The effect of legen treatment on fermentation process in production of nata de legen

N Hendrianie, S N Putri, Y I Satria
Department of Chemical Engineering, Faculty of Industrial Technology and Systems Engineering, Sepuluh Nopember Institute of Technology, Surabaya, East Java, 60111, Indonesia.
nuniek@chem-eng.its.ac.id

Abstract. Palmyra palm (Borassus flabellifer) is one of the most widely cultivated plants in Indonesia, especially in Gresik, East Java. The number of Palmyra palm trees in that area is estimated to be approximately 5,000,000. The Palmyra palm tree produces a large amount of legen obtained by tapping the palm flower and its use now is only limited as a beverage. By processing the legen into the nata, it would increase its selling price and the local people's income. In this research, the method used to produce the nata de legen was by fermenting the legen using the microbial Acetobacter xylinum. This method was the same as for producing the nata de coco from coconut water. There were various methods of pre-treatment of the legen before the fermentation process. The legen was stored at room temperature 30°C, cooled in a refrigerator for 2 hours, or heated at 100°C for 10 minutes, with incubation time was 10 days respectively. The results showed that the quality of the nata de legen was affected by the pre-treatment of the legen. The best quality of the nata obtained was its thickness of the 4 mm and the cellulose content of 23.25% under pre-treatment condition at 100°C

1. Introduction
The Palmyra palm tree (Borassus flabellifer) is a versatile type of palm. Almost all parts of the plant can be utilized. The flowers of Palmyra palm produce water drops that are usually used as a beverage (Legen) in the dry season and brown sugar in the rainy season. Legen is the liquid that comes out of the filter which is tapped on the flower tank, both the male and female flowers derived from the Palmyra palm tree. The Legen palm has a relatively high sugar content of about 10-15g/100 ml [1]. Legen contains complex nutrients consisting of sugar, protein, nitrogen, minerals, vitamin B complex that is useful for the growth of microorganisms. Usually, legen is used as raw material for the manufacture of traditional alcoholic beverages that are known as jaggery, Tuak, and vinegar [2]. In this study utilizing legen which is abundant in East Java, especially in Gresik, which so far only made tuak (an alcohol beverage) into Nata de Legen which is a fibrous drink and beneficial for health. Fresh Legen cannot be stored for a long time, only a few hours (± 24-36 hours) since the tapping process because it will undergo some changes marked with the presence of bubbles and sour taste.

The fermentation process is a chemical change in organic substrates, namely carbohydrates, proteins, fats, etc. through biochemical activities carried out by enzymes of specific types of microorganisms [3]. The process of making nata is an example of a non-alcoholic fermentation process. The nata fermentation process is complete when there is no fluid left in the tray except for the nata sheet. Nata with good quality have transparent white color, smooth surface, the same thickness in all parts, a thin membrane on the top surface that can be easily separated, and a thin, soft membrane on
the bottom [4]. The thickness and yield of nata are influenced by the duration of the fermentation process and the height of the media in the fermenter. The longer the fermentation time positively affect the thickness and yield of nata. The shallower the media in the fermenter, the better the air circulation for the growth of the microorganism Acetobacter xylinum [5].

Acetobacter xylinum is a type of Acetobacter bacteria that has features such as rod-shaped, gram-negative, aerob, with the width of 0.5-1 μm and the length of 2-10 μm. During the fermentation process of making nata, the Acetobacter xylinum bacteria will form cellulose as a nata product [6]. Acetobacter xylinum bacteria are able to oxidize glucose into gluconate acid and other organic acids at the same time. The most prominent trait of this bacteria is having the ability to convert glucose into cellulose by the polymerization process. The next cellulose formed a matrix known as nata [7].

2. Experimental

2.1. Material
Aquadest and alcohol 70% were used to sterilize the tools used in this study. The materials used to make the nata media solution were vinegar (dixi brand), food grade ZA, granulated sugar, legen, and starter of Acetobacter xylinum.

2.2. Method
There were 4 stages in making nata in this research which consists of legen treatment, making nata media solution, bacteria inoculation, and fermentation.

2.2.1. Legen treatment
The production of nata de legen began with analyzing the pH, sucrose content, and cellulose content in the legen before treatment. Then, there were 3 legen treatment variables, consisted of storage at room temperature (30°C), cooling in the refrigerator for 2 hours, and heating at 100°C for 10 minutes. Then, the treated legen was analyzed again for its pH and sucrose content.

