Simultaneous Saccharification and Fermentation of Oil Palm Empty Fruit Bunch for Bioethanol Production by *Rhizopus oryzae*

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Abstract: Over the past years, lack of energy fossil has become important issue around the world, especially in developing countries such as Indonesia. Because of that, alternative energy is needed to replace utilization of fossil fuels. Bioethanol is providing one window of potential alternative energy. Lignocellulosic from oil palm empty fruit bunch (EFB) could be a promising bioethanol raw material because it will not interfere food supply. Simultaneous saccharification and fermentation process (SSF) is known as a technique which reduces the number of process for effectiveness of reactor amount and finally reduces the cost production. *Rhizopus oryzae* is a filamentous fungus, which can produce L(+)-lactic acid and ethanol as the main by-product. The SSF experiments were carried out anaerobically at 37 °C, 150 g/L dry matter (DM) solid substrate concentration and 20 filter paper unit (FPU)/g DM of a commercial enzyme cellulase. The experiments were stopped after four days to achieve higher ethanol concentration. Strain was cultivated on pre-treated EFB with variation of pH, and the production of glucose and ethanol was investigated. The ethanol yields by *Rhizopus oryzae* on pH 4.5, 5.0 and 5.5 were 20.51, 33.08, and 26.78 g/L medium, where the glucose concentration at the first day were 56-62 g/L medium. The ethanol yields based on substrate were 0.14, 0.22 and 0.18 g/g DM pretreated EFB.

Keywords: oil palm; empty fruit bunch; bioethanol; *Rhizopus oryzae*; fermentation.
1. Introduction

The future risks of global warming and shortage of petroleum as well as the superior environmental characteristics of ethanol as an oxygenate for fuels promote the production and usage of bioethanol. Ethanol from renewable resources as an alternative fuel or oxygenated additive to the current fossil fuels has been interest issues in recent decades. Lignocellulosic materials, which are relatively cheap and plentiful, are considered the main source of feedstock for low-cost bioethanol production. Oil palm empty fruit bunch (EFB) is one of the abundant lignocellulosic waste materials in the world. In 2008, production of crude palm oil achieved more than 17.5 million ton and each ton of it generally produces 1.1 ton EFB (Wiloso, 2010). EFB material contains cellulose, hemicelluloses, lignin, ashes and the extractive. In terms of chemical composition, the EFB predominantly contains cellulose (33.64%), hemicelluloses (15.22%) and lignin (37.84%). Cellulose as the major fraction of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes and then glucose can be fermented to ethanol by *Saccharomyces cerevisiae* (Badger, 2002).

The cellulosic fraction of lignocelluloses can be converted to ethanol through two processes. Firstly, enzymatic saccharification converts cellulose to monomer glucose, and secondly, fermentation converts glucose to ethanol. The ethanol can be produced by either simultaneous saccharification and fermentation (SSF) or separate enzymatic hydrolysis and fermentation (SHF). SSF is more favored because of its advantages. SSF eliminates the need for complete hydrolysis of carbon substrates prior to the fermentation. In this process, enzymatic hydrolysis, cell growth and microbial production occur simultaneously. A direct benefit of the SSF is a decrease in the inhibition caused by mono or disaccharide accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital costs (Jin et al., 1999). Because of that SSF is more preferable. Furthermore, many factors, such as pH, temperature, substrates and product concentration of glucose and ethanol can affect the efficiency of the SSF (Huang et al., 2005). Empty fruit bunch is simultaneously converted to oligosaccharide or glucose by enzyme cellulase, disaccharide hydrolysis to monomeric glucose by enzyme-glucosidase, and glucose is catabolized primarily to ethanol, cell mass and carbon dioxide by a fermentative microorganism (Huang et al., 2005).

