IBJ
Indonesian Biodiversity Journal
http://ejurnal.unima.ac.id/index.php/ibj

indonesian.biodivers.j.
Vol. 5, No. 1. April 2024
ISSN : 2722-2659

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM RED DRAGON FRUIT (*Hylocereus polyrhicus*) AS EXOPOLYSACCHARIDE PRODUCERS

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Received: October 11, 2023 *Accepted: April 12, 2024*

Abstract

Cherry tomatoes are a type of Red Dragon Fruit that has various benefits, including lowering cholesterol levels, preventing colon cancer, and strengthening the working power of muscles. Isolation of LAB isolated from Dragon Fruit as a production material for EPS. This study aims to isolate and identify LAB as a producer of EPS from red dragon fruit (*Hylocereus polyrhiruz*), which can produce exopolysaccharides. This research uses a descriptive research method. Data from experimental research in the laboratory obtained 10 LAB isolates, namely isolates NG1, NG2, NG3, NG4, NG5, NG6, NG7, NG8, NG9 and NG10. Based on the identification results, isolate NG1 has similarities with the genus Lactococcus (spherical, gram-positive, nonmotile, non-spore). While isolates NG2, NG3, NG4, NG5, NG6, NG7, NG8, NG9 and NG10 have similarities with the genus Lactobacillus (rod form, nonmotile, gram-positive). Based on the morphological characteristics, which are gram-positive, catalase-negative and non-spore. The ten isolates of lactic acid bacteria are capable of producing EPS. these are the results of EPS acquisition, namely: NG1(152.1 mg/L), NG2(127.9 mg/L), NG3(134.6 mg/L), NG4(130.9 mg/L), NG5(137 mg/L), NG6(139.2 mg/L), NG7(204.9 mg/L), NG8(156.2 mg/L), NG9(136.4 mg/L), and NG10 (157, 3 mg/L). The highest amount of EPS was isolated NG7 at 204.9 mg/L. Meanwhile, the lowest EPS was isolated NG4 at 130.9 mg/L.

Keywords: *Lactic Acid Bacteria, Hylocereus polyrhiruz, Exopolysaccharides*

INTRODUCTION

The traditional use of plants to make medicinal food requires scientific research to provide knowledge of the facts and their benefits. Red dragon fruit contains chemical flavonoids, phenolics, and polyphenols (Jafar *et al*., 2009). Flavonoids are found in all green plants and are secondary metabolites that provide all their pharmacological roles (Rohyami, 2008). Exopolysaccharides (EPS) have essential values for various health benefits for the body. Microbes that produce EPS can attach to the mucosa of the small intestine, thereby increasing the potential to suppress the development of typhogenic microbes in the digestive tract.

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Vol. 5, No. 1. April 2024
ISSN : 2722-2659

Cherry tomatoes are a type of Red Dragon Fruit that has various benefits, including lowering cholesterol levels, preventing colon cancer, and strengthening the working power of muscles. Isolation of LAB isolated from Dragon Fruit as a production material for EPS. Much scientific research has been carried out regarding the potential of LAB in producing EPS. However, it is still focused on milk-based products, for example, in research, which isolated LAB from enrichment results from fruit, seeds, and vegetables as production materials. EPS. This research aims to isolate and identify red dragon fruit (*Hylocereus polyrhiruz*) that can produce exopolysaccharides.

RESEARCH METHODS

Place and time of research

This research was carried out from October to November 2022 in the Microbiology Laboratory, Biology Department, Faculty of Mathematics, Natural Sciences, Manado State University in Tondano, North Sulawesi.

Tools and materials

The tools used in this research were knives, microscopes (*Euromex*), autoclaves (*Gea*), bottles, refrigerators, micropipettes (*Accubiotech*), glass objects, incubator shakers, analytical scales (*Fujitsu*), scissors, bunsens, measuring cups, measuring cups (*Pyrex*), Dropper Pipette (*Brand*), Ose Needle (*Mico*), Spiritus Lamp, Test Tube (*Pyrex*), Tube Rack, Laminar Air Flow (*B-One*), Matches, Oven (*Memmert*), and camera for documentation.

