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ORIGINAL RESEARCH PAPER

ENZYMATIC HYDROLYSIS AND BIOETHANOL PRODUCTION FROM *SAMANEA SAMAN* USING SIMULTANEOUS SACCHARIFICATION AND FERMENTATION BY *SACCHAROMYCES CEREVISIAE* AND *PICHIA STIPITIS*

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Abstract: This study investigated the possibility rain tree pods (*Samanea saman*) to produce ethanol. The production of ethanol from lignocellulosic material involves four steps, namely: pretreatment, hydrolysis, fermentation, and product separation. The pretreatment and hydrolysis are important steps to improve the production rate and yield of total sugar as well as the reducing sugar. The alkaline and acid pretreatments using H_2O_2 and H_3PO_4 were studied at different concentrations (2, 4, 6, 8 %, v / v). From this study, the highest total sugar (18 %) and reducing sugar (9.4 %) contents were generated by 8 % H_2O_2 pretreatment in compared with those generated by H_3PO_4. Enzyme loadings (10, 20, 30, 40 FPU·g^{-1}) were studied in hydrolysis step. The effect of enzyme loading show that, the highest total sugar (18 %) and reducing sugar (9.4 %) were obtained at 40 FPU·g^{-1} cellulase when 8 % H_2O_2 was used for pretreatment. In fermentation step, two kinds of yeast were used for fermentation (*Saccharomyces cerevisiae* and *Pichia stipitis*). Furthermore, the effect of yeast only and the mixture of cellulase and *S. cerevisiae* yeast were studied. The mixture of cellulase and *S. cerevisiae* can produce higher ethanol precentage (0.8 %) and (0.46 %) when H_2O_2 and H_3PO_4 treatments were used, repectively as compared with mixture of cellulase and *Pichia sp.* which produced (0.3 %) and (0.12 %) when H_2O_2 and H_3PO_4 treatments were used, respectively.

Keywords: *bioethanol, fermentation, cellulose, lignin, S. cerevisiae, Pichia sp.*

INTRODUCTION

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized [1]. In view of the environmental benefits and the decreasing supply of crude oil, industry has been moving towards greater ethanol fuel usage. Automobile manufacturers such as Ford, Honda, and Chrysler have begun to manufacture limited supplies of E85 (15 % gasoline with 85 % ethanol) and E95 (5 % gasoline with 95 % ethanol) cars [1, 2].

Bioethanol can be produced from biomass thus the accessibility to raw material is virtually unlimited. For example, the agricultural by products like rice straw, sugar cane bagasse and corn stover provide a readily available and cheap biomass source. However, the production of ethanol from lignocellulosic raw materials is more difficult than those from sugar or starch [3]. Lignocellulosic materials predominantly contain a mixture of carbohydrate polymers (cellulose and hemicellulose), lignin, extractives, and ashes [4].

Bioethanol from lignocellulosic materials can be produced using pretreatment followed by enzymatic hydrolysis and fermentation. Several different pretreatment methods can be used to facilitate the enzymatic hydrolysis of lignocellulosic material [5]. Subsequently, the hydrolysis of cellulose and hemicellulose from lignocellulosic materials can be carried out chemically and enzymatic processes to produce ethanol [6].

Rain tree (*Samanea saman*) is easily known by its characteristics particularly its umbrella-shaped canopy. It is widely cultivated and grown naturally throughout the tropics. The mature pods of rain tree are black-brown, oblong, lumpy, 10-20 cm in length, 15-19 mm in width, slightly curved, which are not dehiscing but eventually cracking irregularly and filled with a sticky, sweet and edible brownish pulp [7]. This species is generally used only as protective trees in Indonesia despite of its extensive functions and utilizations. Its wood can be used as commercial wood because of its soft, bright, and strong texture [8]. Furthermore, the pods of rain tree contain high crude protein (15.31 %), nitrogen free extract (69.93 %), total sugar (10.0 %), low crude fibre (10.07 %), neutral detergent fibre (42.86 %), acid detergent fibre (32.33 %), silica (0.20 %), lignin (4.50 %), and tannin (2.50 %). The pods also contain higher hemicellulose (10.53 %) than cellulose (9.77 %) [9]. In addition, they contain calcium (0.84 %), phosphorus (0.77 %), iron of 140 and copper of 9.8 $mg \cdot kg^{-1}$ [9].

The pretreatment step is aimed to separate the lignin and to break the lignocellulose structure, which is usually performed through thermo-chemical processes [10]. Previous study examined the influence of H_2O_2 pretreatment on Douglas fir wood chips and rice hulls. This study indicated that H_2O_2 pretreatment (4 % wt, 60 min) could remove lignin by 22 % [11]. The highest global yield (sum of pretreatment and hydrolysis yield) was obtained at a peroxide concentration of 7.5 %, at *p*H 11.5, 90 °C, and 1 hour that resulted 86.48 % [12]. Another study investigated the effect of H_3PO_4 (0.20 %, $w \cdot v^{-1}$) pretreatment on sugarcane bagasse which resulted the best pretreatment conditions were at temperature of 186 °C, time 24 min with the solubilized hemicellulose of 97.63 % was obtained [13].

