Screening of Lactic Acid Bacteria from Oysters for Antibacterial Activity Against Selected Pathogens

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Abstract. Lactic acid bacteria (LAB) are among the important groups of bacteria and considered as probiotics development. LAB is mostly isolated from the gastrointestinal tract (GIT) from organism such as oyster. Oyster belongs in to class *Bivalvia* which live in estuaries area. The purpose of research was to isolate and screened for antibacterial activity against selected pathogens. The LAB was isolated by pour plate method using MRSA (De Man, Rogosa, Sharpe Agar) as selection media for LAB. Then, LAB was screened by an agar diffusion assay for antibacterial activity. The result showed that four isolates (TR-01; TR-02; TR-03 and TR-04) have inhibitory activity against pathogens including *E. coli, Salmonella enterica, Staphylococcus aureus, Bacillus cereus*. Therefore, it is needed further assay to selected 4 isolates as potential probiotics.

1 Introduction

The problem of diseases caused by pathogenic bacteria can be solved by using antibiotics as chemotherapy to eliminate the disease. Antibiotics have long been used in aquaculture to prevent disease. However, this leads to various problems such as the presence of antibiotic residues in animal tissues, the emergence of bacterial resistance mechanisms and an imbalance in the microbiota of the digestive tracts of aquatic species, which affects their health. In order to overcome these problems, probiotic bacteria can be used as biocontrol agents in aquaculture as an alternative solution. Probiotics are defined as "live microbial food supplements that benefit the host (human or animal) by improving the body's microbial balance" [1]. The approach is based on the activity of microorganisms that can suppress or inhibit the growth of bacterial pathogens without negatively affecting the bacterial ecological balance system. This application is well established and widely used in aquaculture and livestock.

Lactic acid bacteria (LAB) have been used as probiotic agents due to their ability to produce a variety of beneficial compounds and their characterization as safe microorganisms. Lactic acid bacteria are the most common probiotics proposed as biological control agents in aquaculture [2]. Lactic acid bacteria (LAB) constitute an ubiquitous bacterial group that is

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widespread in nature in niches of dairy (fermented), meat and vegetable origin, the gastrointestinal and urogenital tracts of humans and animals, and soil and water.

Estuarine is a combination of freshwater and saltwater, resulting in the formation of chemical constituents and a unique set of microorganisms. Due to the high organic matter and nitrogen content of estuarine, this can influence the characteristics and diversity of the microorganisms produced. *Crassostrea gigas*, commonly known as the meat oyster, has a relatively high environmental tolerance, especially in estuarine waters, and a relatively wide population distribution [3]. Oysters are present in diverse ecosystems, including estuarine waters, coral reefs, seagrass beds, and mangroves [4]. The oyster is of ecological and economic importance. Ecologically, oysters are considered an important biota, contributing to ecosystem formation and serving as an indicator of water quality. Fresh and marine waters are sources of LAB [5]. Therefore, the gut of oysters and other aquatic animals is a natural reservoir for LAB.

The use of probiotics in aquatic organisms has been encouraged for this research. Further research is required to test the probiotic potential of LAB isolates in vivo and in vitro, due to the need for sustainable aquaculture. Many study to isolate and screen probiotic microorganisms for aquaculture, e.g. in fish farming [6]; mollusks [7]; *Penaeus monodon* and *Penaeus merguensis* [8]. The objective of this study was to isolate and characterize LAB isolates from oysters and to analyze their antimicrobial activity against selected pathogens.

2 Materials and methods

2.1 Sample preparation and isolation bacteria Lactic Acid Bacteria (LAB)

Bacteria were isolated from organ digestive tract such stomach and small intestine of Oyster (*Crassostrea gigas*). Oyster was collected in Kuala Gigieng, Aceh Besar Regency. The samples stored in an ice chamber, immediately transported to the laboratory. Twenty-five gram stomach and small intestine of Oyster were crushed aseptically and dissolved in sterile phosphate buffered saline solution. The samples obtained were then serially diluted, from concentrations of 10-1 to 10-3. Then 0.1 ml of each dilution was taken and placed in a Petri dish. de Man, Rogosa, and Sharpe medium (MRS) Agar then poured into a Petri dish and flattened. The Petri disks then incubated anaerobically at 37°C for 24 hours. After incubation, isolates that were catalase-negative and gram-positive bacteria were considered to be LAB.

