Enhancing the efficiency of ethanol production from molasses using immobilized commercial *Saccharomyces cerevisiae* in two layer alginate-chitosan beads

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Abstract. The commercial *Saccharomyces cerevisiae* has been successfully inoculated and immobilized in the present study. The immobilization was conducted through a double layer encapsulated method in which 3% of alginate and 1% of chitosan become the first and second layer, respectively. The light microscopy confirmed that the inoculated-cell was dispersed and spread well in the alginate layer as the first layer. The interaction between alginate-chitosan was confirmed by Fourier Transform Infra-Red (FTIR), and the results demonstrated the physical interaction without forming any chemical interaction and binding in the immobilized cell. The immobilized cell was then used for producing ethanol from molasses through the batch fermentation process. The immobilized cell can successfully convert the glucose to ethanol and used for three cycles of fermentation before finally being saturated. The saturated was generated by breaking down of the chitosan layer generated by cell proliferation. The layer breakage forms the fouling as the effect of absorbed molasses in the surface of layer. However, the fermentation using immobilization cells provides the high-efficiency process by converting high percentages of ethanol yield production and solve the post-treatment process.

1. Introduction

World growing population has generated excessive consumption of fossil fuel applying in industries, transportation, etc., particularly in the developing country such as Indonesia. The fossil fuel consumption has also reported in the generation of high-level pollution and reduce the environmental level during the last few decades. For this reason, the renewable energy or green fuel may make a great solution to substitute the fossil fuel and reduce the fossil consumption. Ethanol (C₂H₅OH) was attracting growing interest all over the world due to its physicochemical properties which show a promising to be a future green fuel [1–3]. Ethanol has many advantages compared to fossil-based fuel such as non-toxic, providing an unlimited source, having high-density energy, high octane number, and low particulate emission [4,5]. Thus, the substituting fossil fuel to ethanol could not only solve the problem of fuel demand but also generating the effective and environmentally friendly fuel.
In general, ethanol is commonly produced using chemical synthesis and biochemical synthesis [6,7]. In chemical synthesis, the ethylene gas (C\textsubscript{2}H\textsubscript{2}) is reacted with water vapor using phosphoric acid in high combustion temperature resulting in high purity ethanol. This process has been applied to synthesize most of the commercial and high purity ethanol. However, the chemical synthesis has some disadvantages such as relatively high cost, using ethylene gas which basically produced from fossil fuel, and need high energy consumption. In the other way, ethanol could be synthesized using biochemical synthesis through the fermentation process. This process is composed by enzymatic hydrolysis of polysaccharide to produce a simple sugar (i) followed by fermentation of simple sugar such ideally glucose which generates ethanol as the main product and carbon dioxide as a byproduct [1,3,4] Figure 1). This process is conducted in room temperature with the facultative condition which provides an environmentally benign process. However, the biochemical synthesis commonly used a free cell which could easily death due to the inhibition of as-generated ethanol from the fermentation. The as-generated ethanol becomes the inhibition product which reduces the bioactivity of cell or yeast resulting low efficiency of substrate utilization and ethanol production as the product.

![Figure 1. The main mechanism of hydrolysis and fermentation of Polysaccharides](image)

The immobilization of cell has been reported as the way to solve the commercial fermentation and improve the efficiency of fermentation process resulting high ethanol production. The immobilization cell provides the cell close together and forced to aggregate by providing the limited space to a cell to move and grow up [3]. Therefore, it could affect their metabolism and growth. In addition, the study of immobilization has been proved to solve the recovery process of fermentation and increases the cell tolerance, indicated by high yield production compared to the free cell fermentation production.

The immobilization could be conducted by using polysaccharides such as Alginate and Chitosan. Alginate is a natural copolymer composed of two types of monosaccharide, 1,4-linked-\beta-D-mannuronic acid (M-block) and 1,4-linked-\alpha-L-guluronic acid (G-block). One of alginate salt which commonly used as the polymer to immobilize cell is sodium alginate (Na-Alginate). Na-Alginate, a water-soluble salt, is gelled when it is reacted with a bi-valent cation such as calcium ion (Ca\textsuperscript{2+}). There are many reports about the detailed experimental in the formation of alginate as the immobilized
material for entrapped cells [8,9]. However, the ability of alginate to immobilize the growth cell have to be enhanced to form the strength bead to trap the cell for long time uses.

