Bioethanol produced from *Moringa oleifera* seeds husk

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Abstract. This paper presents the potential of bioethanol production from *Moringa oleifera* seeds husk which contains lignocellulosic through Simultaneous Saccharification and Fermentation (SSF) process by using *Saccharomyces cerevisiae*. This paper investigates the parameters which produce optimum bioethanol yield. The husk was hydrolyzed using NaOH and fermented using *Saccharomyces cerevisiae* yeast. Batch fermentation was performed with different yeast dosage of 1, 3, and 5 g/L, pH value was 4.5, 5.0 and 5.5, and fermentation time of 3, 6, 9 and 12 hours. The temperature of fermentation process in incubator shaker is kept constant at 32°C. The samples are then filtered using a 0.20 μm nylon filter syringe. The yield of bioethanol produced was analysed using High Performance Liquid Chromatography (HPLC). The results showed that the highest yield of 29.69 g/L was obtained at 3 hours of fermentation time at pH of 4.5 and using 1g/L yeast. This research work showed that *Moringa oleifera* seeds husk can be considered to produce bioethanol.

1. Introduction

Day by day, population is increasing causing decreasing in conventional energy. Increase on world’s energy demand and the progressive depletion of oil reserves are motivating the researchers to find alternative energy resources, especially for those derived from renewable materials such as biomass (Saxena et al., 2009). Agricultural raw material and biomass can be utilized to produce renewable energy in the form of liquid/gaseous fuel which can reduce CO₂ emission on combustion unlike fossil fuels which can cause greenhouse effect. The climate change and the consequent need to diminish greenhouse gases emissions is a global concern. Because of that, it has been encouraged to use bioethanol as a gasoline replacement or as an additive (Mustafa et al., 2008). Bioethanol may also be used as raw material for the production of different chemicals, thus driving a full renewable chemical industry.

Research on bioethanol production has been investigated recently as this fuel is efficient, biodegradable, environmentally friendly and cost effective alternative for the conventional fossil fuel. Bioethanol derived from lignocellulosic material is a potential renewable energy source. There are countless lignocellulosic materials that have been used in recent years to produce bioethanol such as sugarcane bagasse, paper and pulp, cassava waste, saw dust and corn (Pandey et al., 2000; Akinyele et al., 2011; Amarnath & Balakrishnan, 2007).

*Moringa oleifera* seeds husk is considered as new initiative to replace conventional energy resources. *Moringa oleifera* is a tropical plant belongs to the family of *Moringaceae*. It also have fourteen different species which has been identified – all of them possessing varying degrees of coagulation activity (Jahn, 1988). *Moringa oleifera* is the most widespread species, which grows quickly at low altitudes in the whole tropical belt, including arid zones (Julia, 1991; Verdcourt, 1985).
It is generally known in the developing world as a vegetable, a medicinal plant and a source of vegetable oil. However, in the Sudan, it has been traditionally used in water purification. These multiple uses of the *Moringa oleifera* plant have greatly promoted its widespread application. *Saccharomyces cerevisiae* yeast is used to produce bioethanol from *Moringa oleifera* seeds husk. *Saccharomyces cerevisiae* is commonly known as baker’s yeast. It has a single–celled eukaryote that is frequently used in scientific research. *Saccharomyces cerevisiae* is an excellent model microbe in biological research, and has been used for metal biosorption research (Jianlong & Can, 2006, 2009). This research work was carried out to find optimum yield of bioethanol that can be produced from *Moringa oleifera* seeds husk.

2. Material and Method

2.1 Chemicals and baker’s yeast
Sulphuric Acid (H$_2$SO$_4$) and Sodium Hydroxide (NaOH) were obtained from R & M Chemicals. The fermentation was done by using baker’s yeast (*Saccharomyces cerevisiae*), obtained from supermarket. Apart from that, the ultrapure water obtained from Milli–Q® Integral Water Purification System and HPLC–grade sulfuric acid obtained from Merck (Darmstadt, Germany) were mixed in order to prepare the mobile phase for HPLC. HPLC–grade ethanol was obtained from Merck (Darmstadt, Germany) for ethanol standard calibration curve.

2.2 Preparation of *Moringa oleifera* seeds husk
The dried *Moringa oleifera* pods were collected at Batu Berendam, Melaka, Malaysia (August 2015) (figure 1). The *Moringa oleifera* seeds (figure 2) were extracted from its pods, and the seeds were de–husked manually to get the husk for experimental work (figure 3). Around 30 g of *Moringa oleifera* seeds husk for each sample were immersed in 450 mL of pure water in 500 mL conical flasks for an overnight before experiment was started.

