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ISOLATION AND MOLECULAR IDENTIFICATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA FROM FISHERIES WASTE

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Abstract – The present study aimed to isolate and identify the bacteriosin-producing lactic acid bacteria (LAB) from fisheries waste by using molecular identification. LAB was isolated from fisheries waste (smoked fish, presto milk and frozen fillets) by dilution series-pour-plate techniques. The obtained isolate were then screened by qualitative, semi-quantitative and quantitative analysis. Bacterial characterization was carried out using morphology, biochemistry, vitex, physiology, morphology, pathogenic, antimicrobial and molecular test analysis. Molecular identification of LAB was carried out using the neighbor-joining method by MEGA 5 Software. All analyses were performed on a bootstrapped date 1000 replicates. Total of 40 isolates was obtained from screening test of LAB and these isolates consist of 12 isolates from smoked fish waste, 11 isolates from presto-milkfish waste and 17 isolates from freezing fish waste. The screening of bacteriocin extract was found 2 isolates of smoked fish waste, 2 isolates of presto-milkfish waste and 2 isolates of freezing fish waste. Molecular identification using 16S rRNA sequences showed that LA01 isolates had the closest relationship with *Pediococcus accidilactici* DSM 20284 with nucleotide similarity of 98.63%. These results suggested that bacteriocin from LAB fishery waste (smoked fish, presto milk and frozen fillets) has the potential activity as a food preservative.

INTRODUCTION

Preservatives and synthetic antioxidants have been used to inhibit food deterioration. In general, there are two kinds of food preservation method such as physical and chemical. Dehydration, radiation, freeze-drying and refrigeration are kinds of the physical preservation method. While, chemical preservation is generally using a chemical compound like BHA (Butylated Hydroxyanisole), BHT (Butylated Hydroxytoluene) dan TBHQ (Tertiary Butylated Hydroxyanisole) (Ito and Tsuda, 2008). However, since this compound is categorized as a carcinogenic compound, thus Indonesian Government must be monitored the use of this compound.The increasing demand of safe food has become more interestingto use natural products in food preservation (Riyadi *et al*., 2007).

One of natural product that can be used as food preservation is bacteriocin (Singh *et al*., 2013). Bacteriocins are ribosomal synthesized antibacterial peptide produced by bacteria either to inhibit or kill the bacterial growth (Maqueda *et al*., 2008). Either gram-positive or gram-negative bacteria can produce bacteriocins. Some bacteriocins that have been isolated and characterized from lactic acid bacteria and have potential use as food preservation are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins and plantaricins (Ali *et al*., 2006). Each bacteriocin has specificity to killing bacteria such as nisin only kill gram-negative bacteria, while bacteriocin from *Lactobacillus* only effective to kill mesophilic bacteria (Ogunbanwo *et al*., 2003).

The direct use of bacteriocinfor food not only prevents the spoilage of the food but also extend their shelf-life (Gautam and Sharma, 2009; Yusuf, 2013). The application of bacteriocin as food preservation has many advantages. Bacteriocin is not toxic and biodegradable, sobacteriocin did not affect the intestinal microflora because it is easily digested by enzymes in the digestive system. Bacteriocin have relatively simple biosynthetic mechanisms compared with conventional

antibiotics (Gautamand Sharma, 2009). Furthermore, bacteriocin also can be applied in high and low temperature and reduce the use of chemical additives (Galvez *et al*., 2007).

Bacteriocins are generally produced by lactic acid bacteria isolated from fermented and non-fermented fish products (Matamoros *et al*., 2009). Recently, some bacteriocin produced by local microbial from Indonesia have been published, and the majority was isolated from fermented food like Rusip (Kusmawarti *et al*., 2014), fermented meat (Rahayu *et al*., 2000) and shrimp intestine (Jini *et al*., 2011). However, there is limited information about bacteriocin isolated from fisheries waste. Therefore, this study aimed to determinethe characteristics of bacteriocin produced from fishery waste.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from fisheries waste by using dilution series-pour-plate techniques in MRS agar media. The dilution was performed by using sea water and sterile distilled water (70% dan 30%). Several dilutions were then made in 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. At 10^{-1} to 10^{-4} dilutions, 0.1 mL of each dilution was transferred to agar plate containing 20 mL MRS that was added before with $CaCO_3$ 1% and Na azide 0.01% and incubated at 30 °C for 24-48 h. Isolates that produce clear zones were then added with skim milk and glycerol 1:1 and stored at -80 °C.

