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バージョン：

Ethanol Production by Encapsulated *Rhizopus oryzae* from Oil Palm Empty Fruit Bunch

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Abstract: Oil-palm empty fruit bunch (EFB) is abundant from palm-oil industries. One potential utilization of *Rhizopus oryzae* is the saccharification and fermentation process (SSF) for ethanol and lactic acid production. However, there some problems related to the optimum temperature and pH tolerance of *R. oryzae*. Encapsulating *R. oryzae* can help to improve the SSF process. The purpose of this research was to encapsulate the *R. oryzae* with a calcium alginate polymer to improve the tolerance of *R. oryzae* cells, which are required to convert EFB to bioethanol, in varying pHs and temperatures. The capsules were tested for their adaptability in high temperatures and varying mid-range pH. The resulting data from the experiment showed that the encapsulation of *R. oryzae* increased the production of bioethanol, from pretreated EFB through SSF, by 17 % compared to that produced by *R. oryzae* free cells. The highest yield of ethanol from pretreated EFB was approximately 0.43 g/g cellulose, with a maximum ethanol yield of 75.89%, theoretically.

Keywords: *cellulase, empty fruit bunch, encapsulation, lignocellulose, Rhizopus oryzae.*

1. Introduction

The cellulosic fraction of lignocelluloses is biologically converted to ethanol via two processes: first, enzymatic saccharification converts cellulose to glucose monomers, and then, glucose fermentation by fungi converts glucose to ethanol. These two steps either occur separately in different chambers, in a process referred to as separate enzymatic hydrolysis and fermentation (SHF) processes, or simultaneously in a fermentor, via a process referred to as simultaneous saccharification and fermentation (SSF). The SSF process has some direct benefits. The fermentation of saccharides immediately after saccharification reduces fermentation retardation caused by monosaccharide or disaccharide accumulation [1, 2, 3]. This increases the saccharification and fermentation rates, which improve productivity, thus reducing the reactor volume and capital costs [4]. However, other factors, such as differences between saccharification and fermentation for the optimum pH and temperature, affect the efficiency of SSF [5]. The optimum temperature for saccharification is 40 °C–45 °C, whereas that for fermentation is 30 °C [4]. Furthermore, bare yeast is sometimes exposed to harsh external conditions during the industrial fermentation [6].

Several attempts have been made to solve those problems. One of these is the cell encapsulation technique [6, 7, 8, 9], which isolates the microorganisms and creates a barrier between them and their environment. Liquids, semi-liquids (gels) or solid films are often used to shroud individual cells or tissues to protect them and to achieve greater stability in the laboratory and industrial applications. Encapsulation uses polymers and biopolymers such as sodium alginate. The main reason for the use of polymer encapsulation is its ability to withstand different phases, such as liquid, gel, or solid, which enables it to have sufficient physical and mechanical strength [7]. Encapsulated cells have been shown to increase the production of ethanol from dilute acid wood waste [9]. This technique does not affect the metabolism and viability of the cell used [6]. Additionally, in

comparison with free cells, encapsulated *R.oryzae* have been shown to increase the production of lactic acid [10] and ethanol [11]. Some researchers have successfully increased the ethanol production from rice straw to produce ethanol in anaerobic and aerobic conditions using *R. oryzae*, *Mucor indicus* and *Saccharomyces cerevisiae* [12, 13].

R. oryzae has several advantages over other microorganisms; it is capable of producing ethanol from spent sulfite liquor, assimilates xylose and all major hexoses in the liquor, and is tolerant to inhibitors in acid hydrolysates–lignocellulose [12]. In this study, we aimed to produce ethanol from oil palm empty fruit bunch (EFB) via SSF using *R. oryzae*. The goal of this research was to increase the temperature tolerance of *R. oryzae* cells by encapsulating them with calcium alginate polymer. These capsules were examined for their adaptive ability to high temperature and different mid-range pH.