![Figure 1. *Moringa oleifera* tree.](image_url)
2.3 Pretreatment of Moringa oleifera seeds husk

Sodium hydroxide (NaOH) was added drop by drop into the samples of immersed Moringa oleifera seeds husk until the pH 11 was attained. The flasks were covered with cotton wool and aluminium foil (figure 4). The samples were pre-treated in an autoclave (Hirayama HV–85) at a temperature of 121°C for 2 hours.
2.4 **Fermentation of Moringa oleifera seeds husk**

For the first batch of experiments, 1 g/L of *Saccharomyces cerevisiae* was added into the samples. The samples were let cooled to room temperature, then sulphuric acid was added drop-by-drop in order to adjust the pH parameter which is 4.5, 5.0 and 5.5. Then, the samples were kept in a stackable incubator shaker (INFORSH Ecotron) at 180 rpm for 12 hours at 32°C to allow a complete fermentation. The fermentation temperature was chosen based on (Marina, et al., 2009) who stated that 32°C is the ideal fermentation temperature. The fermentation temperature represents a critical step in ethanol production, since higher temperatures affect yeast behaviour, diminishing the ethanol content, which increases the consumption of energy during distillation of ethanol produced (Marina, et al., 2009).

2.5 **Collection of samples**

Sample of 4 mL was taken aseptically at different fermentation periods of 3, 6, 9, and 12 hours. All the collected samples were immediately stored in a freezer at -20°C before used for chromatography analysis. The samples were filtered using 0.20 μm nylon filter syringe into 1.5 mL Agilent vials for HPLC analysis. The same procedure was followed to produce bioethanol using 3 g/L and 5 g/L of yeast.

2.6 **Analysis of ethanol content using HPLC**

The analysis of ethanol concentration from the sample was performed using Agilent 1200 HPLC equipped with Rezex ROA column (150 X 7.80 mm) and refractive index (RI) detector. The chromatography grade 0.005 N sulphuric acid was used as the mobile phase and the flow rate was set at 0.5 ml/min. The column temperature was set at 60°C and RI detector temperature at 40°C. The injection volume of samples to HPLC was 10 μL. The calibration of HPLC was done using ethanol standard. Six dilutions of ethanol standard were prepared with concentration of 5, 10, 15, 20, 25, and 30 g/L. Then, the ethanol standards were filtered using 0.20 μm nylon filter syringe before analysis. Calibration curve of the peak area (obtained from HPLC analysis) versus ethanol concentration was plotted using Microsoft ExcelTM 2013.

3. **Results and Discussion**

3.1 **HPLC Calibration**

The ethanol standards were injected to HPLC and the results are shown in figure 5 shows the calibration curve which represent the relationship between peak area and concentration of ethanol standards. The concentration of ethanol was calculated using the equation y= 70594 x + 218677 with regression correlation of $R^2=0.9928$.
Table 1 summarizes the bioethanol yield during twelve hours of fermentation period, by varying the yeast dosage and pH value. Yeast dosage is evaluated at 1, 3, and 5 g/L and pH value was varied at 4.5, 5.0 and 5.5. Fermentation temperature is kept constant at 32°C with agitation speed of 180 rpm. The fermented broth is collected at each 3 hours for twelve hours of fermentation period of 3, 6, 9 and 12 hours. The results of using 5g/L are not shown in Table 1, because there was no bioethanol produced at that yeast concentration.

Table 1. Concentration of bioethanol produced

| Yeast Dosage, g/L | pH     | 1     | 3     | 1     | 3     | 1     | 3     | 1     | 3     | 1     | 3     | 1     | 3     |
|------------------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                  | 4.5    | 5     | 5.5   | 4.5   | 5     | 5.5   | 4.5   | 5     | 5.5   | 4.5   | 5     | 5.5   | 4.5   |
| Time (hours)     |        |       |       |       |       |       |       |       |       |       |       |       |       |
| 3                | 29.69  | 17.34 | 21.6  | 1.98  | 0.18  | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| 6                | 20.39  | 13.67 | 7.69  | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| 9                | 2.06   | 1.18  | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| 12               | 0      | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |

3.2 Effect of yeast dosage on bioethanol yield
The bioethanol yield using 1 g/L of *Saccharomyces cerevisiae* is shown in figure 6 at different pH values and fermentation time. The highest bioethanol yield was 29.69 g/L produced at pH 4.5 and 3 hours fermentation time. The bioethanol produced was decreasing to reach 0 g/L at 12 hours of fermentation time. The same trend goes by using 3g/L yeast (figure 7). In case of adding 5g/L yeast there was no bioethanol produced.

