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Physical Characteristics of Immobilized Cells *Acetobacter xylinum* of Various Concentrations of Na-alginate

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Abstract

Immobilization of *Acetobacter xylinum* cells by trapping technique is considered one way to maintain cell viability and can be used repeatedly. The purpose of this study was to determine the physical characteristics of immobilized cells in different concentrations of Na-alginate and apply them to the manufacture of nata de coco, as well as to see the surface and pore shape of immobilized cells after fermentation. This study used a descriptive method with visual observation. This study used one factor, namely various concentrations of Na-alginate, namely 2%, 3%, 4%, and 5% w/v. The immobilized cells applied to the manufacture of nata de coco had only a Na-alginate concentration of 3% resulting in a nata fiber layer close to that of a control. Immobilized cells with a Na-alginate concentration of 2% have a cracked, slightly pore surface with a size of 60.37 µm, while a Na-alginate concentration of 5%, have a smooth surface, many pores, with a size of 10.55 µm. Thus, the concentration affects the characteristics of immobilized cells. Application of immobilized cells with a Na-alginate concentration of 3% produces nata de coco with a fairly thick and stable fiber layer characteristic (close to control).

Keywords: *Acetobacter xylinum*; cell immobilization; entrapment; nata de coco; natrium alginate.

INTRODUCTION

Acetobacter xylinum (*A.xylinum*) is a gram-negative bacterium that produces extracellular polysaccharides in the form of cellulose that make up the bacterial cell wall (Wang et al., 2019). Abundant cellulose production is located on the surface of the substrate due to the process of glucose metabolism by *A. xylinum* to produce cellulose under aerobic conditions. The intertwined cellulose of the bacterial cell wall forms the nata structure and the bacterial cells are trapped inside. Nata is composed of cellulose as a fermentation product by *A. xylinum* with the main nutrients of carbon and nitrogen sources. So far, nata fermentation has been carried out by using the technique of direct inoculation of *A. xylinum* starter into the substrate, which is easy to do and inexpensive. However, the direct inoculation method results in starter cultures that are easily contaminated, easily die, and are vulnerable to changes in environmental conditions if the handling is inadequate.

Starter inoculum is available in the market in liquid form packaged in bottles with lids. Liquid cultures are easily contaminated, cell viability decreases during storage, and are difficult to manage (Dewi, 2009). Meanwhile, the sustainability of the fermentation process requires starter cultures that are continuously available, with maximum potential, easy to manage and long shelf life with high production activity. One way that can meet these needs is cell immobilization.

Cell immobilization is defined as a method to confine or physically place cell microbes in a certain space, where the cell still has catalytic activity and can be used continuously and many times (Darmawan and Pradipta, 2015). In this study, immobilization was carried out using entrapment techniques, namely by allowing cells to penetrate into the porous matrix until their mobility (space) was blocked by other cells or porous materials formed in situ into cell culture (Pratiwi et al., 2021).

In this study, sodium alginate (Na-alginate) as a matrix material that traps cells (Ratnasari et al., 2014). Alginate in its salt form, sodium alginate, has the property of being able to form a gel by binding with calcium salts, one of which is calcium chloride ($CaCl_2$), which acts as a cation. Ca^{2+} ions in calcium chloride function to strengthen the alginate gel structure (Mubarokah, 2018). This study aims to determine the characteristics of immobilized cells made using different concentrations of Na-alginate and apply it to the manufacture of nata de coco.

MATERIALS AND METHODS

Materials and Tools

The materials used in this study were *Acetobacter xylinum* bacterial starter, Na- alginate, calcium chloride ($CaCl_2$), peptone solution, phosphate buffer solution, vinegar acid, ZA (*Zwavelzur ammoniac*) food grade from the online shop, granulated sugar, young coconut water from nearest seller, distilled water taken from waste processing laboratory, in the agricultural products technology department, NA (Nutrient Agar) media (Merck), and NB (Nutrient Broth) media (Merck) from online shop.

