Ethanol Production from Pineapple Waste by Co-culture of *Saccharomyces cerevisiae* TISTR 5339 and *Candida shehatae* KCCM 11422

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Abstract

The ethanol production from alkaline pretreated pineapple waste (core and peel) by using co-culture process of *S. cerevisiae* TISTR 5339 and *C. shehatae* KCCM 11422 was evaluated. The potential of ultrasound-assisted alkaline pretreatment was applied to enhance total reducing sugar concentration. The highest yield of total reducing sugar was obtained at 21.84 g/g dried sample after pretreatment by 2% NaOH with ultrasonic-assistance for 60 min subsequently by enzymatic hydrolysis. The compositions of pretreated pineapple waste were   40.52±0.99% cellulose, 24.03±1.52% hemicellulose and 2.27±0.23% lignin. However, the dry matter loss reached to 44.67±3.21% after pretreatment. The structural changes of pretreated pineapple waste were observed by scanning electron microscopy (SEM). The maximum ethanol concentration and ethanol yield after 24 h fermentation were 9.73±0.15g/L and 0.45±0.01 g/g, respectively.
Keywords : *Ethanol production, pineapple waste, ultrasonic, Saccharomyces cerevisiae TISTR 5339, Candida shehatae KCCM 11422*

1. Introduction

Lignocellulosic material, one of the most abundant renewable resources available, represents approximately 50 billion tons per year around the world (1). Lignocellulosic material can be converted to fermentable sugars (such as glucose and xylose) which are substrates for ethanol production. Lignocellulosic agricultural wastes in Thailand were about 100 million per year considering for bioconversion (2). Thailand is the major leader of canned pineapple exporter. The wastes from the process, namely peel, core and crown, are found nearly 0.62 million tons per year (3).

Lignocellulose is mainly composed of cellulose, hemicelluloses and lignin. Since lignin acts like a barrier to obstruct cellulose and hemicellulose accessibility, the pretreatment step is necessary before acid or enzymatic hydrolysis step. The

pretreatment is not only able to breakdown lignin structure but also disrupt the crystallinity of cellulose structure in order to enhance enzymatic hydrolysis.

Alkaline pretreatment of lignocellulose using sodium hydroxide (NaOH) can break the ester bond linked between xylan and lignin, modify lignin structure, swell cellulose, and partial decrease the crystallinity of cellulose (4,5), thus improving the efficiency of enzymatic hydrolysis. The advantage of alkaline pretreatment process is the ability to carry out under lower temperature and pressures comparing with other pretreatment technologies (6). Ultrasound waves generate the difference of pressure within a solution which has physical and chemical effects on lignocelluloses composition (7). The physical effects of ultrasound on lignocelluloses were occurred by shear forces, mass transfer and surface erosion whereas the chemical effects take place via highly reactive radical species such as $\cdot OH$, $\cdot O$, and $HO_2 \cdot$ causing delignification. The goal of pretreatment is to remove lignin and separate cellulose and hemicellulose from lignin and also less time-consuming process. Therefore, alkaline and ultrasound, which have similar principles of biodegradation of lignocellulose, was combined to improve the efficiency of pretreatment and yield of sugar production.

S. cerevisiae is widely used for ethanol production because of its high ethanol productivity. However, S. cerevisiae cannot utilize xylose in biomass hydrolysate. The xylose-utilizing yeast is required in order to enhance substrate utilizing and improve ethanol productivity. C. shehatae is a xylose-utilizing yeast which can ferment xylose to ethanol. It has been reported that the co-culture of S. cerevisiae ICV D254

and C. shehatae HM 52.2 in rice hull hydrolysate effectively converted glucose and xylose to ethanol. The co-culture was maximizing substrate utilization rates, increasing ethanol yields and production rates (8).

The objective of this study is to evaluate the effect of alkaline combined with ultrasound for pineapple waste pretreatment on chemical composition and yield of reducing sugar. Co-culture fermentation was applied for the effective conversion of pineapple waste into ethanol.

