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Effect of combined pretreatment of lignocellulose and the kinetics of its subsequent bioconversion by *Aspergillus niger*

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ABSTRACT

Lignocellulose is the most abundant material to be a renewable raw material for bioethanol production. However, the high percentage of lignin content makes it resistant to be hydrolyzed thus inhibits the production of reduced sugar. This problem is overcome by performing a proper pretreatment prior to hydrolysis.

This study examined the feasibility oil palm empty fruit bunch (OPEFB) as the source of lignocellulose and Aspergillus niger as cellulase-producing fungus respectively. The hydrolysis was performed by growing *A. niger* on the OPEFB. Prior to hydrolysis, OPEFB was pretreated with NaOH at different percentage and were heated simultaneously at 170 °C to destroy lignin that hindered cellulase from contacting cellulose. *A. niger* was further grown on pretreated OPEFB and the reducing sugar was determined via dinitrosalicylic acid method with spectrophotometer reading at 540 nm periodically every 24 h for 9 consecutive days. Kinetics study showed that the yield of the reducing sugar is relatively higher for OPEFB pretreated with 1% NaOH due to the reduction of the cellulose crystallinity which further increase the hydrolysis rate.

1. Introduction

The increasing worldwide ethanol demands due to rapid growth in population and the expansion of industrialization had made bioethanol as one of the attractive alternatives to reduce the consumption of crude oil. One of the paramount predicaments in the production of bioethanol is the availability of the raw materials (Zabed et al., 2016; Sarkar et al., 2012). The advancement of technology in this field is moving towards minimizing the derivation of bioethanol from conventional crops such as corn and sugar cane. This is to avoid the conflicts between the usage of these corps in food and fuel production (Sarkar et al., 2012). Hence, lignocellulosic agricultural waste biomass has been receiving a widespread interest as one of the promising feedstocks for bioethanol production due to its abundant availability and low-cost as compared to the conventional bioethanol source.

Lignocellulose is a complex carbohydrate polymer of cellulose, hemicellulose, and lignin. As it is widely known, cellulose is a polymeric compound consisting of sugar monomers which theoretically can be used as raw material for bioethanol (Piarpuzán et al., 2011). Unfortunately, the lignin content in lignocellulose may hamper the hydrolysis process. Lignin, which is hydrophobic in nature, disturbs the hydrolysis by hindering the enzyme to reach the cellulose and by binding to the enzyme and acting as inhibitors (Li et al., 2016). Therefore, efficient pretreatment method towards total delignification of lignocellulose material is crucial to alter and break down the biomass recalcitrance and further liberate cellulose and hemicellulose for the efficiency enhancement of subsequent enzymatic hydrolysis process (Bommarius et al., 2008, Zheng et al., 2009). Lignin can also reduce cellulose hydrolysis by passively bind to the cellulolytic enzyme (Esteghlalian et al., 2001). The disconnection of celluloses from the lignin will further provide pathways for enzymatic hydrolysis.

Cellulase designates the group of enzymes belong to the hydrolase class that play a role in the hydrolysis reaction (Bhat, 2008). Consisting of endoglucanase, exoglucanase, and β-glucosidase, they work simultaneously to hydrolyze cellulose (Deswal et al., 2011). There are many organisms that act as cellulase producers, mainly from groups of fungi and bacteria, but filamentous fungi are preferred because of their ability to adapt to new environments (Abd-Aziz et al., 2008). Some researchers have reported that fungi of the genus Aspergillus are able to produce cellulase (Shahriarinour et al., 2011; Sun and Cheng, 2002; Bakar et al., 2012).