The material used in this research was red dragon fruit (*Hylocereus polyrhiruz*) from the city of Tondano, North Sulawesi Province. The chemicals used for the isolation and identification of LAB are MRS broth, MRS agar, distilled water, CaCO3, NaCl, Hucker's crystal violet paint solution, Lugol's iodine mordant solution, 70% alcohol, safranin solution and H2O2. Other materials used for sterilization purposes include detergent, aluminum foil, cotton, plastic, rubber and newspapers.

Research Method

The method used in this research uses descriptive research methods to test the activity of lactic acid bacteria which can produce exopolysaccharides. Research data was obtained through laboratory experiments.

Red Dragon Fruit Enrichment (*Hylocereus polyrhicuz*)

Enrichment was carried out sequentially with controlled temperature: 35^{o}C at the beginning of the first 12 hours, 35^{o}C at the beginning of the second 12 hours, 35^{o}C at the beginning of the third 12 hours and 35^{o}C at the beginning of the fourth 24 hours to the 48th hour. The total enrichment time was 2 days. This treatment is based on research by Mussa (2014) which states that bacteria grow more dominantly in 8 hour fruit enrichment.

Isolation of Lactic Acid Bacteria

Spontaneous samples are taken of 1 ml at each enrichment for the first 12 hours, second hour, third hour and fourth hour. Then serial dilutions from 10-1 to 10-6 were carried out, namely by diluting 1 ml of

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ISSN : 2722-2659

sample suspension into 9 ml of 0.85% physiological salt solution and homogenizing with an automatic mixer and this was done until the dilution was 10-6. Dilution was carried out to reduce bacterial solids. which is planted. BAL isolation was carried out using a pour plate. 1 ml of each dilution series was taken starting from dilution 10-4 to 10-6 and put into a petri dish and leveled by moving the petri dish in a figure of eight so that the sample was evenly distributed.

Then ± 15 ml of MRS agar medium was added to which 0.2 g of CaCO3 was added. After the MRS medium hardened, the petri dish was placed in an incubator at 37°C and incubated for 48 hours. Colonies that form clear zones on MRS agar media (suspected to be LAB) are taken with a loop needle and inoculated on the same medium using the spread plate method, then incubated at 37°C for 48 hours. The scratching method is carried out repeatedly until colonies with a uniform and separate shape are obtained.

Identification of Lactic Acid Bacteria

Characterization of lactic acid bacteria includes colony morphology (shape, elevation, edge shape, size, surface and color) and cell morphology (cell shape and Gram stain) (Fachrial *et al.*, 2018). Macroscopic characteristics include: colony diameter, colony color, colony elevation, colony edges and the presence or absence of exudate drops and concentric circles. Microscopy includes: hyphae shape, hyphae pigmentation and spores.

Characterization of Cell Morphology of Lactic Acid Bacteria Isolates

All pure isolates that have been obtained will then be gram stained. The first stage of gram staining is to sterilize the test preparation using alcohol. Bacterial glass slides are made by aseptically taking 1 dose of potential bacterial culture suspension and then spreading it over the surface of the glass slide and then passing it over a Bunsen flame for a while. When it is cold, sprinkle it evenly with 2 – 3 drops of crystal violet solution and let it sit for 1 minute. Washed with running water for 5 seconds then dripped with Lugol's solution on a glass object and dried for 1 minute.

After that, wash the slide again with running water, then wash with 96% alcohol for 30 seconds until there is no more Lugol dye, then wash again with flowing distilled water. Then dripped with safranin solution for 10 – 30 seconds, then washed again using running water, then filtered by placing the slide on top of filter paper, then observing the preparation under a microscope. The glass objects were observed with a strong magnification microscope using immersion oil. Gram-positive bacteria are purple in color, while gram-negative bacteria are red (Christoper and Bruno, 2003).

Production of Crude Exopolysaccharides

15 mL of LAB inoculum grown in MRSA media for 24 hours was centrifuged at 5000 rpm at 4º C for 30 minutes and 10 mL of supernatant was obtained. The supernatant was added with cold ethanol (95%) and stored in a refrigerator, then centrifuged at 5000 rpm at 4º C for 20 minutes to obtain pellets. The pellets are dried at a temperature of 100℃, so that a constant weight is obtained (Purwijantiningsih, 2014).