The other polysaccharide which commonly used to entrap the cell is chitosan. Chitosan is the second abundant biopolymer in nature after biomass which arranged by an empirical structure of β-(1,4)-linked-D-glucosamine and N-acetyl-D-glucosamine which randomly distributed in complex structure and derivate by deacetylation of chitin which covers the surface body of arthropods and also coral, algae, and nematode [10,11]. Chitosan is reported as the excellent material for immobilizing the cell due to its physicochemical properties such as adsorption capacity, high degree crystallinity, solubility, reactivity, hydrophobicity, and acid-base properties [12–14].

Herein, the present study aimed to immobilize the commercial yeast *Saccharomyces cerevisiae* using the combination of the alginate-chitosan bead. The immobilized cell would be used in the fermentation of molasses in order to produce ethanol. The bead stability as the function of cell use would be detailed determined. Some parameters such as bead surface and the number of the released cells are well controlled. The successful immobilization of *Saccharomyces cerevisiae* using alginate-chitosan could be an innovative way to improve the ethanol production from waste-based fermentation to support the independence of energy fuel support

2. Method

2.1. Materials

Molasses was obtained from the local sugar factory in North Sumatera, Indonesia and become the main nutrient (substrate) to produce the ethanol. Commercial yeast used for this study was obtained from commercial baked yeast (Fermipan). The growth cell mediums such as Potato Dextrose Agar, yeast extract, and peptone were purchased from Merck with high purity without further purification. Alginate, K2HPO4, KH2PO4, MgSO4.7H2O, Acetic acid, Citrate acid were purchased by E-Merck with analysis purpose classification. The chitosan was supported by the Research Laboratory of Mathematics and Natural Science Faculty of Universitas Sumatera Utara).

2.2. Yeast strain and medium preparation

The instant yeast was dissolved by the sterilized water and well dispersed using vortex mixer (BioRad-2000) for 1 minute. The yeast solution was cultured by dispersing the suspended cell solution into the potato dextrose agar medium. The medium was kept for 48 hours at 30°C. The growth cell was further regrowth into liquid growth medium of yeast peptone glucose for 48 hours in a rotary shaker (150 rpm) at ambient temperature. The cultured cells were collected by centrifugation at 7500 rpm for 10 minutes at 4°C. The obtained product was washed for several times using sterilized water and dissolved into 0.9% of Sodium Chloride solution to form approximately 15 x 10⁶ cell/mL. The fermentation medium was contained by 142.95 g/L molasses as carbon and energy source, 1.00 g/L K2HPO4, 1.00 g/L KH2PO4, and 0.20 g/L MgSO4. The fermentation medium was adjusted to pH 4 using 2M HCl and kept to pH 4 using citrate buffer. The medium was further sterilized at 121°C for 30 minutes.

2.3. Immobilization of *Saccharomyces cerevisiae* in Alginate-chitosan beads

The as-prepared cells were immobilized into 2 layers of alginate-chitosan bead using encapsulation method. In the first layer, 10 ml the mixture of cultured cells solution was added onto 50 ml of 3% sodium alginate solution and stirred for 30 minutes at 200 rpm. Furthermore, the mixture solution was carefully dropped to 2% of CaCl2 solution using a sterilized syringe. The dropped solution formed the hard and porous beads and kept to the CaCl2 solution for 30 minutes. To form the chitosan layer, the beads were further immersed into 100 mL of 1% of chitosan-CaCl2 solution and kept for 12 hours without stirring. Finally, the bead was filtered and washed with 0.9% of NaCl solution and stored at 0.2 % of yeast extract solution at 4°C of temperature.