Equipment used in cell immobilization were 20 ml syringe from online shop, analytical balance, beaker, stirring rod, test tube, filter in laboratory, ruler, SEM (*Scanning Electron Microscope*), McFarland DEN-1B densitometer, plastic cup container, and refrigerator in laboratory. Equipment used in the preparation of working culture and culture suspensions were ose needle, bunsen, vortex, measuring cup, test tube, test tube rack, hot plate, autoclave, erlenmayer, incubator, petri dish, laminar air flow, and centrifuge in laboratory. The tools used in making nata de coco were square plastic containers, knives, pH meters, stoves, pans, stirrers, filters in laboratory, paper, and rubber bands.

Making *Acetobacter xylinum* Culture on Agar Cups

This process was carried out because the inoculation on the oblique agar does not grow well. Making culture begins with preparing nutrient agar (NA) as much as 100 ml. Next, nutrients to be weighed as much as 2 grams and put into erlenmayer, then add aquades as much as 100 ml. Then heated with a hot plate until NA dissolves. Then the NA solution was sterilized using autoclaf for 15 minutes with a temperature of 121ºC. After the substrate cools, it was poured on 4 petri dishes, then covered and leave to solidify. After solidifying, one pure culture of *A.xylinum* was taken and etched on the agar to be angled with aseptic. Next, the culture was incubated at a temperature of 37ºC for 24 - 48 hours (Sujaya, 2017).

Manufacture of *Acetobacter xylinum* Broth Culture

Making broth culture (liquid) begins with preparing nutrient broth (NB) as much as 100 ml. Next, nutrient broth was weighed as much as 0.8 grams and put into erlenmayer, then added aquades as much as 100 ml. Then heated with a hot plate until the nutrient broth dissolves. Then the media was sterilized using autoclaf for 15 minutes with a temperature of 121ºC. Next, the substrate is poured on 10 test tubes with each tube containing 10 ml, and closed using cotton stoppers. Test tubes containing sterile NB media and placed on the test tube rack and allowed to cool. After cooling, one pure culture of *A.xylinum* from agar saucer was taken and inserted on NB media with aseptic. Furthermore, NB media containing cultures were mixed using vortexes. After that, the culture was incubated at a temperature of 37ºC for 24 - 48 hours, until the media looks cloudy. (Sujaya, 2017).

Calculation of the Number of *Acetobacter xylinum* Bacteria

In this study, the calculation of the number of bacteria was carried out by turbidimetry or looking at the turbidity compared to the Mc Farland (MCF) standard. Such turbidity indicates the presence of bacterial formation. The turbidity standard is intended to replace the calculation of bacteria one by one, making it more efficient in time and effort. The calculation of the number of bacteria is done by comparing the turbidity (turbidity) of bacterial culture with the MCF standard 0.5-10, which is contained in the MCF standard table, so that it can be seen which bacterial culture turbidity corresponds to the table.

Manufacture of *Acetobacter xylinum* Culture Suspension

Making culture suspense begins by incorporating *A.xylinum* broth culture aged 24 – 48 hours into a centrifuge tube. Then culture centrifuged at a speed of 3000 rpm for 5 - 10 minutes. The resulting supernatant was discarded and the pellets were recentrifuged. Furthermore, it was resuspensioned with 2 ml of phosphate buffer so that a culture suspension (Ifadah et al., 2016) was modified.

Preparation of Immobilized Cells

Preparation of immobilized cells began with making Na-alginate solution with a concentration of 2%, 3%, 4%, and 5% w/v, and $CaCl_2$ solution with a concentration of 3% w/v. The next step was to mix the Na-alginate solution with A. xylinum culture suspension until homogeneous. Next, it was dripped into the $CaCl_2$ solution using an injection slowly, and allowed to stand for 20-30 minutes so that the beads were not destroyed. The process was carried out on each Na-alginate concentration. The immobilized cells obtained were then stored in peptone solution (Cahyono, et al., 2021).