Screening of lactic acid bacteria

Qualitative screening

The qualitative test was conducted to select the isolates which produce bacteriocin using the paper disk method. This method used the indicator of bacterium *Pediococcus accidilactici* LB42.

Semi-qualitative screening

The semi-qualitative screening aimed to select isolates from bacteriocin-producing lactic acid bacteria using well diffusion. The inhibitory activity was determined by measuring the bacteriocin inhibition zone after 24 h incubation, while bacteriocin inhibition zone calculated by measuring the area of clear zone around the well. The inhibition zone was calculated using the following formula:

$$Bacteriocin\ activity\ \left(\frac{mm^2}{mL}\right) = \frac{1AU}{mL} = \frac{D_z - W_d}{v} \quad ..(1)$$

Where :

D_z : Diameter of the clear zone (mm^2)
W_d : Wells diameter (mm^2)
V : Volume (mL)

Quantitative screening

The quantitative screening was determined using well diffusion. The obtained supernatant from each isolate was diluted in serial dilution (10x,20x,30x). The inhibitory activity is expressed as Arbitrary Unit (AU/mL) which is the area of inhibition per unit volume of the sample bacteriocintested (mm^2/ mL). This is obtained from the highest dilution which still shows the presence of inhibitory activity (clear zone).

Characterization of lactic acid bacteria

Bacterial characterization was carried out using morphology test, biochemistry test, vitex test, physiology test, pathogenic and antimicrobial and molecular test. Morphological criteria include observations of growth, color, elevation, the edge of colonies, cell shape, cell composition, gram staining and motility tests. Biochemical tests include catalase, gas formation and fermentation types. Catalase test was carried out by adding 3% H_2O_2 solution in bacterial culture. The presence of air bubbles indicated the positive catalase.

Vitex test was performed to identify the types of lactic acid bacteria isolates chemically. Physiological tests include observing bacterial growth on the effect of temperature, pH, and salinity (% NaCl). Bacteriocin-producing lactic acid bacteria test was carried out using several bacteria, including *Aeromonas* sp., *Escherichia coli, Staphylococcus, Vibrio harveyi* and *Bacillus* sp.

Molecular identification was performed only in bacteriocin-producing isolates (SFE-7(33) and P12A(25)). DNA extraction used CTAB method and amplification of 16S rRNA was carried out using Polymerase chain reaction (PCR) with universal primer 27F (5′-AGAGTTTGATCCTGGCT-CAG-3′) and 1592 (5′-GGTTACCTTGTTACGACTT-3′). PCR mixture consists of 30 µL including 15 µL PCR mastermix, 6 µL distilled water, 3 µL primers forward and reverses respectively and 3 µL DNA template. Amplified DNAwere then sequenced using ABI Prism.

Data Analysis

All morphology, physiology and biochemistry data were analyzed descriptively. Molecular identification of lactic acid bacteria was carried out

using the phylogenetic tree. The obtained sequence was aligned using CLUSTAL W software. The phylogenetic tree was derived from distance matrices method using the neighbor-joining method trough MEGA 5 Software. All analyses were performed on a bootstrapped date 1000 replicates.

RESULTS

LAB isolation

The total of 603 isolates was obtained in the present work. These isolates consist of 189 isolates from smoked fish waste, 267 isolates from presto-milkfish waste and 147 isolates from freezing fish waste (Table 1). The isolates were then purified (2 times) on MRS agar media with strike method.

