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Molecular Identification of Lactic Acid Bacteria Producing Antimicrobial Agents from Bakasang, An Indonesian Traditional Fermented Fish Product

Helen Joan Lawalata[1*], Langkah Sembiring[2], and Endang Sutriswati Rahayu[3]

[1]Departement of Biology, Faculty of Mathematics and Natural Science, Manado State University, Manado, Indonesia
[2]Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3]Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Twenty seven strains of lactic acid bacteria (LAB) were isolated from bakasang, Indonesian traditional fermented fish product. In general, LAB have inhibitory activity againts pathogenic bacteria and spoilage bacteria. Screening for antimicrobia activity of isolates were performed with well-diffusion method. One isolate that was designed as *Pediococcus* BksC24 was the strongest against bacteria pathogenic and spoilage bacteria. This strain was further identified by 16S rRNA gen sequence comparison. Isolates LAB producing antimicrobial agents from bakasang were identified as *Pediococcus acidilactici*.

Keywords : Bakasang, LAB, antimicrobial, phenotypic characteristics, 16S rRNA gene

Introduction

Bakasang is fermented fish products traditionally made from the guts of big fish (*Katsuwonus pelamis* L.), small fish and fish eggs which is the typical food of North Sulawesi (Manado). In fermented food fish, Lactic Acid Bacteria (LAB) is a bacterium which played important role in the process of fermentation. One role of lactic acid bacteria is the ability to produce antimicrobial components that can inhibit the growth of pathogenic bacteria and spoilage bacteria. Components that are antagonistic can be found in the form of organic acid, hydrogen peroxide, and diasetil bacteriocin (Daeschel,1989; Ouwehand,1998; Park *et.al*., 2005). Natural antimicrobial produced by lactic acid bacteria have been widely used as chemotherapeutic agents that can control the growth of pathogenic microbes (Liasi *et al*.,2009). LAB included as safe microorganisms when added to food because its not toxic and does not produce toxin, so-called food-grade microorganisms and are designated as "Generally Recognized as Safe" (GRAS). These microorganism is not at risk to health, even some types of bacteria are useful for health. In Indonesia, it has been widely reported data that revealed the potential of lactic acid bacteria as producers of antimicrobial substances from fermented foods (Savadogo, 2004; De Vuyst and Vandamme, 1994). The objectives of the experiment was to isolate and identify lactic acid bacteria on bakasang (fermented Cakalang fish) and to test the inhibitory activity against pathogenic bacteria and spoilage bacteria by using well-diffusion method.

***Correspondence author**:
Helen Joan Lawalata
Departement of Biology, Faculty of Mathematics and Natural Science, Manado State University, Kampus UNIMA Tondano. Indonesia.
Email: lawalata_helen@yahoo.com

Material and Methods

Indicator Strains and Growth Conditions

Escherichia coli ATCC 35218 and *Stapylococcus aureus* ATCC 25923 were obtained from the collection of Laboratory of Microbiology, Faculty of Medicine, Universitas Gadjah Mada, while *Pseudomonas fluorescens* FNCC 0070 and *Pediococcus acidilactici* PAF

11 were obtained from the collection of Laboratory of Microbiology, Center for Food and Nutrition, Universitas Gadjah Mada. All strain, except *Pediococcus acidilactici* PAF 11, were grown in NA (Nutrien Agar) at 37°C.

Pediococcus acidilactici PAF 11 (positive control) was grown in De Man Rogosa Sharpe (MRS) at 37° C.

Isolation of Lactic Acid Bacteria

Guts of Big fish, and egg fish were collected from local market in Manado. These samples were transported to the laboratory using cool box (4°C) and cut into small pieces and mashed. Salt and rinse reagent were added and mix thoroughly. The mixture was packed into bottles, corked and then incubated at 37°C for 7 days. LAB were isolated from sample of bakasang. Ten grams of samples were taken aseptically and homogenized in 90 mL of NaCl solution. Serial dilutions up to 10^{-7} were prepared and appropriate dilutions were plated onto de Man Rogosa and Sharpe Agar supplemented with $CaCO_3$ 1%, Na Azida and cyclohexamide. All plates were incubated at 37°C for 48 h. Only lactic acid producing bacterial colonies were selected. This can be observed from clear zones around the colonies which indicated the dissolving of $CaCO_3$ by an acid. Colonies with different morphology were counted, picked up and purified by restreaking on the same medium.

Cell morphology, Gram staining and catalase test, motility, non-spore forming were performed as a preliminary screening for lactic acid bacteria (Holt *et al*, 1994). The selected lactic acid bacteria were maintained as stock cultures at -80 °C in 10% skim milk and 20% glycerol.

