Bioethanol production from sugarcane bagasse by 
*Saccharomyces cerevisiae* ATCC 9763 immobilized in Na-alginate

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**Abstract.** Currently the supply of fossil energy is running low; besides, it contributes very significantly to environmental pollution and climate change. Therefore, it is necessary to develop new and renewable alternative energy, one of which is bioethanol. One of the potential raw materials used in bioethanol production is sugarcane bagasse which is an abundance of agricultural waste. This study aims to determine the concentration of Na-alginate and the density of cell suspension with the best activity and stability of immobilized *Saccharomyces cerevisiae* ATCC 9763 cells in the bioethanol production process. This research is an experimental study using a randomized block design (RBD) with a factorial pattern consisting of two factors, namely the first factor is the concentration of Na-alginate and the second factor is the concentration of cells suspension in making immobilized *S. cerevisiae* cells. The first factor consists of 5 levels, namely 0%, 2%, 3%, 4%, and 5% (w/v). The second factor consisted of 3 levels, namely cells with OD$_{660}$ 20, 25, and 30. The parameters observed were the activity of immobilized *S. cerevisiae* cells including several variables, namely reducing sugar content, pH, and ethanol content. The stability of immobilized *S. cerevisiae* cells was seen from the level of cell turbidity (OD$_{660}$ nm). The results showed that the concentration of Na-alginate, the concentration of *S. cerevisiae* cells, and the interaction between treatments had a very significant effect on the activity and stability of immobilized *S. cerevisiae* cells. The treatment of Na-alginate 2% (w/v) with cell density 25 was able to produce ethanol with a higher level of 63.87 ppm compared to treatment with 0% Na-alginate (free cells) with the level of cell density 25 was 56.97 ppm.

1. Introduction

The energy crisis, air pollution and climate change are driving the development of non-fossil energy. One of the alternative energy which is environmentally friendly and renewable is bioethanol [1]. Bioethanol is not only an alternative to gasoline substitution, it is also able to reduce CO$_2$ emissions that endanger human health. CO$_2$ produced by engine waste will be absorbed by plants for their growth process. Furthermore, these plants are used as raw material for the bioethanol production so that there is no accumulation of carbon in the atmosphere [2].

Bioethanol can be produced from lignocellulosic materials such as agricultural waste, industrial waste, household waste, and other sources. The sugar cane industry produces waste in the form of bagasse, a potential raw material for bioethanol production, because it contains high cellulose and is already concentrated at the factory site. Bagasse has the highest cellulose content (52.7%) when
Bioethanol production from lignocellulosic biomass of agricultural waste includes delignification (pretreatment), hydrolysis (saccharification), ethanol fermentation and purification [4]. The conversion process of simple sugars from the saccharification process to ethanol generally uses microbes, both yeast and bacteria in the form of free cells. The use of free cells has several disadvantages including: it requires a lot of cells amount, cell viability decreases rapidly, separation of products and media is more difficult, and cannot be used repeatedly. To overcome this problem as an alternative is to use immobilized cells. In this study, immobilization of \textit{S. cerevisiae} cells was carried out using the entrapment method. Materials that can be used in the manufacture of immobilized cells include: Na-alginate, carrageenan, chitosan, pectin, polyvinyl alcohol (PVA), etc. [6]. In this case Na-alginate is used because in the manufacture of immobilized cells it is relatively easy and fast to form a uniform gel, the material is quite a lot available, especially in Indonesia which is rich in marine products, namely brown algae which is the basic material for making alginates, so that it is expected to increase the economic value of seaweed products [7].

In this experiment, a combination of treatments was carried out between variations in the concentration of Na-alginate and variations in the concentration of \textit{S. cerevisiae} cells. The size of the beads used is 2 mm where the immobilized cells are generally able to work optimally [7], [8], [9]. The experiment was carried out to determine changes in reducing sugar levels, changes in pH, ethanol produced and the stability of immobilized cells during fermentation. Research using the immobilized cell technique is expected to produce optimal ethanol by utilizing bagasse as a raw material so that it can produce an environmentally friendly renewable bioenergy.

2. Materials and methods

2.1. Materials

The materials used in ethanol production are bagasse taken from sugar factories in Sidoarjo, East Java, pure culture of \textit{Saccharomyces cerevisiae} ATCC 9763 obtained from the Bogor Agricultural University, \textit{Aspergillus niger} FNU 6018 obtained from the Microbiology Laboratory of Inter-University Center of Food and Nutrition, Gadjah Mada University. The chemicals used are: CaCl$_2$, Na-alginate, NaCl, HCl, NaOH, (NH$_4$)$_2$SO$_4$, KCl, MgCl$_2$, MgSO$_4$, urea, dinitrosaliclyc acid, potato dextrose agar (PDA), glucose, yeast extract, peptone, 70% alcohol, NPK, distilled water. All chemicals used are grade for analysis.