Nowadays, yeast is commonly used to generate ethanol fuel from renewable energy resources. The yeast strains of *Pichia stipitis*, *Saccharomyces cerevisiae*, and *Kluyveromyces faligis* have been reported to produce ethanol from total sugar [14]. The

mannose sugar fermentation is normally efficient in *S. cerevisiae*, whereas high xylan materials are widely used in *Pichia sp.* [15].
The aim of this study was to investigate the effect of steam, hydrogen peroxide (2, 4, 6, 8 %, v/v), and phosphoric acid (2, 4, 6, 8 %, v/v) for a pretreatment of rain tree pods to produce reducing and total sugar. The best pretreatment of H_2O_2 and H_3PO_4 were also converted into ethanol by *Saccharomyces cerevisiae* and *Pichia stipites*.

MATERIAL AND METHODS

Raw material

The fruits of rain tree (*Samanea saman*) were obtained from Blitar, East Java. The fruit consist of pods and seed where the pods and the seed were separated by dissolving them in water at 50 °C. Subsequently, the pods were oven-dried at temperature of 80 °C for 24 hours to reach 10 ± 1 % moisture content. A commercial cellulase enzyme from Suntaq International lmtd was also used in this work. Other materials used in the research included malt extract (Merck), D(+)-glucose (Merck), yeast extract granulated (Merck), agar bacteriological (Oxoid), peptone p (Oxoid), urea (Merck), 2,6-diaminopimelic acid (Merck), H_2O_2 (Merck, 50 wt %), H_3PO_4 (Merck, 98 wt %), natrium acetate (Merck), acetic acid (glacial) (Merck, 100 wt %), H_2SO_4 (Mallinckrodt, 96.1 wt %).

Experimental methods

Agar plates
The strains of *S. cerevisiae* and *Pichia sp.* were obtained from Microbiology Laboratorium Gadjah Mada University. The bacterial strains were cultivated at 30 °C for 24 hours. The sterile medium contained of: 20 $g \cdot L^{-1}$ agar, 3 $g \cdot L^{-1}$ yeast extract, 3 $g \cdot L^{-1}$ malt, 5 $g \cdot L^{-1}$ peptone, and 100 $g \cdot L^{-1}$ glucose [16].

Preculture and Mainculture
The preculture was prepared by cultivated the bacterial strains from agar plates at 30 °C in a rotary shaker at 200 rpm for 48 hours in 10 mL sterile medium containing of: 3 $g \cdot L^{-1}$ yeast extract, 3 $g \cdot L^{-1}$ malt, 5 $g \cdot L^{-1}$ peptone, and 100 $g \cdot L^{-1}$ glucose [16]. Subsequently, they were stored in a refrigerator until further use.
The mainculture of *S. cerevisiae* and *Pichia sp.* were prepared by adding a preculture (10 mL) in 20 mL sterile medium containing: 1 $g \cdot L^{-1}$ urea, 0.5 $g \cdot L^{-1}$ 2,6-diaminopimelic acid [16]. The maincultures were incubated at 30 °C in a rotary shaker for 24 hours.

Pretreatment of rain tree pods
The pretreatment involves mechanical and steam or acid pretreatments. Mechanical pretreatment aims to increase the total accessible surface area and size of lignocellulosic pores, while acid pretreatment allows cellulose to have better accessibility to the enzymes during hydrolysis and has little effect on degrading lignin. The rain tree pods were physically treated using a high speed home blender and screen to a size of 420 µm.

The pretreated rain tree pods were subsequently stored in a container at room temperature for further step.

Nine sets of mechanical pretreated samples with concentration of 30 % (w/v) were weighed and transferred into 250 mL Erlenmeyer. The samples were labeled accordingly which include four sets for hydrogen peroxide (H_2O_2) pretreatment, four sets for phosphoric acid (H_3PO_4) pretreatment and one sample for steam pretreatment. The concentration of H_2O_2 (2, 4, 6, 8 %, v/v) and H_3PO_4 (2, 4, 6, 8 %, v/v) were prepared. The samples were autoclaved (1 atm, 121 °C) for 60 min for sterilization. After the autoclave process, each flask was neutralized and it was dried at 80 °C for 24 hours to reach the 10±1 % moisture content.

Enzymatic hydrolysis of pretreated rain tree pods

Eight samples of pretreated rain tree pods (H_2O_2 and H_3PO_4 pretreatment) and one sample of steam pretreated were used in the enzymatic hydrolysis. The 10 % (w/v) rain tree pods pretreated were added to 100 mL of 0.2 M acetic buffer at *p*H 5.0. The samples were autoclaved (1 atm, 121 °C) for 20 min. Subsequently, 10 FPU·g^{-1} of cellulase enzyme was added into each flask. The samples were incubated in a rotary shaker incubator at 100 rpm, 50 °C for 24, 48, and 72 hours. The procedures were repeated for 20, 30, 40 FPU·g^{-1} of cellulase enzyme, respectively.

