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Enzymatic Hydrolysis Using Ultrasound for Bioethanol Production from Durian (*Durio zibethinus*) Seeds as Potential Biofuel

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The appealing second generation bioethanol production brings a good promise to achieve a fuel production that is renewable and sustainable; this makes durian (*Durio zibethinus*) seed interesting to take advantage of, especially for a tropical country like Malaysia. This paper aims to produce bioethanol from durian seed by utilizing ultrasound technique in its enzymatic hydrolysis process. 9 % (w/v) pre-treated durian seed was brought into the ultrasound-assisted glass reactor to begin the liquefaction and saccharification processes. *Bacillus licheniformis* Type XII-A was employed, and ultrasound at 50% amplitude for 60 min was set for liquefaction process; while amyloglucosidase from *Aspergillus niger* was used, and ultrasound at 40% amplitude for 120 min was run for saccharification process. The sum of both processes in hydrolysis yielded 41.07 g/L of reducing sugar, which was immediately brought to fermentation stage. *Saccharomyces cerevisiae* was employed for fermentation and resulted 18.48 g/L (0.44 g ethanol/g glucose), which is equivalent to 86.27 % of theoretical ethanol yield (0.51 g ethanol/g glucose) after 84 h of fermentation at 37 °C with 150 rpm incubator shaker. The ethanol purity was improved in the next stage, distillation. Using zeolite as adsorbent, ethanol with purity of 95.7% (v/v) was produced. From the acquired results, durian seed shows a justifiably potential as a second generation bioethanol feedstock. To further improve its potential, studies of optimization using this feedstock is highly encouraged.

1. Introduction

There are two visible problems in fulfilling our dependency of fossil fuel: depleting fossil fuel reserves and environmental sustainability, and fossil fuel is known to exhibit many negative 'side-effects', which leads to global warming. Many evidences have revealed how greenhouse gas emissions emitted by fossil fuel could affect the ecosystem, including glaciers melting (which leads to the rise of ocean level), extreme climate change, natural biodiversity loss, etc (Hansdah et al., 2013). In addition, petroleum-based products were one of the main causes of increasing carbon dioxide (CO_2) emission to the atmosphere. One-fifth of global CO_2 emissions was created by the transport sector (Shadidi et al., 2014). Replacing fossil fuels with biofuels can be as a solution to reduce vehicle exhaust emissions. Moreover, they have also been seen as a way to reduce exhaust emissions from road transport because they were considered contributor CO_2 to the atmosphere (Suarez- Bertoa et al., 2015). This is due to the product of fossil fuel combustion which contributes 73 % of the total CO_2 emission (Lokhorst and Wildenborg, 2005).
Biofuel is understood to be a promising solution to tackle fossil fuel environmental issues. Because of its sustainable and renewable source of production, biofuel is tactical to restrict emissions and improve the air quality (Sebayang et al., 2016). Bioethanol, as one form of biofuel, carries practical benefits since alcohol fuel is able to surrogate gasoline in spark-ignition engines (Nigam and Singh, 2011).