2.2. Experimental design

This research is an experimental study designed with a randomized block design (RBD) with a factorial pattern experiment consisting of two factors, namely first factor is the concentration of sodium alginate and second factor is the cells concentration of \textit{S. cerevisiae} in the manufacture of immobilized cells. In this study, observations were made on the effect of the combined treatment of sodium alginate concentration and \textit{S. cerevisiae} cell concentration on the stability and activity of immobilized \textit{S. cerevisiae} cells. First factor, the concentration of sodium alginate consists of 5 levels, namely: 0%, 2%, 3%, 4%, and 5% (w/v). Second factor, the concentration of \textit{S. cerevisiae} cells consisted of 3 levels, namely: OD$_{660}$ 20, 25, and 30. Thus, 15 treatment combinations were obtained and each was carried out in 2 groups based on the processing time so that a total of 30 experimental unit.

2.3. Preparation of substrates for ethanol production

The substrate for ethanol production was obtained from previous studies which had been modified. The substrate was prepared in the following manner: the bagasse was crushed using a grinding machine and
then sieved (passed 60 mesh), so that uniform size bagasse powder was obtained. The bagasse powder was delignified using 6% NaOH solution and soaked for 12 h at room temperature [4][10]. Delignified bagasse powder was then hydrolyzed using crude enzymes from Aspergillus niger FNU 6018. This cellulase enzyme was produced by growing A. niger for 5 days of incubation at 50°C, in media containing nutrients in the form of (NH₄)₂SO₄, KH₂PO₄, CaCl₂, MgCl₂, MgSO₄, urea, and the pH was adjusted to pH 4.8. After the saccharification process was complete, the fermented sugars were filtered used Whatman No. 1 filter paper to obtain glucose liquid, then used it as a substrate in the ethanol production process.

2.4. Cells propagation
The rejuvenated culture of S. cerevisiae on PDA media, then taken as much as 2-3 ose to be grown again in 50 mL of yeast extract peptone glucose (YPG) media. The YPG media was made by taking 5 g/L of yeast extract, 5 g/L of peptone, 10 g/L of glucose dissolved in 50 mL of distilled water then sterilized at 121°C for 15 min. S. cerevisiae cultures were transferred aseptically into YPG media and incubated at 30°C for 24 h and shaken at 100 rpm [1], [11].

2.5. Immobilization of cells
The results of cell multiplication were then centrifuged at a speed of 5000 rpm for 10 min to separate the cells from the media. The obtained cell pellets were then washed with a sterile 0.85% (w/v) NaCl solution, then homogenized and centrifuged at the same condition, this washing these cells was carried out twice. Furthermore, the clean cells were added with 0.85% (w/v) NaCl solution and then adjusted the cell concentration based on the desired optical density according to the experimental design at a wavelength of 660 nm.

Cells with a certain OD according to the treatment, then mixed with a sterile Na-alginate (w/v) solution with a ratio of 1: 2, namely 3 mL of cell suspension (OD₆₆₀ 20, 25, and 30) and 6 mL of Na-alginate solution at a concentration 0%, 2%, 3%, 4%, and 5% (w/v). The mixture of cell suspension and Na-alginate was transferred to the falcon to be homogenized. Then the mixture is inserted into an injector which can produce beads with a size of 2 mm. The droplets that come out of the injector are collected in a beaker containing a sterile 4% (w/v) CaCl₂ solution. CaCl₂ solution can make the droplets form a gel (beads) at room temperature. Beads that have been formed are hardened again by storing them at 4°C for 6 h, the beads are then cleaned with 0.85% (w/v) NaCl solution [6]. Before being used for fermentation, the immobilized cell beads were activated using a 2% glucose solution with a pH of 4.5 and shaken at 100 rpm for 12 h at a temperature of ± 30°C then cleaned again with 0.85% (w/v) NaCl solution, and ready to use for fermentation.

2.6. Alcohol fermentation process
The fermented sugars obtained from the enzymatic cellulose saccharification process, then added nutrients, namely 0.15 g/100 mL ammonium sulfate and 0.04 g/100 mL NPK, the pH was adjusted with 1N HCl solution to become 4.5, then pasteurized at 70°C for 30 min and cooled. Next, immobilized cells of S. cerevisiae (according to treatment) were inserted into Erlenmeyer containing 300 mL of saccharified sugars solution. The fermentation process was carried out at ± 30°C for 96 h and was shaken at 125 rpm. During the fermentation process, reducing sugar levels and pH were observed every 24 h of incubation. The fermentation product was distilled off to obtain ethanol. Immediately after the distillation process was complete, an analysis of the ethanol content of the sample was carried out.