The capability of zygomycetes was recently explored for production of ethanol (Milati et al., 2005). Classes of filamentous fungi are saprophytic organisms, which are able to produce several metabolites including ethanol. Among three genera of *Rhizopus*, *Mucor* and *Rhizomucor*, *Mucor indicus* and *Rhizopus oryzae* showed good performances on ethanol production from glucose, xylose and wood hydrolyzate (Milati et al., 2005). *R. oryzae* is one of the most economically important
members of zygomycete group of fungi and represents the first squenched fungus from the early liniages of the fungal phylogenetic tree and thus the genome sequence sheds light on the evolution of the entire fungal kingdom (Ghosh et al., 2011). *R. oryzae* is a filamentous fungus, which is an active component in some traditional foods of Indonesia, China and Japan. Its asexual spores are contained within sporangia borne on sporangiophores.

Millati et al. (2005) reported that ethanol yield by *R. oryzae* was higher on xylose, but lower on glucose and the hydrolyzate than by *M. indicus*. Sues et al. (2005) obtained 0.43 g/g ethanol from glucose under anaerobic condition by *R. oryzae*. These fungi may have several industrial advantages compared to baker’s yeast such as (a) capability of utilizing xylose, the major fraction of hemicelluloses of rice straw, (b) having a valuable biomass for, e.g. production of chitosan, and (c) optimum temperature of the baker’s yeast is in the range of 28 - 35 °C, where as these fungi showed ethanol production with comparable yield and productivity at 37 °C (Chatterjee et al., 2005; Sues et al., 2005). Another advantage using the fungi is the low cost, due to (1) use of raw and/or waste materials, (2) no need of specific nutrients, and (3) easy and unexpensive separation of filamentous or pellet biomass from the fermentation broth (Rosenberg and Kristofikova, 1995; Soccol et al., 1994).

The current study aimed at production of ethanol from oil palm empty fruit bunch by simultaneous saccharification and fermentation with *Rhizopus oryzae*. Optimum pH for ethanol production was also investigated. The profiles of glucose as well as the formation of ethanol were examined.

### 2. Materials and Methods

#### 2.1. The Fungal Strains

The organism used in this study, *R. oryzae* was maintained on potato dextrose agar. It was incubated at 32 °C. After growth and sporulation, spore suspensions were prepared by adding 10 mL of sterile distilled water to the slant and shaking it vigorously. This spore suspension was centrifuged and dilute with sterile distilled water to obtain initial spore concentration $1 \times 10^8$ spores/mL of the media. Spore concentration was determined by counting the spores on a *haemocytometer*.

#### 2.2. Empty Fruit Bunch

Oil palm empty fruit bunch (EFB) used in this experiment was obtained from PTPN VIII oil palm field (Banten, Indonesia). EFB was milled and sieved to reduce the size prior to pre-treatment. This EFB was pre-treated by 10% NaOH solution in reactor with 150 °C temperature and four bar pressure for 30 min and the materials were then collected from the reactor. The solid fraction was
washed five times with tap water and stored at room temperature prior to SSF. Pre-treated EFB containing 11.52% hemicellulose, 60.34% cellulose, and 20.00% lignin, were measured according to NREL Chemical Analysis & Testing Procedure (Ruiz and Erhman, 1996).

2.3. Enzyme

A commercial cellulase and β-glucosidase enzyme supplied from NOVOzym were used in all the experiments as sole enzymatic complex. We have analyzed it by measuring its activity as FPU. The enzyme showed activity of 70 FPU/mL. One unit of activity in each case is equal to 1 mole of glucose produced per minute per milliliter of the enzyme.

2.4. SSF Experiment

A media contains (g/L): yeast extract, 2.5; peptone, 2.5; KH$_2$PO$_4$, 1.0; MgSO$_4$·7H$_2$O, 0.25; pre-treated oil palm empty fruit bunch, 150.0 and 0.05 M buffer citrate were used in 250 mL Erlenmeyer flasks. Medium pH was adjusted to 4.5, 5.0 and 5.5 to see the effect of pH medium to ethanol production by NaOH (2 N) and acetic acid (1 N). The medium except pretreated EFB was then autoclaved in 121 °C, and the pretreated EFB, strain R. oryzae, and the required enzyme were added to each flask aseptically. The final volume in each flask was 100 mL. All the SSF experiments were performed at 37 °C, 150 rpm rotary shaker. The enzyme loading was 20 FPU/g DM pretreated EFB. The experiment was carried out under anaerobic condition. The flask was covered with elastic cup in anaerobic conditions. This cup was used to anticipate the gas production during processing, and to prevent entrance of air into flask. Pure nitrogen was purging into the media at the beginning of the fermentation and during the sampling. The process terminated after 96 hours SSF.