Simultaneous saccharification and fermentation (SSF)

SSF was conducted using a mixture of cellulase enzyme and *S. cerevisiae* or *Pichia sp.* mainculture which were added to pretreated rain tree pods sample. The SSF samples (10 %, w/v) were prepared in 100 mL acetic buffer solution (*p*H 5.0) at 250 mL Erlenmeyer. The enzyme was added at 0, 20, 40, 60, and 80 FPU·g^{-1}. The 20 % (v/v) of yeast was incubated in anaerobic condition at 37 °C for 72 hours. At the end of the fermentation, the sample was distilled and the ethanol content was measured.

Analysis

Determination of lignin

The lignin contents were measured by a solid residue that remains after hydrolysis process with 1.25 % H_2SO_4 for two hours and 72 % H_2SO_4 for four hours. Subsequently, the samples were filtered and rinsed out with distilled water and oven-dried at 105 °C to obtain constant weight samples. The lignin content is represented by the equation of the lignin weight (g) divided by the samples weights (g) [17].

Determination of cellulose and hemicellulose

The cellulose and hemicellulose contents of untreated and pretreated rain tree pods were determined by using a Chesson method [18]. One gram of the sample was suspended in 100 mL distilled water at 100 °C for 2 hours in water bath and then filtered. The residue was dried to constant weight. Two grams of dried rain tree pods was suspended in 100 mL of 0.5 M H_2SO_4 at 100 °C for 2 hours in a water bath. Subsequently, the samples were filtered, dried, and weighed to a constant weight. The hemicellulose content was determined by the weight loss of samples. The analysis of cellulose content was done by added 10 mL of 72 % (v/v) H_2SO_4 into the dried residue and then put them at rotary shaker at 200 rpm, 30 °C for one hour. The mixture was diluted up to 4 % (v/v)

of H_2SO_4 and autoclaved at 1 atm for 40 min after the incubation. The contents were filtered, dried, and weighted. The weight loss was represented as cellulose.

Determination of total sugar and reducing sugar

The procedure was determined by modified Nelson Somogyi method [19]. The total sugar was measure by mixed 25 mL of lead free filtrate, 15 mL of distilled water, and 5 mL of HCl into Erlenmeyer and boiled at 67 °C for 10 min. The mixture of solution was neutralized with 45 % NaOH and diluted to 100 mL volume. The Standard solution was served by dissolved anhydrate glucose (2, 4, 6, 8, and 10 mg) in 100 mL volumetric flask with distilled water. Mixtured of 1 mL of each standard solution, 1 mL of distilled water for blank, and 1 mL of sample were pipetted into test tube for analyzed. Each test tube was mixed with 1 mL of Nelson reagent and boiled for 20 min at 100 °C. After cooled, 1 mL of arsenomolybdate reagent and 7 mL of distilled water (H_2O_2) were mixed until homogeneous. The absorbance was measured at 540 nm. The reducing sugar was measured the same as the previous method, without mixed the lead of free filtrate, distilled water and HCl, boiled and neutralized.

Determination of ethanol contents

The determination of ethanol concentration was carried by using gas chromatography (Shimadzu GC 8) and a polar capillary column (GP 106 SP 1200). Nitrogen was used as the carrier gas with the pressure set at 2 $kg \cdot cm^{-2}$ and flow rate was set at 1 $mL \cdot min^{-1}$. The column temperature was set at 150 °C, while the detector and injector temperature was set at 250 °C. The volume was injected at 1 µl using a splitless injection. Measuring the quantity of alcohol using a polar capilarry gas chromatography was also reported by Brill and Wagner [20].

RESULTS AND DISCUSSION

Lignocellulosic composition of rain tree pods

The lignocellulosic composition of rain tree pods consisted of lignin, cellulose, and hemicellulose. As shown in Table 1, rain tree pods consisted of a high amount of cellulose (24.34 %) and hemicellulose (25.71 %). This component is essential to convert raw material to bioethanol.

Table 1. *Rain tree pods composition*

Parameter	Composition [%]			
	Untreated rain tree pods	**Steam pretreatment**	**8 % H_2O_2 pretreatment**	**8 % H_3PO_4 pretreatment**
Lignin	56.69	55.26	43.66	45.66
Cellulose	24.34	26.88	28.31	26.06
Hemicellulose	25.71	18.23	17.48	19.84

Pretreatment was performed to remove lignin and hemicellulose, increase the porosity of surface materials, and reduce cellulose crystallinity [1]. The cellulose content of rain tree pods increased from 24.34 to 28.31 % using 8 % H_2O_2 treatments and 26.06 %

using 8 % H_3PO_4 treatments, respectively in contrast with the decline of lignin content. H_2O_2 was better to be used for pretreatment than H_3PO_4. Previous study reported the combination of 2 % H_2O_2 and 1.5 % NaOH pretreatment increased the cellulose content up to 1.2 times and decreased the hemicellulose up to 8.5 times at 121 °C for 15 min [10]. According to de Vasconcelos *et al.* [13] the H_3PO_4 pretreatment can reduce the hemicellulose content from 27.49 ± 0.67 to 7.68 ± 0.28 % and increase the cellulose content from 40.10 ± 0.93 to 56.02 ± 0.02 % at 165 °C for 16 min. Therefore, the removal of hemicellulose and lignin using H_2O_2 pretreatments lead to high cellulose content of substrate which is useful to facilitate for the subsequent SSF to obtain high ethanol content.

