Utilization of Molasses to Produce Lactic Acid by Using \textit{Lactobacillus} \textit{Delbrueckii} and \textit{Lactobacillus} \textit{Plantarum}

S Nurkhamidah*, A Altway, Susianto, Y Rahmawati, F Taufany, N Hendrianie, H Ni’mah, I Gunardi, S Zulaikah, E O Ningrum, R D Nyamiati, and A Ramadhani

Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Kampus ITS Sukolilo, Surabaya 60111, Indonesia

*E-mail: dst_eureka@yahoo.co.uk

\textbf{Abstract.} Molasses is the final liquid from the process of crystallization of sucrose in a sugar factory. In general this liquid is only disposed to the surrounding environment or used as a fertilizer mixture. However, this fluid still contains glucose that can be fermented into lactic acid. Lactic acid is a water-soluble compound commonly used in food, pharmaceutical and chemical industries. Through the polymerization process, lactic acid changes into polylactic acid (PLA) which is a biodegradable plastic. In general, PLA is made from corn. If PLA can be synthesized from molasses, it can reduce the use of corn as a raw material for making PLA and reduce waste in the sugar industry. This study aims to determine the optimal conditions of lactic acid fermentation by using sugarcane drops from Tulung Agung as a source of glucose and its purification so that it can obtain high levels of lactic acid. The results showed that fermentation with \textit{Lactobacillus plantarum} could produce more lactic acid with faster fermentation time than the fermentation using \textit{Lactobacillus delbrueckii} with a concentration of lactic acid 19.68 g/L.

1. Introduction

Lactic Acid (2-hydroxycaboxylic acid) is an organic compound that can dissolve in water and can be found in nature in the form of L - lactic acid [1]. Lactic acid is widely used in industry as a chemical solvent and as a detergent. In addition, lactic acid is also widely used as an additive in cosmetics that serves to maintain moisture. Lactic acid is also used in the food industry as an additional ingredient to give food a sour taste. Another use of lactic acid is when it is in the form of potassium lactate that has properties to control pathogenic bacteria in fish and animal feed products [1]. Lactic acid can be obtained either by the action of fermented microorganisms or chemical synthesis. The fermentation process has the advantage of being more cost-effective [2]. Lactic acid demand continues to grow every year. However, the production of the lactic acid industry does not follow market demand. That happened for several reasons, including the price of raw materials. The substrate used by the industry is glucose and starch, both of which have been processed, which eventually ends the process [3]. Lactic acid fermentation can also be carried out by fungi such as \textit{Rhizopus oryzae} in aerobic conditions. However, fungi such as \textit{Rhizopus oryzae} have a slow growth and lower production rates compared to lactic acid bacteria. Therefore, fermentation using lactic acid bacteria is more widely used [4].

According to the product produced by lactic acid bacteria, there are two types of lactic acid bacteria, first homofermentative lactic acid bacteria which are lactic acid bacteria that can produce L- lactic acid
or D-lactic acid from glucose with a yield of 85% and above. Whereas, heterofermentative lactic acid bacteria are lactic acid bacteria which only produce L-lactic acid or D-lactic acid from glucose with a 50% yield and the rest are byproducts [5]. In the lactic acid fermentation process, there are several raw materials that can be used as sources of monosaccharides and disaccharides, for example flour and lignocellulose [4]. In addition, lactic acid bacteria also require other nutrients such as vitamin B, amino acids, fatty acids, peptone and yeast extract [5]. The addition of some of these nutrients will increase production costs, but the process is expected to produce purer lactic acid. Lactic acid bacteria can grow under anaerobic conditions and can grow well in the temperature range between 5-45 °C and in the pH range 5.5-6.5 [4]. The polymerization process of lactic acid is initiated by dehydration of the monomer, which generates a prepolymer chains consisting of oligomers and low molecular weight PLA. This process, starting from lactic acid involves three distinct stages: polycondensation, obtaining lactide and ring-opening polymerization [6].