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2.4. Fermentation of Molasses using immobilized Saccharomyces cerevisiae

150 g of immobilized S. cerevisiae was incubated into 2% of yeast extract solution for 15 minutes at 30°C and filtered. The bead was mixed to 250 mL sterile fermentation medium and fermented for 12 hours in ambient temperature. After the fermentation test, the bead was separated and washed using sterilized water. The used bead was incubated in 2% of yeast extract solution for 15 minutes before reused for the further fermentation.

2.5. Characterization

The cell distribution and surface images of the as-prepared bead and the used bead were characterized using light microscopy. The interaction between chitosan, alginate, and the cell was investigated using Fourier Transform Infrared spectroscopy (FTIR). The conversion of glucose during the fermentation was confirmed as the disappearance of glucose concentration controlled by Lane-Eynon method. The ethanol production was measured using Gas Chromatography (Shimadzu QP2010).

3. Results and Discussions

3.1. The calculation of immobilized cell

The amount of immobilized cell was calculated using the calculation of cell which contained in the culture of S. cerevisiae medium, CaCl2 solution, and Chitosan-CaCl2 solution confirmed by Haemocytometer (Marienfield). This approach was based on the possibilities of cell released during the bead formation. First, the mixed solution of culture and alginate solution was dropped to the CaCl2 solution, and there were the cells trapped to alginate beads and also release to the CaCl2 solution. The entrapped cell was calculated by measuring the total cell in culture media minus the released cell which contained to CaCl2 solution (Equation 1)

Total cell in Alginate Bead = Total cell in the culture medium – Total cell in CaCl2 solution (1)
Total cell in Alginate Bead = 15.8 x 10^9 cells – 0.704 x 10^9 cells
Total cell in Alginate Bead = 15.096 x 10^9 cells

The second path was that alginate beads were immersed in chitosan-CaCl2 solution. The released cells were predicted to release when the alginate bead was immersed and stirred in chitosan-CaCl2 solution. Therefore, the cell which contains in the chitosan-CaCl2 solution should be calculated as the released cell. The final amount of trapped cell was calculated by measure the total cell that trapped to alginate bead minus the released cell which contained to chitosan-CaCl2 solution (Equation 2)

Total immobilized cell = Total cell in alginate bead – total cell in Chitosan-CaCl2 solution (2)
Total immobilized cell = 15.096 x 10^9 cells – 0.544 x 10^9 cells
Total immobilized cell = 14.522 x 10^9 cells

All the immobilized cell was used to the fermentation of glucose to produce ethanol. The total cells which released during the fermentation were not calculated because the total cell was not constant due to their proliferation and metabolism.

3.2. Characterization of immobilized S. cerevisiae

The bead interaction of alginate-chitosan was further analyzed using FTIR spectroscopy. The FTIR spectra showed the fingerprint broadband of alginate chains in 3247.13 cm⁻¹, 1594.95 cm⁻¹, 1408.04 cm⁻¹, 1018.80 cm⁻¹ that correspond to hydroxyl (OH) group, the antisymmetric stretch of carboxylic (COO) group, the symmetric stretch of carboxylic (COO) group, and carbonyl (C=O) group, respectively [15,16]. The fingerprint of chitosan chain was also detected in 3361.17 cm⁻¹ which
correspond to the primary amine (NH₂), 1588.93 cm⁻¹ to the amine group in NHCOCH₃ group, 1376 cm⁻¹ to the methyl (CH₃) group in NHCOCH₃ group, and 1025.25 cm⁻¹ correspond to carbonyl (C=O) group [11,16]. The investigation was further conducted in the alginate-chitosan bead and the result showed no significant shifts of each compounds peaks indicating the physical interaction between alginate-chitosan beads. However, there was the decreasing intensity of broadband in the FTIR spectrum was generated by the water contain the sample in which the bead sample containing high water content compared to the chitosan and alginate samples.