2.5. Analytical Methods

The samples from SSF were taken and stored in a freeze. They were then analyzed by a HPLC. Glucose and ethanol were analyzed on an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) at 65 °C with 0.6 mL/min eluent of 5 mM sulfuric acid along 25 min retention time.

3. Results and Discussion

3.1. SSF by R. oryzae

Simultaneous saccharification and fermentation (SSF) of empty fruit bunch was examined. The experiments were carried out under anaerobic conditions, where the cellulase enzymes producing
glucose from the cellulose and *R. oryzae* simultaneously assimilated the glucose to ethanol. We have summarized the SSF experiment with *R. oryzae* in Fig. 1.

The results presented in Fig. 1 indicate that *R. oryzae* can produce ethanol from oil palm empty fruit bunch. The production of ethanol has occurred after 24 h. In the first 24 h, *R. oryzae* still in the lag phase and trying to adaptable with EFB, in this time only small ethanol concentration can be produced. This figure also shows increasing of glucose concentration during the first 24 h. It is mean that cellulase enzyme was converting cellulose in to glucose monomer and only small glucose concentration was assimilated by *R. oryzae*. After 24 h, *R. oryzae* was growth in log phase, and then ethanol concentration was increased and reached maximum concentration at 96 h with 33.08 g/L ethanol concentration. Furthermore, decreasing of glucose concentration caused by consumption coincided with increasing of ethanol concentration.

![Figure 1. Ethanol and glucose concentration profile during SSF of pretreated EFB with *Rhizopus oryzae*.](image)

Glucose concentration remained less than 1.0 g/L within the 96 h in which ethanol reached its maximum concentration. The similar result was obtained by Karimi *et al.* (2005) that the production of ethanol has occurred in the first 2-3 days. Furthermore, *R. oryzae* also produce lactic acid as other metabolite products (data was not shown). *R. oryzae* produced ethanol from different sugar resource, under anaerobic or oxygen limiting conditions (Scory *et al.*, 1997; Taherzadeh *et al.*, 2003). *R. oryzae*
converted glucose to ethanol by *Embden-Mayerhof* pathway or often referred as glycolysis. In glycolysis one mole of glucose is converted to two moles of pyruvate via 10 enzymatic steps. Pyruvat in *R. oryzae* can be converted to three metabolites, which were ethanol by *Pyruvate decarboxylase* (PDC) and *Alcohol dehydrogenase* (ADH) enzymes, fumarat by *Fumarase* enzyme and lactic acid by *Lactate dehydrogenase* (LDH) enzyme (Buyukkileci et al., 2006). Ethanol was preferred producing in anaerobic conditions (Scory et al., 1997).

The selected filamentous fungi have some advantages, which are able to grow at higher temperature than *Saccharomyces cerevisiae* (Milati et al., 2005). Another advantage of the used strains of filamentous fungi is the valuable products that could be produced from their biomass, e.g. a super absorbent material or glucosamine (Milati et al., 2005).

### 3.2. Effect of Growth pH

In order to determine the impact of growth pH on the saccharification and fermentation of ethanol by the *R. oryzae*, the growth pH was adjusted in the range at 4.5, 5.0, and 5.5 by adding NaOH (2 N) solution. The previous investigation proved that this fungus species demonstrated a very active enzymatic saccharification during the first 8 h cultivation (Huang et al., 2005). Therefore, glucose and ethanol concentration in culture were measured every 24 h. The results presented in Fig. 2 indicate that *R. oryzae* can produce ethanol from empty fruit bunch in the pH range from 4.5 to 5.5, and the SSF process were influenced obviously by the growth pH.