In Indonesia and Malaysia, palm oil is one of the major profitable agricultural commodities. This industry generates huge quantities of

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Nomenclature

C_E	Enzyme concentration, g/L
C_S	substrate concentration, g/L
C_X	mold concentration, g/L
K_H	constant for half-velocity hydrolysis, $g_{substrate}$/L
r_c	mold consumption rate, g_{sugar}/h
r_H	rate of mold hydrolysis, g_{sugar}/day
r_{main}	Rate of cell maintenance, g_{sugar}/day
$Y_{(P/S)}$	ratio of produced sugar according to substrate consumed, $g_{sugar}/g_{substrate}$
$Y_{(X/P)}$	ratio of produced mold according to consumed sugar, g_{mold}/g_{sugar}
β	constant, g_{enzyme}/g_{mold}
μ_H	constant of hydrolysis rate, $g_{substrate}/(g_{mold}.day)$
μ_{main}	Constant for rate of cell maintenance, $g_{sugar}/(g_{mold}.day)$
μ_{maxH}	constant for maximum hydrolysis rate, $g_{substrate}/(g_{mold}.day)$

biomass including the oil palm empty fruit bunch (OPEFB) which is resilient and hard to be degraded naturally (Bakar, 2012). The accumulation of the OPEFB had triggered waste problems. The abundance of this material had put OPEFB as a prominent candidate for the source of lignocellulose. The conversion of this waste into bioethanol not only could alleviate the pollution load but also drive the palm oil to its fullest potential. It is reported in some literatures that the main setback of lignocellulose hydrolysis using living microorganism to produce the enzyme is the poor yield of glucose (Chinedu et al., 2001). As it is known, glucose is the main nutrient for the majority of living things so there is a high possibility that the resulted glucose from hydrolysis will be re-consumed by the enzyme-producing microorganisms. Therefore, in this research, the effect of combined pretreatment of lignocellulose towards the enzymatic hydrolysis by *Aspergillus niger* and the kinetics analysis of the reactions included were analyzed.

2. Methods

2.1. Pretreatment

Five grams of OPEFB was cut into smaller pieces around 1–2 cm and was further undergone a combination of chemical and thermal pretreatment. The OPEFB was soaked in sodium hydroxide at various percentage (0%, 0.5%, 1% and 2%) with simultaneous heating at 170 °C The optimized NaOH concentration are crucial to determine the effect of the pretreatment towards the total delignification of the lignocellulose. This process lasted for 2 h. The pretreated OPEFB was then washed with water several times until the pH was around 7. The OPEFB was further dried in an oven at 50 °C overnight. The composition of lignocellulose was assessed using Chesson-Datta Method (Chesson, 1981).

2.2. Hydrolysis

A. niger was grown in Potato Dextrose Agar for 7 days. The spores were then harvested in physiological salt 0.85% using centrifuge at 6000 rpm and at a room temperature. The spores were immediately used without further purification within 15 min. Hydrolysis was done by growing *A. niger* on the sterilized, pretreated OPEFB. The microorganism growth was supported using basal medium. For every 100 g of basal medium it contained KH_2PO_4, 5 g; corn steep liquor, 5 g; industrial yeast extract, 0.5 g; $CoSO_4{\cdot}7H_2O$, 0.1 g; and $CuSO_4{\cdot}5H_2O$, 0.5 g. The pH of the basal medium was adjusted to 7.0 with the addition of acetic acid buffer (Kang et al., 2004). The medium was sterilized at 121 °C for 30 min. The moisture was adjusted to 80% by adding sterile medium. The experiment was done in series of Erlenmeyers with various sampling times. Samples were taken every 24 h for 9 consecutive days. The resulting sugar was analyzed using dinitrosalicylic acid (DNS) method (Ghose, 1987).

In this case, the portion of sugar re-consumed as cell growth and maintenance must be determined at first. These phenomena were observed by two separate experiments. The first experiment was growing *A. niger* on medium with filter paper as the only carbon source in attempt to trigger the cellulase production. The amount of filter paper hydrolyzed into sugar was measured. The second experiment was growing *A. niger* on the medium with glucose as the carbon source. The amount of glucose consumed was also measured.

