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Research Article

Chemical Compound Identification of Lactic Acid Bacteria (LAB) From Porang Tubers Waste *(A. muelleri blume)* as Probiotic

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Abstract: The production of amylase produced by lactic acid bacteria (LAB) has better stability. The purpose of this study was to utilize and determine the characteristics of LAB isolates from porang skin waste which have the potential as probiotics and the activity of amylase enzymes. Isolation was done by fermentation method for 48 h with the addition of starter *Pediococcus acidilactici* that had been cultured. The results showed that the number of colonies of N1 isolates was 49 $\times 10^8$ CFU/mL with regular small round colonies, having a convex elevation, the edges of the colonies were round, yellowish white, and Gram positive bacteria because they produced a purple color on Gram staining. Acid resistance test at pH 2.0; 3.0; 4.0; 5.0 and 6.0, the isolates N1 was able to survive at each pH because it showed turbidity in the growth medium. The antimicrobial activity test showed a strong zone of inhibition on *S. aureus and S. pyogenes* of 10.33 and 11.17 mm and a moderate zone of inhibition on *K. pneumoniae*, *S. dysentriae*, and P. *aeruginosa*, respectively, which was 6.33; 7.7 and 7.5 mm. The positive N1 isolate contained alkaloids as bioactive compounds from the results of phytochemical screening and the results of the FTIR analysis showed that specific groups of alkaloids were N-H, C-N, and C=O at wave numbers 3423.30 cm^{-1}; 1235.45 cm^{-1}; and 1634.81 cm^{-1}.

Keywords: Lactic acid, Amylase, Probiotic, Porang Tubers

Introduction

The development of biotechnology-based food products is progressing very rapidly. Biotechnology products are divided into two types namely conventional such as traditional food products processed through a fermentation process, namely tempe, tape, yogurt, while modern biotechnology products such as enzymes glucose resulting from enzymatic hydrolysis, and genetic engineering [1]. The processing of food product cannot be separated from the role of microorganisms in the form of bacteria and fungi. Microorganisms are able to interact with fellow microorganisms which will then have an effect on food products, both beneficial and detrimental [2] the microbial growth can be obtained through the fermentation process.

The use of amylase enzymes in the industrial sector is quite extensive, so it is very important to maximize natural materials that have the potential to generate amylase enzymes [3]. Porang tubers (A. muelleri blume) is a type of tuber that is often found in tropical and subtropical zone like Indonesia. The nutritional content of porang tubers are glucomannan (3.58%), starch (7.65%), protein (0.92%), fat (0.02%) and fiber (2.5%). The use of porang tubers in Indonesia is still very limited to flesh of tubers, while the skin

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is still not used properly so it becomes waste because its high starch content so porang tuber skin has big potential as a source of probiotics to generating amylase enzymes and LAB.

Untill now, only the flesh of porang tuber has been used as an ingredient for making flour and chip, while outer part of tuber (skin) is peeled and discarded [4]. Therefore porang tuber skin has the potential as a renewable material that can be used in various industries involving microorganisms, one of which is as a bioethanol generate [5]. The amylase enzyme activity test of LAB isolates obtained from porang tuber skin will be carried out from the research. In this research, porang tuber skin is used do that it does not just become waste and pollute the environment but can be used as an ingredient to generate probiotics and amylase enzymes. The LAB fermentation process in this study was carried out for 36 and 48 h at room temperature and under anaerobic conditions [6]. The LAB obtained was then subjected to an antibacterial test against bacteria such as *S. pyogenes, S. aureus, P. aeruginosa, S. dysentriae*, and *K. pneumoniae.*

Materials and Methods

Materials

The research was conducted in the chemistry research laboratory of Universitas Islam Indonesia for 3 months. The materials used in this research are porang tuber skin (*A. muelleri blume*) obtained from Yogyakarta, *Pediococcus acidilactici*, distilled water, nutrient agar (Oxoid), nutrient broth (Oxoid), mueller hilton broth (Oxoid), peptone water (Oxoid), HCl 37%, alcohol 70%, phenol solid Na_2SO_3, NaOH 2 M, Ka-Na Tartrate 40%, starch, glucose, Gram staining solution, wrap, aluminium foil, universal pH, ethyl acetate p.a, paper disc, antibiotic disc (erythromycin).

