncob hydrolysates. The fermentation of rice straw, oil palm EFB, sugarcane bagasse and corncob hydrolysates by *Candida shehatae* TISTR 5843 resulted in 9.8, 9.3, 7.9 and 10.9 g/l of ethanol respectively.

**Keywords:** ethanol, *Candida shehatae*, agricultural wastes, acid pretreatment, enzyme hydrolysis

# 1. Introduction

The depletion of petroleum-based fuels has stimulated the development of alternative energy sources including biofuels, of which bioethanol has been one in the focus (1). Current interest in bioethanol production has been turned into the use of non-food based materials such as lignocellulosic materials as the feedstocks to ethanol production. The materials are an interesting choice as a substrate for biofuels production especially for agricultural and agro-industrial based countries since large amount of biomass are generated as wastes products. Upon hydrolysis of lignocellulosic material, fermentable monosaccharides could be obtained from hemicellulose and cellulose fractions of the material. Various methods including physical, physico-chemical, chemical and biological have been reported in preparation of lignocellulosic materials for biofuels production (2, 3, 4).

There have been reports on the use of various lignocellulosic biomasses such as sugarcane bagasse (5, 6, 7), corncob (8), rice straw (9), wheat straw (10), softwood (11) and yellow poplar (12) for ethanol production. Ethanol productions of 1.8-45.7 g/l were reported, from which the amount produced varied depended on amount of sugars obtained from hydrolysis and microorganism used.

Being one of the world leaders in agricultural products and export of agricultural products, Thailand also has abundant biomass resources especially those from agricultural residues. Agricultural crops and plants that could be considered as economically important include sugarcane, rice, oil palm and corn with the production of 103.3, 38.5, 12.2 and 4.7 million tons in 2013 (13).

In this study, we evaluated ethanol production potentials from the four agricultural wastes mentioned above. The waste materials were heat-treated by diluted acid and then hydrolyzed by cellulolytic enzyme. Finally, we investigated the ethanol production from their hydrolysates by *Candida shehatae* TISTR 5843, a native strain of xylose fermenting yeast.

# 2. Materials and methods

### 2.1 Raw materials and their preparation

Four agricultural wastes were obtained from Khon Kaen and Surat Thani provinces. Rice straw and corncob were collected from cultivation sites in Khon Kaen University, Khon Kaen. Oil palm EFB was obtained from Thai Tallow and Oil Co. Ltd., Surat Thani. Sugarcane bagasse was obtained from Mitr Phu Viang Sugar Mill, Khon Kaen. All materials were dried at 60 $^{o}$C for 48 h. They were then cut and milled to the size smaller than 10 meshes except for corncob which was sieved to 10- and 4- mesh particle sizes. Corncob of each size was mixed in equal fraction to make up the final weight used in further step.

All materials were then subjected to alkali pretreatment in order to reduce their lignin content. Each material was suspended in 15% (v/v) $NH_4OH$ at the ratio of 1 gram solid to 30 ml of alkali solution. The mixture was incubated at room temperature for 24 h with manual stirring periodically. Solid fraction was separated from the liquid by filtration using muslin cloth. It was washed 3 times and dried at 60°C for 48 h before being used.

### 2.2 Acid pretreatment

All materials were pretreated by suspending the dried materials in 2% (v/v) sulfuric acid at the ratio of 1g dry solid (DS) to 20 ml of acid and autoclaved at 121 $^{o}$C, 15 lb/in$^2$. The autoclaved time was varied at 10-35 min. Solid residual was washed 3 times and dried at 60 $^{o}$C for 48 h before being tested for susceptibility to cellulose hydrolysis

In the susceptibility to cellulase hydrolysis, 1 g of acid-treated solids was suspended in 20 ml of 0.05 M sodium citrate buffer (pH 4.8). Cellulase enzyme (Sigma, USA) was added at 10 FPU/g DS and the mixture was

incubated at 50 °C for 72 h. The amount of reducing sugar liberated indicated the level of susceptibility of material to cellulase hydrolysis.

**2.3 Enzyme hydrolysis**

Acid-treated solid was suspended in 0.05 M sodium citrate buffer (pH 4.8) at the ratio of 1 g solid to 20 ml of buffer. In the case when the slurry from acid pretreatment was used, pH of the slurry was adjusted to 4.8. Accellerase 1500® (Genencor International, Inc., USA) was added at 60, 110, 160 and 210 FPU/g DS. The mixture was incubated at 50 °C for 72 h. Liquid fraction was analyzed for reducing sugar concentration.