2.7. Observed variables and statistical analysis
The parameters observed were the activity of immobilized S. cerevisiae cells which included reducing sugar levels using the Nelson-Somogyi method [12], pH [13], and ethanol content using Gas Chromatography [13]. The stability of immobilized S. cerevisiae cells was seen from the level of turbidity of cells or cells separated or loose from the beads (OD₆₆₀ nm) in the activation process for 12 h [6]. The data obtained were then analyzed by ANOVA and if the treatment had a significant effect.
(P<0.05) on the observed parameters, it was continued with the Duncan test. All experiments were carried out in two replications.

3. Results and Discussion

3.1. Reducing sugar content

The results of the analysis of variance showed that the treatment of sodium alginate concentration, *S. cerevisiae* cell concentration, and the interaction between treatments had a very significant effect (P < 0.01) on reducing sugar levels in fermentation media.

![Figures](a) Changes in reducing sugar levels during the fermentation process at different sodium alginate concentrations (a) 0%, (b) 2%, (c) 3%, (d) 4%, (e) 5%.
The results showed that there was a decrease in reducing sugar levels during the fermentation process. The reduction in reducing sugar levels rapidly occurred at the beginning of fermentation until the 48th h, while at the 72nd to 96th h, the decrease in reducing sugar levels was not significant (Figure 1). The initial reducing sugar content in the medium was 72.3039 mg/100 mL (723.039 ppm). In the treatment with a concentration of 0% sodium alginate (free cells) the reduction in reducing sugar levels rapidly occurred until the 24th h of fermentation. The reduction in reducing sugar levels that takes place so fast is caused by the presence of cell activity in the media which also utilizes the substrate for growth in addition to being converted to ethanol [14]. In addition, treatment with sodium alginate concentration of 0% (free cells) is less stable when compared to treatment with sodium alginate concentration of 2-5% [6], [7]. Figure 1b showed that the one with the lowest reducing sugar content at the 96th is the treatment of sodium alginate concentration 2% OD660 25, namely 0.250 mg/100 mL (2.50 ppm), which means that the treatment has the least remaining reducing sugar while the treatment which has the highest residual reducing sugar is sodium alginate concentration treatment 5% OD660 20 of 0.951 mg/100 mL (9.51 ppm) (Figure 1e). The decrease in reducing sugar levels during fermentation is due to the consumption of glucose by S. cerevisiae cells through the Embden-Meyerhof-Parnas Pathway (EMP), the glucose is fermented to produce ethanol (ethyl alcohol). Pyruvic acid which is formed from glucose is decarboxylated into acetaldehyde with the help of the enzyme pyruvate decarboxylase which is then converted into ethanol [15].

3.2. pH change during fermentation

The results of the analysis of variance showed that the treatment of sodium alginate concentration, S. cerevisiae cell concentration, and the interaction between treatments had a very significant effect (P <0.01) on the pH of the fermentation medium. The average pH value observed every 24 h can be seen in Table 1.

Table 1. The average pH during fermentation (0-96 h).

| Time of observation (h) | Sodium alginate concentration (% w/v) | Cell concentration (OD660) |
|-------------------------|--------------------------------------|----------------------------|
|                         |                                      | 20 | 25 | 30 |
| 24                      | 0                                    | 4.4 c | 4.4 c | 4.2 e |
|                         | 2                                    | 4.2 d | 3.8 g | 3.7 h |
|                         | 3                                    | 4.3 d | 4.1 f | 3.9 g |
|                         | 4                                    | 4.3 d | 4.4 c | 4.0 f |
|                         | 5                                    | 4.5 b | 4.4 c | 4.5 a |
| 48                      | 0                                    | 4.2 a | 4.2 ab | 4.1 c |
|                         | 2                                    | 4.1 bc | 3.6 h | 3.5 i |
|                         | 3                                    | 3.9 e | 3.8 e | 3.6 gh |
|                         | 4                                    | 4.0 d | 3.9 e | 3.7 fg |
|                         | 5                                    | 4.0 cd | 3.7 f | 3.9 e |
| 72                      | 0                                    | 4.2 a | 4.0 b | 3.4 g |
|                         | 2                                    | 3.8 c | 3.4 g | 3.4 g |
|                         | 3                                    | 3.6 ef | 3.7 def | 3.6 f |
|                         | 4                                    | 3.8 c | 3.7 def | 3.7 cd |
|                         | 5                                    | 4.0 b | 3.7 def | 3.7 de |
| 96                      | 0                                    | 3.8 bcd | 3.4 f | 3.1 g |
|                         | 2                                    | 3.8 bcd | 3.6 e | 3.4 f |
|                         | 3                                    | 3.7 d | 3.9 b | 3.6 e |
|                         | 4                                    | 3.6 e | 3.8 bc | 4.1 a |
|                         | 5                                    | 4.1 a | 3.7 d | 3.8 cd |