2.3. Kinetics analysis

Reaction mechanisms for hydrolysis and sugar consumption were also observed in analogue with Monod equation. The steps of the whole mechanism were:

1. *A. niger* secreted cellulase (E) to hydrolyze cellulose.
2. Cellulase (E) hydrolyzed pretreated lignocellulose to produce simple sugars. The reaction rate constant for this step was assumed to follow Monod equation:

$$\mu_H = \frac{\mu_{maxH} C_S}{C_S + K_H} \tag{1}$$

Hydrolysis rate was approached by an assumption that its rate was in comparison with the mold concentration:

$$r_H = \mu_H . C_X \tag{2}$$

3. Parts of produced sugar were re-consumed by *A. niger* for growth. The consumption rate constant was approached by Monod equation:

$$\mu_C = \frac{\mu_{maxC} C_P}{C_P + K_c} \tag{3}$$

Next, the consumption rate was also assumed to be in comparison with the concentration of mold:

$$r_C = \mu_C . C_X \tag{4}$$

The sugar consumed was also used for several cellular processes including cell maintenance. The consumption rate could be approached by equation (5):

$$r_{main} = \mu_{main} . C_X \tag{5}$$

Cellulose mass balance could be written as follow:

$$\frac{dC_S}{dt} = -\mu_h . C_E \tag{6}$$

Enzyme concentration was assumed in comparison with the concentration of mold.

$$\frac{dC_S}{dt} = -\mu_H . \beta . C_X = -\frac{\beta \mu_{maxH} . C_S}{C_S + K_H} . C_X \tag{7}$$

Sugar mass balance modeling was derived from equations (3), (6) and (7) as follow:

$$\begin{pmatrix} Rate\ of\ sugar \\ accumulated \end{pmatrix} = \begin{pmatrix} Rate\ of\ sugar \\ produced \end{pmatrix} - \begin{pmatrix} Rate\ of\ sugar \\ consumed \\ for\ growth \end{pmatrix} - \begin{pmatrix} rate\ of\ sugar \\ consumed \\ for\ maintenance \end{pmatrix}$$

$$\frac{dC_P}{dt} = \frac{\beta \mu_{maxH} . C_S}{C_S + K_H} . C_X . Y_{P/S} - \frac{\mu_{maxC} . C_P}{C_P + K_C} . C_X - \mu_{main} . C_X \tag{8}$$

The mass balance of *A. niger* could be written on the base of equation (9) as follow:

In this case, $Y_{(P/S)}$ and $Y_{(X/P)}$ were determined by two separate experiments as described in section 2.2.

$$\frac{dC_X}{dt} = \mu_C.C_x.\ Y_{X/P} = \frac{\mu_{maxC}.C_P}{C_P + K_C}.C_X.Y_{X/P} \quad (9)$$

From the description of the above equations, there are several parameters that must be sought, i.e. μ_{maxH}, μ_{maxC}, K_H, K_C.

3. Results

Based on the preliminary assessment, OPEFB contained 35.2% of cellulose, 17.9% of hemicellulose, 22.8% of lignin and the rest was ash content. After the pretreatment, the composition of OPEFB was presented on Table 1.

According to Table 1, the percentage of cellulose in OPEFB pretreated by 1% of NaOH is relatively higher as compared to the one using 0.5% of NaOH. This result is in agreement with previous literature where NaOH was reported to cause swelling, boosting the internal surface of cellulose and diminishing the degree of crystallinity and further enhancing the lignin disruption (Taherzadeh and Karimi, 2008; Carvelheiro et al., 2008). Further, by visual observation it was seen that the fiber treated by 1% NaOH possessed a suppler structure which can pre-concluded to have lower crystallinity due to the disruption of the lignin (Mulyaningtyas et al., 2017).

The concentration of sugar obtained from the enzymatic hydrolysis was plotted against time and the resulting graphs were shown in Fig. 1.

As depicted in Fig. 1, the concentration of sugar initially rose then decreased. This phenomenon occurred due to the consumption of sugar for the growth and production of other metabolic products by *A. niger*. The further depletion of sugar concentration was due to the growth of *A. niger* in aerobic phase before the anaerobic stage which would further convert the sugar to ethanol.