Preparation of Bacteria from Porang Tuber Skin

The porang tubers are cleaned and peeled the skin of 0.5 cm, the fermentation was carried out under anaerobic conditions with various times namely 36 and 48 h at room temperature. After that, 1 g of fermented porang tuber skin was put into nutrient broth and added of *Pediococcus acidilactici* starter and then incubated for 1 x 24 h at a temperature of 37 ^{o}C. The fermentation product obtained then carried out in a multi-stage dilution process using peptone water media and then that it was grown in nutrient agar [6].

Biochemical Identification

Macroscopic identification is carried out by observing the shape and size of the colony, elevation of bacteria which is cancave or convex, the shape of edge from colony is circular or irregular and color from colony and then, write the results of observations [7]. Moreover, microscopically by using microscope with size 40 or 100X after the gram staining process like morphological form of bacteria contained in the sample [8].

Acid Resistance and Antibacterial Activity Test

The bacteria resulting from 36 and 48 h fermentation were grown in nutrient broth with pH various add HCl 37% to pH 2, 3, 4, 5, and 6. And then, incubated for 24 h at 37 ^{o}C. the turbidity level of nutrien broth was observed with indicates that bacteria have growth from the medium [9].

Antibacterial activity was carried out using mueller hilton broth of 1.68 g and 2.24 g nutrient agar on which pathogen bacteria had been applied. Then the antibiotic (Erythromycin) disc on the medium. The sample isolates werw grown on test medium using paper disc and observed for 3x 24 h at a temperature of 37 ^{o}C. The clear zone formed every 24 h is measured [10]. And then compound identification using Fourier Transform Infra Red (FTIR).

Results and Discussions

Preparation of Bacteria from Porang Tuber Skin

Fermentation is process of producing energy by microbial body cells with breaking down complex compounds being simple compounds. The skin of porang tubers is washed using ditilled water to minimize contamination during fermentation process. The medium used in the preparation and isolation process is selective medium that is nutrient broth. Nutrient broth is a standard medium that can be used to grow various types of microorganisms because the content in the medium is quite complete like carbohydrate components, amino acids, peptone, and complex vitamins especially vitamin B. Then the cleaned porang tuber skin is put into the nutrient broth and then added the *Pediococcus acidilactici* starter. The fermentation process in the anaerobic condition (without oxygen) because microorganismssuch as LAB can only carry out the respiration process or energy mobilization in conditions without oxygen is due to not having a acomplex metabolic system [11].

The fermentation process was carried out at room temperature for 36 and 48 h. Variations in fermentation time are based on the growth phase of the bacteria. The fermentation time of 36 h is the beginning of the stationary phase, while the fermentation time of 48 h is the peak of the stationary phase. The stationary phase is a condition of balance between the number of living bacteria and the number of bacteria that die due to lack of nutrition [12]. Isolation of LAB by fermentation in the stationary phase can maximize the production of primary metabolite compounds such as lactic acid, organic acid production which causes a decrease in the surrounding pH so that it can inhibit the growth of pathogens, enzyme production and the production of bioactive compounds.

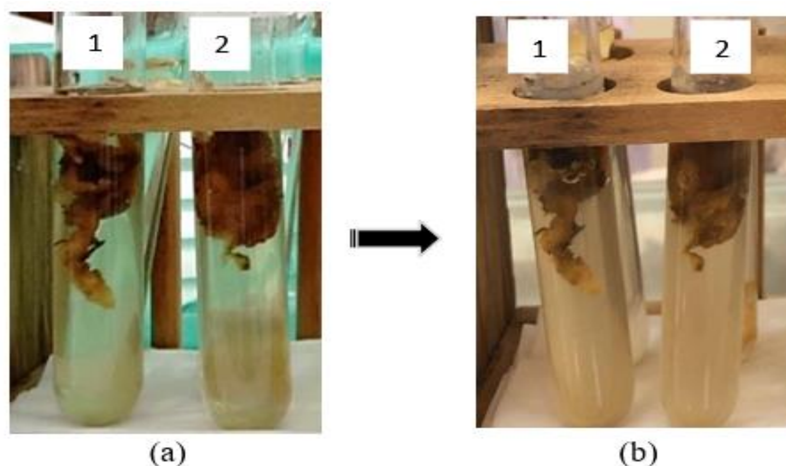
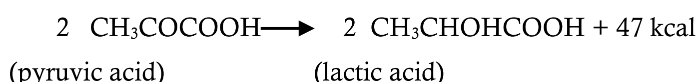


Figure 1. Fermentation process of porang tubers: a. before fermentation; b. after fermentation, 1. sample 36 h; 2. sample 48 h

