Isolation And Characterization Of Lactic Acid Bacteria From Rice Washing Water Waste

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Abstract.

Rice washing water is still considered as household waste because its benefits for plants are not widely known. Rice washing water is obtained from water that is washed many times before cooking which is rich in carbohydrate content in the form of starch and glucose and vitamins. Lactic acid bacteria (LAB) require sugar or carbohydrates from rice washing water which is used as food to support metabolic activity and microbial cell proliferation. LAB is also included in a large group of microorganisms that physiologically produce lactic acid as the main metabolic product. LAB are classified as good bacteria so they are widely used as food processing products, especially those related to human digestion. The use of lactic acid bacteria in the agricultural sector is still very minimal, especially as a biodecomposer, even though LAB has the ability to produce cellulase enzymes which are very important in the breakdown of lignin in organic matter. This study aims to determine the presence of lactic acid bacteria in rice washing water fermentation. Isolation of lactic acid bacteria using pour plate method with MRSA (de Man Rogosa Sharpe Agar) media. Lactic acid bacteria isolates obtained were then characterized morphologically, physically, and biochemically. The results of isolation and characterization obtained four isolates with the codes AL-01, AL-02, AL-03, and AL-04. Four LAB isolates showed gram-positive, catalase-positive, non-motile, non-H2S, and IAA-positive results.

Keywords: *Isolation, Rice Washing Water, Lactic Acid Bacteria,and Fermentation.*

I. INTRODUCTION

Rice is one of the most important staple foods in most societies around the world, playing a crucial role in human life throughout a long history [1]. Its rich nutritional content and diverse cultural uses have made rice a staple food in many countries around the world. Rice has been the main source of carbohydrates in Asian cultures for thousands of years, and over time, its cultivation and production have expanded around the world [2]. According to [3] carbohydrates are nutrients that provide energy for the body, and in some societies, rice is a staple food that accounts for the majority of daily energy intake.Rice produces water from soaking the rice before the cooking process, known as rice washing water [4], [5]. Rice-washing water is included in household waste because it is a by-product that is not used anymore and has not been managed for human needs [6]. Rice washing water is a household liquid waste generated from the repeated washing of rice. The high level of consumption in Indonesia causes a lot of unutilized and wasted rice-washing water. Rice washing water waste contains organic and mineral compounds. From several studies, it has been found that the content of rice washing water in the form of protein, vitamins, and carbohydrates is quite high [7], [8]. The content of rice washing water can be a microbiological potential by utilizing carbohydrates as a food source for microbes. One of the microbes that can be found in rice washing water is the lactic acid bacteria group [9].Lactic acid bacteria (LAB) are known for their ability to convert carbohydrates into organic acids [10], including lactic acid, which has a wide range of applications in the food, health, and environmental industries [11].

Research related to the ability of lactic acid bacteria has been widely identified in food fermentation products [12] as well as drinks [9] but still little in the way of organic matter decomposers [13].Lactic acid bacteria (LAB) are a group of microorganisms that play an important role in biogeochemical cycles and as decomposers in natural ecosystems [14]. The main function of LAB is to break down complex organic matter into simpler components such as organic acids, carbon dioxide, and water [15]. Lactic acid bacteria have been shown to participate in the decomposition of various organic materials, including crop residues,

animal manure, and other natural organic materials. The decomposition process carried out by lactic acid bacteria involves several biochemical reactions, with lactic acid as the main product. This lactic acid can then contribute to a decrease in the pH of the surrounding environment, which in turn can affect the activity of other microorganisms and other environmental factors [12]. Therefore, the identification of lactic acid bacteria as decomposers is crucial in understanding the nutrient cycling and transformation of organic matter in nature.

II. METHODS

2.1 Preparation of Rice Washing Water Solution

The preparation of rice washing water solution is done by taking 150 grams of rice samples that have been washed with 300 ml of clean water, then the first washing water is collected in a sterile glass bottle as much as 2/3 of the volume of the bottle [9]. The rice-washing water that has entered the bottle is then closed using tissue or porous cloth by tying it with rope or rubber. The rice-washing water samples were then stored in a room temperature place or room that was not exposed to sunlight for three days. The sample used in this study is a cloudy liquid that is in the middle layer of rice water resulting from fermentation.