The effect of dilute acid and alkaline concentration of pretreatment on hydrolysis

Prior to enzymatic hydrolysis, pretreatment is necessary for the conversion of cellulose to glucose, and hemicellulose to become xylose and arabinose. In this study, rain tree pods were treated by two different parameters which were concentration of H_2O_2 and H_3PO_4 and pretreatment time. The total sugar yield from hydrolysis of pretreated rain tree pods with different parameters are shown in each parameter is shown in Figure 1. All the curves show positive trends which resulted the highest total sugar yield of 9.4 % that obtained from 8 % H_3PO_4 for 72 hours and 18.2 % that obtained from 8 % H_2O_2 for 72 hours, respectively. The results indicated that acid pretreatment improve the total sugar in the hydrolysis process and these results are aligned with previous work reported by Saha, *et al.* [21], that studied the effect of the pretreatment with H_2SO_4 (0.25-1.0 %, v/v) of rice hulls at 121 °C for 15-60 min on enzymatic (cellulase and *β*-glucosidase) saccharification which resulted the production of glucose of 47 ± 0 to 81 ± 1 $mg \cdot g^{-1}$ was obtained after enzymatic hydrolysis. Pretreatment with H_2SO_4 on rice straw was also reported by Aditya *et al.* [22], where pretreatments at 1-2 M H_2SO_4, 90 °C for 30-90 min indicated the increasing of the concentration and the retention time of pretreatment increase the sugar yield with the highest sugar yield (9.71 $g \cdot L^{-1}$ or 32.37 g / g %) was obtained at 2.0 M for 60 min.

The effect of H_2O_2 and H_3PO_4 concentration on reducing sugar are depicted in Figure 2. It shows that the reducing sugar increased with the increase in concentration (2-8 %) and reaction time (24-72 h). The maximum reducing sugar was obtained by 8 % H_2O_2 pretreatment (9.4 %) and 8 % H_3PO_4 (5.1 %) for 72 hours, respectively. From previous studies [23], the acid concentration has affected on the reducing sugar of water hyacinth treated with H_2SO_4 at 108, respectively 121 °C, 0.11 mPa for 1-3 hours. The reducing sugar obtained was from 1.76 ± 0.07 to 26.26 ± 1.19 $g \cdot L^{-1}$ using pretreatment condition at 3.0 % H_2SO_4, 3 hours and 121 °C. Based on studied reported by Timung *et al.* [24], the pretreatment of sugarcane bagasse and spent citronella biomass with 0.1-0.9 M H_2SO_4 at 80-120 °C showed an increased of total reducing sugar yield with increased in the acid concentration and reaction time with the maximum reducing sugar of 296.64 and 274.93 $mg \cdot g^{-1}$ were obatined at 0.1 M H_2SO_4, 80 °C for 30 min for sugarcane bagasse and 0.9 M H_2SO_4, 80 °C for 120 min for spent citronella biomass, respectively.

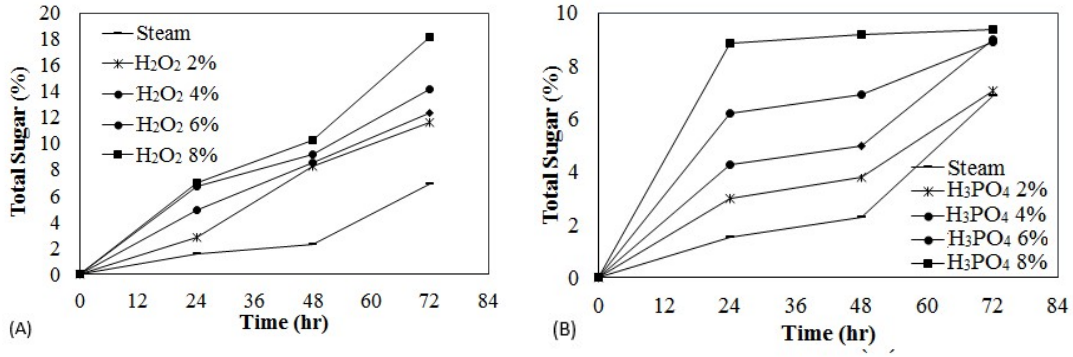

Figure 1. *Total Sugar for H_2O_2 pretreatment (A); H_3PO_4 pretreatment (B). Hydrolysis at 40 FPU·g^{-1} enzyme cellulase, 50 °C, 100 rpm*