2. Materials and Methods

2.1 Raw material

The pineapple waste was obtained from Kamphaeng Saen market, Nakhon Pathom province, Thailand in year 2014. The samples were chopped, washed several times with tap water, sun-dried, and dried in oven at 60 °C for 72 h. It was further blended and kept at room temperature in a desiccator before determination of acid and neutral detergent fiber by the method of Van Soest (9,10)

2.2 Combination of alkaline and ultrasonic pretreatment for reducing sugar production

Five grams of dry pineapple waste were added to 100 ml of 1 or 2% NaOH solution in a 250-ml bottle. All samples were pretreated as follows: (A) 1% NaOH with ultrasound for 60 min; (B) 1% NaOH with ultrasound for 30 min followed by standing for 30 min and then ultrasound for 30 min; (C) 2% NaOH with ultrasound for 60 min; (D) 2% NaOH with ultrasound treatment as in method B; (E) 1% NaOH by standing in the oven at 60 ºC for 90 min; (F) 2% NaOH by standing in the oven at 60 ºC for 90 min. After pretreatment, samples

(3 out of 6 flasks) were analyzed the chemical composition by centrifugation, wash with distilled water until neutral pH and dried at 60 °C. The other samples (3 flasks) were further used for reducing sugar production by adjusting to pH 4.8 by 1 N sulfuric acid and subsequently being hydrolyzed by cellulase (Accellerase 1500, Genecor, CA) with loading of 20 FPU/g biomass in a shaking incubator (180 rpm) at 50 °C for 24 h. The total reducing sugar concentration was analyzed by dinitrosalicylic acid (DNS) method (10). The hydrolysate from the best condition was supplemented with 1% yeast extract (w/v) and then autoclaved for 15 min at 121°C.

2.3 Fermentation
2.3.1 Yeast strain and inoculum preparation

A commercial S. cerecisiae TISTR 5339 and C. shehatae KCCM 11422 were purchased from Thailand Institute of Scientific and Technology (TISTR), Bangkok, Thailand and Korean Culture Center of Microorganisms (KCCM), South Korea, respectively. The inoculum was prepared by cultivation of yeast in 250-mL Erlenmeyer flask containing 100 mL of YM medium (yeast extract, 3 g/L; peptone, 5 g/L; malt extract, 3 g/L and glucose 10 g/L). The flask was incubated at 30°C for 24 h with shaking at 180 rpm. The cell concentration was diluted to obtain at 1×10^8 cells/ml prior to using.

2.3.2 Ethanol production by mono and co-culture fermentation

The fermentation was carried out in 250-ml Erlenmeyer flask using the best hydrolysate which was initially inoculated with the culture suspension of S. cerevisiae (5% v/v) for mono and co-culture fermentation. The culture suspension of C. shehatae (3% v/v) was inoculated after 15 h fermentation for co-culture fermentation. The fermentation was operated at 30°C under static condition. The samples were collected until the sugar and ethanol concentration were stable.

2.4 Analytical method

The chemical composition, namely cellulose, hemicellulose and lignin content were analyzed according to the method of Van Soest (9,10). The total reducing sugar concentration was determined by dinitrosalicylic acid (DNS) method (11). The glucose, xylose and ethanol concentration were analyzed using high performance liquid chromatography (Waters 2695 system, MA, USA) outfitted refractive index detector with SugarPax column (Waters, MA, USA). The column temperature was set at 85°C. Samples were eluted with deionized water at a flow rate of 0.5 mL/min. Filter paper assay (FPU assay) was determined according to the standard IUPAC (International Union of Pure and Applied Chemistry) procedure. One FPU unit of activity liberates 1 µmol of reducing sugar (expressed as glucose equivalents) in one minute under specific assay conditions of 50 °C and pH 4.8. A scanning electron microscopy (Camscan, MX2000, UK) was used to observe the physical changes in pretreated pineapple waste.

All experiments were carried out in triplicate. The data were analyzed for statistical significance using one-way analysis of variance (ANOVA) at the significance level of 0.05 followed by Duncan's multiple range test.

3. Results and discussion

3.1 The effect of different pretreatment methods on the chemical composition of pineapple waste

The chemical compositions of unpretreated and pretreated pineapple wastes were determined for cellulose, hemicelluloses and lignin (Table 1). The main chemical composition was cellulose and hemicellulose before pretreatment. After pretreatment, the maximum cellulose content of $40.52 \pm 0.99\%$ was obtained from sample with pretreatment method C which was higher than that of unpretreated pineapple waste about 1.74 times. The maximum cellulose content of $40.52 \pm 0.99\%$ was obtained from sample with pretreatment method C. The orders of cellulose content was C>D>A>B>F>E>-control. The lignin content significantly decreased after pretreatment. The alkaline and ultrasound-assisted alkaline pretreatment increased the cellulose content to 26-28% and 31-40%, respectively. In addition, more than 63% of delignification was found in all pretreated samples. Other compounds in pineapple waste are composed of crude protein, fat, ash and extractive compounds (12). The cellulose and hemicellulose of ultrasound-assisted alkaline pretreatment were higher than those of alkaline pretreatment sample.