Optical purity of lactic acid is very important for the physical properties of poly-lactic acid (PLA), and pure lactic acid can be polymerized to highly-crystalline poly-lactic acid (PLA) which is suitable for commercial use [7]. As a monomer for the production of poly-lactic acid (PLA) it has become famous for its broad usefulness and beneficial characteristics, such as biodegradability, biocompatibility, elasticity and well-controlled drug release profiles [8]. Poly-lactic acid (PLA) is considered to be one of the most promising biodegradable plastics, mainly due to its high chemical resistance, which is advantageous for the manufacture of fibers and films, while heat resistance is beneficial for the production of many equipments [9]. Molasses is an industrial byproduct of the process of sugar and alcohol and rich in fermented sugar [10], nitrogen and vitamins. This substrate is not expensive and highly available in Brazil, with annual production of 17.9 million tons during the sugar manufacturing process.

The purpose of this study is to determine the optimal conditions of lactic acid fermentation using molasses as a source of glucose by using Lactobacillus plantarum and Lactobacillus delbruckii bacteria to obtain high levels of lactic acid.

2. Materials and Methods
The main materials used were molasses from waste in the sugar industry. Lactobacillus plantarum and Lactobacillus delbruckii were used in this study. These strains are efficient lactic acid producing bacteria, which can convert glucose into Lactic acid through the fermentation.

2.1. Starter Preparation
Starter Preparation begins with the addition 10 mL of molasses, 4 gr of urea, and Na-Asetat buffer solution reaches pH 4.5. Finally the pH was set the pH between 6.5-7 by addition of NaOH, a total volume of 100 mL, added Lactobacillus plantarum or Lactobacillus delbruckii. These were incubated at 37 °C for 24 hours in a incubator.

2.2. Fermentation Lactic Acid
Fermentation begins with the addition of 100 mL molasses, 240 gr urea, and Na-acetate buffer solution reaches pH 4.5. Finally the pH was set to pH 6.5-7 with the addition of NaOH, a total volume of 1 L, put into 5 erlenmeyer, added with a starter of Lactobacillus plantarum or Lactobacillus delbruckii for 10 mL, incubated at 37 °C for 24 hours in a shaking incubator.

2.3. Fermentation Result Analysis Method
Growth in the amount of microbes is observed through the counting chamber. The fermentation results were autoclaved at 121 °C then reduced sugar test using spectrophotometry after 10 times dilution, and lactic acid test with the addition of 1 mL of fermentation after autoclave, 6 mL of concentrated H2SO4, heated 10 minutes, cooled at room temperature, added 100 μL CuSO4 and 200 μL PHF was then measured using spectrophotometry after dilution of 100 times with a wavelength of 560 nm.
3. Results and Discussion

3.1. Analysis of Amount Microbes

Molasses is a waste from the sugar processing process in the sugar industry which means it still contains glucose. Therefore, the level of reducing sugar in molasses were firstly analyzed before fermenting using the DNS method and obtained a result of 208.73 g/L. In the process of glucose fermentation to lactic acid, there are two types of microorganisms used, namely Lactobacillus plantarum and Lactobacillus delbrueckii. The fermentation was carried out for eight days including the observation of the number of microorganisms, reducing sugars, and lactic acid produced.

The fermentation process was carried out in batches. First, microorganisms Lactobacillus plantarum and Lactobacillus delbrueckii were reactivated and stored in an incubator at 37 °C for approximately 24 hours. Then a starter was made with 0.4 gr of urea added; 1 mL molasses; and added Na-Acetate buffer then set the pH between 6.5-7, the pH is adjusted in such a way because the pH in the fermentation process must be controlled and maintained at a slightly acidic pH close to neutral so that lactic acid bacteria can grow [4]. Then added 1 microbial Lactobacillus plantarum and Lactobacillus delbrueckii which had been activated and incubated at 37 °C for 24 hours, because according to [4] lactic acid bacteria were able to grow at a temperature range of 5-45 °C.

Table 1. Result of Calculation Amount Lactobacillus plantarum and Lactobacillus microorganisms

| Time (day) | Amount of Lactobacillus delbrueckii Microbes | Time (day) | Amount of Lactobacillus plantarum Microbes |
|------------|---------------------------------------------|------------|-------------------------------------------|
| 2          | 48000000                                   | 2          | 19333333                                   |
| 5          | 59280000                                   | 5          | 19280000                                   |
| 6          | 38960000                                   | 6          | 15520000                                   |
| 7          | 55760000                                   | 7          | 14880000                                   |
| 8          | 47840000                                   | 8          | 27200000                                   |

After the starter was incubated for 24 hours, a fermentation medium was made followed by starter addition into it. The starter medium that had been added to the microbe was stirred evenly and divided into 5 erlenmeyers for fermentation. Fermentation was carried out anaerobically, because bacteria can grow well in anaerobic conditions [4].

