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Application of *Saccharomyces Cerevisiae* ATCC 9763 Immobilised Cells in Nanocellulose Based Matrix for Bioethanol Production

Nuril Puspita Rahayu[1], Bambang Piluharto[2], Nurhayati Nurhayati[3], Jay Jayus[3,4]

[1]Graduate School of Biotechnology, University of Jember, 37 Kalimantan street, Jember 68121, Indonesia
nurilpr92[at]gmail.com

[2]Department of Chemistry, Faculty of Math and Natural Science, University of Jember, 37 Kalimantan street, Jember 68121, Indonesia
bampito.fmipa[at]unej.ac.id

[3]Department of Agricultural Product Technology, Faculty of Agricultural Technology, University of Jember, 37 Kalimantan street, Jember 68121, Indonesia
nurhayati.ftp[at]unej.ac.id

[4]Center of Excellent for Industrial Plant Biotechnology, University of Jember, 37 Kalimantan street, Jember, 68121, Indonesia
jayus.ftp[at]unej.ac.id

Abstract: ***Attempts to improve the bioethanol productivity of S. cerevisiae ATCC 9763 were made using the entrapment method of cell immobilisation for its culture fermentation. The cells were entrapped using several types of matrix (beads) materials including alginate/cellulose, alginate/CMC and alginate/nanocellulose, which had the potential to differ in their mechanical strength charge and particle size and thus affect the value of water absorption of beads and the viability of the cells within them. To evaluate the productivity of the yeast, fermentation studies were carried out under repeated-batch fermentation using free and immobilised cells systems. Despite the relative similarity of the concentration of bioethanol produced by the yeast in both the free cell and immobilised systems (approximately 1% w/v higher in immobilised culture), the two culture systems had different productivities. The highest bioethanol productivity (1.38 g/L/h) was observed for the immobilised culture system using alginate/nanocellulose as beads. This was higher than the productivity of the free cells culture system, which reached only 0.76 g/L/h. Furthermore, cell reusability was maintained in the immobilised culture system for up to nine fermentation cycles, with only 26% of the beads damaged after nine such cycles. Interestingly, there was a lower breakage rate among the alginate/nanocellulose beads (18%), which may indicate the greater mechanical strength of this nanomaterial.***

Keywords: Bioethanol, immobilised cells, nanocellulose, nanoparticle, repeated-batch fermentation

1. Introduction

Bioethanol is a renewable fuel alternative that is able to contribute to a reduction in worldwide problems such as global warming and pollution that arise from the use of fossil fuels [1]. Bioethanol is generally produced by the fermentation of starch, sugar and cellulose sources of biomass with microorganisms. In traditional fermentation systems, bioethanol production uses freely suspended yeast cells in a batch bioreactor. The batch fermentation of free cells is characterised by greater difficulty when it comes to separating the cells from the component mixture containing ethanol, glucose and other impurities, and the reusability of the cells is low [2]. To overcome such problems, immobilised cells using inert support has been reported to present many advantages over free-cell fermentation such as simplification of the separation process and an increase in the reusability of cells (by five to ten cycles of repeated-batch fermentation) [3–5]. In addition, there is increased productivity due to the shorter fermentation time. According to [6], the fermentation time of immobilised cells is 26 h shorter than that for free cells.

A widely used immobilisation method in ethanol production is cell entrapment in gel such as Ca-alginate and carrageenan due to the fact that the material is non-toxic, simple, biocompatible and low-cost. However, alginate also has several disadvantages: low mechanical strength, fragility of the gel particles [7] and low stability when used in repeated-batch fermentation [3]. According to [3], 40% of beads were damaged after eight fermentation cycle, and the ethanol production during the eight successive cycles were fluctuated. Therefore, [8] attempted to improve both the mechanical strength and stability of alginate gel beads by reinforcing them with an interpenetrating network (IPN) of ionic and covalent cross-links, which resulted in more stable ethanol production during repeated-batch processing. Moreover, [9-10] also reported that incorporating polymer such as cellulose (loofa sponge)-alginate matrixes resulted in improved stability, mechanical strength and reusability in repeated-batch ethanol fermentation. Cellulose is a type of natural polymer that is often used as the matrix for enzyme or cell immobilisation due to its good biocompatibility properties, robustness, biodegradability, ability to absorb water, non-toxicity and low cost [11-12]. In recent studies, cellulose derivatives such as carboxyl methyl cellulose (CMC), cellulose acetate (CA), cellulose nitrate and nanoparticles have been used most often as support material for the immobilisation of enzymes due to the increased biocatalytic efficiency that arises from their functional

groups through increased enzyme loading [13] and high surface areas [14]. In this research, the effect of combining alginate/microcrystalline cellulose (MCC), alginate/ carboxyl methyl cellulose (CMC) and alginate/ nanocellulose (NC) will be observed with respect to the protection of cells during ethanol production.