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Despite the similar outcome, second generation bioethanol seems to be more reassuring than first generation bioethanol. This is because the feedstock used in second generation does not contradict the feedstock sustainability, unlike the first generation which uses food crops as the feedstock, including sugar-contained crops, starchy and grains crops (Vohra et al., 2014). Therefore, much effort is being made to produce bioethanol from non-edible feedstocks. Second generation bioethanol is produced from non-edible bio-resources, for instance wheat straw, wood shavings, perennial grasses (Miscanthus, switch grass, and reed canary grass), etc. (Nigam and Singh, 2011). In addition, second-generation bioethanol is also produced from non-edible agricultural lignocellulosic raw materials, and therefore, one way that promises to reduce human dependence 'on fossil fuels. (Aditiya et al., 2016a). With this, the debate on food for fuel can be avoided.
Ultrasound method is a method using ultrasound waves that is acoustic waves with frequencies greater than 20 kHz (Subhedar and Gogate, 2013). The advantages of ultrasound method are it is quick, easy, and does not result in significant changes in the chemical structure (Gonçalves et al., 2014). Using ultrasound as a method of hydrolysis has been widely studied and reached the same conclusion that ultrasound treatment can increase the concentration of reducing sugar. Subhedar et al. (2015) observed that the enzymatic hydrolysis process with the ultrasound-assisted resulted in reducing sugar was 2.4-fold higher than without ultrasound. Ramón et al. (2015) concluded in their research that the results of acid and enzymatic hydrolysis, using ultrasound is better than without ultrasound. The concentration of reducing sugar increased by around 31 % in the enzymatic hydrolysis process with the ultrasound-assisted. Lunelli et al. (2014) analyzed the enzymatic hydrolysis of sugarcane bagasse to ultrasound-assisted, and they concluded that the concentration of reducing sugar increased 2-fold compared to without ultrasound-assisted.
According to a study by Aditiya et al. (2016a), Malaysia is the home of more than 370 kt of durian production, of which more than 87 kt of durian seeds are produced. This abundant amount of food waste is appealing to second generation bioethanol production since in durian seed the high content of carbohydrate, as the main key-element in bioethanol production, holds the practical potential as a sustainable feedstock for bioethanol production. Amin and Arshad (2009) characterized carbohydrate content in durian seed, which is 76.8 % for peeled durian seed. Surprisingly, utilizing durian seed as the feedstock in bioethanol production is rather an uncommon practice. However, using starchy material in bioethanol production is quite widespread.
The advantageous ultrasound-assisted enzymatic hydrolysis method, coupled with the potential of durian seed as the feedstock in second generation bioethanol production drives this study upfront, so that a favourable quality of bioethanol can be produced as the outcome of this study. The result from this study also contributes to the development of second generation bioethanol production since it is such rarity to find durian seed used as the feedstock.

2. Materials and methods

The durian seeds were collected from a local durian stall in Seri Serdang, Selangor, Malaysia with no price. The collected durian seeds were cleanly washed with water then peeled to remove the lignocellulose substrate from the peel, which is not useful in this experiment since the focus is to convert the starch substrate of the durian seed waste.

2.1 Pre-treatment

A mechanical pre-treatment was performed by shredding the peeled durian. The seeds were then dried in an oven at 80 ºC for 7 h. Further refining process was performed by grinding the dried seeds using mortar. Following this, the durian seeds were ground and sieved to obtain starch. The durian seeds were homogenized, having size smaller than 1 mm (125–150 µm) and it has an effect on reducing sugar concentration in hydrolysis process. According to Barcelos et al., (2011) the decrease of the particle size diminished the effect of diffusion limitation and led to a higher sugar yield in comparison with higher particle sizes. The durian seeds were dried so that they can be stored for longer periods at a temperature of 25 °C.

2.2 Microorganism

The yeast *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich (Saint Louis, MO, USA). It was used for the fermentation of hydrolyzed durian seed waste into ethanol and was maintained on yeast peptone dextrose (YPD) medium. YPD was used for cultivating and maintaining dry yeast from S. *cerevisiae*. The yeast peptone dextrose was prepared by 2 g of yeast extract, 4 g bacterial peptone, 4 g of glucose and 12 g agar in 200 mL of distilled water. Then, dry yeast was activated by adding 100 mL of distilled water in Duran flask. The solution was sterilized in an autoclave for 35 minutes and it was placed and maintained on a glass petri dish. The yeast is kept in an incubator at a temperature of 37 ºC for 48 h in order to make it occulated before being used for bioethanol production.

2.3 Enzymes

Enzyme α-amylase from *Bacillus licheniformis* Type XII-A and amyloglucosidase from *Aspergillus niger* are used as a catalyst liquefaction and saccharification. Both enzymes were purchased from Sigma-Aldrich (Saint Louis, MO, USA) with enzymatic activity of more than or equal to 500 U/g protein for α-amylase and greater than or equal to 300 U/mL for amyloglucosidase.

2.4 Ultrasound intensified liquefaction and saccharification of durian seed waste

The general procedure of enzymatic hydrolysis was initiated by preparing the respective substrates in a flask filled with distilled water. A solution of 9 %(w/v) durian seed flour was initially prepared, then was introduced to each the respective enzyme of the hydrolysis process. Liquefaction was performed first, and the solution was introduced to α-amylase enzyme (90, 100, and 105 U/g) in a 250 mL flask and was put to the ultrasonic generator at 20 kHz frequency and 500 W power. Here, the amplitude of sonication was 50 % retained and impulses on 4 s off 2 s at 60 min for the liquefaction process. Saccharification was performed next with introducing amyloglucosidase 45 (U/mL) to the liquefied solution. The process was run also assisted by ultrasound at settings 40 % amplitude impulses on 4 s off 2 s to 120 min. At the end of this stage, the hydrolysate was tested for its reducing sugar content using DNS (2, 3-dinitrosalicylic acid) method (Miller, 1959).