![Figure 1. Effect of fermentation time on amount of microbes produced](image-url)
The calculation of the amount of microorganisms was carried out on the second day. The observation shows that the amount of microorganisms was still fluctuating. For Lactobacillus delbrueckii, on the 5th day it increased when compared to the second day. However, on the 6th day it decreased and the 7th day rise again and the 8th day declined again. Where in Lactobacillus plantarum, the amount of microorganisms decreased until the 7th day but increase on the 8th day. From Figure 1 it can be seen that the amount of Lactobacillus delbrueckii is more than Lactobacillus plantarum although the number of bacteria added to the starter was the same. According to [4] the commercial fermentation process of lactic acid bacteria takes 3 to 6 days to complete at concentrations between 5-10 wt%.

3.2. Effect of Fermentation Time on Amount of Reduced Sugar

Besides observing amount of microorganisms during the fermentation process, the remaining reducing sugar was also observed. In theory, the amount of reducing sugar will decrease with the increasing of fermentation time, because the sugar has been converted into a product by microorganisms through the fermentation process.

![Figure 2. Effect of Fermentation Time on Amount of Reduced sugar](image)

3.3. Analysis Lactic Acid

Table 2 shows the absorbance results of lactic acid fermentation with Lactobacillus delbrueckii and Lactobacillus plantarum by using spectrophotometer analysis.

| Time(day) | Absorbance Lactobacillus plantarum | Lactid Acid Concentration of Lactobacillus plantarum (ppm) | Absorbance Lactobacillus plantarum | Lactid Acid concentration of Lactobacillus plantarum (ppm) |
|-----------|-----------------------------------|----------------------------------------------------------|-----------------------------------|----------------------------------------------------------|
| 2         | 0.119                             | 13.98                                                    | 0.111                             | 13.29                                                    |
| 5         | 0.154                             | 16.67                                                    | 0.126                             | 14.52                                                    |
| 6         | 0.143                             | 15.83                                                    | 0.193                             | 19.67                                                    |
| 7         | 0.178                             | 18.52                                                    | 0.103                             | 12.75                                                    |
| 8         | 0.205                             | 20.60                                                    | 0.135                             | 15.21                                                    |
The fermentation results were autoclaved at 121 °C then reduced sugar test using spectrophotometry after 10 times dilution, and lactic acid test with the addition of 1 mL of fermentation after autoclave, 6 mL of concentrated H₂SO₄, heated 10 minutes, cooled at room temperature, added 100 μL CuSO₄ and 200 μL PHF was then measured using spectrophotometry after dilution of 100 times with a wavelength of 560 nm. After that the absorbance data was calibrated using a calibration curve and obtained the concentration of lactic acid (ppm) in fermentation each microbe.

![Figure 3. Effect of Fermentation Time on Lactic Acid Concentration](image)

In Figure 3, it can be seen that the concentration of lactic acid for Lactobacillus delbrueckii has an upward tendency, only on the 6th day it experienced a slight decrease. Whereas in Lactobacillus plantarum, the results of fluctuating lactic acid concentration have not been read. The highest lactic acid concentration for Lactobacillus delbrueckii was 20.60 g/L on day 8. Whereas the highest lactic acid concentration for Lactobacillus plantarum is 19.67 g/L on the 6th day. The commercial lactic acid bacteria fermentation process takes 3 to 6 days to complete at a glucose concentration of between 5-10 wt% [4]. Research [11] also the concentration of lactic acid resulting from fermentation using Lactobacillus plantarum was analyzed using HPLC to produce lactic acid concentration of 5 wt%. From the results of lactic acid obtained, it can be concluded that lactobacillus plantarum can produce more lactic acid and a shorter time than Lactobacillus delbrueckii and with fewer microorganisms can see in Table 1. According to research [12] a with lactobacillus plantarum can produce lactic acid more, more quickly and using molasses as a carbon source it was achieved the highest lactic acid yield considering among the alternative sources of carbon, to know 88 %. The productivity using molasses was the highest, even considering the glucose control: 3.17 g L⁻¹ h⁻¹ [13].
3.4 Effect of Observations Time on Amount of Microbes Produced

Figure 4. Effect of Observations Time on Amount of Microbes Produced

To ascertain whether the starter with incubation for 24 hours was in the phase log of the growth of microorganisms, then the starter was observed as shown in table 3, the starter observation was done for 9 days.