Table 1. Lactic acid bacteria isolates from fisheries waste

Sample type	Total of lactic acid bacteria
Smoked fish waste	189 isolates
Presto-milkfish waste	267 isolates
Freezing fish waste	147 isolates

Screening of LAB isolates using indicator bacteria

Total of 40 isolates was obtained from screening test of lactic acid bacteria using paper disk method and *Pedioccocus acidilaktici* LB 42 indicator. These isolates consist of 12 isolates from smoked fish waste, 11 isolates from presto-milkfish waste and 17 isolates from freezing fish waste (Table 2). The inhibitory activity was determined by measuring the bacteriocin inhibition zone after 24 hours incubation. The resulted isolates were then screened.

Table 2. The number of isolates suspected of producing bacteriocin

Sample type	Total of lactic acid bacteria
Smoked fish waste	12 isolates
Presto-milkfish waste	11 isolates
Freezing fish waste	17 isolates

Bacteriocin screening for indicator bacteria

The candidate isolates was isolated thebacteriocin until the resulting inhibition is derived from bacteriocin. The screening of bacteriocin extract using well method was found 2 isolates of smoked fish waste, 2 isolates of presto-milkfish waste and 2 isolates of freezing fish waste (Table 3). The previous study showed that screening of *Lactobacillus plantarum*with indicator *Lactobacillus bulgaris* was performed in 37°C for 48 h (Moraes *et al*., 2010). Furthermore, screening of lactic acid bacteria from

Table 3. The isolates resulting from bacteriocin screening using the well method

Sample type	Total of lactic acid bacteria suspected of producing bacteriocin	Diameter
Smoked fish waste	2 isolate	>10 mm
Presto-milkfish waste	2 isolate	>10 mm
Freezing fish waste	2 isolate	>10 mm

Table 4. Morphological, biochemical, and sugar fermentation characterization of bacteriocin-producing lactic acid bacteria

Character	Smoked fish waste		Presto-milkfish waste		Freezing fish waste	
	LA01	LA02	LB01	LB02	IB01	IB02
Morphology						
Coccus shape	+	+	+	+	+	+
Tetrad cell arrangement	+	+	+	+	+	+
Gram-positif	+	+	+	+	+	+
Motility	-	-	-	-	-	-
Biochemistry						
Catalase	-	-	-	-	-	-
Homofermentatif	+	+	+	+	+	+
Gas formation	-	-	-	-	-	-
Fermented glucose						
Gliserol	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	+
D-Glucosa	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+

fermented fish was also performed in 37 °C for 48 h (Pringsulaka *et al*., 2012).

Isolates characterization of bacteriocin-producing LAB

The morphological characteristic of candidate isolates was coccus shape, tetrad cell arrangement, including of gram-positive bacteria and have negative motility. Furthermore, biochemistry characterization showed that candidate isolates have no catalase activity, homo-fermentative and no gas formation observed (Table 4).

Physiologically the growth of bacterial isolates from fishery waste can grow at a temperature range of 4 °C to 50 °C, in a pH range from 4 to 10 and capable of growing at 5-10% NaCl levels (Table 4). Based on phenotypic characteristics when compared with BAL characteristics in the reference isolates, isolates were identified as *Pediococcus acidilactici.* This is supported by a Vitek test which indicates that bacterial isolates are *Pediococcus* (Table 5).

Based on the antimicrobial test of the three pathogenic bacteria (*Escherichia coli*, *Staphylococcus* and *Vibrio harveyi* showed that the isolates were able to inhibit the growth of these three pathogenic bacteria (Table 7), but in *Escherichia coli* the inhibition zone appeared thin due to bacteriocins produced from gram-positive can only effectively inhibit related bacteria strain. This condition was in accordance with the opinion of Liasi (2009) who stated that bacteriocins are generally stable to temperature, sensitive to several proteolytic enzymes, and generally effective against gram-positive bacteria.

Table 5. Physiology characterization of bacteriocin-producing lactic acid bacteria

	Smoked fish waste		Presto-milkfish waste		Freezing fish waste	
	LA01	LA02	LB01	LB02	IB01	IB02
Growth temperature						
4°C	+	+	+	+	+	+
10°C	+	+	+	+	+	+
30°C	+	+	+	+	+	+
40°C	+	+	+	+	+	+
45°C	+	+	+	-	+	+
50°C	+	+	+	-	+	+
pH						
4.0	+	+	+	+	+	+
8.0	+	+	+	+	+	+
8.5	+	+	+	+	+	+
9.0	+	+	-	_	+	+
NaCl						
5%	+	+	+	+	+	+
6,5%	+	+	+	+	+	+
10%	+	+	+	+	+	+
18%	-	-	-	-	-	-

Table 6. Vitek analysis in suspected isolates.