Note: a different notation behind the mean value indicates a significant difference (P <0.05).
The results showed that there was a decrease in pH during fermentation (Table 1). Just like the decrease in sugar content, the pH also decreased rapidly at the beginning of the fermentation until the 48th h. In Table 4, it can be seen that the treatment that has the lowest pH at 96 h is the treatment of sodium alginate concentration 0% (free cells) OD_{660} 30 with a pH of 3.1 while the treatment that has the highest pH at 96 h is the treatment of sodium alginate concentration 5% OD_{660} 20 with pH 4.1. There are several treatments that experience an increase in pH at the 96th h. The decrease in pH during fermentation occurs due to the accumulation of organic acids formed during the fermentation process. These organic acid compounds include pyruvic acid and acetic acid. Pyruvic acid is a compound that is formed during the glycolysis process. During the glycolysis process, every one mole of glucose will be broken down into two moles of pyruvic acid and releasing two moles of H^+ ions. The presence of H^+ ions is thought to reduce pH during the fermentation process [1], [11]. The decrease in pH during fermentation was also caused by the addition of nutrients in the form of (NH_4)\_2SO_4 as a nitrogen source. Ammonium in solution (below pH 9) exists as NH_4^+: microorganisms incorporate it into cells as R-NH_2, with R as the carbon skeleton. In the process, H^+ is left in the medium, so the pH of the medium tends to decrease [16]. According to Neelakandan et al. [17], ethanol formed in the fermentation process can increase the pH of the fermentation solution so that the pH at the end of fermentation has increased.

### 3.3. Ethanol content

At the end of the fermentation, an analysis of ethanol content was carried out to determine the ethanol content produced during the fermentation process. Analysis of ethanol content was carried out using Gas Chromatography (GC). Prior to analysis, a distillation process was carried out which aims to separate the alcohol from water. In this study, the sample analyzed for ethanol content was the sample that had the lowest remaining reducing sugar content, this can be seen in the results of the analysis of reducing sugar levels. From the results of the analysis of reducing sugar levels, it can be seen that the treatment that has the least remaining reducing sugar content or the lowest reducing sugar content at the 96th h is the treatment of 2% sodium alginate concentration and its cell turbidity level (OD_{660}) 25 with the remaining reducing sugar content of 0.250 mg/100 mL (2.5 ppm) it is assumed that the existing sugars are converted into alcohol so that the alcohol content in the treatment with the lowest remaining reducing sugar is higher when compared to the treatment with higher residual reducing sugar. In this study, a comparison of the treatment with sodium alginate concentration of 0% which has a cell turbidity level (OD_{660}) of 25 was carried out to compare the ethanol content resulting from the use of immobilized S. cerevisiae cells and the use of free cells. The ethanol content can be seen in Table 2.

| Sodium alginate concentration (%) | Cells concentration (OD_{660}) | Ethanol content (ppm) |
|----------------------------------|-------------------------------|----------------------|
| 0                                | 25                            | 56.974               |
| 2                                | 25                            | 63.874               |

From the analysis of ethanol content, it is known that treatment with sodium alginate concentration of 2% OD_{660} 25 has a higher ethanol content when compared to treatment with sodium alginate concentration of 0% OD_{660} 25 (free cells). This shows that the use of immobilized cells is better than free cells because immobilized cells are more stable than free cells [6], [7]. The use of immobilized cell techniques can increase ethanol productivity [7], [15], [18]. According to Najafpour et al. [19], the optimal concentration of sodium alginate in immobilized cell activity to produce ethanol is sodium alginate with a concentration of 2%. The high concentration of sodium alginate causes low ethanol to be produced, because the diffusion of glucose fluids into cells is inhibited. In the glycolysis pathway, each glucose molecule will produce 2 molecules of ethanol, 2 moles of carbon dioxide, and 2 molecules of ATP. The two ATP molecules formed are used for energy needed during cell growth [20]. Fermentation efficiency is the percentage ratio between the ethanol concentration obtained (actual) and the theoretical ethanol concentration. The theoretical ethanol concentration is obtained by means of the
initial reducing sugar content in the substrate, minus the residual reducing sugar content then multiplied by 0.51 because 100% glucose is converted to 51.1% ethanol and 48.9% to CO₂ [1], [11].