2.5 Ethanol fermentation of durian seed waste hydrolysates

Yeast S. *cerevisiae* was employed to ferment the sugars formed after hydrolysis stage. The yeast was precultured overnight in 100 mL of yeast extract peptone dextrose (YEPD) medium. Hydrolysis durian seed starch solution was transferred to a 500 mL glass bottles. The hydrolysate was brought into fermentation process by adding nutrients: 1.6 g of potassium dihydrogen phosphate (KH_2PO4), 0.8 g of ammonium chloride (NH_4Cl) and 4 g of yeast extract for 400 mL of hydrolysate. The chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Using incubator shaker, hydrolysate was fermented for 96 h at agitation speed 150 rpm and a temperature of 37 °C. Samples were withdrawn after 12, 24, 36, 48, 60, 72, 84 and 96 h. Ethanol concentration was estimated from the distillate by the dichromate method . (Caputi et al., 1968).

2.6 Distillation and dehydration

Due to the water-ethanol azeotrope, separation of ethanol from water can only be achieved less than 95 % (Sebayang et al., 2016). Hence, the water-ethanol separation process was performed in two different stages to maximize the ethanol extraction: distillation and dehydration. In the first stage in water-ethanol separation, distillation was performed using a rotary evaporator at temperature, pressure and rotary speed of 60 °C, 175 mbar and 100 rpm. Subsequently, dehydration process used 90 % (w/v) zeolite in the distillate produced from the previous stage. The ethanol content was determined based on the density of alcohol distillate at 20 °C and expressed in % (v/v) by using DMA 35.

2.7 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy is a powerful analytical technique to examine the functional groups of a alcohol, therefore FTIR (Bruker Tensor 27) was utilized to identify the limits of the chemical structure of durian seeds. The produced bioethanol durian seeds was analyzed by ATR (attenuated total reflection) sample compartment with MIR (mid-infrared) spectra in the wavenumber range of 4000-400 cm^{-1}

3. Results and discussions

3.1 Hydrolyzed durian seeds

The load of durian seed starch and amyglucosidase enzyme used in this study were set constant as 9 % (w/v) and 45 U/mL respectively, while the load of the enzyme α-amylase was varied at 75 U/g, 90 U/g and 105 U/g. **Figure 1** shows the increasing reducing sugar yield as the concentrations of the enzyme α-amylase is also increased. The most starch conversion was occurred by 105 U/g enzyme α-amylase, where it yielded reducing sugar yield of 41.07 g/L. This is explained since α-amylase is responsible in breaking longer sugar polymer chain and leaving random, shorter sugar chains. Specifically, this enzyme attacks α-1, 4 glycosidic bond, of which in charge in the formation of linear, long-chain of amylose polymer. A thorough review of α-amylase reaction in amylose is described in our comprehensive review (Aditiya et al., 2016b). The end result from both liquefaction and saccharification is simpler sugar monomer, which in our work only collections of reducing sugars are accounted.