2. Materials and Method

2.1. Preparation

Oil palm EFB was acquired from a state-owned company PTPN VIII in Malimping, Banten province, Indonesia. The EFB was then reduced in size to 1–3 mm before autoclaving in 10% NaOH solution under 150 °C and 4 atm for 30 minutes. To neutralize the alkaline solution, the EFB was then cleaned with tap water. This pretreated EFB was kept at room temperature until required for SSF.

R. oryzae, acquired from SITH-ITB, Indonesia, was incubated for 48 h on potato dextrose agar at 32 °C, and preserved as a culture stock at 4 °C. *R. oryzae* from the culture stock was mixed into 100 mL pre-sterilized medium of glucose (10 g/L), yeast extract (1.0 g/L), KH_2PO_4 (0.1 g/L), $MgSO_4.7H_2O$ (0.1 g/L), and $(NH_4)_2SO_4$ (0.1 g/L), and then cultivated in incubation at 32 °C for 7 d on an orbital shaker at 150 rpm to instigate sporulation. The medium was sterilized in a 250 mL Erlenmeyer flask by autoclaving at 121 °C for 15 min.

Commercial enzymes Ctec2 (Novozymes) were the single enzymatic complex used in this analysis. Its activity was measured as FPU. The enzyme displayed 70 FPU/mL of activity. One unit of activity is specified as the number of enzymes generating 1 μmol of glucose per minute from filter paper [14].

2.2. Encapsulation of *R. oryzae*

Spores were centrifuged and diluted after cultivation to achieve the optimal concentration of spores which was measured using a hemocytometer. *R. oryzae* spores were suspended in 1% $CaCl_2$ solution at a concentration of 1×10^8 cells/cm^3. This mixture was added drop-wise into a sterile solution of 0.5% sodium alginate, which was stirred using a magnetic stirrer to produce capsules with an average diameter of 2–3 mm.

2.3. SSF process

Medium containing 2.5 g/L yeast extract, 2.5 g/L peptone, 1.0 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 150.0 g/L pretreated oil-palm EFB and 0.05 M citrate buffer was used for the SSF process in 250 mL Erlenmeyer flasks. The pH of the medium was adjusted to 4.5, 5.0, and 5.5 by introducing 2 N NaOH or 1 N acetic acid. After the sterilization of the media, encapsulated *R. oryzae* and the required enzyme were added aseptically to each flask. The protein loading was 20 FPU/g pre-treated EFB. Each flask had a final volume of 100 mL. All experiments were carried out in triplicate. The SSF process was performed under anaerobic conditions. During the process, the flasks were covered with a plastic cup in anticipation of the gas emitted and to prevent air from entering the flask. At the outset of the fermentation, and during processing, pure nitrogen gas was purged into the media. The SSF process was performed at 37 °C and 150 rpm in a shaker incubator for 96 h.

2.4. Glucose and ethanol analysis

The composition of the EFB was tested in conjunction with NREL Chemical Analysis and Testing Procedure [15]. Samples were collected every 24 h from the SSF process and analyzed through high-performance liquid chromatography (Waters 2695, Milford, MA). An Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) and RI Detector (Waters, 2414) were used to analyze glucose and ethanol at 65 °C with 0.6 mL/min eluent of 5 mM sulphuric acid along 25 min retention time.

2.5. Statistical analysis

The resulting data was analyzed statistically using an alysis of variance to evaluate the effects of pH on ethanol production while using *R. oryzae*.

3. Result and Discussion

3.1. Pretreatment of oil palm EFB

The chemical composition of oil palm EFB in this study is listed in Table 1. Before the total pretreatment of carbohydrates in the oil palm, EFB was 48.86% and lignin fraction was 26.53% of dry biomass. The cellulose content after pretreatment increased from 33.64% to 60.34%, whereas the lignin content decreased from untreated EFB to 47.14%. The high cellulose content in pretreated EFB facilitates its conversion to bioethanol [15].