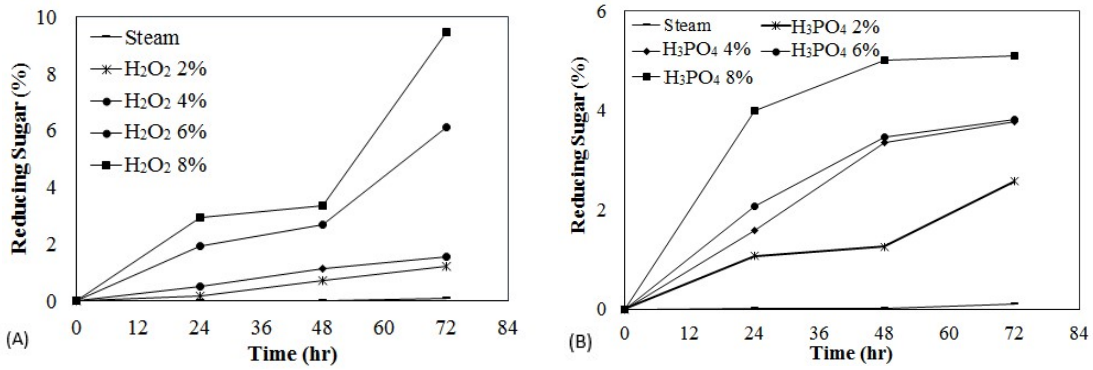

Figure 2. *Reducing Sugar for H_2O_2 pretreatment (A); H_3PO_4 pretreatment (B). Hydrolysis at 40 FPU·g^{-1} enzyme cellulase, 50 °C, 100 rpm*

The effect of enzyme cellulase concentration on hydrolysis

The yield of total sugar from hydrolysis process can be seen in Figure 3. The enzyme concentration of 10 FPU·g^{-1} shows that the lowest value of total sugar 9.5 % was obtained for H_2O_2 treatment and 5.7 % for H_3PO_4 treatment at 72 hours hydrolysis time, respectively. In the beginning of hydrolysis, the yield of total sugar was increased slightly for the concentration of enzymes of 10, 20 and 30 FPU·g^{-1}, however at the end of hydrolysis the total sugar increased significantly. The enzyme concentration of 40 FPU·g^{-1} produced the highest total sugar content at 72 hours, approximately 18.2 % was obtained for H_2O_2 treatment and 9.4 % for H_3PO_4 treatment, respectively. The result indicated that enzymatic hydrolysis is affected by enzyme concentration. In addition, Dahnum *et al.* [25] reported that the addition of enzyme will increase the sugar from hydrolysis. Their research work used the combination of enzyme i.e. Cellic® Ctect2 and Cellic® HTech2 (50 °C, 150 rpm, 72 hours, *p*H 4.8) with enzyme concentration of 10-40 FPU which produced the glucose concentration of 6.2 % at 10 FPU and 10.67 % for 40 FPU, respectively. The results indicate that the higher the concentration of enzyme, the higher the amount of cellulose converted. Therefore, it will increase the glucose produced.

The effect of different initial enzyme concentrations and reaction time on enzymatic hydrolysis of pretreated rain pods on the increase of reducing sugar content was shown

in Figure 4. The maximum reducing sugar of 9.4 and 5.1 % were obtained when 8 % H_2O_2 and 8 % H_3PO_4 was used, respectively at 40 FPU·g^{-1} cellulose enzyme at 72 hours. This result in accordance with previous studies reported by Kolusheva and Marinova[26], the hydrolysis of starch with *α*-amylase at 90 °C, *p*H 7 for 30 min increased the precentage of strach hydrolysis from 8.2 to 30.2 % with increasing of enzyme concentration from 6 to 14 units per mL suspension. The study also in line with study reported by Kusmiyati [16], who showed that the reducing sugar increased with the increased of *β*-amylase concentration. The study reported that the highest reducing sugar concentration (12.5 % v/v) was obtained at 6.4 mL of *β*-amylase and 65°C, *p*H 5, for 4 hours with liquefaction conditions at 95-100 °C, *p*H 6, *α*-amylase concentration of 3.2 mL·kg^{-1} flour for 1 hours. Akuzuo *et al.* [27], reported the influence the addition of malted barley as the source of enzyme and hydrolysis time on reducing sugar from tacca and tigernut starch. The highest reducing sugar yield of 208.33 mg·mL^{-1} was obtained at 3.5 hours, 0.2 g·g^{-1} enzyme, and 3.0 mL·g^{-1} water content for tacca starch and 86.21 mg·mL^{-1} at 3 hours, 0.3 g·g^{-1} enzyme, and 4.5 mL·g^{-1} water content for tigernut starch, respectively.

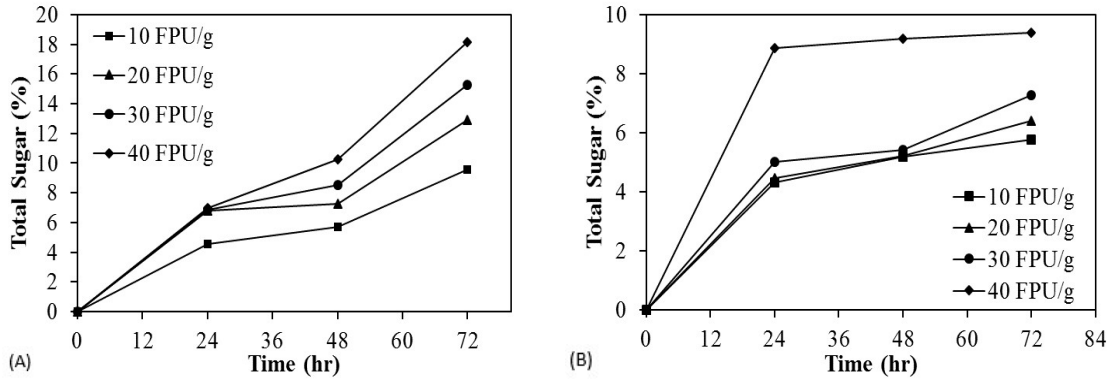

Figure 3. *Total Sugar for 8 % H_2O_2 pretreatment (A); 8 % H_3PO_4 pretreatment (B). Hydrolysis at 50 °C, 100 rpm*

