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Isolation and Characterization of Lactic Acid Bacteria from Sago Wastewater as Antibacterial

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Abstract

Background: Foodborne diseases are infections of the gastrointestinal tract caused by food containing microbiological agents and usually, these infections affect groups of individuals who have low immune systems. Lactic acid bacteria (LAB)are bacillus-shaped Gram-positive bacteria that work by inhibiting pathogenic bacteria that cause foodborne diseases. Maluku is a region in Indonesia rich in sago production. Sago ihur (*Metroxylon sylvestre*) and tuni (*Metroxylon rumphii*) are types of sago commonly found in Maluku. Sago is rich in carbohydrates and therefore has the opportunity to produce LAB. This study aimed to isolate and characterize LAB from sago wastewater was a laboratory experimental research with descriptive method using primary data. **Methods:** Bacterial isolation was carried out using a selective media. Characterization carried out by macroscopicly characterization by observing shape, margin, elevation, pigmentation, appearance, optical properties, and texture. Microscopic characterization was performed using Gram and spore staining. **Results:** The results of this study showed that five bacterial isolates (I1, I2, T3, T4, and I5) were successfully isolated in a round shape, flat edges, yellowish white color (not pigmented), shiny appearance, and mucoid texture, but isolate T3 showed a raised elevation and translucent to light (transparent), while for other isolates it was convex and translucent, and non-spore forming gram positive. **Conclusion:** Antibacterial tests against *S. aureus* and *E. coli* showed no inhibition zones. LAB isolates from sago wastewater showed no significant antibacterial effect

Keywords: lactic acid bacteria, maluku, sago, wastewater

Original Research Article

INTRODUCTION

Foodborne diseases are infections or irritations of the digestive tract caused by food or drink containing bacteria, parasites, viruses, or harmful chemicals. Common symptoms of foodborne diseases include vomiting, diarrhea, abdominal pain, fever, and chills (Moore & Bell, 2018). Foodborne diseases affect individuals of various age groups, especially children under the age of five and residents of low-income regions (Grace, 2023). Food-associated pathogens capitalize on the weaknesses of the immune system. Infants, children, pregnant women, the elderly, and immunocompromised individuals are particularly vulnerable to foodborne diseases (Lund & O'Brien, 2011). They are at a high risk of contracting and dying from diseases commonly associated with food, especially when hot food is not hot enough or cold food is not cold enough, bacteria can reproduce (WHO, 2015).

Several food preservation techniques have been used to prevent food spoilage. Food preservation methods, such as chemical or physical treatments (e.g., heating or UV radiation), have been used to inhibit the growth of microorganisms in food (Chittora et al., 2022). However, the increasing prevalence of antibiotic-resistant pathogenic bacteria in the food industry has become a serious global public health issue requiring control through effective antibacterial agents (Prestinaci et al., 2016) . The microbiological safety of food products is becoming an important focus in the food supply worldwide. Therefore, it is important to reduce the use of chemical additives in food and switch to more natural and microbiologically safe food products (Mechai et al., 2020). Biopreservation can be defined as the use of non-pathogenic microorganisms and their metabolites to improve microbiological safety and extend the shelf life of food (Chittora et al., 2022). With the advancement of time, many studies have shown that Lactic Acid Bacteria (LAB) can inhibit the growth of bacteria (Aritonang et al., 2017; Ayivi et al., 2020; Fadila et al., 2022).

LAB are Gram-positive bacteria, bacilli, or cocci that do not produce spores, are anaerobic, and are catalase-negative(Edy, Harmileni, & Anggraini, 2022). As a probiotic, it must have several probiotic properties and can produce antimicrobials that function as growth inhibitors against pathogens in the gastrointestinal tract; for example, organic acids and bacteriocins, which have non-pathogenic properties, can survive in acidic environmental conditions and bile salts and can colonize the gastrointestinal tract(Alang, Kusnadi, Ardiyati, & Suharjono, 2019; Detha, Datta, Beribe, Foeh, & Nemay, 2019).