Table 1. Chemical composition of untreated and pretreated oil palm empty fruit bunch (EFB)

Composition	Untreated EFB (%)	Pretreated EFB (%)
Lignin	37.84	20.00
Cellulose	33.64	60.34
Hemicellulose	15.22	11.52
Other	13.30	8.14

3.2. Ethanol production via SSF

The SSF of pretreated EFB was carried out under anaerobic conditions. Cellulose fraction in EFB was converted to glucose using cellulase enzymes, and *R. oryzae* simultaneously converted glucose to ethanol. At the beginning of SSF, the mixture contained 20 g/L glucose, which was derived from glucose by the cellulase enzymes, and β-glucosidase was added to the SSF process (Fig. 1). Glucose concentration increased in the first 24 h and then decreased until the end of the SSF process, indicating the saccharification/hydrolysis of the long carbon chains of cellulose in pretreated EFB to glucose monomers by cellulase enzymes and β-glucosidase [12]. Enzymatic saccharification works specifically for cellulose to break down long chains of glucose monomers so that the resulting glucose production would be more optimal. Glucose concentration decreased to 1 g/L by the end of the SSF process, i.e., at 96 h, indicating that glucose was converted by *R. oryzae* during SSF process.

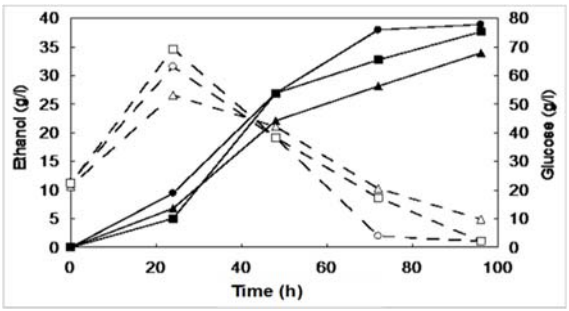

Fig. 1. Glucose and ethanol production using encapsulated *R. oryzae* at variable pH. Empty symbols indicate the glucose concentration, and filled symbols indicate ethanol concentration. Triangles, circles, and squares represent pH 4.5, 5.0, and 5.5, respectively.

Ethanol production began before 24 h. In the first 24 h, ethanol concentration was low possibly because *R. oryzae* cells were in the process of adapting to pretreated EFB media. After 24 h, ethanol production increased and reached a maximum at 96 h. Ethanol concentration at pH 4.5, 5.0, and 5.5 was 20.51, 33.08, and 26.78 g/L, respectively. The highest ethanol concentration was obtained at pH 5.0, which is the optimum pH for saccharification [7,16].

In the encapsulated cells, glucose is metabolized by *R. oryzae* present within the calcium alginate capsules. Glucose diffuses into the capsule through the calcium alginate walls, and ethanol produced from the fermentation process diffuses out through the capsule wall. Alginate concentrations are optimized to ensure proper diffusion of glucose and ethanol in or out of the capsule through the calcium alginate wall.

Encapsulation protects the *R. oryzae* cell from high ethanol concentration as well as the metabolites and toxic byproducts of saccharification and fermentation. The production of glucose and ethanol using encapsulation *R. oryzae* is shown in Fig. 1. The concentration of ethanol produced by encapsulated *R. oryzae* was 38.92 g/L at pH 5.0, 33.92 g/L at pH 4.5, and 37.66 g/L at pH 5.5. Ethanol concentrations at various pH were significantly different ($P < 0.05$). These results are higher than those using free *R. oryzae* cells during the SSF process [17].

3.3. Effect of pH on ethanol production using encapsulated *R. oryzae*

The concentration of ethanol produced from pretreated EFB via the SSF process using encapsulated *R. oryzae* was higher than that using free *R. oryzae* cells of in our previous work (Fig. 2). The concentrations of ethanol produced by encapsulated *R. oryzae* at pH 4.5, 5.0, and 5.5 in a row were 33.92, 38.92, and 37.66 g/L. These concentrations were approximately 17% to 65% higher than those using free *R. oryzae* cells.