The excellent pretreatment should extremely remove lignin and sustain cellulose and hemicelluloses. The major consistent advantages between alkaline and ultrasound are break down lignin structure and improve digestibility of cellulose (8,13). The combination of alkaline and ultrasound not only shorten processing time but also decrease the requirement of alkaline (14). Moreover, the ultrasound assistance improved efficiency of pretreatment compared to without ultrasound (15) which was consistent with the result of this study.

Table 1. The effect of different pretreatment methods on the chemical composition of pineapple waste

Method	Cellulose (%)	Hemicellulose (%)	Lignin (%)
A	33.44 ± 1.27^{c}	21.64 ± 1.24^{d}	3.07 ± 0.17^{c}
B	31.84 ± 0.43^{d}	19.35 ± 0.94^{e}	3.42 ± 0.33^{c}
C	40.52 ± 0.99^{a}	24.03 ± 1.53^{bc}	2.27 ± 0.23^{d}
D	37.16 ± 1.25^{b}	24.68 ± 1.16^{b}	2.25 ± 0.17^{d}
E	26.45 ± 0.82^{f}	26.72 ± 0.83^{a}	4.54 ± 0.12^{b}
F	28.11 ± 0.76^{e}	22.45 ± 0.62^{cd}	4.23 ± 0.19^{b}
Control	23.32 ± 0.43^{g}	22.90 ± 0.67^{bcd}	12.24 ± 0.46^{a}

*Values in a column with different letters are significant difference at $p<0.05$.
*Control is the untreated sample.

3.2 The effect of different pretreatment method on total reducing sugar concentration

Enzymatic hydrolysis was carried out by loading at 20 FPU/g biomass at 50°C for 24 h. The effect of different pretreatment method on total reducing sugar concentration after enzymatic hydrolysis is shown in Table 2. The maximum total reducing sugar concentration was obtained at 218.41 ± 1.51 mg/g dried sample from the sample pretreatment by method C which was about 7.3 times higher than that of the control. The ultrasound assistance exhibited the potential to increase total reducing sugar concentration comparing to without ultrasound. The results of total reducing sugar concentration were agreed with the cellulose content of pretreated sample. The total reducing sugar augmentation clearly caused by the lignin removal and the increase of cellulose accessibility to enzyme (14). The sample with pretreatment method C was selected for the subsequent experiment.

Table 2. The effect of different pretreatment method on total reducing sugar concentration

Method	Total reducing sugar concentration (mg/g dried sample)
A	185.34 ± 3.07^{c}
B	177.54 ± 0.95^{d}
C	218.41 ± 1.51^{a}
D	203.19 ± 1.32^{b}
E	140.69 ± 2.26^{e}
F	127.99 ± 4.03^{f}
Control	27.66 ± 0.67^{g}

*Values in a column with different letters are significant difference at $p<0.05$.
*Control is the untreated sample.

3.3 SEM analysis

The sample from pretreatment method C was selected for SEM analysis due to the highest cellulose content and reducing sugar concentration. In Figure 1A, the untreated sample showed rigid surface and compact structure. The structure of pineapple waste after ultrasound-assisted alkaline pretreatment severely damaged, fractured, occurred rough surface, and generated pores (Figure 1B). It suggested that the lignin was removed leading to the change of external and internal structure, the increase of porosity, and cellulose accessibility to enzyme. The result of SEM analysis of this study resembles to alkaline pretreatment of oil palm fronds (16). Thus, the removal of lignin and alteration of external and internal structure may improve cellulose and hemicelluloses accessibility to enzyme which contributes to increase in the efficiency of enzymatic hydrolysis.
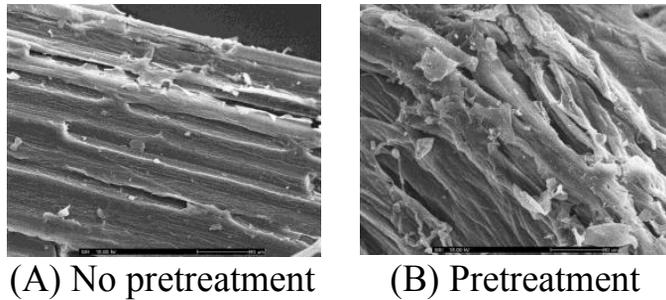
(A) No pretreatment   (B) Pretreatment

Figure 1. Scanning electron micrographs of untreated and pretreated pineapple waste.