2. The Material and Method

2.1 Materials

The materials used in the research were microcrystalline cellulose (Sigma-Aldrich, St. Louis), CMC (Carboxyl Methyl Cellulose), Na-alginate (Sigma-Aldrich, St. Louis), nanocellulose, $CaCl_2$, Bacteriological Agar (Oxoid), Malt Extract Broth (Merck) and molasses from a local sugar cane factory (PTPN XI Djatiroto, Lumajang, Indonesia). The microorganism used was *Saccharomyces cerevisiae* ATCC 9763 from the Laboratory of Food and Agricultural Products Microbiology, Faculty of Agricultural Technology, University of Jember.

2.1 Gel Beads Preparation

The The nanocellulose preparation method has previously been described by [15]. Four types of gel beads were made using alginate powder by mixing alginate/cellulose, alginate/CMC and alginate/nanocellulose in the ratio of 2:1. The mixture was dissolved in 100 ml of distilled water, stirred using a magnetic stirrer until homogeneous before 5 ml were added to a syringe. This was then dropped into a 2% $CaCl_2$ solution to form beads. The beads were washed twice using distilled water and then dried in order to determine the bead characterisations.

2.2 Bead Characterisations

The morphology of the beads was captured using a Hitachi TM3030Plus *Analytical Scanning Electron Microscope*, while their water absorption capabilities were measured using the method from [16].

2.3 Immobilisation of S. cerevisiae

The inoculums were prepared with a modification of glucose concentration using the method previously described by [3]. The immobilised cells were prepared by mixing 2.5% (w/v) of inoculums (cell concentration of ± 1.6 x 10^8 cells/ml) into a sterilised solution of alginate, alginate/cellulose, alginate/ CMC and alginate/ nanocellulose. The solution was then stirred until homogeneous. Furthermore, the solution was aspirated in a syringe and dropped into a 2% calcium chloride solution. The beads were stored at 4^{o}C for 24 hours in the fresh calcium chloride solution prior to use.

2.4 Analysis of Cell Viability

1 g of immobilised cells was inserted into 9 mL 0.85 % saline solution and stirred for 30' until the polymer capsules ruptured. Furthermore, 1 ml of the cell solution was diluted from 10-1 -10-6. 1 ml of serial dilution 10-4-10-6 was then poured onto a blank Petri dish. Next, liquid malt extract agar media was added and it was incubated for 48 hours at 28^{o}C. The number of colonies was analysed using the formula contained in the Bacteriological Analytical Manual (BAM) (Food and Drug Administration, 2001). The cell viability was calculated from the percentage of log population of immobilized cell per log population of cell culture.

2.5 Analysis of Cell Viability

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2.6 Production of Bioethanol

The preparation method for fermentation medium has previously been described by [18]. Repeat-batch fermentation experiments were carried out in 500 ml Erlenmeyer flasks using 300 ml molasses. The fermentation medium was inoculated with either 10% (g/v) free or immobilised cells starter culture. It was then incubated at room temperature and agitated at 150 x g. At the end of the fermentation cycle, the fermentation broth was collected, with new, fresh media added to the remaining cells for the next cycle.

2.7 Analytical Methods

The bioethanol content was measured using the 3,5-dinitrosalicylic acid method (DNS) for reducing sugar [19], while ethanol concentration was analysed using the Chamber Conway method as described by [18].

2.8 Statistical Analysis

Analysis of the water absorption and the viability of the cells data obtained from the results was performed using analysis of variance. If there were further differences, a Duncan's New Multiple Range Test (DNMRT) was subsequently performed at the test level $\alpha \leq 5\%$.