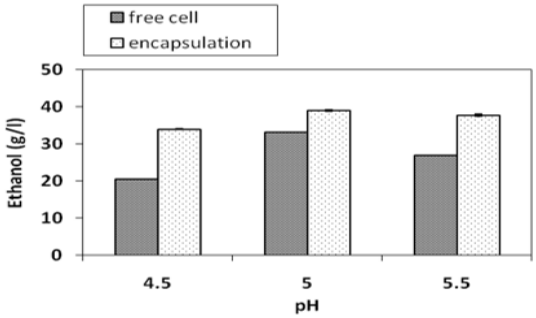

Fig 2. Comparison of ethanol production using free and encapsulated Rhizopus *oryzae* at variable pH.

3.4. Glucose and ethanol analysis

R. oryzae is a filamentous fungus, which forms hyphae during growth. Although *R. oryzae* is known as the fungus of Tempe (Indonesian fermented food), the metabolism of *R. oryzae* through the glycolysis produces lactic acid and ethanol. *R. oryzae* converts glucose to pyruvic acid via the Embden–Meyers pathway. Glycolysis breaks down 1 mol of glucose to 2 mol of pyruvic acid via 10 enzymatic steps in the cell. Pyruvic acid is converted to three types of metabolites using different enzymes: fumarate by fumarase, lactic acid by lactate dehydrogenase (LDH) and pyruvate decarboxylase enzyme of ethanol with (PDC) and alcohol dehydrogenase (ADH). In anaerobic condition, *R. oryzae* tends to produce ethanol [19].

Cell encapsulation of *R. oryzae* proved to be effective in increasing the concentration of ethanol produced from pretreated EFB via the SSF process. Encapsulated cells are more resistant to adverse environmental conditions than free cells; in this study, adverse environmental conditions were represented by the pH during the SSF process. Encapsulation also protects the cells using artificial cell walls, which provide resistance to the cells against acidic conditions in solution. High pH induces

stress on microorganisms, thus affecting their metabolism, whereas acidic pH slows down their metabolism run slower [18]. Methods using free cells of *R. oryzae* are very susceptible to changes in pH; a little change in pH lowers ethanol production, whereas encapsulated cells are more resistant to pH changes.

The results of this study approached those of Karimi *et al.* (2006) to get the max theoretical yield of about 60% of the rice straw substrate [13]. Theoretically, the maximum glucose concentration generated by cellulase enzymes is 90 g/L, whereas the maximum ethanol content is 45 g/L. In this study, the maximum ethanol concentration was 33 g/L. It is good results if for fermentation using *R. oryzae*.

The production of ethanol from cellulose using encapsulated *R. oryzae* was higher than that using free cells; cell encapsulation increased ethanol production by 17% compared with free cells. Comparison of ethanol produced by research with theoretical reached 75.89% at pH 5.0; the result is higher than the free cells as well as research by Karimi *et al.* [13]. However, the encapsulation of cells is still slightly smaller than the result of cell encapsulation of *S. cerevisiae* by Ylitervo *et al.* (2011) [9]. It conducted fermentation with encapsulated *S. cerevisiae* in 30 g/L glucose medium and produced ethanol (13.5 g/L) and the yield of 0.44 g ethanol/g glucose.

The effect of pH of the medium on the production of ethanol was similar between encapsulated and free cells. Ethanol production was the highest ethanol production at pH 5.0, regardless of the type of cells used. This suggests that pH 5.0 is optimum for the SSF of pretreated EFB. At optimum pH, *R. oryzae* cells consume glucose at a faster rate, and the rate of ethanol production is higher.

The yield of ethanol using free and encapsulated *R. oryzae* is shown in Table 2.