The glucose concentration profiles are shown in Fig. 2. Every growth pH gave the increasing glucose in the first 24 h, and started to decrease after 24 h until 96 h. The higher glucose reduction was in growth pH 5.0. Same like the ethanol concentration, in the lowest pH (pH 4.5) the glucose consumption by *R. oryzae* was less than other growth pH. The production of ethanol decreased as pH decreased from 6.0 to 4.0 (Zhang et al., 2007). The highest ethanol yields was reached in pH 5.0 in the range pH 4.5 to 5.5 with ethanol concentration was 33.08 g/L medium, and the ethanol yields on pH 4.5 and 5.5 were 20.51 and 26.78 g/L medium.

Fig. 3 shows the ethanol concentration in variation of growth pH. *R. oryzae* had a higher ethanol concentration at growth pH 5.0. It was interested to note that the ethanol concentration increased with the increase in the growth pH from 4.5 to 5.0, but it was decreased in the growth pH 5.5. The ethanol concentration had a peak in the pH 5.0 means that growth pH 5.0 is the optimal pH for SSF process for ethanol production from oil palm empty fruit bunch with *R. oryzae* in the range pH 4.5 to 5.5. In the growth pH 5.5, ethanol yield has decrease 19.05% from yield in the growth pH 5.0, but it’s still higher than ethanol yield in the growth pH 4.5 reached 38.01%. The growth pH 4.5 is
too acid for SSF process with *R. oryzae*, so its pH is unfavorable for *R. oryzae* to convert glucose to ethanol (Huang *et al.*, 2005).

![Figure 2](image1.png)

**Figure 2.** Ethanol concentration (filled marker) and glucose concentration (blank marker) profile during SSF of pretreated EFB with *R. oryzae* in pH 4.5 (▲/▲), pH 5.0 (■/□) and pH 5.5 (●/○).

![Figure 3](image2.png)

**Figure 3.** Ethanol concentration by SSF of pretreated EFB with *R. oryzae* in variation of growth pH.

The summary data process SSF of pretreated EFB in variation growth pH was shown in Table 1. The yield ethanol concentration based on cellulose content in the EFB was 0.23, 0.37, and 0.30 g/g cellulose at growth pH 4.5, 5.0 and 5.5. In growth pH 5.0, the result is similar with one of the *R. oryzae* strain in Millati’s result that ethanol yield with *R. oryzae* reached 0.37 g/g from glucose, but it’s higher
than Abedinifar’s result that ethanol yield was 0.33 g/g using rice straw (Abedinifar et al., 2009; Millati et al., 2002). The maximum theoretical ethanol yield describes the comparison of ethanol concentration by experiment to ethanol concentration based on theoretical calculation. The higher result is only reached 64.05% growth pH 5.0 which is the highest result than other growth pH (4.5 to 5.5).

Table 1. The yield of ethanol in simultaneous and fermentation (SSF) of pretreated empty fruit bunch with *R. oryzae*

<table>
<thead>
<tr>
<th>pH</th>
<th>Max. ethanol concentration (g/L)</th>
<th>Ethanol yield based on cellulose (g/g cellulose)a</th>
<th>Ethanol yield based on pretreated EFB (g/g pret-EBF)</th>
<th>Max theoretical ethanol yield (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>20.51</td>
<td>0.23</td>
<td>0.14</td>
<td>39.98</td>
</tr>
<tr>
<td>5.0</td>
<td>33.08</td>
<td>0.37</td>
<td>0.22</td>
<td>64.50</td>
</tr>
<tr>
<td>5.5</td>
<td>26.78</td>
<td>0.30</td>
<td>0.18</td>
<td>52.21</td>
</tr>
</tbody>
</table>

Note: a ethanol yield based on cellulose = max ethanol/cellulose fraction in EFB (0.6034); b ethanol yield based on EFB = max ethanol/DM EFB (150 g/l); c max theoretical ethanol yield = [max ethanol]/(0.51 × 1.111 × dry weight of EFB × F) × 100, F = cellulose fraction in biomass (0.6034).

4. Conclusions

We may conclude that oil palm empty fruit bunch can be successfully converted to ethanol by the SSF process. *R. oryzae* are able to produce ethanol in the high yield. Therefore, this species seems to be a good alternative to *Saccharomyces cerevisiae* in production of ethanol from lignocelluloses and particularly oil palm empty fruit bunch.

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