Screening of Lactic Acid Bacteria for Antimicrobial Activity

The antimicrobial activity of LAB (Culture) against *Escherichia coli* ATCC 35218, *Stapylococcus aureus* ATCC 25923, 0070, *Pseudomonas fluorescens* FNCC 0070 was performed by the well diffusion assay (Davidson and Paris, 1989). Lactic acid bacteria culture were grown in MRS broth at 37° C for 24 h. Indicator bacteria were grown in Nutrien Broth at 37° C for 24 h. Ten mililiters of Nutrien soft agar was inoculated with 50 µl broth culture of pathogenic bacteria. MRS agar was poured on petri dish and allow to solidify, then overlaid with nutrient broth prepared previously and then incubated at 4 ° C for 1 h. Wells were made and filled with 50 µl LAB culture Incubation petri dish at 37° C for 24 h. LAB isolates which gave clear zones are indicated as isolates that have antimicrobial activity against indicator bacteria. The diameter of the inhibition zone was measured. The antimicrobial activity was determined by measuring the clear zone around the wells.

Identification of Lactic Acid Bacteria Isolates

The isolated LAB showing the highest antimicrobial activity were identified based on phenotypic and genotypic characterization. Phenotypic characterization was performed by examining cell morphology, motility and spore forming of isolate. Isolate was Gram stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties such as gas production from glucose, growth at different temperatures as well as the ability to grow in different concentrations of sodium chloride and pH in De Man, Ragosa, Sharpe (MRS) broth (Dykes *et al.*, 1994). Genotypic characterization was conducted by 16S rDNA sequence comparison.

Genomic DNA Extraction

Extraction of genomic DNA were done as described by Pospiech and Neumann (1995) with minor modifications to the method versatile quick prep for gram positive bacteria. Bacteria were cultivated on MRS broth. After 2-3 days of cultivation at 30°C, 1.5 mL of biomass was collected by centrifugation (5 min, 13.000 rpm). The pellet was resuspended in 400 µL SET buffer (75mM NaCl, 25mM EDTA, 20 mM Tris, pH 7,5), 50 µL Lysozyme (10 mg/mL), 20 µL Proteinase

K (15mg/mL) were added and incubated at 37°C for 1 h. Fifty microliters SDS 10% was added and incubated at 65°C for 1 h. 400µL cooled chloroform was added and centrifuged (13.000 rpm 10 min). The aqueous layer was re-extracted with isopropanol (1:1 v/v) and then incubated at -20°C overnight. DNA was centrifugated and washed with cooled ethanol 70% and then supernatant was removed. The purify of DNA solution was resuspended with TE buffer and checked spectrophotometrically at λ_{260} and λ_{280} nm.

Amplification and Sequencing 16S rDNA

The 16S rRNA genes were amplified from purified DNA of the strain using a commercial kit (Mega Mix Blue ® and universal primers 27f (5'-AGAGTTTAGTCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') for 16S rDNA (Thomas, 2007). The condition of amplified gene fragment : pre-denaturation of the target DNA at 96°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 51,5°C for 1min and 30 sec and primer extension at 68°C for 8 min. PCR was completed with 10 min elongation at 68°C followed by cooling to 4°C. PCR product was visualised by electrophoresis on a 2 % (w/v) agarose gels, stained with ethidium bromide in the presence of a 1 kb ladder The parameters for the electrophoresis were 90 V for 30 min.

The amplified of DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to manufacturers' instructions (ABI PRISMA 3100 Genetic Analyzer User's Manual).

Phylogenetic analysis

For phylogenetic analysis, sequences BksC24 strain and BksJ43 strain were aligned by using CLUSTAL X software (Thompson, 1997). The phylogenetic tree of the 16S rDNA sequences was constructed by the neighbor-joining algoritm (Saitou, and Nei, 1987). The root position on the unrooted tree was estimated by using *Bacillus subtilis* DSM 10 as the outgroup strain.

Results and Discussion

Isolation of Lactic Acid Bacteria

Samples of bakasang were used for isolation of lactic acid bacteria. 45 isolates of LAB in which production clear zone around theirs colonies were obtained from *bakasang*. The clear zone appearance is due to the dissolution of $CaCO_3$ on MRS medium by acid agent (Panthavee *et al.,* 2007). Among the 45 isolates were rearranged and confirmed as LAB 27 isolates. All of these isolates were Gram positive, rods and cocci, appeared singly, in pair, chain, tetrad. Cell were non motile and non spore forming and gave negative reaction for catalase. These strains were then classified into genus level using profile matching method. Based on the profile matching method these 27 isolates separated into three groups.

Table 1. Identification of lactic acid bacteria isolates into genera level by profile matching method.

	Group					
Characteristics	I	II	III	*Lactobacillus**	*Pediococcus**	*Leuconostoc**
Number of isolates	5	16	6			
Gram stain	+	+	+	+	+	+
Shape	Rods	Cocci	Cocci	Rods	Cocci	Cocci
Cell arrangement	Single/pair/chain	Tetrad	Pair/chain	Single/pair/chain	Tetrad	Pair/chain
Production gas from glucose	+/-	-	+	+/-	-	+
Catalase	-	-	-	-	-	-
Spore formation	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Fermentation type	Hetero/homo	Homo	Hetero	Hetero/homo	Homo	Hetero

+positive reaction or growth; - no reaction or no growth

Group I were putatively identified as genus *Lactobacillus*. Group II were represented as cocci (tetrad) homofermentative which were identified as genus *Pediococcus*. Finally, group III were identified as genus *Leuconostoc*. It was concluded that lactic acid bacteria isolated from *bakasang* are dominated by *Lactobacillus, Pediococcus*, and *Leuconostoc* (Table 1).