Research variable

- The single variable is the genus of lactic acid bacteria isolated from red dragon fruit (*Hylocereus polyrhiruz*).

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- The independent variables in this research are temperature and Ph value.
- The dependent variable in this study is the number of bacteria that produce expolysaccharides per milligram (*mg*).

Data analysis technique

Data obtained from the identification results are presented in a qualitative descriptive manner including the morphological and biochemical characteristics of each isolate of lactic acid bacteria (LAB) isolated from fermented red dragon fruit (*Hylocereus polyrhiruz*) and the crude exopolysaccharide value produced.

RESULTS AND DISCUSSION

Results of Isolation of Lactic Acid Bacteria

Lactic acid bacteria were successfully isolated from red dragon fruit (*Hylocereus polyrhiruz*). which was enriched for 2 days. Isolation of red dragon fruit samples (*Hylocereus polyrhiruz*) was carried out based on the dilution method using a physiological salt solution (*Aquades*). Next, a 10-1 to 10-7 dilution series was made and then inoculated on MRSA media so that incubation was carried out by covering it in laminar air flow at room temperature. The colonies that grow are then separated based on colony morphology.

Figure 1. Red Dragon Fruit From Tondano, North Sulawesi

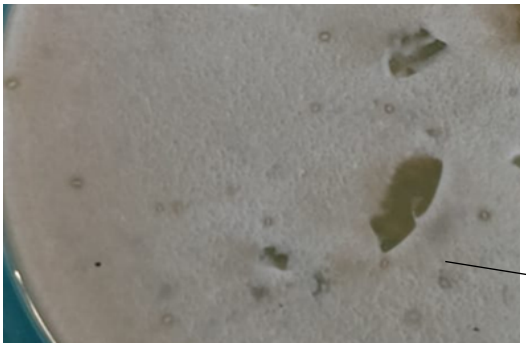

Figure 2. Around the LAB colonies growing in 1% MRSA + CaCo3 media, it is indicated by the presence of a clear zone around the colonies.

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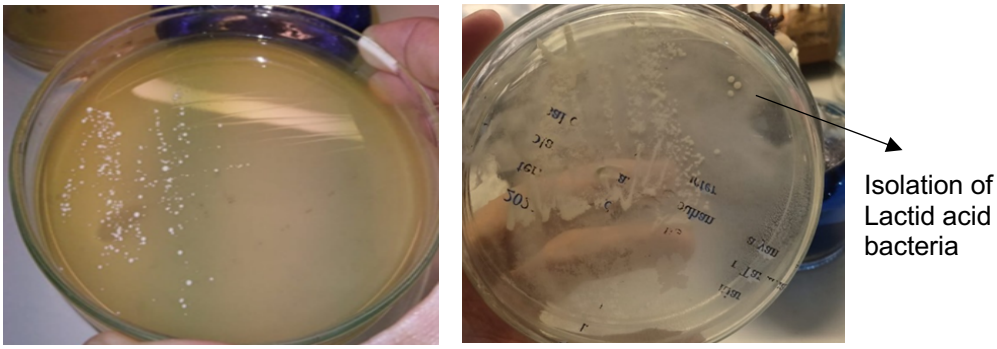

Figure 3. shows that lactic acid bacteria from Red Dragon Fruit (*Hylocereus polyrhiruz*) grow on MRSAgar. At 10 -6 lactid acid bacteria, and at 10-7.

Identification of Colony Morphology of Lactic Acid Bacteria

The results of identifying the morphology of LAB colonies have various shapes, colors and colony elevations. The following results of the morphology of LAB colonies can be seen in table 1.