Preparation of Nata de coco

The process of making nata de coco was carried out using *A.xylinum* starter as a control and also immobilized cells with various concentrations of Na-alginate that have been determined. The process of making nata begins with filtering coconut water to separate it from impurities. The coconut water was then boiled to a boil and vinegar, sugar, and ZA were added. The boiling coconut water is poured into a container and allowed to cool.The cold medium was then added to the starter and immobilized cells in each container, then covered with paper and tied with rubber bands. Next, fermentation was carried out for 14 days. After the nata fiber layer is formed, it will be

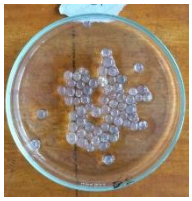
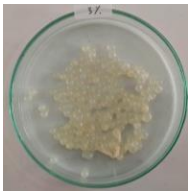
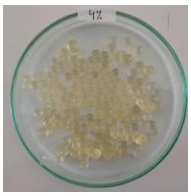
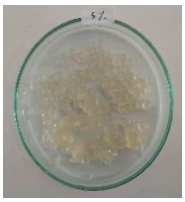
separated from the immobilized cells (Azhari, 2014) modified.

RESULTS AND DISCUSSION

Immobilized Cell Characteristics

The results obtained from the preparation of immobilized cells using different concentrations of Na-alginate produce different characteristics. The immobilized cells obtained were then applied to the manufacture of nata de coco. The application of immobilized cells aims to see whether the immobilized cells experience changes in characteristics (shape, size, texture, and color) after being used for making nata de coco. The characteristics of immobilized cells can be seen in Table 1.

Table 1. Characteristics of immobilized cells before and after being applied to the manufacture of nata de coco.

Treatment of Na-alginate Concentration	Immobilized Cells Before Fermentation	Description	Immobilized Cells After Fermentation	Description
2%		▪ Texture: Solid slightly soft ▪ Shape: Round ▪ Size: 3 mm ▪ Color: White bening		▪ -Texture: Solid slightly soft ▪ -Shape: Round ▪ -Color: slightly yellowish
3%		▪ -Texture: Solid ▪ -Shape: Round ▪ -Size: 4 mm ▪ -Color: White murky		▪ -Texture: Solid ▪ -Shape: Round ▪ -Color: Yellowish
4%		▪ -Texture: Solid ▪ -Shape: Round ▪ -Size: 6 mm ▪ -Color: White slightly ▪ Brownish		▪ -Texture: Solid ▪ -Shape: Round ▪ -Color: Yellowish
5%		▪ -Texture: Solid ▪ -Shape: Round and there is like a tail ▪ -Size:6 mm ▪ -Color: Brownish		▪ -Texture : Solid ▪ -Shape: Round and there is like a tail ▪ -Color: Yellowish

Based on the images of immobilized cell products in Tables 1, the characteristics (texture, shape, size and color) of immobilized cells with different concentrations of Na-alginate before and after being applied for nata fermentation did not change.

Shape and Size

The 2% Na-alginate concentration has a perfectly round shape with a size of 3 mm, while the 3% Na-alginate has a round shape with a larger size, which is 4 mm. The 4% Na-alginate concentration produced a round shape with a larger size than the 2% and 3% concentrations, which was 6 mm, and immobilized cells with 5% Na-alginate concentration produced a round shape and there was a tail at the bottom of the beads. Immobilized cells with 5% Na-alginate have the same size as the 4% concentration, which is 6 mm. The 4% Na-alginate concentration formed a solution with a high viscosity, but it was still easy to be dripped by injection. The tail shape on immobilized cells with 5% Na-alginate concentration may be caused by the high viscosity of the Na-alginate solution, so that when dripped with an injection it forms a tail. Viscosity that is too high is not ideal to form a perfectly round beads (Lee et al., 2013).

Texture

The 2% Na-alginate concentration produced a dense but slightly soft texture, while the 3%, 4% and 5% Na-alginate concentrations had a dense and firm texture. The difference in texture produced in this study was due to

the use of different concentrations of Na-alginate, causing the immobilized cells to have different textures and firmness. The firm texture of immobilized cells is due to the large amount of mannuronic acid (M) and gulluronic acid (G) contained in alginate. (Mubarokah, 2018).