2.1.1 Catalase test

A 24-hour old bacterial isolate was dropped onto the microscope slide with 2 drops of 3% H_2O_2. A positive catalase test reaction, characterized by the formation of oxygen bubbles, indicates that the bacteria produce the enzyme catalase, which converts H_2O_2 into water (H_2O) and oxygen (O_2).

2.1.2 Preparation subculture from stock bacterial cultures

Indicator organisms were collected from the Microbiology Laboratory, Biology Department, Syiah Kuala University. In the research*, Staphylococcus aureus* and *Bacillus cereus* were used as indicator organisms for the group of gram-positive bacteria, and *Escherichia coli* and *Salmonella enterica* were used as representatives of the group of gram-negative bacteria. Antibacterial assay was prepared by streaking one loop from fourth regeneration stock bacterial cultures, namely *Staphylococcus aureus*, *Bacillus cereus*, *E. coli,* and *Salmonella*

enterica on Mueller Hinton Agar (MHA) (Merck, Germany), which was stored at 4°C. The indicator organisms were cultured overnight (24 hours) at 37°C before the antibacterial assay.

2.2 Lactic Acid Bacteria characterizations

Morphological characterization of LAB colony was done after getting a pure culture in a petri dish. Characteristics of a colony such as shape, edge, elevation, color and texture. Observation is often made with the naked eye. The surface of colonies can be seen from the side and the edge of the colony can be seen from the above.

2.3 Production of crude bacteriocin

The potency test of the crude bacteriocin extract (antibacterial activity) was carried out with disk diffusion method (Kirby-Bauer method). Tetracycline Antimicrobial Susceptibility discs (30µg) were used as a positive control due to their broad spectrum and ability to inhibit both gram-positive and gram-negative bacteria. On the other hand, pure MRSB as negative control in this treatment.

The isolate was inoculated into Man Ragosa Sharpe Broth (MRSB) media and incubated in a shaker incubator for 24 hours at 37°C. One millilitre of bacterial culture was centrifuged at 10,000 rpm for 5 minutes, separating the liquid from the sediment. The resulting liquid, called the cell-free supernatant, is a crude bacteriocin extract.

Four indicator bacterial cultures, namely *Staphylococcus aureus*, *Bacillus cereus*, *E. coli* and *Salmonella enterica* were matched bacterial concentration 0.5 McFarland. A total of 100 µL of liquid isolate was applied to the surface Mueller Hinton Agar (MHA) media and scratched with a sterile swab to spread it evenly. Sterile commercial blank discs (Oxoid) are soaked in the BAL supernatant for 30 minutes. The soaked test paper is placed on the four solid cultures of indicator bacteria previously isolated. Each solid culture of indicator bacteria is also given tetracycline as positive control and pure MRSB as negative control. Then, the plate incubated at 37°C for 24 hours. The inhibition zone/clear zone visible around the paper test is observed and the diameter of the inhibition zone is measured.

3 Results and discussion

3.1 Isolation and characterization Lactid Acid Bacteria (LAB)

A total four isolates Lactid Acid Bacteria were obtained from the digestive tract of oysters in the form of the stomach and the intestines of oysters (*Crassostrea gigas*). Different codes are given to growing isolates. The isolate LAB obtained from oysters was coded TR-01; TR-02: TR-03 and TR04. The macroscopic and microscopic characterization of the bacterial isolates can be seen in (Figure 1).

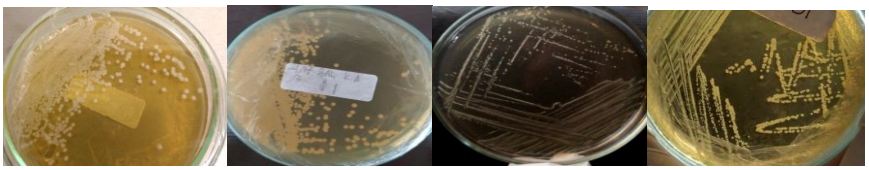

Fig. 1. The Growth of LAB isolates on MRSA.