Table 1. Anova Test Table for Number of Leaves

Kode Koloni	Morfologi				
	Color of Colony	Shape Of Colony	Edge of Colony	Surface of Colony	Texture of Colony
NG 1	White like Milk	*Punctiform*	Choppy	Arise	*Glossy*
NG 2	Cream	Round	Intact	Arise	*Glossy*
NG 3	White	*Punctiform*	Choppy	Arise	*Glossy*
NG 4	White	Round	Intact	Arise	*Dull*
NG 5	Cream	*Punctiform*	Choppy	Flat	*Glossy*
NG 7	White like Milk	*Punctiform*	Intact	Arise	*Dull*
NG 8	Cream	*Punctiform*	Choppy	Arise	*Glossy*
NG 9	White	*Punctiform*	Intact	Arise	*Dull*
NG 10	Cream	*Punctiform*	Intact	Flat	*Glossy*

Identification of Cell Physiology and Biochemistry of Lactic Acid Bacteria Isolates

In the process of isolating lactic acid bacteria, the morphology of the bacterial isolates was not the same and was different from other isolates. Because of this, observations were made covering the color, shape, edges and surface of the colony. In the suspected species, five species were obtained according to Bergey's Manual of Determinative Bacteriology Seventh Edition (2005). The results of the morphology and physiology of lactic acid bacteria isolates can be seen in Table 2.

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Table 2. Picture of Cell Isolate Lactid Acid Bacteria

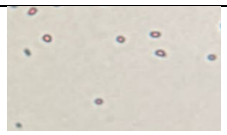
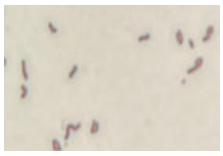
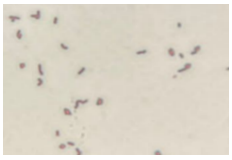
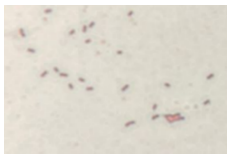
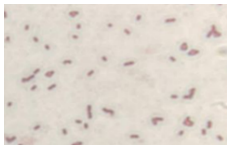
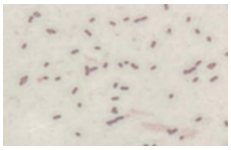
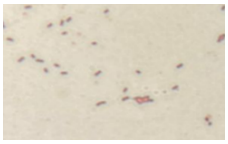
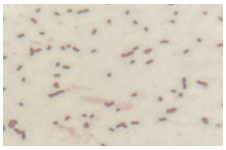
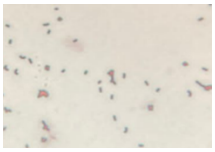
Code Of Isolate	Picture of Isolate	Result Of Coloring Gram
NG1		Positive
NG2		Positive
NG3		Positive
NG4		Positive
NG5		Positive
NG6		Positive
NG7		Positive
NG8		Positive
NG9		Positive

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Table 3. Results of Biochemical Characterization and Morphology of BAL Cells of Red Dragon Fruit (*Hylocereus polyrhiruz*)

Code Isolate	Morfologi Koloni: Shape Of Cel	Arrangement Of Cell	Gram	SIM Spore	Catalase	Temperature 10°	Temperature 37°	Temperature 45°	TSIA	PH	Conjecture Genus
NG 1	Coccus	Singular	+	- -	-	+	+	-	-	5,0	*Lactococcus sp*
NG 2	Basil	Forming a Palisade	+	- -	-	+	+	-	-	5,0	*Lactobacillus sp*
NG 3	Basil	Form A Pair	+	- -	-	+	+	-	-	5,0	*Lactobacillus sp*
NG 4	Basil	Singular	+	- -	-	+	+	-	-	6,0	*Lactobacillus sp*
NG 5	Basil	Forming a Shape V	+	- -	-	+	+	-	-	6,0	*Lactobacillus sp*
NG 6	Basil	Singular	+	- -	-	+	+	-	-	6,0	*Lactobacillus sp*
NG 7	Basil	Singular	+	- -	-	+	+	-	-	5,0	*Lactobacillus sp*
NG 8	Basil	Singular	+	- -	-	+	+	-	-	5,0	*Lactobacillus sp*
NG 9	Basil	Forming a Palisade	+	- -	-	+	+	-	-	5,0	*Lactobacillus sp*
NG 10	Basil	Forming a Shape V	+	- -	-	+	+	-	-	5,0	*Lactobacillus sp*

Testing of Lactic Acid Bacteria That Produce Exopolysaccharides

Testing of lactic acid bacteria containing exopolysaccharides showed that ten isolates of lactic acid bacteria had the ability to produce exopolysaccharides, namely isolates NG1, NG2, NG3, NG4, NG5, NG6, NG7, NG8, NG9 and NG10. From the results of weighing the size of the exopolysaccharide results, the complete data can be read in table 4.