Table 2. Concentration of ethanol produced from oil palm EFB via simultaneous SSF using *R. oryzae* cells encapsulated in calcium alginate polymer.

pH	Maximum ethanol concentration (g/L)	Ethanol yield based on cellulose (g/g cellulose)[a]	Ethanol yield based on pre-EFB (g/g pre-EFB)[b]	Maximum theoretical ethanol yield (%)[c]
4.5	33.9	0.4	0.2	66.1
5.0	38.9	0.4	0.3	75.9
5.5	37.7	0.4	0.3	73.4

[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] Maximum theoretical ethanol yield = [max ethanol]/(0.51 × 1.111 × dry weight of EFB × F) × 100, F = cellulose fraction in biomass (0.6034).

The highest concentration of ethanol was produced after 96 h, and the ethanol yield was based on cellulose content of pretreated EFB. Table 2 also compares the ethanol produced in this study with that produced according to a theoretical calculation. The results of the conversion of an existing substrate by free cells *R. oryzae* showed that the conversion is still quite small compared to the theoretical; mm cell *R. oryzae* only reached 65% at pH 5.0 and 4.0 and 52% at pH 4.5 and 5.5.

The utilization of waste and side products of EFB production into bioethanol was the implementing sustainability in the industry, as it ensures the continuation of the fulfillment of human's basic needs [20]. In Japan, various types of research have been performed using biomass to overcome the crisis of energy [21]. EFB has the potential to be developed as bio-oil [22], production of xylose [23], the fiber as sustainable acoustic absorber [24], and hydrogen production [25].

4. Conclusion

Pretreated EFB is converted to ethanol via enzymatic hydrolysis followed by fermentation using *R. oryzae*. Encapsulation increases the resistance of the fungus against adverse conditions, such as pH, chemical and temperature. This study investigates the influence of *R. oryzae* encapsulated in calcium alginate on ethanol produced. Ethanol concentration was the highest at pH of 5.0 (38.92 g/L). Encapsulation of *R. oryzae* also increased its resistance to the temperature during fermentation, as the SSF process was carried out at 37 °C, which is higher than the commonly used temperature during fermentation (32 °C). Additionally, the encapsulation of *R. oryzae* cells increased the production of ethanol by 17% compared with that produced by free cells of *R. oryzae*.

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References

1) Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P., & Ballesteros, I, Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromyces marxianus CECT 10875, *Process Biochem*, **39**(12), 1843–1848 (2004).
2) Bélafi-Bakó, K., Koutinas, A., Nemestóthy, N., Gubicza, L., Webb, C, Continuous enzymatic cellulose hydrolysis in a tubular membrane bioreactor, Enzyme Microb. *Technol.*, **38**(1-2), 155–161 (2006).
3) Sakimoto, K., Kanna, M., & Matsumura, Y, Kinetic model of cellulose degradation using simultaneous saccharification and fermentation, *Biomass and Bioenergy*, **99**, 116–121 (2017).