2.2 Isolation of Lactic Acid Bacteria from Rice Washing Water Solution

The sample used is the turbid liquid from the fermentation of rice washing water and then aseptically inserted into a sterile sample bottle. Samples were taken as much as 1 ml and then added 9 ml of 0.9% NaCl and homogenized using a vortex. The resulting suspension (10-1 dilution) was diluted to 10-7 using the multilevel dilution method by taking 1 ml of the previous dilution and then adding 9 ml of 0.9% NaCl. The results of the dilution were put into a Petri dish containing 15ml MRS Agar media which had been added with 1% CaCO3 using the pour plate method. Then inoculated in an incubator at 37°C for 48 hours (2 days). Isolates that had formed a clear zone on MRS Agar media (suspected as LAB) were then taken with an ose needle and inoculated on a new medium (MRS Agar) using the streak plate method, then inoculated at 37°C for 48 hours. The streak method was repeated until a pure isolate (single colony) was obtained in a uniform and separate form.

2.3 Morphological Characterization

Lactic Acid Bacteria (LAB) were characterised macroscopically including morphology of bacterial isolates (color, edge, elevation, size, and surface shape observed when in MRSA media), while microscopic observations included gram test.

Gram Test

Gram test is done to see the presence of bacteria obtained in Gram positive or Gram-negative bacteria. The Gram test was carried out by mixing one ose of bacterial isolates on a glass object that had been dripped with 3% KOH as much as 10 µL, then observed whether mucus was formed or not. If mucus is formed, the bacteria are classified as Gram negative, but if no mucus is formed, they are classified as Gram positive. The Gram test is carried out by following the working procedure of the [16].

2.4 Physiological Characterization

Catalase Test

Isolates obtained from oblique agar media were taken using an ose needle and then transferred to a glass slide that had been cleaned with 70% alcohol. Then drip 1-2 drops with 3% H2O2 solution. The catalase test is positive if gas bubbles form after hydrogen peroxide is given. The formation of oxygen bubbles indicates that the body produces the enzyme catalase, which converts hydrogen peroxide into water and oxygen. LAB is a catalase-negative bacterium, so the catalase test reaction results do not form gas bubbles, this indicates that LAB does not produce the catalase enzyme, only negative Gram-positive staining and negative catalase results are further identified because these two test results are common characteristics of LAB. The catalase test was performed by following the working procedure of the method of [16].

Motility Test

Bacterial isolates were taken from the culture stock as much as 1 ose needle and then circulated by puncture into the Sulfide Indole Motility (SIM) semi-solid medium in a test tube and incubated for 24 hours at 37°C. A positive test is indicated by bacterial growth that spreads (motile), while a negative test is

indicated by bacterial growth that does not spread and is only a single line (non-motile) in the puncture area. This test aims to determine the movement of growth in bacteria. The motility test is done by following the working procedure of [16].

2.5 Biochemical Characterization

H2S Production Test

For H2S analysis, each LAB isolate was inoculated into semi-solid sulfide-indole mobility (SIM) media, and incubated for 24-48 hours. After incubation, 3 drops of Ehrlich's reagent were dripped onto the surface aseptically. A black precipitate was present in the culture medium, indicating that the infected bacteria could produce H2S. The H2S test is carried out by following the working procedure of the method [16].

Indole Acetid Acid Production Test

Bacterial isolates that grow on MRSA media are tested by dropping Salkowski's solution. A positive result after Salkowsky's solution is indicated by a color change to pink after incubating in the dark for 30 minutes [17].

III. RESULT AND DISCUSSION

Isolation of Bacteria from Rice Washing Water

Isolation is a method in separating one type of microbe from another type of microbe that comes from rice washing water. The bacteria that will be isolated are lactic acid bacteria using selective media in the form of MRSA (Łepecka et al., 2023). Selective media is media that contains inhibitors to inhibit bacterial growth so that it is only used to grow certain bacteria. Lactic acid bacteria have the ability to degrade carbohydrates into lactic acid which can grow at low pH (Tahir et al., 2023). The isolation process was carried out by multistage dilution method on MRSA media and incubated for 2 days. The images of the growing isolates are presented in Figure 1. The growing isolates were then purified to obtain a single colony.