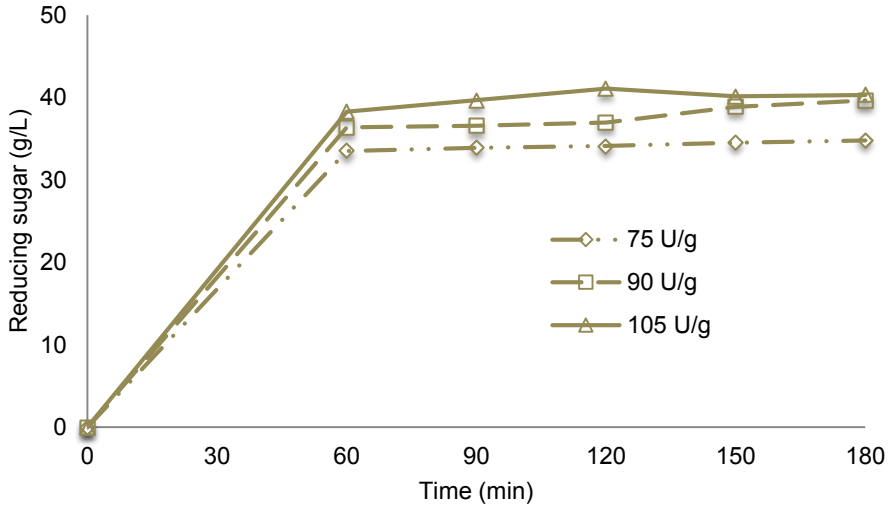

Figure 1: Effect of α-amylase concentrations over time on the enzymatic hydrolysis.

3.2 Fermentation durian seeds

From the hydrolysis process (Section 3.1), the highest reducing sugar yield was selected and brought into the subsequent stage of fermentation. Figure 2 shows the rising ethanol production trend with the dropping reducing sugar trend over fermentation period. The occurrence is logical since the consumption of sugar by yeast S. *cerevisiae* for its growth produces ethanol at the same time throughout the fermentation period, hence the longer the fermentation period the lower the concentration of sugar resided within the hydrolysate. This fermentation process yielded ethanol of 0.43-0.46 g ethanol/g glucose, which is equivalent to 85.78-90.21 % of theoretical ethanol yield (by assuming all glucose production and theoretical conversion ratio 0.51 g ethanol/g glucose). In this study, ethanol yield seemed to be higher than that by Moshi et al. (2014), where they yielded 82-84 % of theoretical ethanol yield. In addition, the results of research by Sivamani et al. (2015) were 83% of theoretical ethanol yield in which they used cassava peel as feedstock. Besides that, the study by Gumienna et al. (2016) with the feedstock of corn and ethanol yield was 81.33 % of theoretical ethanol yield. The results of this study were slightly better than both researchers. The weakening ethanol concentration after 84 h fermentation was due to the accumulated ethanol production which eventually inhibited the growth of yeast. In this study, the maximum ethanol yielded was 18.48 g/L which occurred at 84 h of fermentation.

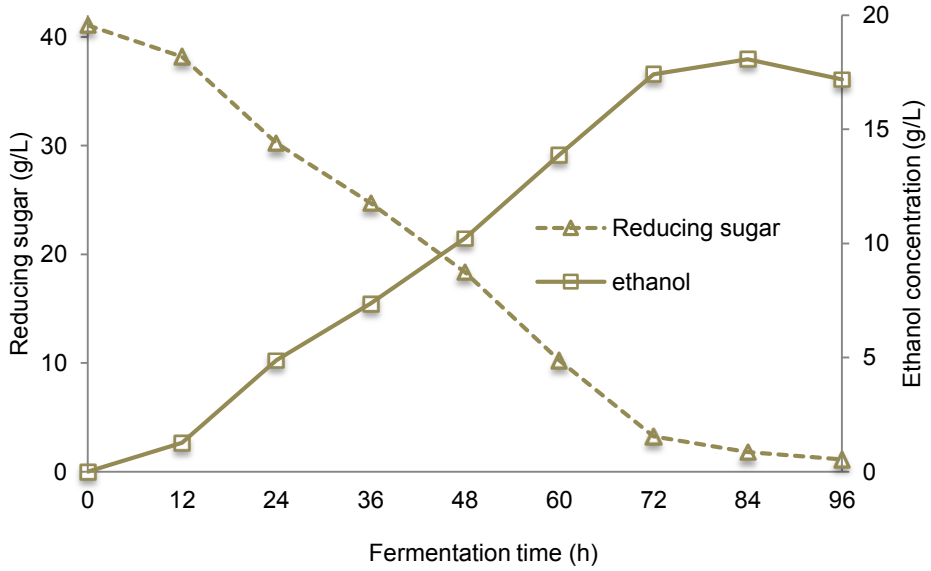

Figure 2: Ethanol and reducing sugar concentration changes during batch fermentation with S. cerevisiae

3.3 Distillation and dehydration durian seeds

The produced ethanol from fermentation was then brought into distillation to improve its ethanol quality, and it yielded ethanol with quality 90.23 % (v/v). Additional dehydration was also performed to further upgrade its quality. Using 90 % (w/v) zeolite as absorbent in the dehydration column, the ethanol purity was upgraded to 95.7 % (v/v). The final ethanol concentration from this study is 3.9 % higher than study by Moshi et al. (2014).