To find the parameters values included in the reactions, the resulting data from hydrolysis with OPEFB treated at 170 °C with two concentrations of NaOH were plotted.

From Table 2, the consumption rate constant, μ_{maxc}, was much higher than the hydrolysis rate constant, μ_{maxh}. It indicated that the sugar consumption rate was expressively faster than the hydrolysis rate. The phenomenon of sugar consumption by the mold had made the sugar concentration restricted. But, in overall, to compare both rates, the amount of substrate must be considered. Hydrolysis rate had more amount of substrate, i.e. OPEFB, than sugar consumption rate as it relied on the sugar production from hydrolysis. Hence, the hydrolysis rate was faster than sugar consumption.

From this study it was known that the phenomenon of sugar reconsumption by cellulase-producing mold had suppressed the sugar production. To overcome this, there were several ways that could possibly be done. First, the sugar harvesting time must be carefully determined in order to obtain the optimal concentrations. The second way was to design a separation process that was simultaneous with the hydrolysis. This could minimize the consumption of sugar by the mold so that the whole process produced higher sugar concentration. Based on the pattern of Fig. 1, it was better to harvest the sugar on day 8–9. Different substrate, microorganism and operation conditions would give different time although it should make the same curve pattern.

4. Conclusion

Lignin content existed in OPEFB structures decreased after combined pretreatment to make it feasible for *A. niger* to grow. Combined pretreatment was done at 170 °C with a series of NaOH concentrations. Both pretreatment resulted comparable lignocellulose compositions as shown in Table 1 as it was shown that the cellulose content were 62.4% and 71.0% for 0.5% and 1% NaOH respectively. Hydrolysis using OPEFB pretreated at 170 °C using NaOH 1% produced more sugar due to lower crystallinity of OPEFB. From the results of this study, it was known that the constant of sugar consumption was much higher than constant of the hydrolysis rate which meant that *A. niger* consumption rate was greater than the hydrolysis rate. However, the hydrolysis rate had much more substrate that makes the hydrolysis rate became substantial.

As it had been explained above and stated in Fig. 1, the rate of sugar consumption was faster than the hydrolysis rate. However, in general, with higher substrate availability, hydrolysis rate was higher than sugar consumption rate. This was reasonable considering sugar is the main source for microorganisms. To obtain relatively high sugar concentrations in this case, it was proposed to harvest the results of hydrolysis on day 8–9.

Table 1
The composition of OPEFB before and after pretreatment.

Composition (%)	No pretreatment	NaOH concentration	
		0.5%	1%
Cellulose	35.2	62.4	71.0
Hemicellulose	17.9	11.4	10.6
Lignin	22.8	7.0	7.4
Others	24.1	19.2	11.0

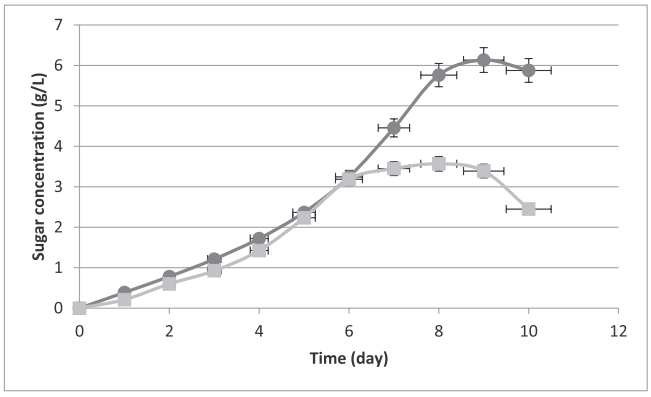

Fig. 1. Two different sugar concentrations between OPEFB. treated by NaOH 0.5% (square marks) and NaOH 1% (round marks).

Table 2
Resulting parameters for hydrolysis and sugar consumption rates.