2.2.2. Making nata media solution
The treated legen was put into 3 different beaker glasses. Then, the granulated sugar was added into the legen as much as 15% of the total volume of legen. Granulated sugar serves as a carbon source in the growth of Acetobacter xylinum because sucrose is the best compound for the growth of Acetobacter xylinum. After the addition of granulated sugar, the sucrose content was analyzed again.

Next, 4 ml of vinegar and 0.5 gram/liter of food grade ZA were added to the legen. Food grade ZA functions as a source of nitrogen and sulfur for Acetobacter xylinum, where nitrogen and sulfur are important elements in the growth and proliferation of Acetobacter xylinum. Nitrogen and sulfur are the main building blocks of protein. If non-organic nitrogen was compared with organic nitrogen (such as protein and yeast), the cost of using non-organic nitrogen is cheaper and the quality is quite good.

Food grade ZA or (NH4)2SO4 is a non-organic nitrogen which is very good when used as an additive to make nata because it is very economical, it dissolves easily in other solutions, and it is very selective for the growth of other microbes. Meanwhile, vinegar serves to maintain the solution of Nata de Legen media to remain acidic, because the optimum pH required by Acetobacter xylinum to produce cellulose as a product of Nata de Legen is around 3-4 [7]. Legen that has been mixed with granulated sugar, food grade ZA, and vinegar is called nata media solution.

2.2.3. Starter of Acetobacter xylinum
In this research, the starter of Acetobacter xylinum reached the log phase at the 6th hour. In the log phase, Acetobacter xylinum grows and divides at maximum speed and releases extracellular-polymerase enzymes which function to compose glucose polymers into cellulose or nata matrix [8]. Based on calculation of the number of cells every 2 hours using the counting chamber method, the Acetobacter xylinum in this research had a growth curve as shown in Figure 1.
2.2.4. Fermentation

When the nata media solution was ready, it was cooled to room temperature (30°C) and was inoculated with the starter of *Acetobacter xylinum* which had been in the log phase. Then, the nata media solution that had been inoculated with *Acetobacter xylinum* was covered using newspaper with a hole and rubber. *Acetobacter xylinum* is an aerob bacteria that needs oxygen for its growth, so the cover paper must be perforated for the air needed by *Acetobacter xylinum*. The final step is nata media solution fermentation at room temperature (30°C) for 10 days because the optimal temperature for nata incubation was 28-30°C.

During the 10 days of the fermentation process, cellulose will be formed as a product of nata yields because of *Acetobacter xylinum* activity. The mechanism of cellulose formation by *Acetobacter xylinum* is a series of biochemical processes consisting of four reaction stages. The first stage is hydrolysis of the main content of granulated sugar (sucrose), which produces fructose and glucose. At this stage, sucrose is hydrolyzed by the enzyme sucrase or invertase enzyme, which is a type of protein that acts as a catalyst in converting sucrose to glucose and fructose [9]. The hydrolysis reaction of sucrose occurs such as Figure 2.

![Figure 2. Sucrose hydrolysis reaction](image)

The second stage is the reaction of intramolecular changes from α-D-glucose to β-D-glucose using the isomerase enzyme which is produced by *Acetobacter xylinum*. A glucose in the form of β played a role in the formation of cellulose [9]. The change reaction of α-D-glucose to be β-D-glucose occurs such as Figure 3.
Figure 3. The change reaction of α-D-glucose to be β-D-glucose

The third stage is the intermolecular reaction of glucose through 1,4 β-glycoside bonds [9]. This reaction occurs such as Figure 4.

Figure 4. The form reaction of 1,4 β-glycoside

The fourth stage is the polymerization reaction, which is a reaction for the formation of cellulose by *Acetobacter xylinum*, with the cellobiose as the re-unit. The type of polymerization is condensation polymerization by eliminating water [9]. This polymerization reaction occurs such as Figure 5.