Fermentation that involves bacteria and produces lactic acid is called lactic acid fermentation. The lactic acid fermentation process requires the help of enzymes to convert the glucose contained in the skin of porang tubers into the final product lactic acid. The process starts with the fermentation of pyruvic acid which is produced from the breakdown of glucose molecules. The pyruvic acid produced will undergo an anaerobic fermentation process to produce lactic acid with the help of the enzyme lactic dehydrogenase as in **Figure 2**. This enzyme is reduced by NADH without producing CO_2 and will produce lactic acid [11].



$$2\ CH_3COCOOH \longrightarrow 2\ CH_3CHOHCOOH + 47\ kcal$$

(pyruvic acid) (lactic acid)

Figure 2. Chemical reaction of lactic acid fermentation

The product fermentation carried out multilevel dilution method on peptone water medium and the streak method on nutrient agar media. Multilevel dilutions were carried out up to 10^{-8}. Bacterial dilution is the process of dissolving or releasing bacteria from their substrate into the air with the aim of reducing the

density of isolated bacterial colonies. Sample dilution needs to be done so that the isolated bacterial colonies meet the criteria for probiotic food approximately 10^6-10^8 CFU/mL from World Health Organization [6]. The results of the 10^{-8} dilution in samples 1 and 2 were embedded in nutrient agar media using the pour plate method and then incubated at 37°C for 24 h. Incubate at a temperature of 37 °C because LAB can grow optimally in the temperature range of 25 °C – 37 °C. After that, purification of the bacteria was carried out on nutrient agar media using the zigzag streak method at 37 °C for 24 h with the aim of obtaining a pure culture of bacteria. From the results of the streak, only 1 isolate was obtained from sample 2 because sample 1 was contaminated, then the isolate obtained was given the code N1. The results of purification of N1 isolates are shown in **Figure 3**.

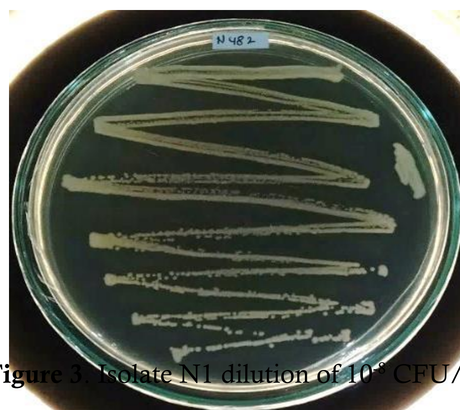


Figure 3. Isolate N1 dilution of 10^{-8} CFU/mL

The number of bacterial colonies of N1 isolates was then calculated based on the Bacteriological Analytical Manual, with the condition that the number of colonies used to calculate total colonies must be in the range of 25-250 CFU/mL which was then multiplied by 1 per dilution factor [13]. From these calculations, the number of LAB isolate N1 colonies was 49×10^8 CFU/mL.

Biochemical Identification

Identification of N1 isolates with Gram staining is carried out to differentiate groups of gram-negative bacteria or gram-positive bacteria. The characteristics of LAB are generally oval, rod or round. All LAB are gram-positive bacteria, meaning that LAB has peptidoglycan walls which are composed of peptides in the form of amino acids and carbohydrates (glycans) [14].

Table 1. Characteristics of N1 isolates

isolates	shape	size	elevation	edge shape	color	Gram test
N1	circular	small	convex	circle	yellowish white	coccus (positive Gram)

Microscopic identification is a further test used to ensure that the isolate obtained is LAB. This test is carried out through the Gram staining process and then observed under a microscope. The results of Gram staining identification showed that the N1 isolates had the shape of a coccus (round) with Gram positive, indicated by the formation of a purple color in the bacterial cells (**Figure 4**). Gram positive bacteria do not have an outer membrane but have a cell wall 20-80 nm thick which is mostly composed of peptidoglycan, while Gram negative bacteria have a double membrane consisting of a plasma membrane then covered by a permeable outer membrane with a thickness of 7-8 nm which is composed of composed of lipids, proteins and lipopolysaccharides, and has a cell wall in the form of peptidoglycan measuring 2-7 nm.