Alginate polymers consist of 3 types, namely mannuronic acid (M), gulluronic acid (G), and the combined polymer of the two (MG) (Picture 1). The M polymer is formed from the equatorial structure of the C-1 and C-4 groups and forms a straight polymer chain, while the G polymer is formed from the axial structure, thus forming an up and down polymer chain.

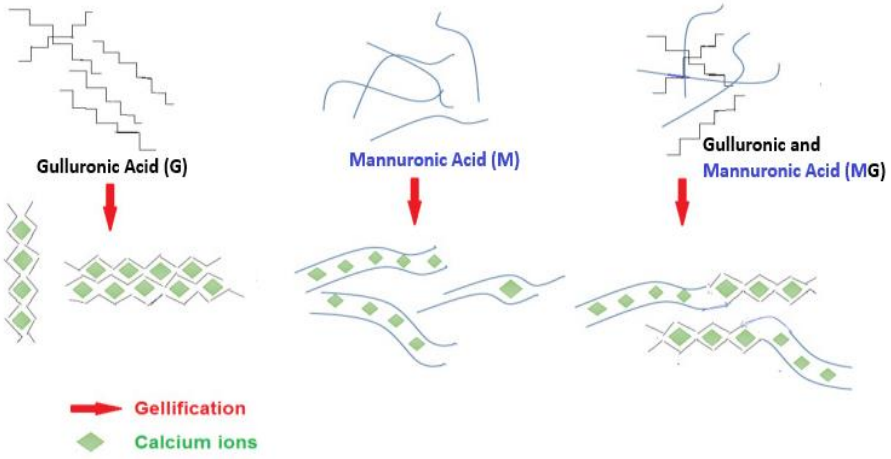


Figure 1. Ca-alginate matrix formation. Source: Khajoui et al., (2022) modified.

This difference in polymer structure causes polymer G to be more widely used for the process of alginate gel formation with the addition of Ca^{2+} ions. Ca^{2+} ions will replace H^{+} ions in the carboxylic group and form a connecting ion bridge between gulluronic acid (G) to form polymer G. The relationship between these G polymers will form an egg-box structure (Picture 2).

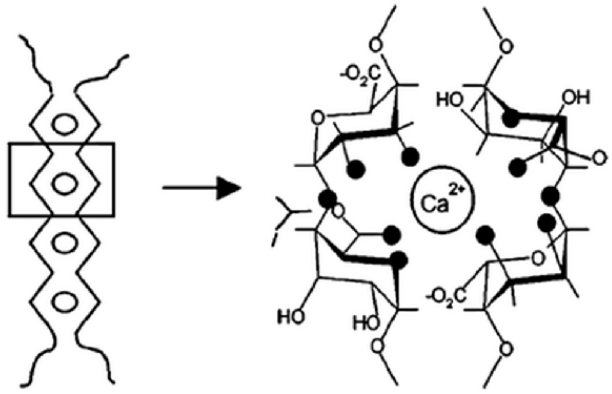


Figure 2. Formation of egg-box structure. Source: Bedê et al., (2017).

The interaction between guluronic acid (G) and Ca^{2+} ions causes the flexibility of the alginate molecule to increase, with the order of flexibility from the lowest, namely gulluronic acid (GG), followed by mannuronic acid (MM) and a combination of mannuronic and gulluronic acids (MG) (Wijffels, 1996). The horizontal ribbon-like chain of mannuronic acid (M) (Picture 3) can bind to Ca^{2+}, but tends to at high cation concentrations. Due to the difference between the structures of mannuronic acid (M) and gulluronic acid (G), the gel formed from mannuronic acid (M) is more elastic, while the gel from gulluronic acid (G) is firm (Bedê et al., 2017).