**2.4 Ethanol fermentation**

All fermentations were carried out in YM-base medium consisted of 3 g/l of yeast extract, 3 g/l of malt extract, 5 g/l of peptone and xylose/hydrolysate. When the hydrolysates were used as carbon sources, they were mixed with YM-base solution at the volume ratio of 9:1.

*C. shehatae* TISTR 5843 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). The yeast was propagated twice in YM media containing 10 g/l and 20 g/l of xylose, respectively at the conditions of 30 °C and 200 rpm for 24 h.

The fermentations were carried out in 250-ml Erlenmeyer flask. The 10% (v/v) inoculum was transferred to 100 ml of fermentation medium with hydrolysate as carbon source. The fermentation conditions were 30 °C with shaking at 100 rpm for 42 h.

**2.5 Analytical methods**

Reducing sugars were analyzed by 3,5-dinitrosalicylic acid (DNS) method (14). The concentration of ethanol was analyzed using HPLC (CTO-10A, Shimudzu, Japan) with a refractive index detector (RID-6A, Shimudzu, Japan). Separation was carried out using an Aminex column (HPX-87H, Bio-Rad, USA) at 40 °C with 5 mM $H_2SO_4$ as eluent at the flow rate of 0.75 ml/min

# 3. Results and discussion

**3.1 Acid pretreatment**

The time used in acid pretreatment was investigated in order to choose the period that was suitable for the treatment. The results in Table 1 showed that the materials resulted differently in their susceptibility to cellulase hydrolysis with rice straw as the most susceptible materials. It showed the most reducing sugar liberated, followed by corncob, sugarcane bagasse and oil palm EFB.

For each material, no significant differences were observed between the autoclaving periods from 10-35 min. As there was a relationship between the cellulose crystalline structure and the susceptibility to cellulase hydrolysis (15), these results indicated that increasing heating time did not affect the crystallinity of the cellulose structure of each material hence no enhance in susceptibility to cellulase hydrolysis. Therefore, 10 min was the suitable period for the pretreatment with dilute acid using autoclave at 121 °C.

**3.2 Enzymatic hydrolysis of acid-treated materials**

The solid fractions from acid pretreatment step were hydrolyzed with the commercial enzyme mixture, Accellerase 1500®. Dosage of Accellerase 1500® to be used with each acid-treated materials was determined. The results in Table 2 indicated that by increasing enzyme dosage, relatively higher amounts of reducing sugar were obtained for each material. Statistical analysis of the amount of reducing sugars in enzymatic hydrolysates showed that there was a limit in the amount of sugar obtained from each material regardless of increasing enzyme dosage. For rice straw, sugarcane bagasse, and corncob, no significant differences in the amount of reducing sugar were observed when using the enzyme dosage between 60-210 FPU/g DS. For oil palm EFB,

there was no significant difference in the amount of sugar obtained when 110-210 FPU/g DS of enzyme was used. As the cost of enzyme contributes significantly to the total cost of ethanol production from lignocellulosic materials (8), the cellulase dosage should be minimized as much as possible. Therefore, the enzyme dosage of 60 FPU/g DS was selected to be used for all acid-treated materials except for oil palm EFB which the dosage of 110 FPU/g DS was selected.

**3.3 Ethanol fermentation from hydrolysates**

The fermentations were carried out to determine the potential ethanol production from each waste material using their hydrolysates as carbon sources. Hydrolysate of each material was prepared using two different sources: solid and slurry from acid pretreatment step. Selected time and enzyme dosages from previous parts of this study were employed in the preparation.