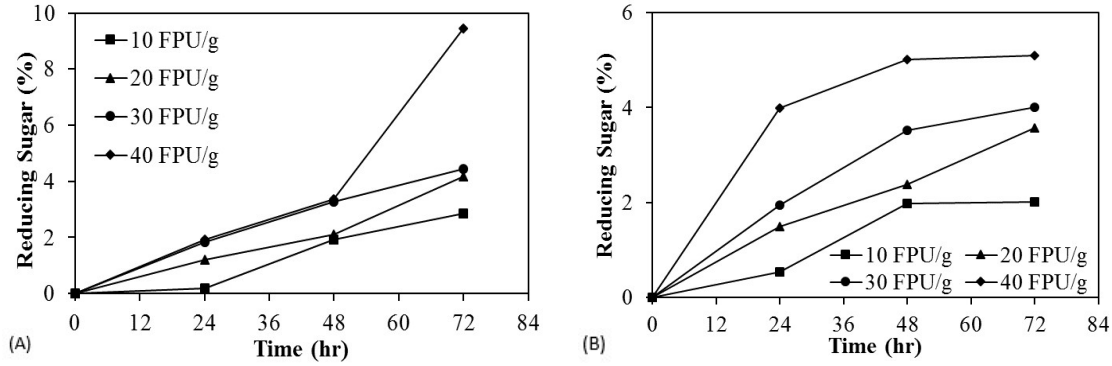

Figure 4. *Reducing Sugar for 8 % H_2O_2 pretreatment (A); 8 % H_3PO_4 pretreatment (B). Hydrolysis at 50 °C, 100 rpm*

Ethanol concentration in *S. cerevisiae* and *Pichia sp.* on SSF

Figure 5a shows four samples consist of fermentation of 8 % H_2O_2 pretreated rain tree pods with *S. cerevisiae*, 8 % H_2O_2 pretreated rain tree pods with mixture of enzyme

cellulase and *S. cerevisiae*, 8 % H_3PO_4 pretreated rain tree pods with *S. cerevisiae*, and 8 % H_3PO_4 pretreated rain tree pods with mixture of enzyme cellulase and *S. cerevisiae.* The highest ethanol concentration of 0.8 % was obtained at 8 % H_2O_2 pretreated rain tree pods with a mixture of enzyme cellulase and *S. cerevisiae*.

Figure 5b shows four samples consist of fermentation of 8 % H_2O_2 pretreated rain tree pods with *Pichia sp.*, 8 % H_2O_2 pretreated rain tree pods with mixture of enzyme cellulase and *Pichia sp*, 8 % H_3PO_4 pretreated rain tree pods with *Pichia sp*, and 8 % H_3PO_4 pretreated rain tree pods with mixture of enzyme cellulase and *Pichia sp.* The highest ethanol concentration 0.3 % was obtained at 8 % H_2O_2 pretreated rain tree pods with mixture of enzyme cellulase and *Pichia sp*.

The comparison between 8 % H_2O_2 pretreated rain tree pods with a mixture of enzyme cellulase and *S. cerevisiae* and 8 % H_2O_2 pretreated rain tree pods with mixture of enzyme cellulase and *Pichia sp*. indicated that ethanol concentration was higher on fermentation with mixture of enzyme cellulase and *S.cerevisiae* as compared to the mixture of enzyme cellulase and *Pichia sp.* This was might be due to *Pichia sp.* was more suitable in SSF that contains materials with high xylose content as a substrate for ethanol production rather than for cell growth [15, 28]. Based on study repoted by Agbogbo *et al.* [28], the ethanol concentration and yield were higher on the 100 % xylose media (24.3±0.34 $g \cdot L^{-1}$, 4.18±0.14 $g \cdot g^{-1}$) than that on 100 % glucose media (22.7±0.96 $g \cdot L^{-1}$ and 3.01±0.16 $g \cdot g^{-1}$) when *Pichia sp.* was used. The highest ethanol concentration using a mixture of cellulase and *S. cerevisiae* than *Pichia sp.* was due to *S. cerevisiae* organism has been proven to be robust to inhibitors, and hence it is suitable for the fermentation of lignocellulosic materials [15].