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Table 4. Table of Results Considering Measurements of Lactic Acid Bacteria Isolates Capable of Producing Exopolysaccharides.

Code Isolate	Amount Of Expolysaccharide mg/L
NG1	152.1
NG2	127.9
NG3	134.6
NG4	130.9
NG5	137
NG6	139.2
NG7	204.9
NG8	156.2
NG9	136.4
NG10	157.3

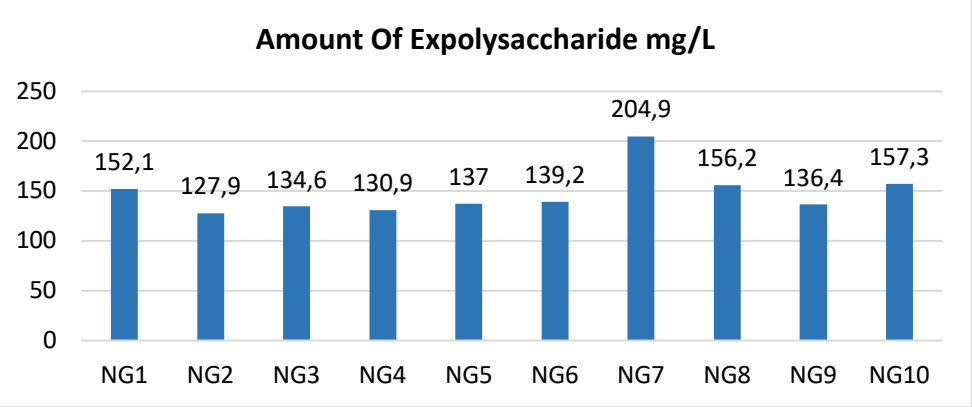

Figure 4. Grafic Of Production Exspolysaccharide

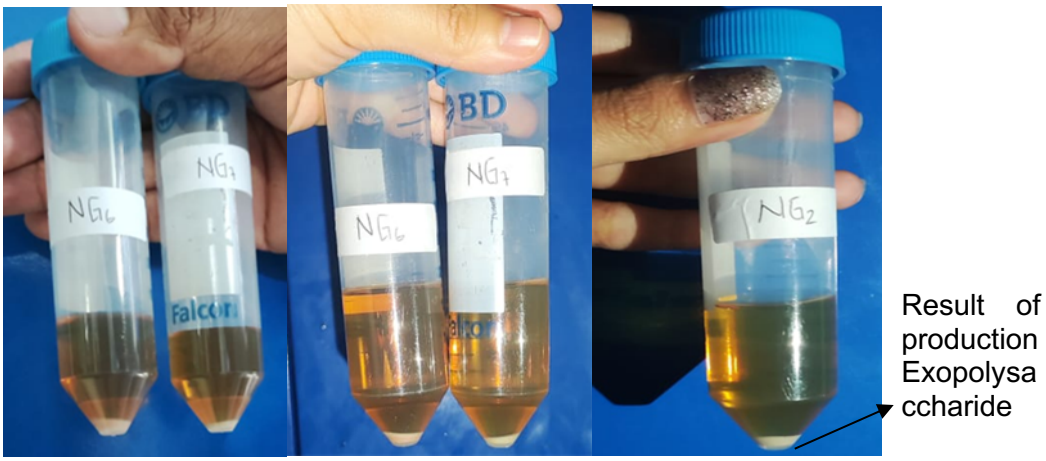

Figure 5. Result Of Production Exopolysaccharides from Dragon Fruit

DISCUSSION

Results In the isolation stage of lactic acid bacteria from red dragon fruit (*Hylocereus polyrhiruz*) through a dilution process using NaCl (0.85%) which acts as a disinfectant. According to Lestari (2014) physiological salt solution is the best medium for maintaining the survival of lactic acid bacteria isolates,