Color

The color of the immobilized cells produced in this study is very likely caused by the concentration of Na-alginate. Visually, immobilized cells with a Na-alginate concentration of 2% produce a clear white color, while at a concentration of 3% they produce a slightly cloudy white color. A Na-alginate concentration of 4% produces immobilized cells with a slightly brownish white color, whereas a concentration of 5% produces immobilized cells with a brownish white color. The color produced in this research is in accordance with research by Mubarokah (2018) which used alginate concentrations of 1%, 2%, 3% and 4% where the 1% concentration had a clearer color when compared to the 2%, 3% and 4% concentrations. Therefore, differences in Na-alginate concentration are very likely to result in changes in the color of immobilized cells.

Number of Bacteria Based on Turbidity Using MCF Standard

Calculation of the number of bacteria using the turbidity method based on Mc Farland standards using 4 tubes containing NB (Nutrient Broth) media and *A.xylinum* culture.

Table 2. Results of bacterial calculations based on turbidity values using McF standards and conversion values.

Tube	Na-alginate Concentration Treatment	Number of bacteria based on turbidity value	Number of Bacteria in (CFU/ml)
1	2%	5.08	1.5 x 10^9
2	3%	5.47	1.5 x 10^9
3	4%	4.17	1.2 x 10^9
4	5%	4.68	1.2 x 10^9

Note: Observation of bacterial turbidity using Densitometer

The difference in turbidity values based on Na-alginate concentration treatments of 2%, 3%, 4%, and 5% w/v (Table 2) shows that the number of microbes in Tube 1, calculated using a densitometer, has a turbidity value of 5.08, equivalent to the standard Mc Farland 1.5 x 10^9 CFU/ml, in Tube 2 with a turbidity value of 5.47, equivalent to the Mc Farland standard, namely 1.5 x 10^9 CFU/ml, Tube 3 with a turbidity value of 4.17, equivalent to 1.2 x 10^9 CFU/ml, in Tube 4 with a turbidity value of 4.68, equivalent to 1.2 x 10^9 CFU/ml. Even though the bacterial population in the tube looks different, it is still at a concentration of 109 CFU/ml. The results of the bacterial calculations carried out will then be used to make immobilized cells.

Application of Immobilized Cells in Making Nata de Coco

The immobilized cells that had been made, with Na-alginate concentrations of 2%, 3%, 4% and 5% (w/v) were then applied to make nata de coco with *A.xylinum* starter as a control. The results of the nata de coco fermentation process using free cells (starter) and also immobilized cells can be seen in Table 3.

Table 3. Nata de coco fermentation results.

Treatment	Picture	Description
Starter *A.xylinum* (kontrol)		-Nata layer: stable and quite thick -Medium color: brownish -Yield: no arrangement is formed so there is no yield
Immobilized cells with Na-alginate 2%		-Nata layer: there is no nata layer because it is contaminated -Medium color: reddish -Yield: no arrangement is formed so there is no yield
Immobilized cells with Na-alginate 3%		-Nata layer: stable and quite thick -Media color: cloudy light brown -Yield: no arrangement is formed so there is no yield
Immobilized cells with Na-alginate 4%		-Lapisan nata: tipis -Medium color: clear light brown -Yield: not formed nata so there is no yield
Immobilized cells with Na-alginate 5%		-Nata layer: very thin -Medium color: murky white -Yield: no arrangement is formed so there is no yield

Note:

- Starter A.xylinum (kontrol) : 100 ml media + 15 ml starter *A.xylinum*
- Immobilized cells with 2% Na-alginate: 100 ml of media + 5 grams of immobilized cells of *A.xylinum* with a concentration of 2% Na-alginate
- Immobilized cells with 3% Na-alginate: 100 ml media + 5 grams of *A.xylinum* immobilized cells with a concentration of 3% Na-alginate
- Immobilized cells with 4% Na-alginate: 100 ml media + 5 grams of *A.xylinum* immobilized cells with a concentration of 4% Na-alginate
- Immobilized cells with 5% Na-alginate: 100 ml media + 5 grams of *A.xylinum* immobilized cells with a concentration of 5% Na-alginate