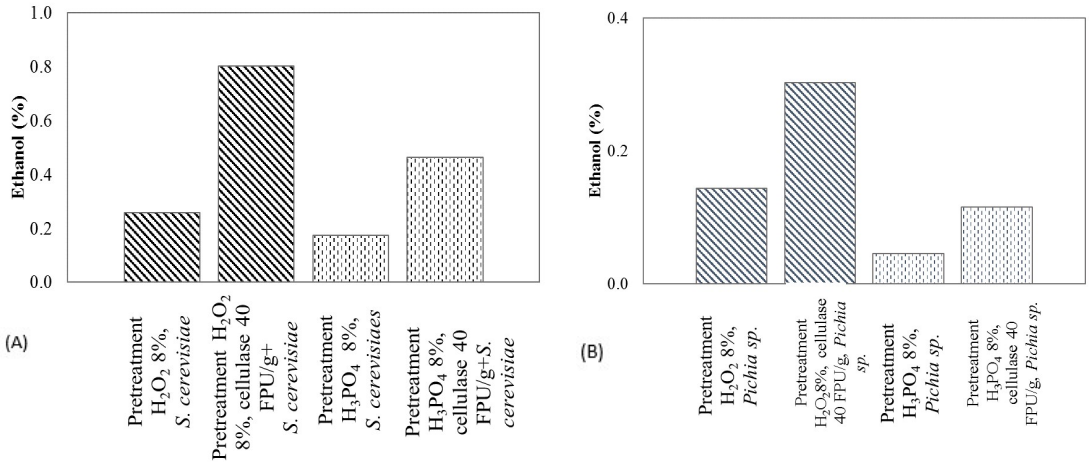

Figure 5. *Ethanol concentration from fermentation of 8 % H_2O_2 and 8 % H_3PO_4 pretreated rain tree pods using concentration of yeast 20 % (v / v) at 72 hours with S. cerevisiae (A) and Pichia sp. (B)*

CONCLUSIONS

It can be concluded that rain tree pods are potential raw material for the production of bioethanol from lignocellulosic material. Rain tree pods pretreated with 8 % H_2O_2 can reduce the lignin content by 13.03 % and increase the cellulose content by 3.97 %. Meanwhile, the H_3PO_4 treatment can reduce the lignin content by 11.03 % and increase the cellulose content by 1.72 %. The highest total sugar concentration of 18.2 % was obtained from 8 % H_2O_2 pretreatment and 9.4 % from 8% H_3PO_4 pretreatment, respectively. Furthermore, a mixture of enzyme cellulase and *S. cerevisiae* for fermentation of 8 % H_2O_2 pretreated rain tree pods can produce higher ethanol concentration of 0.8 % as compared to 0.3 % from a mixture of enzyme cellulase and *Pichia sp.*

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REFERENCES

1. Sun, Y., Cheng, J.: Hydrolysis of Lignocellulosic Materials for Ethanol Production: a Review, *Bioresource Technology*, **2002**, **83** (1), 1-11;
2. Khalil, A.M., Al-shawabkeh, A.F., Mazahreh, A.S., Al-damanhoory, M.S., Quasem, J.M.: Utilization of Soft Wood Wastes as a Feed Stock to Produce Fuel Ethanol, *American Journal of Engineering and Applied Sciences*, **2009**, **2** (2), 451-455;
3. Öhgren, K., Bura, R., Lesnicki, G., Saddler, V., Zacchi, G.: A Comparison between Simultaneous Saccharification and Fermentation and Separate Hydrolysis and Fermentation using Steam-Pretreated Corn Stover, *Process Biochemistry*, **2007**, **42** (5), 834-839;
4. Taherzadeh, M.J., Karimi, K.: Acid-Based Hydrolysis Processes for Ethanol from Lignocellulosic Materials: a Review, *BioResources*, **2007**, **2** (3), 472-499;
5. Öhgren, K., Vehmaanperä, J., Siika-Aho, M., Galbe, M., Viikari, L., Zacchi, G.: High Temperature Enzymatic Prehydrolysis Prior to Simultaneous Saccharification and Fermentation of Steam Pretreated Corn Stover for Ethanol Production, *Enzyme and Microbial Technology*, **2007**, **40** (4), 607-613;
6. Taherzadeh, M.J., Karimi, K.: Enzyme-based Hydrolysis Processes for Ethanol from Lignocellulosic Materials: a Review, *BioResources*, **2007**, **2** (4), 707-738;
7. Staples, G.W., Elevitch, C.R.: *Samanea saman* (rain tree), *Permanent Agriculture Resourse*, **2006**, 1-15;
8. Hidayati, F., Siagian, P.B.: Struktur dan Sifat Kayu Trembesi (*Samanea saman MERR*) dari Hutan Rakyat di Yogyakarta, *Prosiding Seminar Nasional Mapeki XIII*, **2012**, 228-232;
9. Hosamani, S.V., Gowda, N.K.S., Kologi, S.D.: Evaluation of Chemical, Nutritive and Feeding Value of Rain Tree Pods, *Karnataka Journal of Agricultural Sciences*, **2005**, **18** (1), 110-113;
10. Karp, S.G., Woiciechowski, A.L., Soccol, V.T., Soccol, C.R.: Pretreatment Strategies for Delignification of Sugarcane Bagasse: a Review, *Brazilian Archieves of Biology and Technology*, **2013**, **56** (4), 679-689;
11. Alvarez-Vasco, C., Zhang, X.: Alkaline Hydrogen Peroxide Pretreatment of Softwood: Hemicellulose Degradation Pathways, *Bioresource Technology*, **2013**, **150**, 321-327;
12. Diaz, A., Le Toullec, J., Blandino, A., De Ory, I., Caro, I.: Pretreatment of Rice Hulls with