Starter Preparation started with the addition 10 mL of molasses, 4 gr of urea, and Na-Asetat buffer solution reaches pH 4.5. Finally the pH was set to pH 6.5-7 by addition of NaOH, a total volume of 100 mL, added Lactobacillus plantarum or Lactobacillus delbrueckii. These were incubated at 37 °C.

In Figure 4. Observations show that the phase log of microorganisms in the starter until the 3rd day. Therefore, the next experiment will be fermentation with a starter incubation period for 3 days. With this, it is expected to produce even more lactic acid.

4. Conclusion

From the results of experiments that had been carried out it can be concluded that lactic acid fermentation using molasses as a source of glucose found that the Lactobacillus plantarum bacteria can produce more lactic acid than Lactobacillus delbrueckii with a faster fermentation time, with a concentration of 19.68 g/L for 6 days and phase log of microorganisms in the starter between 0 to 3 days.

Acknowledgments

This work has been financially supported by local funding by Institut Teknologi Sepuluh Nopember (ITS) Surabaya with contract’s number 1470/PKS/ITS/2018 to which the authors express their sincerest gratitude.

References

[1] Sin L, Rahmat A and Rahman W 2013 Polylactic Acid (Amsterdam: Elsevier William Andrew)
[2] Silva S S and Mancilha I M 1991 Aproveitamento de Agroindustriais Acido Lático Uma Alternative Tec. De. Alimen. 25 37-40
[3] Bomrungnok W, Sonomoto K, Pinitglang S and Wongwicharn A 2012 Single Step Lactic Acid Production from Cassava Starch by Laactobacillus plantarum SW14 in Conventional Continuous and Continuous with High Cell Density APCBEE. Proc. 2 103-97
[4] Mäki A P, Simakova I, Salmi T and Murzin D 2013 Production of Lactic Acid/Lactates from Biomass and Their Catalytic Transformations to Commodities Chem. Rev. 114(3) 1909-71
[5] Reddy G, Altaf M, Naveena B, Venkateshwar M and Kumar E 2008 Amylolytic Bacterial Lactic Acid Fermentation Rev. Bio. Adv. 26(1) 22-34
[6] Milena S L, Andre L J and Rubens M F 2014 Synthesis and Characterizations of Poly (Lactic Acid) by Ring-Opening Polymerization for Biomedical Applications Chem. Eng. Trans. 38 331-36
[7] Lunt J 1998 Large-Scale Production, Properties and Commercial Applications of Polylactic Acid Polymers and Polymer Degradation Stability 59 145–52
[8] Djukić V A P, Mojović L V, Vukašinović S M S, Rakin M B, Nikolić S B, Pejin J D and Bulatović M L 2012 Effect of Different Fermentation Parameters on L-lactic Acid Production From Liquid Distillery Stillage Food Chem. 134(2) 1038–43
[9] Tanaka T, Hoshina M, Tanabe S, Sakai K, Ohtsubo S and Taniguchi M 2006 Production of D-lactic Acid from Defatted Rice Bran by Simultaneous Saccharification and Fermentation Bio. Tech. 97(2) 211–17
[10] Lima U A, Aquarone E and Borzani W 1975 Biotecnologia Tecnologia das Fermentações (Brasil: São Paulo)
[11] Andrea K, Patricia F M, Johnatt O, Betania H L, Rubens M F and Maria R W M 2014 Purification of Lactic Acid Produces from Sugarcane Molasses Chem. Eng. Trans. 37 367-72
[12] Regiane A O, Andrea K, Carlos E Y R, Maria R W M and Rubens M F 2017 Hybrid Short Part Evaporation as an Option to Lactic Acid Recovery from Fermentation Broth Chem. Eng. Trans. 57 37-42
[13] Regiane A D O, Rubens M F and Carlos E V R 2016 High Lactic Acid Production from Molasses and Hydrolysed Sugarcane Bagasse Chem. Eng. Trans. 50 307-12