Figure 5. The reactions of cellulose formation by *Acetobacter xylinum*

3. Result and Discussion
The characteristics of legen before treatment were shown in Table 1 and the characteristics of legen after treatment were shown in Table 2.
Table 1. The result analysis of legen before treatment

| Legen Treatment                              | Sucrose (%) | Cellulose (%) | pH |
|----------------------------------------------|-------------|---------------|----|
| Stored at room temperature (30°C)            | 6.93        | 0.37          | 4  |
| Cooled in the refrigerator for 2 hours       | 6.93        | 0.37          | 4  |
| Heated at a temperature of 100°C for 10 minutes | 6.93        | 0.37          | 4  |

Table 2. The result analysis of legen after treatment

| Legen Treatment                              | Sucrose (%) | pH |
|----------------------------------------------|-------------|----|
| Stored at room temperature (30°C)            | 6.93        | 4  |
| Cooled in the refrigerator for 2 hours       | 4.21        | 4  |
| Heated at a temperature of 100°C for 10 minutes | 5.23        | 4  |

Based on Table 1 and Table 2, the sucrose content in the legen which was stored at room temperature (30°C) had an equal value with the initial amount before treatment. Meanwhile, the sucrose content in legen which was heated at 100°C for 10 minutes decreased by 1.7% from the initial amount before treatment and the sucrose content in legen which was cooled in the refrigerator for 2 hours decreased by 2.72% from the initial amount before treatment. Both have decreased because the sucrose in the legen will be inverted into invert sugar, namely glucose and fructose which are reductive, when the legen was heated and cooled [10].

Based on the results, the amount of sucrose content in legen was still insufficient to use it as a nata-making material because the optimal sucrose content for the start of fermentation in making nata is at least 16% [5]. Therefore, in order for the Acetobacter xylinum used for making nata have adequate nutrition, granulated sugar was added as much as 15% of the total volume of legen. After the granulated sugar was added, the sucrose content on all of legen was increased shown in Table 3. After the 10 days fermentation process, the nata was formed and the result is shown in Table 4.

Table 3. The result analysis of legen after the granulated sugar was added

| Legen Treatment                              | Sucrose (%) |
|----------------------------------------------|-------------|
| Stored at room temperature (30°C)            | 16.29       |
| Cooled in the refrigerator for 2 hours       | 15.97       |
| Heated at a temperature of 100°C for 10 minutes | 16.33       |

Table 4. The result analysis of nata de legen product

| Legen Treatment                              | Sucrose (%) | Cellulose (%) | Thickness (mm) | pH |
|----------------------------------------------|-------------|---------------|----------------|----|
| Stored at room temperature (30°C)            | 5.34        | 19.79         | 4              | 3  |
| Cooled in the refrigerator for 2 hours       | 6.22        | 18.35         | 4              | 3  |
| Heated at a temperature of 100°C for 10 minutes | 3.69        | 23.25         | 4              | 3  |
Based on Table 2 and Table 4, it can be concluded that the best nata de legen product in this research was obtained when the legen undergoes initial treatment of heating at 100°C for 10 minutes. From 200 ml of legen, the best nata was produced with a yield of 0.114 ml nata/ml legen. This product had nata volume of 22.69 ml with the highest yield of converting sucrose to cellulose.

It happened because the effect of high temperature on legen treatment inhibits the activity of pathogenic microorganisms present in legen so that the sucrose in legen is not damaged much. With heating, the sucrose contained in legen was not fermented properly by microorganisms that are already present in fresh legen, thus inhibiting the decrease in pH due to fermentation. High temperatures can also inhibit the activity of the invertase/sucrase enzyme which plays a role in the breakdown of sucrose in the cellulose biosynthesis process by the *Acetobacter xylinum*. The invertase enzyme can work optimally at 60°C so that with the legen treatment in the form of heating at 100°C, the activity of enzymes and pathogenic microorganisms decreases. Thus, the use of high temperatures in heating legen is one of the best treatment efforts to defend legen from damage. Therefore, legen heated at 100°C was the best medium for making nata de legen in this research so that it can produce the best nata de legen products [6].

The pH of legen before and after treatment was 4, while the pH of legen after undergoing fermentation is 3. In the metabolic process, the *Acetobacter xylinum* forms a cellulose membrane as a nata product due to the activity of the *Acetobacter xylinum* on glucose. The carbohydrates contained in the nata medium are broken down into glucose, then the glucose will bind to fatty acids (guanosine triphosphate) to form a characteristic precursor of cellulose. This activity is assisted by extracellular-polymerase enzymes, then the precursors are released into the environment to form cellulose bonds on the surface of the nata media. During the metabolism of carbohydrates by the *Acetobacter xylinum*, a glycolysis process occurs which begins with the conversion of glucose into glucose 6-phosphate which then ends with the formation of pyruvic acid. The glucose 6-phosphate formed in the glycolysis process is used by *Acetobacter xylinum* to produce cellulose. The cellulose formed has β 1,4-glycoside bonds and is composed of glucose, mannose, rhamnose, and glucuronic acid in a ratio of 3: 1: 1: 1 [11]. During fermentation, the pH decreased from 4 to 3, due to the formation of pyruvic acid as a result of the glycolysis process.