Vitek Analysis	Smoked fish waste		Presto-milkfish waste		Freezing fish waste	
	LA01	LA02	LB01	LB02	IB01	IB02
Sample	*Pediococcus*	*Pediococcus*	*Pediococcus*	*Pediococcus*	*Pediococcus*	*Pediococcus*

Table 7. Pathogen antimicrobial test.

	Smoked fish waste		Presto-milkfish waste		Freezing fish waste	
	LA01	LA02	LB01	LB02	IB01	IB02
Eschericia coli	+	+	+	+	+	+
Staphylococcus aureus	+	+	+	+	+	+
Vibrio harveyi	+	+	+	+	+	+

DISCUSSION

In the present study, we found that there was 603 lactic acid bacteria isolates from fisheries waste at the appropriate environmental conditions (Table 1). The clear bacteriocin inhibition zone is related to the nature of single hit inactivation, which means that one bacteriocin molecule will kill one indicator of the bacterial cell (Singh, 2018). Bacteriocin activity is expressed as arbitrary units per mL (AU/mL) from the culture medium (SimonovaandLaukova, 2007). One AU/mL is the area of inhibition per unit volume of the sample bacteriocin tested (mm^2/mL). Isolates were stated as bacteriocin-producing when producing inhibitory zones in spot tests agar at least ≥ 7 mm (Moraes *et al*., 2010). Bacteriocin activity test using agar spot test was faster and more effective than the well diffusion agar method. However, well diffusion is important to test the crude bacteriocin extract to determine the bacteriocin character produced by LAB.

Several studies have been conducted on bacteriocin-producing lactic acid bacteria found in fish intestines including *Lactobacillus plantarum* with molecular weight 2.5 kDa (Karthikeyan and Santosh, 2009), *Carnobacterium divergence* V41 from the intestine of Salmon with a molecular weight of 4.509 kDa, *Pediococcus pentosaceus* from the intestine of marine fish with a molecular weight of 5 kDa and *Carnobacterium piscicola* VI from the intestines of trout produces bacteriocin with a molecular weight of 4.416 kDa (Buntin *et al*., 2011).

Molecular identification using 16S rRNA sequences showed that LA01 isolates had the closest relationship with *Pediococcus accidilactici* DSM 20284 with nucleotide similarity of 98.63% (Fig. 1). According to Willey *et al.* (Willey *et al*., 2009) sequencing of 16S rDNA and continued with alignment with the data available at GeneBank is one of the molecular detection methods that are ideal for knowing the relationship between bacteria because the 16S rDNA sequence is a gene found in all microbes and is needed in maintaining their lives (Madigan and Martinko, 2006; Vidotti *et al*., 2003). The species only have 3% sequence difference in the 16S rDNA sequence or it can be said to have a sequence homology of ≥ 97%. Sequence homology with a value of ≥ 97% is equivalent to the 70% hybridization equation, the minimum value used to express two bacteria included in one species.

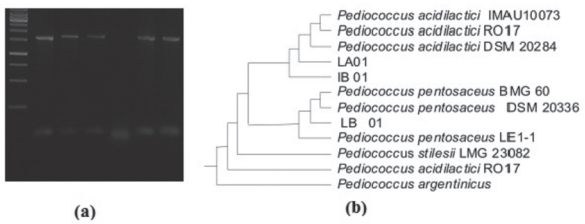

Fig. 1. (a) Electrophoresis gel of isolated DNA; (b) Phylogenetic tree of suspected isolates.

CONCLUSION

Bacteriocin from Lactic Acid Bacteria fishery waste (smoked fish, presto milk and frozen fillets) has the potential as a food preservative. Identification of isolates using 16S rRNA gene sequences showed that the isolates had the closest kinship relationship with *Pediococcus accidilactici*

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