![Figure 6](image-url)
Figure 7. Bioethanol yield produced with 3 g/L of *Saccharomyces cerevisiae* at different pH.

Figure 8 shows the comparison of bioethanol yield using 1, 3 and 5 g/L of *Saccharomyces cerevisiae* at pH 4.5 and 3 hours fermentation time. The highest bioethanol yield was 29.69 g/L produced at pH 4.5 and 3 hours fermentation time for 1 g/L of yeast used. Bioethanol yield is decreased with addition of yeast to reach 1.98 g/L using yeast dosage of 3g/L, and in case of using 5g/L there was no bioethanol produced.

Figure 8. Comparison of bioethanol yield using different yeast dosages

3.3 Effect of pH value on bioethanol yield

Figure 9 shows the bioethanol yield using pH 4.5 with different yeast dosages and fermentation time. The highest bioethanol yield was 29.69 g/L produced at 3 hours fermentation time with 1 g/L of *Saccharomyces cerevisiae*. When the fermentation reached 12 hours onwards, there was no bioethanol produced.
When the pH increased to 5, the bioethanol produced was decreased to reach 17.34 g/L using 1g/L yeast. At the same pH of 5 the bioethanol produced was only 0.18 g/L with 3g/L yeast, reaching to 0.00 g/L bioethanol by adding 5g/L yeast as shown in figure 10.

Figure 9. Bioethanol yield produced with pH 4.5

Figure 10. Bioethanol yield produced with pH 5.0

Figure 11 is showing that by doing fermentation at high pH, the bioethanol will decrease as pH increase. In this work, the bioethanol produced was 11.60 g/L at pH 5.5 and 3 hours fermentation using 1g/L yeast, compared to 29.69 g/L at pH 4.5 and 3 hours fermentation using 1g/L yeast, and 17.34 g/L at pH 5 and 3 hours fermentation using 1g/L yeast. The same trend goes when using 3g/L yeast, where the bioethanol produced was 0.00g/L at 5.5, while it was 1.98 g/L at pH 4.5, and 0.18 g/L at pH 5.
The comparison of bioethanol yield using pH 4.5, 5.0 and 5.5 on 1 g/L of *Saccharomyces cerevisiae* and 3 hours fermentation time is shown in figure 12. The highest bioethanol yield is 29.69 g/L produced at pH 4.5 and 3 hours of fermentation time for 1 g/L of yeast. Bioethanol yield is decreased with increasing pH values.

**Figure 12.** Comparison of bioethanol yield at different pH.

### 3.4 Effect of fermentation time on bioethanol yield

Figure 13 shows the comparison of bioethanol yield at 3, 6, 9 and 12 hours of fermentation time using pH 4.5, 5.0 and 5.5 on 1 g/L of *Saccharomyces cerevisiae*. The highest bioethanol yield is 29.69 g/L produced at pH 4.5 and 3 hours fermentation time for 1 g/L of yeast used. Bioethanol yield is decreased with increasing fermentation time.
Figure 13. Comparison of fermentation time effect on bioethanol yield

4. Conclusion

Based on previous studies, it had been approved that *Moringa oleifera* seeds husk can be considered as a potential substrate for ethanol production due to their high cellulose contents (Martin et al., 2010). The results indicated clearly that the characteristics of the husks can lead to the production of ethanol. The highest amount of the ethanol yield was 29.69 g/L at 3 hours fermentation at temperature of 32°C, agitation speed of 180 rpm, pH 4.5 and yeast concentration of 1 g/L. It can be concluded that bioethanol can be produced with a low dosage of yeast and in a short time, which both considered positive results economically. It is recommended to further this study using lower yeast concentration than 1 g/L and analyse the fermented sample each 1 hour up to 4 hours to find the optimum condition. And more studies need to be done to compare between manual de–husking and mechanical de–husking. It was proved that *Moringa oleifera* seeds husk is a good source for bioethanol production to have biodegradable fuel with no pollution problems.

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