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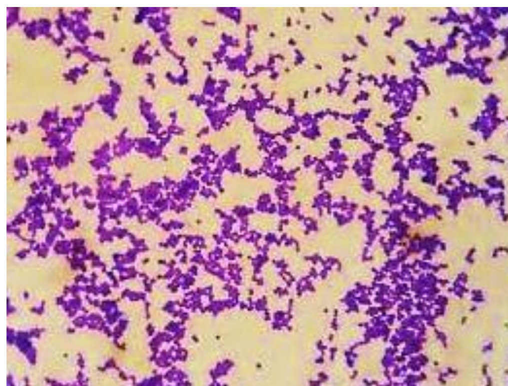

Figure 4. N1 isolates of Gram staining

Acid Resistance and Antibacterial Activity Test

Lactic acid bacteria are generally resistant to extreme pH. One of the requirements for probiotics is that LAB must be resistant to low acid conditions. In the condition of the stomach when producing HCl, the pH value in the stomach ranges from 2.0-4.0. LAB can survive the pH conditions in the stomach that LAB has the potential to be used as a probiotic. The N1 isolates was then tested for resistance to pH, with pH settings ranging from 2.0 to 6.0. At this stage, the test treatment uses liquid media or nutrient broth whose pH is adjusted by adding concentrated HCl, then the pH value is measured using a calibrated pH meter. The results obtained can be seen from the level of turbidity of the test medium, if the medium becomes more turbid, the more bacteria will grow.

Table 2. Acid resistance test

Isolates	Bacteria growth rate at pH variations				
	2.0	3.0	4.0	5.0	6.0
N1	+	++	++	+++	+++

Information : + : little cloudy, ++ : cloudy , +++ : very cloudy

Probiotic bacteria must be at least tolerant to pH 3.0 found in the stomach, or pH 4.0 which is the pH of the gastric mucus layer [15]. The results of the pH resistance test for isolate N1 are presented in Table 2. The results showed maximum bacterial growth in normal media without the addition of HCl, namely pH 6.0 or close to neutral. Furthermore, at pH 5.0, the turbidity of the media is still the same as the turbidity in pH 6.0 media, because pH 5.0 is a weak acid range so it is not yet. As a probiotic candidate, LAB must have the ability to inhibit the growth of pathogenic bacteria. Lactic acid bacteria are known to have the ability to produce antimicrobial compounds called bacteriocins and other bioactive compounds [16]. Antibacterial properties are divided into two, namely bacteriostatic and bacteriocidal [17].

Table 3. Antibacterial activity test result

Isolates	Duration (h)	Clear zone (mm)				
		S. aureus	*S.pyogenes*	*K.pneumoniae*	*P.aeruginosa*	*S.dysenteriae*
N1	24	9	9.5	5	7	7
	48	11	13.5	7	7.5	8.5
	72	10.5	11	6.5	8	8.5
Average		10.167	11.333	6.167	7.5	8

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Antibacterial results were observed from the formation of a clear area around the paper disc [18][19]. In this study, the disc diffusion method was used with a disc diameter of 3 mm. Then the paper disc was dipped into a test tube containing N1 which had been grown in nutrient broth. Next, the discs were placed on nutrient agar media containing pathogenic bacteria, then incubated at 37 °C for 72 h. Clear zone observations are carried out every 24 h. In the first 24 h of incubation, a clear zone had formed around the disc, indicating that the N1 isolates was positive for killing pathogenic bacteria. Then at 48 h, the formation of the clear zone is maximal because it enters the stationary phase. Meanwhile, the clear zone was constant and even decreased at 72 h (Table 3).

These results were analyzed based on the criteria of antibacterial strength from N1 isolates, so it can be stated that the inhibitory power of LAB against Gram-negative pathogenic bacteria, namely *Klebsiella pneumoniae*, *Shigella dysenteriae*, and *Pseudomonas aeruginosa*, is in the medium category, while for Gram-positive pathogenic bacteria, namely *Staphylococcus aureus* and *Streptococcus pyogenes* belongs to the strong category. Gram-negative bacteria have better resistance to antibacterial compounds produced by LAB because Gram-negative bacteria have a more complex outer membrane structure, including a plasma membrane coated with a permeable outer membrane, so that the antimicrobial compounds produced by LAB are difficult to penetrate cell walls [20].