A. Isolate TR-01; B. Isolate TR-02; C. Isolates TR-03 and D. Isolate TR-04

LAB have been isolated by different researchers with different numbers of the isolates. There are 6, 8, and 65 LAB strains isolated from digestive tract of Gourami fish (*Osphronemus goramy*) [9], pomfret fish (*Trachinotus blochii*) [10], and the mud crab (*Scylla* sp.)[11], respectively.

MRSA media is commonly used to isolate LAB growth. MRSA is a selective solid media that can only grow LAB. MRSA contains beef extract and yeast extract as carbon, nitrogen and vitamin sources. MRSA media can only be used to grow and isolate LAB growth in colonies [12]. Dextrose is a fermented carbohydrate that acts as a carbon and energy source. The ammonium citrate has the function of supporting the growth of the LAB in low pH conditions. Dipotassium phosphate and sodium acetate act as buffers to keep the pH low. Magnesium sulphate is used as a source of both ions and sulphate. Peptone, meat extract and yeast extract are sources of nutrients for growth as they contain nitrogen, vitamins, minerals and amino acids [13].

3.2 Morphological characterisation of Lactic Acid Bacteria (LAB)

LAB were purified, then various characterizations were performed on the LAB isolates obtained. The pure colony was morphologically observed under a microscope. Characterization is divided into morphological (macroscopic) and physiological (microscopic) characterization. The characteristics that were observed including colony shape/form, edge, elevation (colony surface shape) and pigmentation. The characteristic cell was observed cell shape, Gram stain and catalase test. Macroscopic morphological and physiological observations of LAB results can be seen in Table 1.

Table 1. Colony characteristics and cell morphology of LAB.

Isolate Code	Colony				Cell		
	Form	**Edge**	**Elevation**	**Pigmentation**	**Form**	**Gram Stain**	**Catalase test**
TR-01	Circular	Smooth	Convex	Cream	Coccus	Positive	negative
TR-02	Circular	Smooth	Convex	Milky white	Coccus	Positive	negative
TR-03	Circular	Smooth	Arise	Creamy white	Coccus	Positive	negative
TR-04	Circular	Smooth	Arise	Cream	Coccus	Positive	negative

Colony morphology of 4 LAB isolates (TR-01, TR-02, TR-03 and TR-04) are circular form (100%), smooth edge (100%). The results also obtained colony characteristic convex (50%) and arise (50%) elevation, and cream (50%); milky white (25%) and creamy white (25%) colour. All of LAB show characteristics cell with positive Gram stain (100%) and catalase negative test (100%). Adde and Endang (2018) were studied about observations macroscopic of the morphology of LAB colonies from fish-based fermented food on MRSA media are shaped bacterial colonies circular and punctiform, with convex, flat and raised elevations. in general, LAB are a diverse group of Gram-positive bacteria. They are usually non-sporulating and exhibit bacilli or coccus morphology, catalase-negative and are either aerobic or facultatively anaerobic also acid-tolerant [14]. Assessing the morphology of LAB is essential because it helps to identify which species of bacteria are growing in MRSA. Different species of LAB have different colony morphologies.

The Gram stain test was carried out on all isolates to confirm their classification as Gram-positive bacteria. This is a characteristic feature of LAB. This is due to their lower lipid content, resulting in easier cell wall dehydration during alcohol treatment. Cell wall

dehydration reduces pore size and permeability, preventing the crystalline purple dye, which is the primary dye, from leaving the cell, thus the cell retains its purple colour [16].

The catalase test was carried out on all isolates to verify that all isolates were catalase negative, which is one of the characteristics of lactic acid bacteria. The presence of the catalase enzyme is indicated by the formation of bubbles. The results of the catalase test showed that all isolates were catalase negative. This is indicated by the absence of bubbles. The catalase test can be used to determine the nature of the bacteria in terms of their requirement for oxygen. Facultative anaerobic bacteria have the enzyme superoxide dismutase but not catalase, but facultative anaerobic bacteria have the enzyme peroxidase. This enzyme can catalyse the reaction between H_2O_2 and organic compounds, producing non-toxic compounds [15]. The results of the catalase test showed that all LAB isolates are a facultative anaerobic bacteria. It is characterized by not producing the catalase enzyme.