Alkaline Peroxide to Enhance Enzyme Hydrolysis for Ethanol Production, *Chemical Engineering Transactions*, **2013**, **32**, 949-954;
13. de Vasconcelos, S.M., Santos, A.Ma.P., Rocha, G.J.M., Souto-Maior, A.M.: Diluted Phosphoric Acid Pretreatment for Production of Fermentable Sugars in a Sugarcane-based Biorefinery, *Bioresource Technology*, **2013**, **135**, 46-52;
14. Azhar, S.H.M., Abdulla, R., Jambo, S.A., Marbawi, H., Gansau, J.A., Faik, A.A.M., Rodrigues, K.F.: Yeasts in Sustainable Bioethanol Production: A Review, *Biochemistry and Biophysics Reports*, **2017**, **10**, 52-61;
15. Olofsson, K., Bertilsson, M., Lidén, G.: A Short Review on SSF - an Interesting Process Option for Ethanol Production from Lignocellulosic Feedstocks, *Biotechnology for Biofuels*, **2008**, **1** (1), 1-14;
16. Kusmiyati, K.: Ethanol Production from Non-Food Tubers of Iles-Iles (*Amorphophallus campanulatus*) by using Separated Hydrolysis and Fermentation, *Bulletin of Chemical Reaction Engineering and Catalysis*, **2014**, **9** (2), 93-99;
17. Irfan, M., Gulsher, M., Abbas, S., Syed, Q., Nadeem, M., Baig, S.: Effect of Various Pretreatment Conditions on Enzymatic Saccharification, *Songklanakarin Journal of Science and Technology*, **2011**, **33** (4), 397-404;
18. Shah, S.R., Ishmael, U.C., Palliah, J.V., Asras, M.F.F., Ahmad, S.S.B.N.W.: Optimization of the Enzymatic Saccharification Process of Empty Fruit Bunch Pretreated with Laccase Enzyme, *BioResources*, **2016**, **11** (2), 5138-5154;
19. Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, *Journal of Biological Chemistry*, **1944**, **153**, 375-380
20. Brill, S.K., Wagner, M.S.: Alcohol Determination in Beverages using Polar Capillary Gas Chromatography-Mass Spectroscopy and An Acetonitrile Internal Standard, *Concordia College Journal of Analytical Chemistry*, **2012**, **3**, 6-12;
21. Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V.: Dilute Acid Pretreatment, Enzymatic Saccharification, and Fermentation of Rice Hulls to Ethanol, *Biotechnology Progress*, **2005**, **21** (3), 816-822;
22. Aditiya, H.B., Sing, K.P., Hanif, M., Mahlia, T.M.I.: Effects of Acids Pretreatment on Enzymatic Hydrolysis in Bioethanol Production from Rice Straw, *International Journal of Technology,* **2015**, **6** (1), 11-21;
23. Idrees, M., Adnan, A., Bokhari, S.A., Qureshi, F.A.: Production of Fermentable Sugars by Combined Chemo-Enzymatic Hydrolysis of Cellulosic Material for Bioethanol Production, *Brazilian Journal of Chemical Engineering*, **2014**, **31** (2), 355-363;
24. Timung, R., Deshavath, N.N., Goud, V.V., Dasu, V.V.: Effect of Subsequent Dilute Acid and Enzymatic Hydrolysis on Reducing Sugar Production from Sugarcane Bagasse and Spent Citronella Biomass, *Journal of Energy*, **2016**, **2016**, 1-12;
25. Dahnum, D., Tasum, S.O., Triwahyuni, E., Nurdin, M., Abimanyu, H.: Comparison of SHF and SSF Processes using Enzyme and Dry Yeast for Optimization of Bioethanol Production from Empty Fruit Bunch, *Energy Procedia*, **2015**, **68**, 107-116;
26. Kolusheva, T., Marinova, A.: A Study of The Optimal Conditions For Starch Hydrolysis Through Thermostable α-Amylase, *Journal of University of Chemical Technology and Metallurgy*, **2007**, **42** (1), 93-96;
27. Akuzuo, U.O., Uchechukwu, C.O., Okechukwu, D.O.: Effect of Hydrolysis Reaction Time on The Reducing Sugar Yield of Tacca (*Tacca involucrata*) and Tigernut (*Cyperus esculentus*) Starch for Bioethanol Production, *Der Pharma Chemica*, **2015**, **7** (6), 324-329;
28. Agbogbo, F.K., Coward-Kelly, G., Torry-Smith, M., Wenger, K.S.: Fermentation of Glucose/ Xylose Mixtures using *Pichia stipitis*, *Process Biochemistry*, **2006**, **41** (11), 2333-2336.