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because NaCl (physiological salt solution made from NaCl salt with a concentration of 0.9%) functions to maintain the balance of microbial cell ions. Red dragon fruit that has been enriched using MRSB isolation media for the next two (2) days will be used for bacterial dilution, namely dilutions 10-2 to 10-7, and the dilutions used are dilutions 10-6 and 10-7 which will be placed into petri dishes using the pour plate method then added with MRS Broth media then incubated for 1 day at room temperature. Colonies that have grown on MRSA media which has been added with CaCo3 1% of LAB isolates that have grown have a distinctive characteristic, namely that the growth of lactic acid bacteria isolates is surrounded by a clear zone which has the role of being a buffer and also the first selection of lactic acid production bacteria, lactic acid then carries out The bond in CaCo3 becomes Ca-Lactate, resulting in a clear zone around the colony, because the lactic acid dissolves the calcium carbonate so that it can be used as the first stage of LAB isolate (Seelly *et al*., 2001). The next isolate was taken from the single isolate, then the results of this stage obtained 5 isolates, namely five isolates from the 10-6 dilution and five isolates from the 10-7 dilution. The ten isolates, namely NG1, NG2, NG3, NG4, NG5, NG6, and NG7, were then purified through 2 purifications. The pure isolates were then made into stock for the next research stage. From the confirmation test by replanting the isolates in the media, the results were that the ten LAB isolates could grow well and produce clear zones around their colonies. After the isolation stage, the next stage is the identification stage of lactic acid bacteria isolates. This identification is carried out macroscopically (*by observing*) and microscopically (*with the help of a microscope*).

The gram staining biochemical test is an effective criterion for classification. The staining results will show basic and complex differences in bacterial cells (*cell wall structure*), so that bacteria can be divided into 2 groups, namely Gram-positive bacteria and Gram-negative bacteria (Jawetz *et al*., 2004). Gram-positive bacteria on Gram staining are purple because the crystal violet-iodine dye complex is retained even when given an alcohol solution, while gram-negative bacteria are red because the complex dissolves when the alcohol solution is applied so it takes on the red color of safranin (Jawetz *et al*., 2004). In figure 4.4. showed the results of microscopic observations that isolates NG2, NG4, NG5 and NG7 had rod-shaped cells (*bacilli*) while isolate BN1 had round-shaped cells *(cocci*) and if we looked at the arrangement of the cells, isolates NG7 and NG8 had single cells (*arranged themselves*). While the BN3 isolate has paired cells. Observation of colony morphology is not sufficient to identify the seven LAB isolates, therefore further characterization is needed to determine the metabolic activity caused by the workings of the enzymes of the seven LAB isolates. Biochemical characterization was carried out by carrying out catalase, starch hydrolysis, hydrogen sulfide, citrate, and gelatin endospore hydrolysis tests, TSIA, sulfate indole motility, catalase, and pH tests. From the results of the biochemical tests that were carried out, 1 isolate was obtained which was similar to the *Lactococcus* genus, namely isolate NG1. *Lactococcus sp* bacteria, like most other lactic acid bacteria, are mesophilic bacteria that grow optimally at temperatures of 20-30°C. Acid production generally decreases or even stops completely when bacteria are incubated at temperatures below 20°C, but the growth of bacteria in the range of 10-42°C is not inhibited (Ahmed *et al*., 2006). And the other nine isolates are thought to have similarities to the genus Lactobacillus. According

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to Yuni (2013), these *Lactobacillus* bacteria are gram positive (+), catalase negative (-), do not have spores, are not motile, facultative anaerobic, sometimes microaerophilic, grow little in the air but good under low oxygen pressure, and some anaerobes in isolation.