![FT-IR spectrum of Chitosan, Alginate, and Immobilized Alginate-Chitosan Bead](image)

Figure 2. FT-IR spectrum of Chitosan, Alginate, and Immobilized Alginate-Chitosan Bead

Glucose as carbon and energy source for immobilized cell had been consumed during the fermentation. The fermentation was carried out three times using the same immobilized cell. The fermentation showed the reducing of glucose concentration after each fermentation. The glucose consumption in pre-fermentation was adjusted to 142.95 g/L and no glucose consumption and ethanol production appeared. In the first fermentation, the immobilized cell showed excellent work by converting 12.2 g/L to 37.54 % ethanol (C₂H₅OH) and carbon dioxide (CO₂). The high glucose consumption and ethanol production were caused by the immobilization of cells that kept the cells close together and were forced to aggregate [3]. However, In the second and third fermentation, there were decreasing in the glucose consumption and ethanol production of the immobilized cell wherein they had only consumed 5.33 g/L and 4.89 g/L for glucose consumption and 26.66 % and 13.29% for ethanol production, respectively (Table 1).

| Cycle | Glucose concentration (g/L) | Glucose consumption rate (g/L) | Retention time (h) | Ethanol production (v/v) |
|-------|-----------------------------|-------------------------------|-------------------|-------------------------|
| 0     | 142.95                      | -                            | -                 | -                       |
| 1     | 130.75                      | 12.20                        | 4.858             | 37.54                   |
| 2     | 125.42                      | 5.33                         | 4.856             | 26.66                   |
| 3     | 120.53                      | 4.89                         | 4.890             | 13.29                   |
3.3. **Effect of fermentation on the surface breakage**

The analysis of surface breakage was carried out after each fermentation. Before fermentation, the bead had a rough surface which caused by the surplus CaCl₂, which not associated with complexation of Chitosan-Alginate (Figure 3A) [17]. There was no hole or breakage on the surface before the fermentation. However, during the fermentation process, the bead surface broke and formed many holes and increase after the third cycle fermentation. The breakage of surface bead during the fermentation was generated by the cell proliferation [18]. In addition, ethanol evolution would also absorb the water of bead and make the bead surface weak and break. For the first fermentation, the outer layer of beads did not show the breakage and only turned to rougher surface area (Figure 3B) which predicted as the beginning of cell proliferation. It could show that the second layer could permit cell growth during the beginning of cell proliferation. After the second and third fermentation (Figure 3C and 3D). The bead surface became rougher and broke not only the outer layer but also the inner layer. The surface breakage and holes would release out the cell which directly contacted to suspension.

![Figure 3. Images of bead surface (A) before fermentation, and after (B) first fermentation; (C) second fermentation; and (d) third fermentation (Magnification at 40x)](image)

The decreasing of the performance of the immobilized cell was predicted as the surface breakage of beads. The surface breakage would release the cell to the medium which contained high glucose and ethanol concentration. These breakings would release more cell and could make ethanol stress in *s. cerevisiae* cell because ethanol was an inhibitor of yeast growth at relatively low concentration and increase cell death by reducing cell vitality [19,20]. The fast surface breakage was caused by the weak interaction between chitosan and alginate (physical interaction) which formed without using any cross-linking agent. The other possibility was predicted by the molasses medium that adsorbed to chitosan layer which could not be consumed by the cells. Thereby, the trapped-molasses in the chitosan layers
would break its surface and formed the hole to release the trapped molasses, it was the reason why the performance of immobilized cell decreased during the second and third fermentation.

4. Conclusions

*Saccharomyces cerevisiae* was successfully immobilized in the alginate-chitosan bead using entrapment method. The interaction between alginate, chitosan, and cell was a physical interaction which showed no shift in the FT-IR broadband. The immobilized cell showed excellent performance in the first fermentation process by converting 12.20 g/L glucose to be 37.54% of ethanol. This high yield of ethanol was not kept for the second and third fermentation due to the surface breakage of alginate-chitosan beads. The surface breakage would release more cell and could make ethanol stress in *s. cerevisiae* cell because ethanol was an inhibitor of yeast growth at relatively low concentration and increase cell death by reducing cell vitality.

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