Consumption of foods that contain LAB is beneficial for health, such as in the gastrointestinal tract, which can relieve diarrhea, reduce the risk of necrotizing enterocolitis (NEC), and reduce symptoms of lactose intolerance in infants (Cruchet et al., 2015). In addition, LAB can affect the immune system because LAB will circulate in the bloodstream to increase the body's recognition of the body's immune system, such as in the respiratory tract, where LAB can help increase immunity and reduce the severity of secondary bacterial infections in the respiratory tract (Vinderola, Ouwehand, Salminen, & Wright, 2019). It can be a preservative because of its antibacterial properties, which can prevent decomposition (Fadila et al., 2022).

Metroxylon sago, also known as sago, is a plant that contains carbohydrates and can usually be found in areas with high water intensity, such as riverbanks and swamps. At least 50% of sago plant in the world is found in Indonesia region, This indicates that sago has a significant impact, one of which is on food security, particularly in the sago-producing regions in Indonesia; West Papua, Maluku, Sulawesi, and Riau (Kasi et al., 2017).Sago is a plant with a myriad of capabilities, such as high production capacity and carbohydrates content, that is not inferior to other foods (Tirta et al., 2013).The declining role of sago in Maluku is the focus of the government to restore the role of sago as a local food, and several efforts, such as expanding sago land and making sago a food ingredient for home industries, have been widely practiced to improve the quality and resilience of local food(Musaid et al., 2019; Siahaya et al., 2021).

The results of previous studies show that many natural sources contain LAB, they are often associated with animal oral cavities and intestines e.g. *Enterococcus faecalis* and plant leaves *Lactobacillus*, *Leuconostoc*, occur naturally in fermented food and have been detected in soil, water, manure and sewage (Adjie & Setyawatiningsih, 2021; Ibrahim et al., 2021; Kasi et al., 2017). The high carbohydrate content of sago makes it a good medium for LAB growth. Therefore, sago not only has benefits as a food item but also as a potential product in the health sector. Maluku is a region in Indonesia rich in sago production. Sago ihur and tuni are types of sago commonly found in Maluku. Sago is rich in carbohydrates and therefore has the opportunity to produce LAB.

LAB can be sourced from various environments, such as wastewater, and is capable of hindering the proliferation of pathogenic microorganisms by generating antimicrobial substances throughout the fermentation process, which persist within food products, thereby impeding the growth of harmful bacteria and pathogens. Despite the underutilization of Sago wastewater, there remains a need for more information regarding potential bacterial resources within it. This study aimed to isolate and

characterize LAB from sago wastewater, and the LAB isolates obtained will then be tested for their ability as antibacterial.

MATERIALS AND METHODS

This research was a laboratory experimental research with a qualitative descriptive method using primary data and was conducted at the Microbiology Laboratory of the Faculty of Medicine, Pattimura University in May 2023.

In this study, samples were collected from Tulehu, Salahutu, Maluku Tengah. Two types of sago wastewater were tested: Ihur sago and Tuni sago. Sago ihur and sago tuni are Metroxylon sago, a kind of sago that grows in wetlands. Sago ihur and sago tuni have different physical characteristics. The Sago Tuni tree is a large tree, and the leaves stick out upwards, while the Sago Ihur tree is a manageable size, the leaves point upwards, and the tips of the leaves have long spines.

Bacterial isolation was carried out using selective media. Characterization is carried out by macroscopic characterization by observing shape, margin, elevation, pigmentation, appearance, optical properties, and texture, and microscopic characterization was performed using Gram and spore staining.

Sample Preparation

Fresh sago wastewaters were collected and stored in sterilized bottled (**Fig.1**). Samples was brought to laboratory for further analysis. Samples were stored to fermentation for 7 days in room temperature

Figure 1. Collecting sample sago wastewater

Isolation

Bacterial isolation is a method for obtaining specific or single colonies from isolated MRS agar media that can be used to maximize the growth of LAB colonies (Delvia et al., 2015). Isolation was carried out with multilevel dilutions up to 10^{-4}, by mixing 9 ml of physiological saline (sterile 0.85% NaCl) with 1 ml of the results of dilutions 10^{-1} to 10^{-4}. Dilution aims to reduce the density of bacteria when cultured in Petri dishes(Delvia et al., 2015; Qonita et al., 2018).The results of dilutions 10^{-1} to 10^{-4}, 1 mL of which each of them was put into a petri dish then homogenized (Harissa & Hasanah, 2019; Qonita et al., 2018). Petri dishes containing the bacterial samples were incubated for 48 hour at 37°C. Colonies with clear zones were re-cultured by scraping method using an ose needle and incubated again until scattered and uniform colonies were obtained (Qonita et al., 2018).