The fermentation results show that nata de coco using *A.xylinum* starter produces layers of nata fiber that do not break, are compact and quite thick (3 mm) with a brownish color. Meanwhile, nata fermentation using immobilized cells with a Na-alginate concentration of 3% produced a nata fiber layer close to control, with the nata fiber layer looking stable and quite thick, with a cloudy light brown color. The nata fiber layer formed during fermentation of nata with a 4% Na-alginate concentration looks thinner than the nata fiber layer using a 3% Na-alginate concentration, and is a clear light brown color. Nata fermentation using immobilized cells with a Na-alginate concentration of 5% produces a very thin layer of nata fiber with a cloudy white color. There was no yield in the fermentation process carried out from control or immobilized cells.

In this research, the nata de coco fermentation process using immobilized cells and *A.xylinum* starter as a control did not produce a yield, because the nata produced was only a layer of white fiber measuring ± 3 mm. However, the fermentation process continued, which was marked by a color change in the fermentation medium and the presence of a sour aroma which was thought to be acetic acid, a metabolic product of *A.xylinum*. According to Hasfita et al., (2015) in their research on the use of Seri fruit (*Muntingia Calabura L*) for making acetic acid using *A.xylinum* bacteria, that the fermentation of acetic acid produced by *A.xylinum* bacteria with fermentation times of 3, 6, 9, and 12 days produced the highest acetic acid levels on day 9, namely 1.369%.

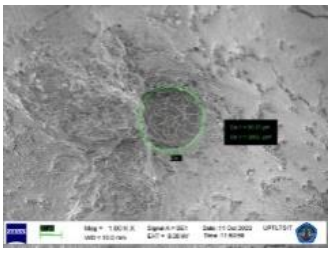
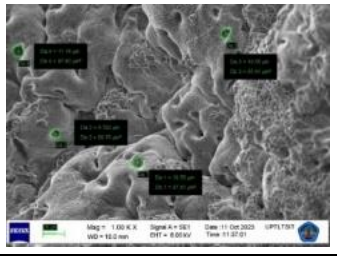
Fermentation of nata coco in this study did not produce an alcoholic aroma. This is likely because during the fermentation process the sugar is oxidized to acetic acid. During its growth, *A.xylinum* releases extracellular enzymes that are able to form cellulose from glucose which resembles a gel matrix called nata (Kuncara, 2017). The process of forming cellulose by *A. xylinum* consists of four reaction stages (Kuncara, 2017). The first stage is the hydrolysis of sucrose which produces fructose and glucose by the sucrase enzyme, the second stage, the intramolecular conversion reaction of α-D-glucose into β-D- glucose with the help of the isomerase enzyme, the third stage, the intermolecular reaction of glucose through 1,4 B-glycoside bonds, and the final stage of formation of cellulose with its repeat units, namely cellobiose.

Several factors cause nata not to form, including inhibiting the growth activity of *A.xylinum* bacteria caused by unfavorable fermentation conditions. Disturbed aeration can affect nata fiber content and produce cellulose, conversely, thin nata is caused by inhibited growth of *A.xylinum* (Muchtadi, 1997). According to Rohmani and Kristianingrum, (2012), the inhibited growth of *A.xylinum* bacteria will result in the resulting nata being soft and thin, and it is even possible that no nata will be formed. The growth phase of *A.xylinum* is in the stationary phase, the cells are unproductive or no longer productive or the cell biomass is small (Brooks et al., 2013). The color difference in the fermentation medium is probably caused by the thickness of the nata. The thicker the nata, the darker the resulting color, it is suspected that there will be more and denser cellulose tissue formation, conversely, the thinner the nata, the lighter the result (Putriana and Aminah, 2013).

SEM Analysis to Observe the Pores and Surfaces of Immobilized Cells

SEM observations were carried out on immobilized cells with Na-alginate concentrations of 2% and 5% to see the differences in pores and surfaces of immobilized cells. Table 5 shows the results of observations of immobilized cells using a *Scanning Electron Microscope* (SEM) with 1000x and 3500x magnification.

Table 4. Observation results using SEM on immobilized cells.