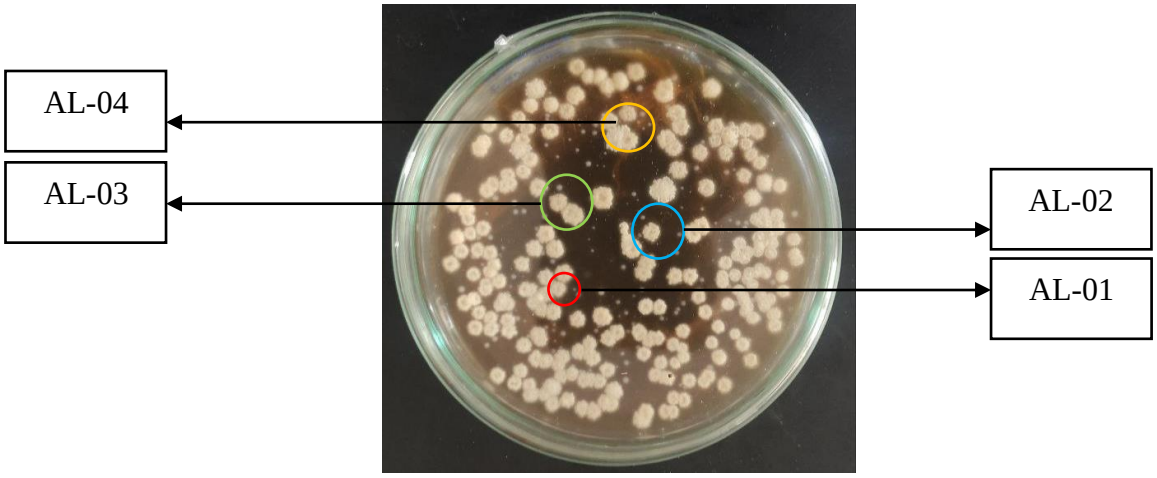
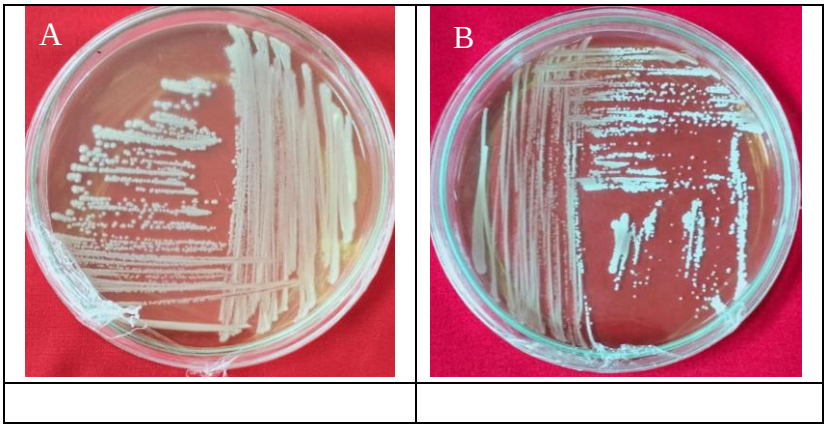


Fig 1. Lactic Acid Bacteria Isolate

From the results of isolate purification, 4 pure isolates were obtained which were suspected to be lactic acid bacteria. The 4 isolates can be seen in Figure 2.