In SNI standards, the cellulose content in nata de coco is 35%. The best nata de legen product in this research contained 23.25% cellulose which did not match with SNI standards. The type of fiber in nata was crude fiber. Crude fiber was the result of sugar reshuffling in the fermentation medium by the activity of *Acetobacter xylinum* [12]. Crude fiber consists of cellulose, hemicellulose, and lignin [13].
Nata does not contain lignin. Therefore, the crude fiber yield was considered to only indicate the level of cellulose nata which was the result of the *Acetobacter xylinum* activity. This was due to the insufficient fermentation time of the nata media, so that to increase the cellulose content in nata, the fermentation time is needed to be more than 10 days.

Meanwhile, for the SNI standard, the sucrose content in nata de coco is at least 15%. However, the best nata de legen product in this research had a sucrose content of 3.69% which did not match with SNI standards. This can be corrected by increasing the concentration of sugar added in the manufacturing process.

The SNI standard for the thickness of nata de coco is 10 - 15 mm which was obtained during the fermentation time of 7-14 days, while the nata de legen product in this research had a thickness of 4 mm which was obtained on the 10th day of fermentation. On the 10th day of fermentation, the thickness of nata de legen has not reached 10 mm, this was influenced by the substrate concentration, material composition, environmental conditions, and the ability of *Acetobacter xylinum* to produce cellulose [12].

If the nata de legen product had a value of sucrose and cellulose (fiber) content and a value of thickness of the nata which matched the SNI standards for nata, the nata de legen product can be consumed. Therefore, it can be concluded that this nata de legen research needs to be carried out and developed again so that it is in accordance with SNI standards and it can be consumed.

References

[1] Silaban B M J and Yuwono L F 2017 *Optimasi Fermentasi Produksi Etanol dari Legen Palmyra palm (Borassus flabellifer) Menggunakan Mikroorganisme Saccharomyces cerevisiae dan Pichia stipitis dengan Response Surface Methodology* (Surabaya: Institut Teknologi Sepuluh Nopember) p 8

[2] Ristriani S, Kuswardani I and Adikaryo M.I.L. 2001 *J. Biota* 4 2

[3] Prescott S C and Dunn C G 1981 *Industri Microbiology* (New York: McGraw-Hill Book)

[4] Pambayun R 2006 *Teknologi Pengolahan Nata de Coco* (Yogyakarta: Kanisius) p 3

[5] Haryatni T 2002 *Pengaruh Komposisi Bahan Terhadap Mutu Fisik dan Stabilitas Warna Nata de Coco* (Bogor: Fakultas Teknologi Pertanian Institut Pertanian Bogor) p 4

[6] Hesse S and Kondo T 2005 *J. International Devoted to Scientific and Technological Aspects of Industrially Important Polysaccharides* 60 1

[7] Brown R M 2006 *The Biosynthesis of Cellulose* (Texas: Botany Department University of Texas)

[8] Malvianie E 2014 *J. Institut Teknologi Nasional* 2 6

[9] Uswatun B 2014 *Pembuatan Nata de Coco* (Palembang: Sukses Bisnis) p 1

[10] Sutedjo V I, Kusumatawi N and Widyawati P S 2015 *J. Teknologi Pangan dan Gizi* 14 86

[11] Sutriah K and Sjahriza A 2000 *Pemanfaatan Limbah Cair Industri Tempe Sebagai Bahan Baku-Alternatif Pengganti Air Kelapa Pada Industri Nata "Segar Sari Mandiri" di Ciheuleut Kecamatan Bogor Utara, Kodya Bogor* (Bogor: Institut Pertanian Bogor) p 5

[12] Putriana I and Aminah S 2013 *J. Pangan dan Gizi* 4 31

[13] Sudarmadj S 2003 *Analisa Bahan Makanan dan Pertanian* (Yogyakarta: Kanisius) p 3