Treatment	Picture		Description
Na-alginate 2%			-Beads surface: *Cracking* or cracking -Pores: Large size with a small amount -Pore size: 60.37 µm
Na-alginat 5%			-Peraccording to beads : Halus -Pores: Small size with a large amount -Pore size: 10.55 µm

Based on the complete cross-section of immobilized cells, it shows that immobilized cells with a Na-alginate concentration of 2% have a different cross-section from immobilized cells with a concentration of 5%, both in terms of size and number of immobilized cell pores. Meanwhile, the surface of the immobilized cells at a concentration of 2% looks cracked, and at a concentration of 5% the surface of the beads is smooth. Low Na-alginate concentrations produce immobilized cells with a slightly soft texture and are likely to be easily destroyed when used. Besides that, low Na-alginate concentrations result in the cell protective layer being thinner and unstable during the fermentation process. Although Na-alginate can still protect cells, the cell sensitivity to the fermentation medium is too low. This results in the amount of living biomass in the Ca-alginate matrix decreasing (Lotfipour et al., 2012).

The results of this research are in line with Andersen's (2015) statement that the lower concentration of Na-alginate in immobilized cells will make the alginate structure more flexible because the guluronic acid and mannuronic acid content is not sufficient to bind water. The SEM results show that on the surface of the immobilized cells there is a collection of *A.xylinum* cells that penetrate out of the beads. According to Luwihana et al., (2010), their research on acetic acid fermentation with immobilized cells of Acetobacter pasteurianus showed that by entrapment immobilized cells are thought to be located on the surface of the beads to form aggregates. This statement was strengthened by Mori et al., (1989) on the Growth Behavior of Immobilized Acetic Acid Bacteria, Barbotin et al., (1990) on Observations of Immobilized Cells in the Physiology of Immobilized Cells, and Osuga et al., (1984) on Production of Acetic Acid by Immobilized *Acetobacter aceti* Trapped in K-Carrageenan Gel, which states that cells that are trapped form aggregates on the surface of beads with a thickness of 20-50 µm, still grow and reproduce. The greater the number of cells in the beads, the motile and aerobic nature of the acetic acid bacteria cells, the more cells will escape from the mobilizing matrix in the fermentation medium when the fermentation process takes place (Fumi et al., 1992).

At 2% concentration it has a size of 60.37 µm, while at 5% concentration the pore size is 10.55 µm (Table 5). These results are in line with research by Halim et al., (2019) concerning the Effect of Percentage of PVA-Alginate Beads on the Decolorization Level of Azo Synthetic Dyes Using the Immobilized Bacteria Consortium, it shows that a PVA-alginate ratio of 8:1 has an average pore size of 6,306 µm, a PVA-alginate ratio of 10:1 has a pore size of 5,089 µm, and a PVA-alginate ratio of 12:1 has a pore size of 2,557 µm . The large pore size is thought to result in a high value of the gel's ability to expand (*swelling*).

CONCLUSIONS

Different concentrations of Na-alginate affect the characteristics of shape, size, texture and color of immobilized cells. Na-alginate concentration of 3% is considered the best concentration compared to other concentrations, resulting in immobilized cells with a shape that is neither too big nor small (4 mm), with an elastic texture. Immobilized cells made at a Na-alginate concentration of 3% were then applied to make nata de coco. The nata that was formed did not provide a yield value because it had a thickness of ±3 mm which was close to the control, but fermentation continued. Different Na-alginate concentrations produce different pore sizes using the SEM test, at low concentrations (2%) produce beads pore sizes of 60.37 µm, and 10.55 µm in beads with a Na-alginate concentration of 5 %.

It is necessary to conduct further research on the storage duration of immobilized cells, cell viability and stability. In addition, this study also needs to calculate the number of bacteria using the Total Plate Count (TPC) method as a comparison with the turbidity method that has been done.

Authors' Contributions: Maria Erna designed the study. Nafiatul Fitriah carried out the laboratory work and analyzed the data. Lathifa Indraningtyas wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing Interests: The authors declare that there are no competing interests.

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