Purification and Characterization

The colonies were then purified using the quadrant streak plate method. Purification aims to purify isolates that are mixed by streaking on de Man, Rogosa and Sharpe (MRS) agar media and incubating for 48 h at 37°C (Azizah et al., 2021). Macroscopic characterization is performed by observing shape, margin, elevation, pigmentation, appearance, optical properties, and texture(Vella, 2022). Microscopic characterization was performed using Gram and spore staining. Gram staining aims

to identify LAB that has Gram-positive by showing purple colour purple. One loop of the bacterial culture was placed on a glass object that was previously cleaned using alcohol, dried in the air, and fixed with a small fire. After that, the glass object was dripped with as much as 2-3 drops and allowed to stand for 1 minute, then rinsed with running water. The Lugol solution was then dripped onto the object glass and allowed to stand for 1 min, diluted with 95% alcohol for 10-20 seconds or until the blue color did not fade. Finally, safranin was given for 10-20 seconds as a dye, rinsed, and dried with absorbent paper. If the coloring has been completed, the sample could seen from a microscope with a magnification of 1000x, The look blue-purple BAL a Gram-positive bacterium (Damayanti et al., 2018; Harissa & Hasanah, 2019).

One of the characteristics of LAB is spore staining, in which there are no spores. For spore staining, the 48-72hour old culture was dissolved in NaCl solution, and then carbolic fuchsin solution was added and heated for 10 min at 80°C. Fixation and immersion were carried out in 1% sulfuric acid for 1-2 seconds, followed by washing with water. The spores were stained with methylene blue for 3 min, washed with water, and observed under a microscope (Damayanti et al., 2018)

Antibacterial Test

The antibacterial test was conducted using the paper disc diffusion method with erythromycin as the positive control and distilled water as the negative control (Roseveld et al., 2022). The petri dishes were then placed in an incubator at 37°C for 48 h to allow for bacterial growth and development (Kasi et al., 2017). After incubation, a clear zone was observed, indicating inhibitory activity (Roseveld et al., 2022).

RESULTS

Lactic Acid Bacteria (LAB) Isolation

Microbial isolation is a technique for developing microorganisms outside their natural environment to obtain pure bacterial cultures that are distinct from other bacteria. The basic principle of microbial isolation is to separate one type of microbe from another type of microbe in the mixture. To achieve this goal, microbes can be grown on solid media, where they form separate colonieser(Jufri, 2020).

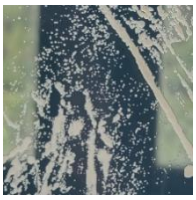
For colony selection, five isolates suspected to be LAB were obtained. The five isolates consisted of three sago ihur and two sago tuni, which were then coded as differentiators, namely I1, I2, and I5, and tuni T3 and T4.

LAB Macroscopic Characterization

Macroscopic identification is performed to recognize colony characteristics, such as shape, edge, elevation, color, appearance, optical properties, and texture. In this study, five bacterial isolates (**Table 1**) were obtained in a round shape, with flat edges, yellowish white color (not pigmented), shiny appearance, and textured sap (mucoid). Isolate T3 showed elevation (raised) and transparency (light can penetrate the colony without obstacles), whereas for other isolates, it was convex and translucent (light can enter).

Table 1. Macroscopic characteristics of LAB isolates from sago wastewater

Isolate code	Pictures	Morphology of colonies
I1		Shape: circular Edge: flat Elevation: convex Color: nonpigmented Appearance: glistening Optical property: translucent Texture: mucoid

I2		Shape: circular Edge: flat Elevation: convex Color: nonpigmented Appearance: glistening Optical property: translucent Texture: mucoid
T3		Shape: circular Edge: flat Elevation: raised Color: nonpigmented Appearance: glistening Optical property: Transparent Texture: mucoid
T4		Shape: circular Edge: flat Elevation: convex Color: nonpigmented Appearance: glistening Optical property: translucent Texture: mucoid
I5		Shape: circular Edge: flat Elevation: convex Color: nonpigmented Appearance: glistening Optical property: translucent Texture: mucoid

LAB Microscopic Characterization

Gram staining was used to identify microorganisms, particularly bacteria. The purpose of this method is to identify bacteria whether they are Gram negative or Gram positive(Agustine et al., 2018), and in this study it was found that all LAB isolates were Gram positive bacteria and their cells were coccus-shaped (**Fig.2**).