4) Sun, Y., & Cheng, J, Hydrolysis of lignocellulosic materials for ethanol production : a review, *Bioresour. Technol.*, **83**(1), 1–11 (2002).
5) Huang, L. P., Jin, B., Lant, P., & Zhou, J, Simultaneous saccharification and fermentation of potato starch wastewater to lactic acid by Rhizopus oryzae and R. arrhizus, *Biochem. Eng. J.* **23**(3), 265–276 (2005).
6) Kampf, N, The use of polymers for coating of cells. Polym. *Adv. Technol.*, **13**(10–12), 895–904 (2002).
7) Talebnia, F., & Taherzadeh, M. J, In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated *S. cerevisiae*, *J. Biotechnol.*, **125**(3), 377–384 (2006).
8) Ullah, M. W., Khattak, W. A., Ul-Islam, M., Khan, S., & Park, J. K, Bio-ethanol production through simultaneous saccharification and fermentation using an encapsulated reconstituted cell-free enzyme system, *Biochem. Eng. J.*, **91**, 110–119 (2014).
9) Ylitervo, P., Franzén, C. J., & Taherzadeh, M. J, Ethanol production at elevated temperatures using encapsulation of yeast, *J. Biotechnol*., **156**(1), 22–29 (2011).
10) Haluk, H., & Ryu, D. D. Y. Production of L (+) - Lactic Acid Using Immobilized Rhizopus oryzae. *Appl. Biochem. Biotechnol*., **44**, 125–133 (1994).
11) Millati, R., Niklasson, C., & Taherzadeh, M. J, Effect of pH , time and temperature of verliming on detoxification of dilute-acid hydrolyzates for fermentation by Saccharomyces cerevisiae, *Process Biochem*., **38**, 515–522 (2002).
12) Abedinifar, S., Karimi, K., Khanahmadi, M., & Taherzadeh, M. J, Ethanol production by Mucor indicus and Rhizopus oryzae from rice straw by separate hydrolysis and fermentation, *Biomass Bioenergy*, **33**(5), 828–833 (2009).
13) Karimi, K., Emtiazi, G., & Taherzadeh, M. J, Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and S. cerevisiae, Enzyme Microb. *Technol.,* **40**(1), 138–144 (2006).
14) Adney, B., & Nrel, J. B, Measurement of cellulase activities: laboratory analytical procedure (LAP). *National Renewable Energy Laboratory*. Golden, Colo. (2008).
15) Kusrini, E., Supramono, D., Muhammad, I. A., Pranata, S., Wilson, D. L., & Usman, A. Effect of Polypropylene Plastic Waste as Co-feeding for Production of Pyrolysis Oil from Palm Empty Fruit Bunches. *Evergreen*, **6**(1), 92-97 (2019).
16) Ruiz, R., & Ehrman, T, Chemical Analysis & Testing Task, Determination of carbohydrate in Biomass by High-Performance Liquid Chromatography (Laboratory). Golden, Colo: *Nat. Ren. Energy Lab*. (1996).
17) Muryanto, Sahlan, M., & Sudiyani, Y, Simultaneous Saccharification and Fermentation of Oil Palm Empty Fruit Bunch for Bioethanol Production by Rhizopus oryzae, *Int. J. Environ. Bioener*. **3**(32), 111-120 (2012).
18) Sues, A., Millati, R., Edebo, L., Taherzadeh, M. J, Ethanol production from hexoses, pentoses, and dilute-acid hydrolyzate by Mucor indicus. *FEMS Yeast Research*, **5**(6), 669–676 (2005).
19) Samsuri, M., Gozan, M., Hermansyah, H., Prasetya, B., Nasikin, M., & Watanabe, T. Ethanol production from bagasse with combination of cellulasecellubiase in simultaneous saccharification and fermentation (SSF) using white rot fungi pre-treatment, *J. Chem. Nat. Resources Eng*, **3**, 20–32 (2008).
20) Zahara, Z. F., Economic Assessment of the Sugarcane-based Bio-refinery in Indonesia, *Evergreen*, **5**(2), 67-77 (2018).
21) Furutani, Y., Norinaga, K., Kudo, S., Hayashi, J. I., & Watanabe, T. Current Situation and Future Scope of Biomass Gasification in Japan. *Evergreen*, **4**(4), 24-29 (2017).
22) Abdullah, N., & Gerhauser, H. Bio-oil derived from empty fruit bunches. *Fuel*, **87**(12), 2606-2613 (2008).
23) Rahman, S. H. A., Choudhury, J. P., & Ahmad, A. L. Production of xylose from oil palm empty fruit bunch fiber using sulfuric acid. *Biochemical Engineering Journal*, **30**(1), 97-103 (2006).
24) Or, K. H., Putra, A., & Selamat, M. Z. Oil palm empty fruit bunch fibres as sustainable acoustic absorber. *Applied Acoustics*, **119**, 9-16 (2017).
25) Sivasangar, S., Zainal, Z., Salmiaton, A., & Taufiq-Yap, Y. H. Supercritical water gasification of empty fruit bunches from oil palm for hydrogen production. *Fuel*, **143**, 563-569 (2015).