From the calculation, it is known that the theoretical ethanol concentration with an initial reducing sugar level of 72.3039 mg/100 mL (723.039 ppm) and a reduced sugar level consumed by 720.539 ppm can produce ethanol of 187.68 ppm for treatment with a sodium alginate concentration of 2% OD₆₆₀ 25, whereas for treatment with a sodium alginate concentration of 0% OD₆₆₀ 25 with an initial sugar content of 72.3039 mg/100 mL (723.039 ppm) and a reduced sugar content consumed of 717.989 ppm it can produce ethanol of 187.15 ppm, then it will be compared with the ethanol concentration obtained (actual). From the analysis of ethanol content using Gas Chromatography (GC), it is known that the ethanol content obtained from the research results is 56.974 ppm for treatment with sodium alginate concentration of 0% OD₆₆₀ 25, while for treatment with sodium alginate concentration 2% OD₆₆₀ 25 is 63.874 ppm (Table 2). From the calculation, it can be seen that the efficiency of fermentation, for the treatment of sodium alginate concentration 2% OD₆₆₀ 25 the efficiency is 34%, while for the treatment of sodium alginate concentration 0% OD₆₆₀ 25 the efficiency is 30.4%. This is closely related to the reduction in reducing sugar levels during the fermentation process. From the results of the analysis of reducing sugar levels, it is known that the reduction in reducing sugar levels takes place rapidly at the beginning of fermentation until the 48th h, while at the 72nd to 96th h the reduction in reducing sugar levels is not significant (Figure 1). From the data resulting from the reduction in reducing sugar content, it is known that the possibility of ethanol production is maximally produced at the 48th h then at the time interval from the 72nd h to the 96th h it is suspected that ethanol oxidation to acetic acid occurs.

### 3.4. Stability of immobilized cells

The stability of immobilized cells can be seen from the level of cell turbidity during the activation process for 12 h in 2% glucose solution. The results of the analysis of diversity showed that the treatment of sodium alginate concentration, cell concentration, and the interaction between treatments had a very significant effect (P <0.01) on the stability of immobilized S. cerevisiae cells. The average OD value of cells observed during the activation process for 12 h can be seen in Table 3.

| Sodium alginate concentrations (%) | Cell concentrations (OD₆₆₀) | 20         | 25         | 30         |
|-----------------------------------|---------------------------|------------|------------|------------|
| 2                                 | 0.009 h                   | 0.044 b    | 0.061 a    |
| 3                                 | 0.037 c                   | 0.015 g    | 0.023 e    |
| 4                                 | 0.015 g                   | 0.035 c    | 0.026 d    |
| 5                                 | 0.018 f                   | 0.002 i    | 0.022 e    |

Note: a different notation behind the mean value indicates a significant difference (P <0.05).

The results showed that each treatment had a different average cell turbidity level. It can be seen in Table 3 that the treatment that has the highest level of cell turbidity is the treatment of 2% sodium alginate concentration with an average value of 0.0377 ± 0.0262, while the treatment that has the lowest level of cell turbidity is the treatment of sodium alginate concentration of 5% with an average value of 0.014 ± 0.0105. The high concentration of sodium alginate causes a low average value of cell turbidity (OD₆₆₀) because the immobilized cell wall becomes thick and strong so that cells trapped in sodium alginate are difficult to escape or release. This shows that the treatment with a concentration of 5% sodium alginate has very good stability, while the treatment with a concentration of 2% sodium alginate has a fairly good stability. At higher sodium alginate concentrations, the diffusion of glucose fluids into cells is also inhibited so that ethanol production is also reduced. In the formation of ethanol, the concentration of sodium alginate which has a sufficiently good stability is chosen so that the ethanol produced can be maximized.
4. Conclusion
Based on the research results, it can be concluded as follows: the concentration of sodium alginate and the concentration of S. cerevisiae cells has a very significant effect on the activity and stability of immobilized S. cerevisiae cells. The interaction between treatments also had a very significant effect on the activity and stability of immobilized cells. The concentration of sodium alginate 2% with a cell density (OD660) 25 can work optimally in converting simple sugars from saccharification of bagasse into ethanol and is able to produce ethanol of 63.874 ppm.

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Acknowledgment
The author would like to thank Udayana University for providing financial assistance through the National Strategic Research Grant with contract No: 0229.0/023-04.2/XX/2009 and laboratory facilities.