The next microscopic characterization was to determine whether bacterial isolates also include microorganisms that can produce spores (Mechai et al., 2020) and and it was found that none of the LAB isolates had spores.

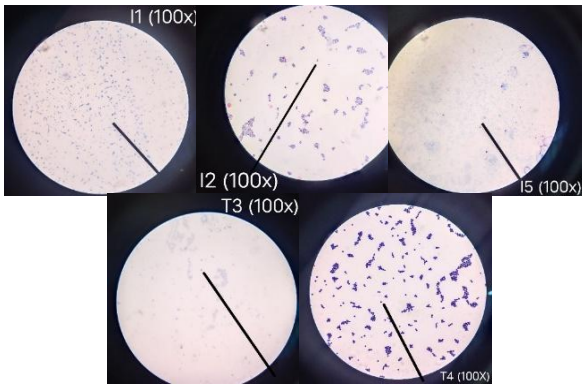


Figure 2. Microscopic characteristics of LAB isolates from sago wastewater. I1, I2, I5, T3, and T4 were Gram positive and coccus-shaped

Antibacterial Test

Inhibition test of LAB isolates showed that the isolates did not have the ability to inhibit the growth of *S. aureus* and *E. coli*. This can be seen by not forming a clear zone around the paper disk of LAB isolates.

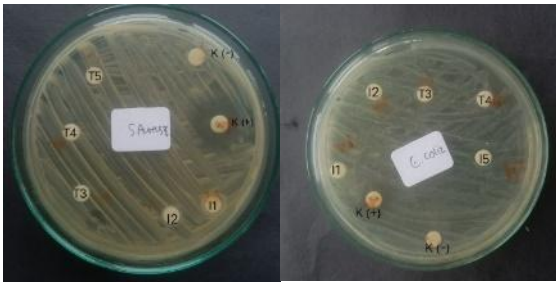


Figure 3. Antibacterial activity of LAB from sago wastewater against *S. aureus*(left) and *E. coli*(right)

DISCUSSION

Macroscopic Identification

Bacteria can grow optimally on media suitable for their nutritional and chemical needs. MRSA media is used for the growth and isolation of LAB that form colonies. MRS media consists of peptone oxide, Lab-Lemco, yeast extract, glucose, Tween 80, Dipotassium Phosphate, Sodium Acetate Trihydrate, triammonium citrate, magnesium sulfate, Manganese Sulfate, and distilled water (Man et al., 1960; Ningsih et al., 2018).Ideally, bacterial growth takes 24-48 hours at 37°C and can be achieved by growing isolated bacteria on selective MRSA media. After the isolate is planted, colonies suspected to be LAB can be observed. According to a study by Kurnia et al., (2020) LAB generally have a basic morphology in the form of white to yellowish-white colonies that are round in shape and have clear edges.

The five isolated bacterial isolates had a morphology of bacterial colonies that grew on growth media that were round, flat edges, yellowish white (not pigmented), looked shiny, and had a sap-like texture (mucoid), but isolate T3 showed a raised elevation (raised) and translucent to light (transparent), while for other isolates, it was convex and translucent. The colony morphology displayed by bacteria on agar media as an auxiliary means to identify bacterial species because of their different and specific growth patterns. Laily et al., (2013) in their research on LAB from salted mustard found that most LAB isolates had a round shape with unclear edges, lacked pigment, and were transparent. Another study by Bounaix et al., (2009) stated that LAB colonies had no pigment and had a slimy texture, while Noman et al., (2020)in their study found that LAB formed cream-colored colonies with a round shape, smooth and shiny surface, and intact edges.

Microscopic Identification

Gram-positive bacteria are a group of bacteria that are classified based on the color seen during the staining process. The Gram staining method was developed by Hans Christian Gram in 1884. This staining method uses a crystal violet dye that attaches to the thick peptidoglycan cell walls of gram-positive organisms. Thus, Gram-positive bacteria are blue when observed under a microscope. Although Gram-negative bacteria generally have an outer membrane, they have a thinner peptidoglycan layer that cannot withstand the blue stain used in the initial process(Sizar &Unakal, 2021).