3.3 Antibacterial activity of LAB isolates

Result show that the activity test of crude bacteriocin extract LAB against selected indicator bacteria showed that there was an inhibition zone formation diameter different between positive Gram stain and negative Gram stain. Inhibition zone of *Staphylococcus aureus* and *Bacillus cereus* higher than *Escherichia coli* and *Salmonella enterica*. The diameter of the inhibition zone formed can be the diameter of the clear zone around the disc which has bactericidal properties (kills bacteria) or the diameter of the semi-zone which has bacteriostatic properties (inhibits microbial growth). The clear zone is formed by the inhibition of microbial cells by antimicrobial compounds. The general mechanism the action of an antimicrobial compound may be mediated by interfering with or damaging by interfering with or damaging the cell wall components, by reacting with the cell membrane causing increased cell permeability, inactivation of enzymes essential for and destruction or inactivation of the function of genetic material [17].

Table 2. Antibacterial activity of LAB isolates.

	Zone of inhibition in mm					
Isolat	*Escherichia coli*	*Salmonella enterica*	*Staphylococcus aureus*	*Bacillus cereus*	Positive control	Negative Control
TR-01	1.4 ± 0.8	2.3 ± 0.0	7.3 ± 1.8	12.05 ± 1.6	31.54 ± 0.0	0
TR-02	1.8 ± 0.0	3.6 ± 0,8	12 ± 0,0	13 ± 1.3	29.09 ± 0.0	0
TR-03	3.6 ± 0.0	5.6 ± 2.4	14.45 ± 2.4	8 ± 0.2	27.09 ± 0.1	0
TR-04	5.8± 2.2	4.8± 1.6	9. 3± 1.6	11 ± 0.8	33.78 ± 0.0	0

The antibacterial activity was expressed as the diameter (mean ± SD, mm) of the mean inhibition zone in the agar plates inoculated with the selected pathogenic bacteria.

Table 2 shows that the positive control (tetracycline antibiotics) is the highest zone of inhibition of the LAB isolates and the negative control. Tetracycline antibiotics have a broad spectrum of activity against many gram-positive and gram-negative bacteria. LAB isolates contain various organic acids and other compounds. One of the most important properties of LAB is its ability to produce various metabolites with antibacterial properties, including antibacterial, including lactic acid, acetic acid, ethanol, diacetyl, CO_2, H_2O_2 and bacteriocin

[18]. In this study, crude bacteriocins from LAB have a potential antibacterial agent for treating indicator microorganism infection. Bacteriosin are antimicrobial peptides produced by non-pathogenic bacteria, mainly lactic acid bacteria, to destroy or inhibit other pathogenic bacteria in the host [19].

A lower zone of inhibition against *E. coli* may be the result of the presence of bacteriocins in the BAL that are specific for inhibiting the growth of Gram-positives. The bacteriocin activity produced by LAB is very effective in inhibiting most of the Gram-positive bacteria, especially the strains based on the phylogeny [20]. This activity is less effective against Gram-negative bacteria. Other factors may be involved, including the lipopolysaccharide (LPS) layer of the cell wall of Gram-negative bacteria. Bacteriocins produced by lactic acid bacteria are ineffective in suppressing Gram-negative bacteria. This is considered to be due to the presence of the lipopolysaccharide (LPS) layer of the cell wall of Gram-negative bacteria, which protects the cell membrane [21]. According to all results, all LAB isolates TR-01, TR-02, TR-03 and TR-04 have antibacterial activity and can develop as potential probiotics.

4 Conclusion

Based on the results of the research, it can be concluded that there are four LAB isolates (TR-01, TR-02, TR-03 and TR-04) that have been isolated from the digestive tract of oysters (*Crasosstrea gigas*). The characteristics morphology colony have different among all isolate LAB and indicate different species. Overall, LAB are a potential antibacterial agent for treating indicator microorganism infection and can develop as potential probiotic bacteria.

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