3. Results and Discussion

3.1 Water Absorption of Beads

Water absorption was used to determine the amount of water absorbed by beads at a particular time. The water absorption process is associated with the ability of the matrix structure of beads to firstly hold water molecules and then retain them. The presence of cellulose, CMC and nanocellulose in alginate beads is able to multiply the cross-links between both and also increase the surface area. Similar to the finding reported by [17], the addition of nanochitosan to alginate beads can increase the surface area and pore volume. The greater the hydrophilic groups on the surface of the beads, the more the beads expanded. The water absorption of various types of beads is shown in Figure 1. Alginate beads

were found to have the lowest water absorption value (DSA) compared to others, at 63.09%. This is due to the weaker interaction of alginate groups such as hydroxyl (-OH) and carboxylate groups (C=O). Alginate/cellulose beads exhibited a similar DSA value to alginate beads. Then, the alginate/CMC beads were found to have a higher DSA than the alginate and alginate/cellulose beads due to the strong interaction of the hydrophilic group in alginate and CMC. Moreover, the alginate/nanocellulose beads were found to have a higher DSA value (229.46%) compared to other non-nanocellulose beads, which may be due to the greater presence of the hydrophilic group between alginate and nanocellulose and the presence of nanoscale particles to give the beads a larger surface area and pore volume. According to [18], high surface area and porosity will increase the swelling power of the polymer. In addition, smaller zeolite-size particles have a large surface area, meaning the absorption of CO2 is greater [19].

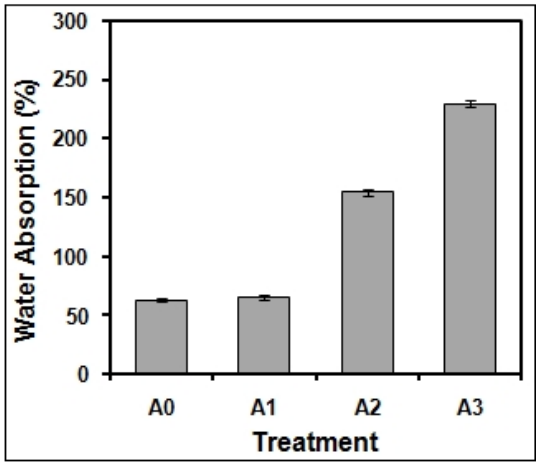

Figure 1: Graphical water absorption in beads. Alginate (A0), alginate/cellulose (A1), alginate/CMC (A2) and alginate/nanocellulose (A3).

3.2 Viability of S. cerevisiae on Beads

In this study, the cell viability was obtained by measuring the surviving cells in each gram of beads from the initial population of cells (1.6 x 10^8 CFU/ml). *Saccharomyces cerevisiae* was able to survive on all beads (Figure 2) with a higher cell viability in alginate/nanocellulose (80.49%) compared to the others. The high viability of the cells demonstrated that all of the beads had good biocompatibility properties to *S. cerevisiae*. Biocompatibility is strongly influenced by the basic structure of the polymer such as its molecular weight, functional side chain, end chain structure, branched-chain length and monomer sequence distribution [20]. Alginate/nanocellulose beads had the highest cell viability as the presence of nanoparticles in beads will create a large surface area compared to others and thus improve the interaction of the cells with the surface of solids. The SEM analysis result shows that *S. cerevisiae* cells are able to survive in alginate/nanocellulose beads (Figure 3). These results are consistent with those obtained by [21], where the cells encapsulated in alginate and alginate/nanofiber (TBOC) beads were alive and the structure of the nanofiber was able to increase cell proliferation, thus entrapping more cells.

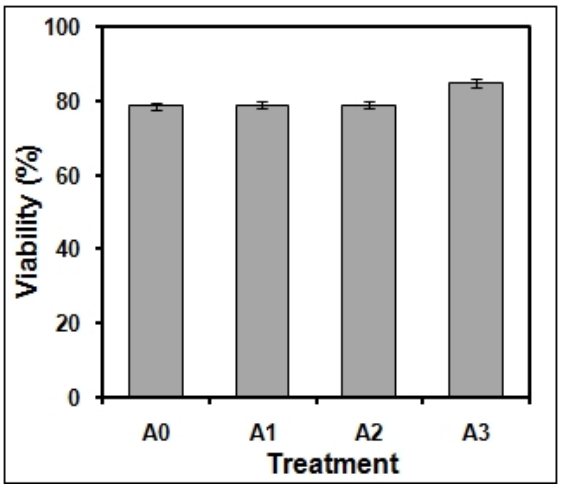

Figure 2: Viability of *S. cerevisiae* on beads. Alginate (A0), alginate/cellulose (A1), alginate/CMC (A2) and alginate/nanocellulose (A3).