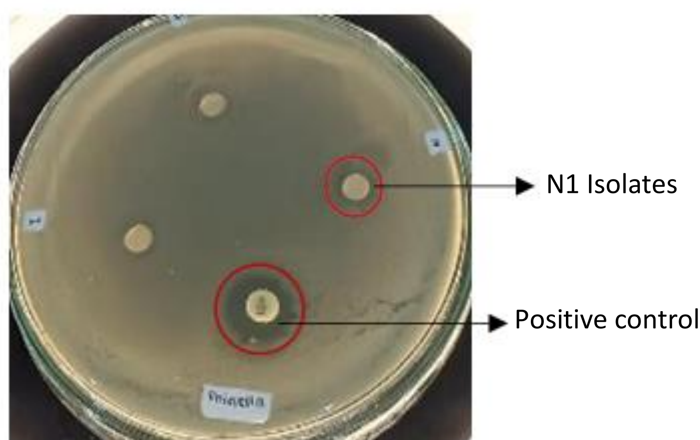
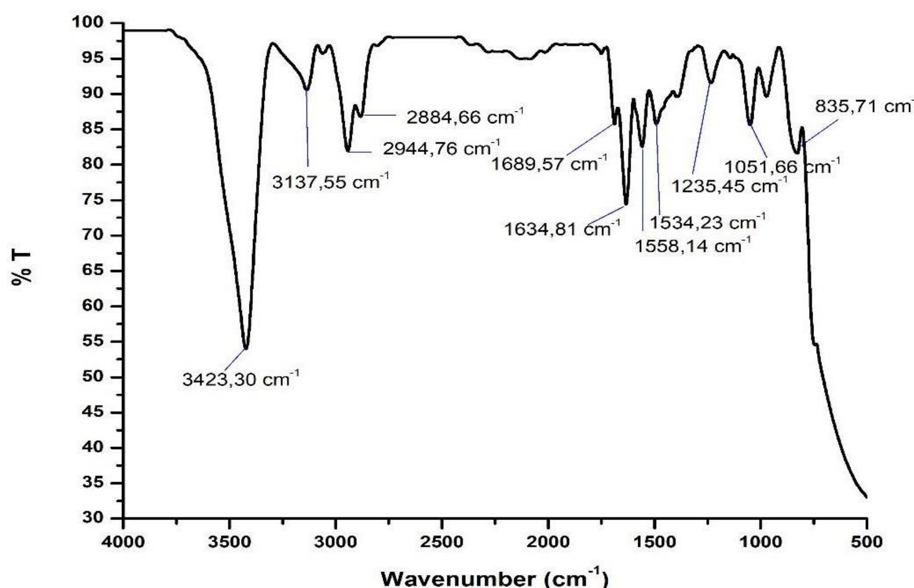


Figure 5. Antibacterial Activity Test

The ethyl acetate extract of N1 isolates was analyzed using FTIR spectroscopy to see the functional groups in the sample. Alkaloid compounds have specific functional groups such as N-H, C-N, and C=O bonds. In Figure 6, the wave number 3423.30 cm^{-1} is the absorption from N-H stretching. The C-N group is



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displayed at a wave number of 1235.45 cm^{-1}. Then the middle C=O group is displayed at the wave number 1634.81 cm^{-}1. The wave number 3137.55 cm^{-1} is the absorption belonging to aromatic C-H. Two peaks at 2944.76 cm^{-1} and 2884.66 cm^{-1} is aliphatic C-H absorption. Then the two peaks at 1558.14 cm^{-1} and 1534.23 cm^{-1} are the absorption of the aromatic C=C group.

Figure 7. FTIR of bioactive compound from N1 isolates

Alkaloid compounds can be antibacterial compounds to kill other bacteria. Alkaloid compounds are able to act as antimicrobials because the presence of these compounds causes the cell wall layer of pathogenic bacteria to not form properly because alkaloids are able to disrupt the peptidoglycan components of bacteria, thereby causing cell death [21]. Alkaloid compounds utilize the reactive properties of their base groups, so that when the base groups in these alkaloids come into contact with pathogenic bacteria, they will react with amino acids which are elements that make up cell walls and are the center for all regulation of cell activities belonging to these bacteria [22]. The explained that the antibacterial mechanism of alkaloids is that the alkaloid component can act as a DNA interchelator and is able to inhibit the topoisomerase enzyme produced by bacterial cells [23].

Conclusion

Fermentation with *Pediococcus acidilactici* starter produced LAB with a colony number of 49×10^8 CFU/mL for 48 h. The bacteria produced are small, regular spherical in shape, have a convex elevation, the edges of the colony are completely round, have a foul white color, and are Gram positive. At pH 2.0 conditions; 3.0; 4.0; 5.0; and 6.0 lactic acid bacteria obtained were able to survive, indicating that the isolate was able to survive in acidic conditions. Antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes*, with an average inhibitory power of 10.167 and 11.333 mm (strong), as well as against Gram-negative bacteria such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Shigella dysenteriae* has an average inhibitory capacity of 6,167; 7.5 and 8 mm (medium). Through phytochemical tests and FTIR analysis, bioactive compounds were obtained that have antimicrobial properties is alkaloid compounds.

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