3.4 Fourier transform infrared spectroscopy of bioethanol durian seeds

Figure 3 illustrates the results of FTIR spectroscopy test of bioethanol durian seeds. Infrared absorption by O-H groups is indicated in 3,346 cm^{-1} absorption wave, this condition shows that the broad O-H stretching vibration is very strong and this is the same as the research of Yadira at al. (2014). Peak at 2,979 cm^{-1} represents a stretching C-H bond. In addition, the existence of absorption wave at 1,644 cm^{-1} region shows the presence of alkene group with variable C=C bonds. Absorption at 1,384 cm^{-1} wave shows there is an alkane group with bonds between atoms C-H in the form of CH_3 with medium intensity. And on the wave of 1,326 – 1,044 cm^{-1} shows the presence of ether groups to stretch C-C bond that is strong enough.

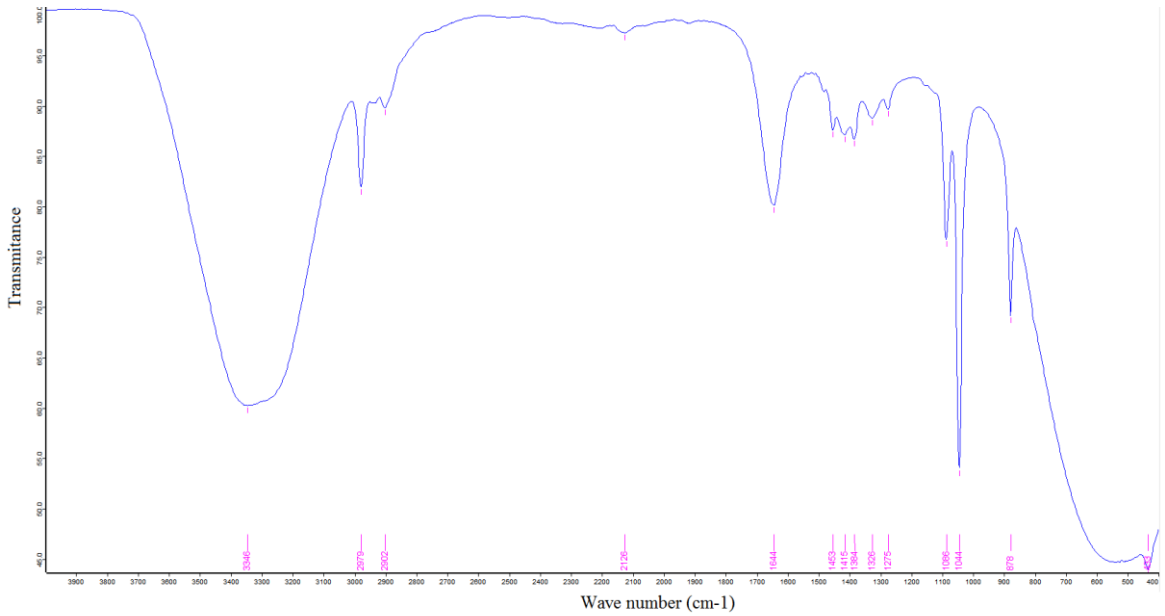

Figure 3. Fourier transform infrared spectrum of bioethanol durian seeds.

4. Conclusions

This study evaluated the use of ultrasound in assisting enzymatic hydrolysis process of durian seed to yield a favourable reducing sugar concentration. Also, the influence of enzyme α-amylase on hydrolysis of durian seed is more prevalent in the higher load. This study resulted ethanol production of 18.48 g/L by fermenting 41.07 g/L of reducing sugar that was obtained by ultrasonic-assisted enzymatic hydrolysis process (using α-amylase 105 U/g + amyloglucosidase 45 U/mL). Further, after distillation and dehydration the ethanol quality was improved to 95.7 % (v/v). Ethanol produced from fermented durian seed indicates that durian seed can be functionally utilized and the production streamline shows a great quality end-product of ethanol.

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