**Table 1.** Susceptibility to cellulase hydrolysis of all materials after pretreatment with diluted acid and autoclaving at various times

| Materials | Time (min) | Susceptibility to cellulose hydrolysis (g/l of reducing sugar) |
|---|---|---|
| Rice straw | 10 | 20.30 ± 1.43[a] |
| | 15 | 20.98 ± 1.17[a] |
| | 25 | 20.73 ± 0.46[a] |
| | 30 | 20.26 ± 2.22[a] |
| | 35 | 19.33 ± 3.53[a] |
| Oil palm EFB | 10 | 6.86 ± 1.14[a] |
| | 15 | 7.52 ± 0.83[a] |
| | 25 | 7.27 ± 1.43[a] |
| | 30 | 7.03 ± 0.77[a] |
| | 35 | 7.22 ± 1.53[a] |
| Sugarcane bagasse | 10 | 8.04 ± 1.01[a] |
| | 15 | 8.37 ± 0.58[a] |
| | 25 | 8.88 ± 0.52[a] |
| | 30 | 8.11 ± 0.52[a] |
| | 35 | 8.40 ± 0.43[a] |
| Corncob | 10 | 10.73 ± 2.04[a] |
| | 15 | 9.60 ± 4.01[a] |
| | 25 | 10.18 ± 3.64[a] |
| | 30 | 11.78 ± 1.27[a] |
| | 35 | 1.43 ± 0.19[a] |

1) Each result was the average value and standard deviation of three independent experiments.

2) Same letter indicates that the values were not significantly different comparing within the same material

**Table 2.** Reducing sugars obtained after hydrolyzing acid-treated solid fractions with various dosages of Accellerase 1500®

| Materials | Enzyme dosage (FPU/g, DS) | Reducing sugar (g/l) |
|---|---|---|
| Rice straw | 10 | 26.38 ± 2.04 [a] |
| | 60 | 31.80 ± 2.80[b] |
| | 110 | 30.84 ± 0.65[b] |
| | 160 | 30.62 ± 0.55[b] |
| | 210 | 29.36 ± 1.12[ab] |
| Oil palm EFB | 10 | 13.30 ± 2.09[a] |
| | 60 | 17.68 ± 1.10[b] |
| | 110 | 19.41 ± 0.50[bc] |
| | 160 | 20.49 ± 1.20[c] |
| | 210 | 19.24 ± 1.68[bc] |
| Sugarcane bagasse | 10 | 12.18 ± 1.21[a] |
| | 60 | 16.85 ± 0.48[b] |
| | 110 | 18.16 ± 1.00[b] |
| | 160 | 18.09 ± 1.98[b] |
| | 210 | 18.87 ± 0.88[b] |
| Corncob | 10 | 14.60 ± 2.14[a] |
| | 60 | 21.31 ± 3.80[b] |
| | 110 | 23.15 ± 2.04[b] |
| | 160 | 23.76 ± 2.41[b] |
| | 210 | 23.26 ± 2.09[b] |

1) Each result was the average value and standard deviation of three independent experiments.

2) Same letter indicates that the values were not significantly different comparing within the same material.

The results in Table 3 showed that reducing sugars in hydrolysates ranged from approximately 19 g/l to 30 g/l. For sugarcane bagasse and corncob, the reducing sugars in hydrolysates prepared from slurry were statistically higher than in those prepared from solid. However, they appeared the same in the case of rice straw and oil palm EFB.

*C. shehatae* could not use all the sugars presented in the hydrolysates. The residual sugars from the hydrolysates prepared from acid-treated slurries were slightly higher than those prepared from solids. The incomplete sugar utilization was not unexpected when lignocellulosic hydrolysate was used as a substrate because the digestion of lignocellulose materials with acid and enzyme released several forms of sugars including oligosaccharides, polysaccharides and monosaccharides, some of which could not be used by microorganisms (16).

**Table 3.** Ethanol productions from enzymatic hydrolysates of all materials by *Candida shehatae* TISTR 5843 (100 rpm at 30°C for 42 h)