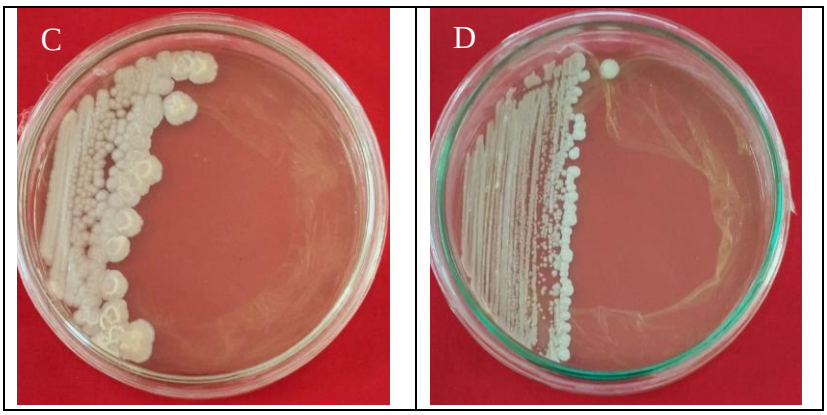


Fig 2. Purification of Lactic Acid Bacteria Isolates; Isolate AL-01 (A); Isolate AL-02 (B); Isolate AL-03 (C); and Isolate AL-04 (D)

Characterization of Lactic Acid Bacteria

Morphological Characters

Morphological characterisation is the first step in identifying bacteria. LAB morphological characterisation is usually done in two ways, namely macroscopic and microscopic characterisation of bacterial morphology. Lactic acid bacteria isolates obtained from the isolation results were continued by performing macroscopic morphological characterisation. Macroscopic morphological characterisation is done by direct observation of the morphology of bacterial isolates that grow on MRSA media. The observation results of macroscopic morphological characterisation can be seen in Table 1.

Table 1. Morphological Characteristics of Lactic Acid Bacteria Isolates from Rice Washing Water

Morphological Characters	Isolate Code			
	AL-01	AL-02	AL-03	AL-04
Shape	Circular	Irregular	Irregular	Circular
Margin	Entire	Undulate	Undulate	Entire
Elevation	Convex	Umbonate	Umbonate	Umbonate
Size	Small	Punctiform	Umbonate	Moderate
Apperance	Glossy	Dull	Dull	Glistening
Optikal Property	Opaque	Opaque	Opaque	Opaque
Texture	Smooth	Rough	Mucoid	Smooth
Pigmentation	Milky white	White	White	Milky white

Based on the results of the research in Table 1 above, 4 isolates of lactic acid bacteria were obtained which had different morphological characters given the isolate code (AL-01, AL-02, AL-03 and AL-04). The morphology of bacterial colonies grown in MRSA media is round, milky white and yellowish white, convex surface and flat and wavy edges. The morphology of lactic acid bacteria colonies produced white and yellowish white color, round shape, flat edges and convex surface [18], [19]. Isolates obtained from isolation results were then further identified to ensure that the isolates obtained were lactic acid bacteria using gram test, catalase test, motility test, indole and H2S production test presented in Table 2.

Table 2. Identification of Lactic Acid Bacteria

Parameters	Isolate Code			
	AL-01	AL-02	AL-03	AL-04
Gram Test	+	+	+	+
Catalase Test	-	-	-	-
Motility Test	Non Motil	Non Motil	Non Motil	Non Motil
IAA Production Test	+	+	+	+
H_2S Test	-	-	-	-

Description: Positive reaction (+); Negative reaction (-)

Gram test is one of the most important and commonly used methods to identify bacteria. This process is performed on a gram-type bacterial isolate, which determines the nature of the isolate based on differences in bacterial cell wall structure. The peptidoglycan layer of the cell wall of gram-positive bacteria is thicker than that of gram-negative bacteria. With isolated bacterial isolates, the cells are characterised microscopically. According to [3] The cell wall of gram-positive bacteria consists of only one layer, which is

a relatively thick layer of peptidoglycan. The cell wall of gram-negative bacteria has two layers of cell wall, namely the outer layer of lipopolysaccharide and protein and the inner layer of peptidoglycan, but it is thinner than the peptidoglycan layer of gram-positive bacteria. The Gram test is carried out by mixing one ose needle of bacterial isolate on a glass object that has been dabbed with 10 µl of 3% KOH and then observed whether there is mucus or not. If there is mucus then the bacteria is classified as gram negative, but if it does not produce mucus then the bacteria is classified as gram positive [20]. Based on the results of the study in Table 2, it appears that the 4 isolates tested by gram showed positive results so that it can be said to be included in the group of lactic acid bacteria. Lactic acid bacteria are bacteria that can convert carbohydrates (Glucose) into lactic acid [10] with morphological characters in the form of Gram positive and not forming spores [21], [22].