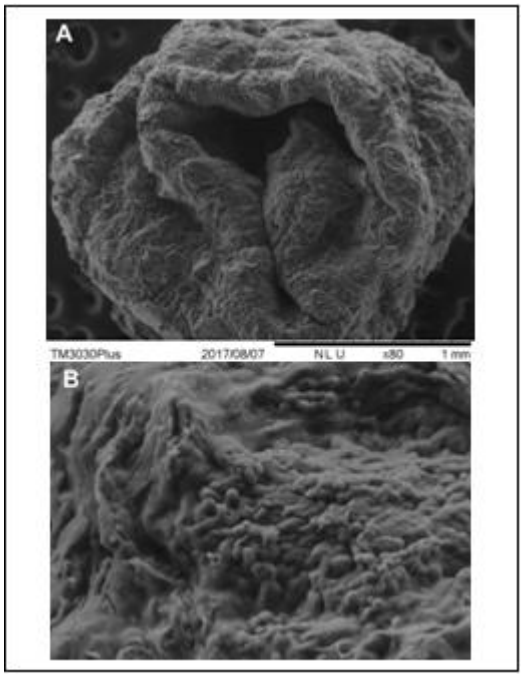

Figure 3: SEM image of alginate/nanocellulose beads

3.3 Application of Immobilised Cells for Bioethanol Production

Water The aim of repeated-batch fermentation in bioethanol production was to determine the stability and mechanical strength of alginate/cellulose and its bead derivatives as a supporting matrix in bioethanol production. Each cycle of repeated-batch fermentation had a constant fermentation time lasting 48 hours. All of the immobilised cells on beads reached nine cycles of repeated-batch fermentation, while the ninth cycle saw a drastic reduction in the ethanol contents (Figure 4). This result indicates that all of the beads were able to maintain stability as supporting matrixes for yeast fermentation. From the first to the eighth fermentation cycles, the concentration of ethanol in alginate/nanocellulose beads was approximately 58.14 ± 0.21 g/L higher than the others with residual reducing sugars were approximately 22.1 ± 0.22 g/L. The initial reducing sugars content for the fermentation of all types of beads was approximately 145.38

± 0.32 g/L, while after the ninth cycle, the ethanol concentrations and residual reducing sugars of all the beads fell to approximately 35.91 ± 0.14 g/L and 36.94 ± 0.78 g/L. This result indicates the growth and development of yeast in the presence of molasses as nutrients during fermentation. Reducing sugar content in molasses acts as a carbon source for *S. cerevisiae* to grow and proliferate. Furthermore, sugar is converted into ethanol for fermentation, so the reducing sugar level decreases and the ethanol concentration increases. As previously described by [22], *S. cerevisiae* will break down disaccharide or polysaccharides into monosaccharide during fermentation for the activation of life. After that, *S. cerevisiae* will transform glucose as a substrate into ethanol through the Entner-Doudoroff pathway using pyruvate decarboxylase and alcohol dehydrogenase enzyme [23].

The four types of beads produced relatively the same concentrations of ethanol, although the ethanol content, productivity and ethanol yield (58.04 g/L; 1.38 g/L/h; 0.51 g/g) of the alginate/nanocellulose beads were greater than the others (Table 1). This result may be due to the presence of more porous space within the alginate/nanocellulose beads (figure not shown), thus allowing a greater bead surface area for cells to grow and also interact more during fermentation. According to a report by [3], cell immobilisation on smaller sizes of corncob was able to increase the surface area, resulting in higher efficiency of ethanol production. In addition, nanocellulose added to alginate can improve mechanical strength of matrixes compared to other beads. Only 18% of the beads were damaged during the course of the nine fermentation cycles. In contrast, more destroyed alginate, alginate/cellulose and alginate/CMC beads were seen after the ninth fermentation cycle, while only 75% of the beads remained after nine successive cycles (data not shown).