| Materials | Hydrolysates from acid-treated solids | | | | Hydrolysates from acid-treated slurries | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial sugar (g/l) | Residual sugar (g/l) | Ethanol (g/l) | Yield (g/g) | Initial sugar (g/l) | Residual sugar (g/l) | Ethanol (g/l) | Yield (g/g) |
| Rice straw | 24.45 ± 3.10$^{A}$ | 2.67 ± 2.83$^{a}$ | 9.81 ± 0.40$^{1}$ | 0.46 | 25.14 ± 3.40$^{A}$ | 2.21 ± 0.74$^{a}$ | 7.78 ± 1.10$^{2}$ | 0.34 |
| Oil palm EFB | 23.46 ± 3.73$^{A}$ | 2.25 ± 0.89$^{a}$ | 9.25 ± 1.66$^{1}$ | 0.43 | 25.25 ± 2.26$^{A}$ | 3.90 ± 2.35$^{a}$ | 9.31 ± 0.47$^{1}$ | 0.44 |
| Sugarcane bagasse | 18.89 ± 1.78$^{B}$ | 1.04 ± 0.11$^{a}$ | 7.19 ± 0.74$^{1}$ | 0.40 | 22.18 ± 1.35$^{A}$ | 2.00 ± 0.85$^{a}$ | 7.90 ± 0.44$^{1}$ | 0.39 |
| Corncob | 21.24 ± 5.19$^{B}$ | 1.78 ± 0.28$^{b}$ | 6.66 ± 0.88$^{2}$ | 0.35 | 29.63 ± 0.98$^{A}$ | 4.48 ± 1.26$^{a}$ | 10.92 ± 0.50$^{1}$ | 0.44 |

1) Each result was the average value and standard deviation of three independent experiments.

2) Initial sugar, residual sugar and ethanol concentrations were compared. Same letter/number indicates that the values were not significantly different comparing within the same material.

**Table 4.** Ethanol productions from rice straw and sugarcane bagasse hydrolysates by C5 utilizing yeasts

| Materials | Organisms | Ethanol (g/l) | Yield (g/g) | References |
|---|---|---|---|---|
| Rice straw | *Pichia stipitis* BCRC 21777 | 9.38 | 0.40 | (17) |
| | *P. stipitis* BCRC 21777 | 20.8 | 0.45 | (18) |
| | *C. shehatae* TISTR 5843 | 9.81 | 0.46 | This work |
| Sugarcane bagasse | *P. stipitis* DSM 3651 | 4.90 | 0.20 | (19) |
| | *C. shehatae* NCIM 3501 | 9.80 | 0.35 | (20) |
| | *C. shehatae* TISTR 5843 | 7.90 | 0.39 | This work |

Ethanol productions in all materials ranged from 7.19 g/l to 10.92 g/l (Table 3). The results obtained were comparable with the results from other studies using the same types of materials especially for the results of ethanol yield (Table 4). Higher ethanol concentration was merely the results of higher sugar concentration obtained from hydrolysis process.

Mixed results were observed in ethanol production when using hydrolysates prepared from solid and slurry. In oil palm EFB and sugarcane bagasse, there were no differences in ethanol production when using the hydrolysates prepared from both sources. Higher ethanol was produced when using hydrolysate from solid in the case of rice straw whereas hydrolysate from slurry resulted in higher ethanol in the case of corncob.

The hydrolysates prepared from the slurries were likely to have inhibitors carried over from the acid pretreatment step. However, the effect of those inhibitors would occur only when certain level of inhibitors was reached. Although the amounts of inhibitors were not analyzed in this study, there were similar studies that had reported low amount of hydroxymethylfurfural (HMF), furfural and acids liberated from acid pretreatment step with mild sulfuric acid and heat (21, 22). Therefore, the inhibitors were unlikely to have any effects on ethanol production in this case especially in bagasse, EFB and corncob hydrolysates.

For corncob hydrolysate, a possible explanation for higher ethanol production and yield obtained when using hydrolysate from slurries was that corncob contained larger fraction of hemicelluloses (22) hence higher amount of xylose was made available for growth and ethanol production after acid pretreatment step. For rice straw hydrolysate, although low level of inhibitors has been reported, inhibitory level of furfural was possible when treated with mild acid and heat (21). Therefore, the decrease in ethanol yield was resulted when using hydrolysate prepared from acid-treated slurry, which could contain inhibitory level of furfural. Similar result was reported in *Pichia stipitis* where ethanol yield was reported to decrease from a non-inhibited level of 0.42 g/g to 0.32 g/g in the fermentation using rice straw hydrolysate supplemented with 1.3 g/l of furfural (23).

## 4. Conclusion

The four agricultural wastes in this study showed their potentials use as substrates for ethanol production. The hydrolysates used in fermentation could be prepared as continuous process without separating the liquid fraction from the acid pretreatment step prior to enzyme hydrolysis. Enhanced ethanol production using those wastes could be further studied especially in the areas of material hydrolysis to obtain higher fermentable sugars and the efficient fermentation process.

## 5. Acknowledgement

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