The catalase test is a way to determine whether bacteria produce the enzyme catalase and tolerance to oxygen by breaking down hydrogen peroxide into water and oxygen [21]. Most bacteria are able to produce the enzyme catalase which can function as aerobic growth because hydrogen peroxide produced with the help of various respiration enzymes is toxic to microbial cells. According to [23], The mechanism of the catalase enzyme breaking down H_2O_2 is that during respiration, bacteria produce various components, one of which is H_2O_2. The bacteria have the ability to break down H_2O_2 immediately using the catalase enzyme and then form a defence system against toxic H_2O_2 produced by themselves. Catalase-positive bacteria break down H_2O_2 into H_2O and O_2 with the parameter being indicated by the presence of bubbles in the form of oxygen bubbles. Catalase-negative bacteria do not produce bubbles which means that negative catalase bacteria do not break down the H_2O_2 given so they do not produce oxygen. Negative catalase bacteria do not have the enzyme catalase, which can break down H_2O_2 [24]. The results of the catalase test in Table 2 show that all isolates give a positive reaction or produce gas bubbles. There are lactic acid bacteria that produce positive catalase and negative catalase. Lactic acid bacteria that showed positive catalase were mostly found in Lactobacillus, Leuconostoc, Streptococcus, dan Pediococcus [25], [26]. According to [27] lactic acid bacteria are bacteria that can produce hydrogen peroxide (H_2PO_2) and compounds that inhibit unwanted bacteria, one of which is Lactobacillus bulgaricus Furthermore, the use of media can also be a trigger in producing enzymes, MRSA media contains hematin compounds that can help bacteria produce apoenzymes that correlate with the activity of hydrogen peroxide separation [25]. [28] said that it is commonly known that lactic acid bacteria have anaerobic properties but are able to tolerate the presence of oxygen and metabolise carbohydrates through the fermentation pathway.

Motility test is one aspect of bacterial testing to determine the ability of bacteria to move or motile. Motile bacteria are characterised by the presence of growth spreading to the area around the ose needle puncture while non-motile bacteria are indicated by the absence of bacterial spread around the ose needle puncture. Based on Table 2, all bacterial isolates showed non-motile results, meaning that there was no bacterial movement during the 2-day incubation time as seen from the absence of propagation around the ose needle puncture. From some research results, it is stated that lactic acid bacteria are bacteria that do not have flagella so that movement is inhibited [29], [30]. In line with the research results [31] that lactic acid bacteria from Kepok Banana Tape showed non-motile bacteria or no bacterial dispersal. The H_2S production test for the four isolates tested gave negative results due to the absence of black sediment at the bottom of the material (Table 2). [32], stated that hydrogen sulphide (H_2S) testing was carried out to determine and monitor the ability of bacteria to convert amino acids alanine and H_2S, while the bacterial isolate could not give a reaction of change from Fe to FeS as indicated by the presence or absence of black or blackish color at the bottom of the test. This indicates that the LAB isolate is not able to transform H_2S and cannot change with Fe to FeS. Lactic acid bacteria obtained from Bamboo shoots also showed similar results with no black precipitate at the bottom as a result of the Fe to FeS conversion [33]. In line with the research results [34] related to the H_2S test results, 60 lactic acid bacteria isolates isolated from different food sources do not have the ability to convert sulfur compounds into sulfide as seen from the absence of darkening of the media. It is possible that sulfide compounds combine with iron compounds to form FeS which is a dark-colored precipitate that makes it difficult for organisms to reduce sulfur compounds to sulfide [32], [35]. The results of the motility test and H_2S production can be seen in Figure 3.