LAB isolates used to produce EPS are colonies that have been grown in MRS Broth media for 24 hours at a temperature of 30^{o}C (Trabelsi *et al.,* 2014). Then the LAB isolate was transferred into a 50 mL centrifuge tube and centrifuged for 30 minutes at a speed of 5000 rpm, then ethanol was added and centrifuged for 25 minutes at a speed of 5000 rpm. The precipitate obtained was then dried at 100^{o}C for 10 minutes. Next, the dry weight of EPS was weighed (Nudyanto and Zubaidah, 2015). LAB which can produce EPS produced by LAB is used for enrichment (Van Hijum, 2006). The research results can be seen in Table 4.5, graphic image in table 4.6. The amount of EPS produced by LAB, observations made on the ten isolates, namely NG1, NG2, NG3, NG4, NG5, NG6, NG7, NG8, NG9, and NG10, showed the potential to produce EPS.

According to (Widayanti, 2014) the results of EPS production are shown by the appearance of precipitate after the centrifugation process which is then separated from the filtrate and then dried. Based on what has been done, all isolates produce EPS with different weights which can be seen in Figure Table 4.6. EPS production is generally influenced by various phenotypic and genetic traits. Phenotypic characteristics tend to be influenced by environmental factors, EPS production can be influenced by several factors such as enrichment conditions, growth media, interactions between strains, the amount of inoculum concentration and fermentation technology (Fatih, 2020). The high and low levels of EPS are influenced by the amount of inoculum and the enrichment environmental situation during the incubation time (Awwaly and Manab 2007). In this study, the highest EPS levels were obtained in the NG7 isolate, namely 204.9 mg/L, while the lowest EPS levels were obtained in the NG4 isolate, which was 130.9 mg/L. In the same species, different exopolysaccharide results were obtained due to differences in bacterial strains due to genetic diversity in bacteria isolated from cherry fruit.

The differences in these strains indicate that the genes contained in these four LAB isolates are also different, each gene has different functions and abilities, resulting in differences in metabolic processes and differences in the results of the metabolites produced. According to (Tallon *et al.*, 2006) apart from the lack of inoculum concentration, there is the possibility influenced by the temperature used, namely 30^{o}C and 40^{o}C. In several studies, LAB isolates such as *Lactobacillus*, at lower enrichment temperatures, have had a positive influence on EPS formation and have a negative influence on LAB growth. This may occur due to the presence of a limiting factor. The limiting factor is the availability of lipid carriers which are needed for the formation of cell walls from the production of EPS. EPS production by BAL has a lower production value compared to EPS produced by industry. Metabolic and structural engineering can be used to increase EPS production for LAB (Papagianni, 2012). In research (Boels *et al.*, 2011) carried out LAB research as an effort to increase EPS production by adding the phosphoglucomutase enzyme to strains of *Streptococcus* thermophilus which cannot grow using a sugar carbon source in the form of galactose due to the presence of a complete gene that codes for the necessary enzyme. In the formation

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of EPS, the main nutritional source is needed, namely the elements C, N and P. Research (Perwitasari, 2008) provides additional compounds (NH4) HPO4 to fulfill the needs of the elements N and P, while the needs for element C are fulfilled by using lactose which is a carbon source for the formation of EPS.

CONCLUSION

From this research, isolation results were obtained, namely isolates NG1, NG2, NG3, NG4, NG5, NG6, NG7, NG8, NG9 and NG10. Isolate NG1 has similarities to the genus *Lactococcus*, while isolates NG2, NG3, NG4, NG5, NG6, NG7, NG8, NG9 and NG10 have similarities to the genus *Lactobacillus*. Based on its morphological characteristics, it is gram positive, catalase negative and does not have spores.

The ten isolates of lactic acid bacteria were able to produce EPS. The highest amount of EPS was isolate NG7 at 204.9 mg/L. Meanwhile, the lowest isolate was isolate NG4 at 130.9 mg/L. This is the result of the ten isolates obtained, namely: NG1(152.1 mg/L), NG2(127.9 mg/L), NG3(134.6 mg/L), NG4(130.9 mg/L), NG5(137 mg/L), NG6(139.2 mg/L), NG7(204.9 mg/L), NG8(156.2 mg/L), NG9(136.4 mg/L), and NG10 (157 .3 mg/L)

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