Table 1 summarises the efficiency of the bioethanol production between free and immobilised cells. Despite the similar levels of ethanol production, the Qp and Yp/s were higher in the immobilised cells compared to those of the free cells due to their shorter fermentation time. According to [24], ethanol fermentation using a molasses medium will need 1–3 days at a temperature of 20–32^{o}C in order to produce an ethanol content of 5–10%. The above results show that the use of immobilised cells in bioethanol fermentation can reduce the inhibition of the final product (approximate difference of 1%); hence, it leads to an improvement in ethanol tolerance and increases productivity, similar to the findings described by [28-29].

3.4 Nomenclature

P	ethanol concentration	gL^{-1}
Qp	productivity of ethanol per hour	$gL^{-1}h^{-1}$
Yp/s	yield of ethanol per biomass	gg^{-1}
t	fermentation time	h

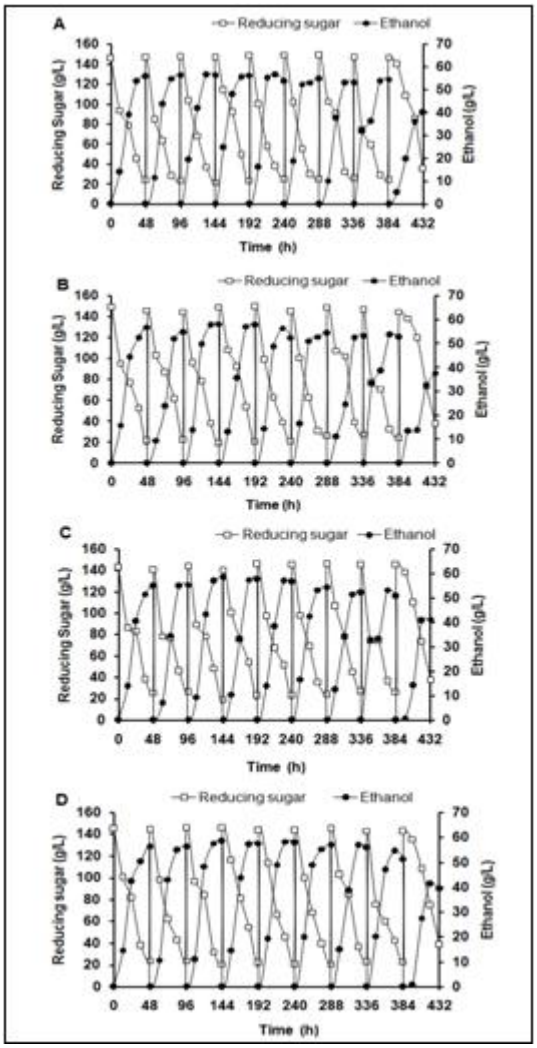

Figure 4: The relationship between reducing sugar and the concentration of ethanol on beads. A) alginate, B) alginate/cellulose, C) alginate/CMC and D) alginate/nanocellulose.

Table 1: Parameters of bioethanol production from molasses with free and immobilised cells of *S. cerevisiae* ATCC 9763

Cell System/ Support Material	Parameter (mean ± SD)			
	P (g/L)[a]	Qp (g/L/h)[b]	Y p/s (g/g)[c]	t(h)[d]
Free cell	45.56 ± 0.22	0.76 ± 0.01	0.39 ± 0.01	60
A0	55.15 ± 0.20	1.31 ± 0.01	0.46 ± 0.03	42
A1	54.80 ± 0.17	1.30 ± 0.01	0.47 ± 0.03	42
A2	55.08 ± 0.20	1.31 ± 0.01	0.49 ± 0.03	42
A3	58.04 ± 0.21	1.38 ± 0.01	0.51 ± 0.02	42

4. Conclusions

In conclusion, alginate/cellulose and its bead derivatives have potential as an alternative material for yeast cell immobilisation. It is obvious that these beads will last for longer during repeated-batch fermentation compared to the free-cell application. Immobilised *S. cerevisiae* using this alginate/nanocellulose matrix can increase bioethanol productivity during fermentation in molasses media (77% higher than for free cell). Immobilised cells can be used repeatedly in all the bead types tested, up to a total of nine fermentation cycles. *S. cerevisiae* entrapped by alginate/

nanocellulose beads has higher P, Qp and Yp/s values for its bioethanol production compared to a free-cell system.

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Author Profile

Mr. Jay Jayus is the Assistant Professor **in the** Department of Agricultural Product Technology, Faculty of Agricultural Technology, University of Jember, 37 Kalimantan street, Jember 68121, Indonesia.