Parameters	1% NaOH	0.5% NaOH
μ_{maxh}	0.62	0.58
K_h	26	7.5
μ_{maxc}	11.8	12
K_c	4	4

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References

Abd-Aziz, S., Hung, G.S., Hassan, M.A., Abdul Karim, M.I., Samad, N., 2008. Indirect method for quantification of cell biomass during solid state fermentation of palm kernel cake based on protein content. J. Asian Sci. Res. 1 (4), 385–393.

Bakar, N.K.A., Zanirun, Z., Abd-Aziz, S., Ghazali, F.M., Hassan, M.A., 2012. Production of fermentable sugars from oil palm empty fruit bunch using crude cellulase cocktail, with *Trichoderma asperellum* and *Aspergillus fumigatus* UPM2 for bioethanol production. BioResources 7 (3), 3627–3639. https://doi.org/10.15376/biores.7.3.3627-3639.

Bhat, M.K., 2008. Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18, 355–383.

Bommarius, A.S., Katona, A., Cheben, S.E., Patel, A.S., Ragauskas, A.J., Knudson, K., Pu, Y., 2008. Cellulase kinetics as a function of cellulose *pretreatment*. Metab. Eng. 10, 370–381.

Carvalheiro, F., Duarte, L.C., Gírio, F.M., 2008. Hemicellulose biorefineries: a review on biomass pretreatments. J. Sci. Ind. Res. 67, 849–864, 2008.

Chesson, A., 1981. Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. J. Sci. Food Agric. 32, 745–758.

Chinedu, S.N., Yah, S.C., Nwinyi, O.C., Okochi, O.I., Okafor, U.A., Onyegeme-Okerenta, B.M., 2001. Plant waste hydrolysis by extracellular enzymes of *Aspergillus niger* and *Penicillium chrysogenum*: effect of ammonia pretreatment. Niger. J. Biochem. Mol. Biol. 23 (1), 1–7.

Deswal, D., Khasa, Y.P., Kuhad, R.C., 2011. Optimization of cellulase production by a brown rot fungus Fomitopsis sp. RCK2010 under solid state fermentation. Bioresour. Technol. 102, 6065–6072.

Esteghlalian, A.R., Svivastava, V., Gilkes, N., Gregg, D.J., Saddler, A.N., 2001. An overview of factors influencing the enzymatic hydrolysis of lignocellulosic feedstocks. In: Himmel, M.E., Baker, W., Saddler, J.N. (Eds.), Glycosyl Hydrolases for Biomass Conversion. ACS, pp. 100–111, 2001.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59 (2), 257–268.

Kang, S.W., Park, Y.S., Lee, J.S., Hong, S.I., Kim, S.W., 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour. Technol. 91, 153–156.

Li, M., Pu, Y., Ragauskas, A.J., 2016. Current understanding of the correlation of lignin structure with biomass recalcitrance. Front. Chem. 4 (45).

Mulyaningtyas, A., Kayati, F.N., Syamsiah, S., Sarto, Sediawan, W.B., 2017. Effects of various pretreatment variables on hydrolysis of lignocellulose to produce sugar in bioethanol production. Mater. Sci. Forum 890, 141–145.

Piarpuzán, D., Quintero, J.A., Cardona, C.A., 2011. Empty fruit bunches from oil palm as a potential raw material for fuel ethanol production. Biomass Bioenergy 35, 1130–1137.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: an overview. Renew. Energy 37, 19–27.

Shahriarinour, M., Wahab, M.N.A., Ariff, A.B., Mustafa, S., Mohamad, R., 2011. Kinetics of cellulase production by *Aspergillus terreus* at various levels of dissolved oxygen tension in a stirred tank bioreactor. Bioresources 6 (4), 4909–49021, 2011.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.

Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int. J. Mol. Sci. 9, 1621–1651.

Zabed, H., Sahu, J.N., Suel, A., Boyce, A.N., Faruq, G., 2016. Bioethanol production from renewable sources: current perspectives and technological progress. Renew. Sustain. Energy Rev. 2–27.

Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. Int. J. Agric. Biol. Eng. 2 (3), 51–68.