3.4 Ethanol production by mono and co-culture fermentation

The ethanol production was conducted from the sample pretreated by method C using mono (*S. cerevisiae*) and co-culture fermentation (*S. cerevisiae* and *C. shehatae*). The initial total sugar concentration without adjustment was 22 g/L. The pineapple waste hydrolysate contains 68% glucose and 32% xylose. The fermentation of pineapple waste hydrolysate using monoculture gave the ethanol concentration and ethanol yield of 7.93 ± 0.18 g/L and 0.51 ± 0.02 g/g after 15 h fermentation whereas the xylose was not utilized by *S. cerevisiae* (Figure 2A). In order to overcome this problem and increase ethanol production, the co-culture fermentation was implemented using hexose and pentose utilized microorganism simultaneously with the ability to convert those sugars to ethanol. The result revealed that the co-culture fermentation had the potential to utilized both glucose and xylose leading to improve the ethanol concentration when compared to monoculture (Figure 2B).

The highest ethanol concentration and ethanol yield after 24 h fermentation were 9.73 ± 0.15 g/L and 0.45 ± 0.01 g/g, respectively. The co-culture fermentation significantly increased the ethanol concentration about 18.5%.

The most effective bioethanol producing yeasts is *S. cerevisiae* that can easily convert hexoses, such as glucose and fructose, into pyruvate through glycolysis. Finally, it reduced to ethanol generating two moles of ATP for each molecule of consumed hexose under anaerobic conditions (17). *S. cerevisiae* ferments xylose hardly because it lacks of enzymes that convert xylose to xylulose (18). On the other hand, natural xylose-utilizing yeast, such as *Pichia stipitis*, *Candida shehatae* can metabolize via the action of xylose reductase (XR) to convert xylose to xylitol and xylitol dehydrogenase to convert xylitol to xylulose that is integrated to the metabolic pathways for pyruvate synthesis and convert pyruvate to ethanol.
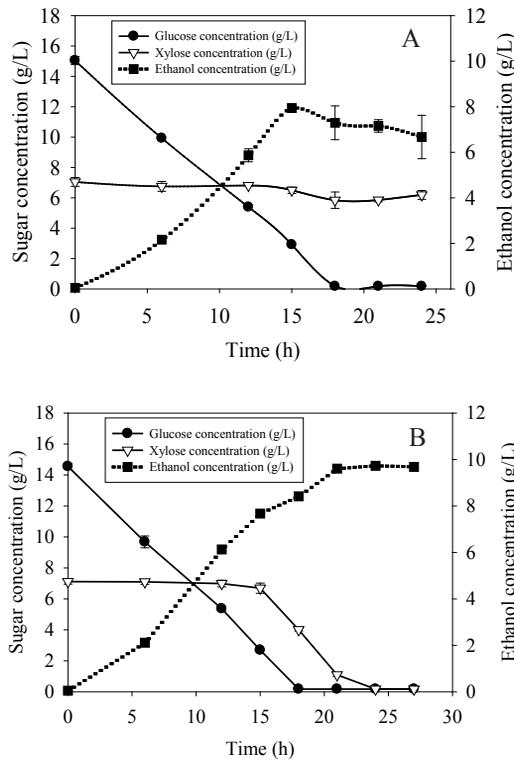
Figure 2. Time course of ethanol production using monoculture (A) and co-culture fermentation (B)

4. Conclusion

Six different alkaline and ultrasound-assistance pretreatments for improving the yield of reducing sugar were compared. All pretreatments increase cellulose content and sugar production. The ultrasound assistance absolutely increases the sugar production. The best pretreatment was using 2% NaOH with ultrasound for 60 min. The co-culture fermentation of *S. cerevisiae* and *C. shehatae* was found to be promising with higher ethanol concentration and maximizing substrate utilization comparing with monoculture fermentation.

5. Acknowledgements

6. References

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