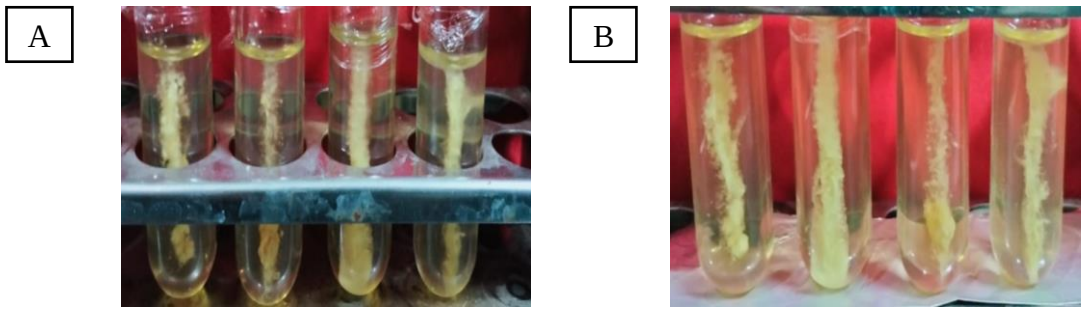


Fig 3. Motility and H_2S Production Test of Lactic Acid Bacteria in Rice Washing Water Before Incubation (A) and After Incubation (B)

IAA hormone production test of lactic acid bacteria gave positive results from all bacteria tested, meaning that lactic acid bacteria produce IAA hormone (Table 2). Indicators of lactic acid bacteria are able to synthesize the hormone IAA is indicated by a change in the color of the isolate to pink to dark when tested with Salkowski. The dark color produced by lactic acid bacteria indicates the greater the content of IAA produced. Of the four isolates of lactic acid bacteria given, it can be seen that isolate AL-01 has a darker color compared to other isolates (Figure 4). The variety of colors produced from these four isolates is a form of response to tryptophan solution. Tryptophan is a precursor or marker of bacteria capable of synthesizing the hormone IAA through the indole pyruvic acid pathway [36]. This indicates that lactic acid bacteria from rice washing water fermentation are able to synthesise the hormone IAA. From the research results [37] stated that lactic acid bacteria are able to produce indole acetic acid (IAA) which is used to spur plant growth. IAA is an auxin hormone that plays an important role in the plant body since, germination, growth and production of plants [38]. [39] said that bacteria capable of producing IAA can be used as plant growth promoters. Different studies have proven an increase in root and shoot length, plant biomass, IAA, and other organic acids after the addition of LAB-treated organic matter compared to the untreated control, and this resulted in increased yield [40].

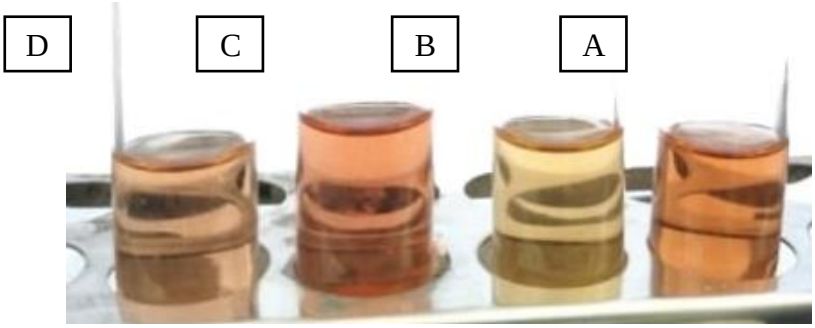


Fig 4. Appearance of IAA Hormone Test Results on Lactic Acid Bacteria; AL-01 (A), AL-02 (B), AL-03 (C) and AL-04 (D)

IV. CONCLUSION

In conclusion, this study shows that there are four isolates of lactic acid bacteria obtained from the fermentation of rice washing water through the process of isolation and morphological, physiological, and biochemical characterization. The results of lactic acid bacteria isolation are expected to be used as biofertilizers in the form of compost and liquid organic fertilizer which are used as plant growth promoters so as to promote sustainable agriculture. Further research is needed to identify the most effective LAB strains and optimize their application in agriculture.

V. ACKNOWLEDGMENTS

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