In Gram staining, the five isolates showed a cocci shape and were blue-purple. According to the theory(Nelsen et al., 2021), Gram-positive bacteria will appear violet or purple because the paint used will stick to peptidoglycan. Iodine was then added to produce a purple color. When washed with alcohol, the purple color faded, but the gram-positive bacteria retained their color. Finally, the sample was stained with safranin liquid so that the gram-positive bacteria appeared purple

The results of research(Laily et al., 2013), also found that LAB isolates from salted mustard greens are Gram-positive bacteria. The results obtained are in line with research conducted by Wibowo in 1988, which was later cited by (Laily et al., 2013), explaining that LAB retains purple color after Gram staining test. This is because LAB have a cell wall rich in peptidoglycan, thus the purple paint attached to the peptidoglycan is not easily released and maintains its purple color.

Spore staining was performed to determine whether the bacteria can produce spores. The spore staining results showed that no spores were observed in the tested bacteria. Therefore, these bacteria can be categorized as spore-negative. In addition, in the painting with methylene blue, there is no visible staining of the spore structure, which usually appears blue. Vanniyasingam et al., (2019), found that the spore staining test on LAB isolated from dairy products did not show any spore reaction; therefore, it was concluded that they were unable to form spores.

However, in contrast to research conducted by Suzuki & Yamasato(1994)which showed that not all LAB did not form spores, especially LAB of the genus Bacillus. This is because spore-forming LAB originate from different phylogenetic positions and their evolution is unified in the Bacillus group.

Antibacterial Test

Antibacterial tests showed that the LAB isolates were unable to inhibit the growth of *E. coli* or *S. aureus,* this could be due to the inability of LAB isolates to produce bacteriocins. Research conducted by Qiao et al., (2022) on the antimicrobial activity of *Pediococcusacidilactici* LAB showed similar results and was unable to inhibit the growth of the test bacteria. This is further explained by the fact that the bioactive compounds produced by *Pediococcusacidilactici* are not broad-spectrum antimicrobials and are only able to inhibit the growth of certain bacterial strains.

Shembil (2016) did not find antimicrobial activity in LAB isolates through the agar diffusion method and concluded that the bacteriocin content in LAB isolates obtained was less, or even did not have bacteriocin to inhibit the growth of the pathogen tested. Another study by Choi & Beuchat, (1994), found that *Pediococcus acidilactici* was not effective in reducing the number of *Listeria monocytogenes* in the intestines of mice. This may be because *Pediococcus acidilactici* failed to compete with other intestinal microorganisms, or the production of pediocin PA-1 was inhibited by other microorganisms in the intestine.

Bacteriocin production by LAB is influenced by growth kinetics and metabolism during the exponential growth phase, and stops once the stationary phase is reached, as well as by specific factors that can limit bacteriocin production. Bacteriocin production rates do not always go hand in hand with high cell yields, as other factors such as specific production rates also play an important role. Therefore, a comprehensive understanding of the relationship between environmental conditions and bacteriocin production is necessary to efficiently optimize the bacteriocin production process (Abbasiliasi et al., 2017). Moreover, complex media have limitations in composition and concentration that are not optimal for bacteriocin production by some bacterial strains, especially LAB. These limitations include the availability of essential molecules, production of organic acids that lower the pH, nutrient deficiencies, lack of essential minerals, and lack of carbon sources. To optimize bacteriocin production, in particularly using sago wastewater, growth media formulation needs to focus on the carbon/nitrogen ratio, the use of media components such as NaCl and ethanol, high carbon source concentration, and pH regulation of the medium. Low nutrient levels can inhibit bacterial growth, while high nutrient levels or water-insoluble components can have negative effects (Abbasiliasi et al., 2017).

CONCLUSION

Five bacterial isolates (I1, I2, T3, T4, I5)were isolated from sago wastewater. Most of the colonies were circular with flat edges and yellowish-white in color (non-pigmented), shiny, and slimy in texture. Isolate T3 has a high elevation and appears translucent (transparent), whereas other isolates have convex and translucent characteristics. All the isolates were Gram-positive cocci and did not produce spores. Antibacterial tests conducted on *S. aureus* and *E. coli* growth revealed no

inhibition zones. LAB isolates from sago wastewater showed no significant antibacterial effect. However, optimization of growth conditions under which the bacteriocin is most effectively produced should be investigated.

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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