Screening of Lactic Acid Bacteria for Antimicrobial Activity

The antimicrobial activity of LAB isolates (culture) were tested against pathogenic bacteria and spoilage bacteria are summarized in (Table 2) by using agar well diffusion assay. The diameters of the inhibition zones were varied and ranged between 3,0 to 15,0 mm. In general, LAB had inhibitory activity againts pathogenic bacteria and spoilage bacteria. Strain BksC24 had the highest diameter of inhibition zones (15 mm). This revealed that the LAB inhibited all the pathogenic bacteria and spoilage bacteria tested. The activity of LAB culture as inhibitory to bacterial pathogens and spoilage bacteria is supported by the acid and the components of metabolites produced. Acid produced by lactic acid bacteria have the effect of antimicrobial against enteric pathogens (Daeschel, 1989). In addition to producing acid, LAB also produce other compounds inhibiting such as diasetyl, hydrogen peroxide and some strains produce bacteriocins.

According to Schillinger and Lucke (1989) inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 0.5 mm or larger. Similar study was carried out in Morocco by Kalalou (2004) whose studied the activity of LAB on some Gram positive and negative pathogenic bacteria such as *Eschericia coli*, *Pseudomonas aeroginosa, Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus cereus* and the inhibition zones were in the range of 1.4 to 2.8 cm.

Many studies were carried out in Nigeria, using poultry meat to isolate LAB and study its antimicrobial activity against several microorganisms. The results showed that LAB inhibited *Staphylococcus aureus*, *Eschericia coli*, *Pseudomonas aerouginosa* with the exception of *Candida albicans* and *Proteus vulgaris* (Adeskan *et al*., 2008).

Table 2. Average of inhibition zone of LAB culture isolated from Bakasang Sample

No	Isolates	Indicator bacteria (50µl)		
		E.coli (mm)	*S.aureus* (mm)	*P.fluorescens* (mm)
1.	BksC1	11,5	6,0	6,0
2.	BksC2	8,0	9,0	9,0
3.	BksC3	11,0	9,0	13,0
4.	BksC4	5,0	11,0	5,5
5.	BksC5	7,0	8,0	6,5
6.	BksC6	8.5	5,0	9,5
7.	BksC7	7,0	8,0	5,5
8.	BksC8	6,0	7,0	7,0
9.	BksC9	10,0	6,5	12,0
10.	BksC10	7,0	9,0	9,0
11.	BksC11	9,0	7,5	11,5
12.	BksC12	7,0	-	9,0
13.	BksC13	8,0	9,0	9,0
14.	BksC14	6,0	7,0	6,0
15.	BksC15	5,0	6,0	7,0
16.	BksC16	8,0	7,0	8,0
17.	BksC17	7,5	8,0	8,0
18.	BksC18	6,0	6,0	5,0
19.	BksC19	7,5	8,0	6,0
20.	BksC20	7.0	7.0	9,0
21.	BksC21	5,0	7,0	5,0
22.	BksC22	3,0	3,0	3,0
23.	BksC23	7,0	8,0	7,0
24.	**BksC24**	9,0	14,0	**15,0**
25.	BksC25	7,5	11,0	7,0
26.	BksC26	-	-	-
27.	BksC27	9.0	9.0	8,0

[-] negative activity

Generally the antimicrobial components produced by LAB inhibited the growth of Gram positive and Gram negative bacteria (De Vuyst and Vandamme ,1994) and the same was reported by Rahayu *et al* (1996). The activity of inhibition varies by LAB due to a combination of many factors produced, including the production of lactic acid which reduce pH of bakasang and also other inhibitory substances such as bacteriocins which are responsible for the most antimicrobial activity (Ogunbanwo, 2005).

Table 3. Phenotypic characteristics of the antimicrobial-producing LAB from Bakasang Sample

Characteristic	*P.acidilactici* FNCC 0110	*P.pentosaceus* FNCC 0019	BksC24
Gram stain	+	+	+
Shape	Cocci	Cocci	Cocci
Cell arrangement	Tetrad	Tetrad	Tetrad
Production gas from glucose	-	-	-
Catalase	-	-	-
Spore formation	-	-	-
Motility	-	-	-
Fermentation type	Homo	Homo	Homo
Growth on :			
- 10ºC	+	+	+
- 40ºC	+	+	+
- 45ºC	+	+	+
- 50ºC	+	-	+
Growth on :			
- pH 4,5	+	+	+
- pH 8,0	+	+	+
- pH 9,0	+	+	+
Growth on :			
- 6,5% NaCl	+	+	+
- 10% NaCl	+	+	+
- 18% NaCl	-	-	-

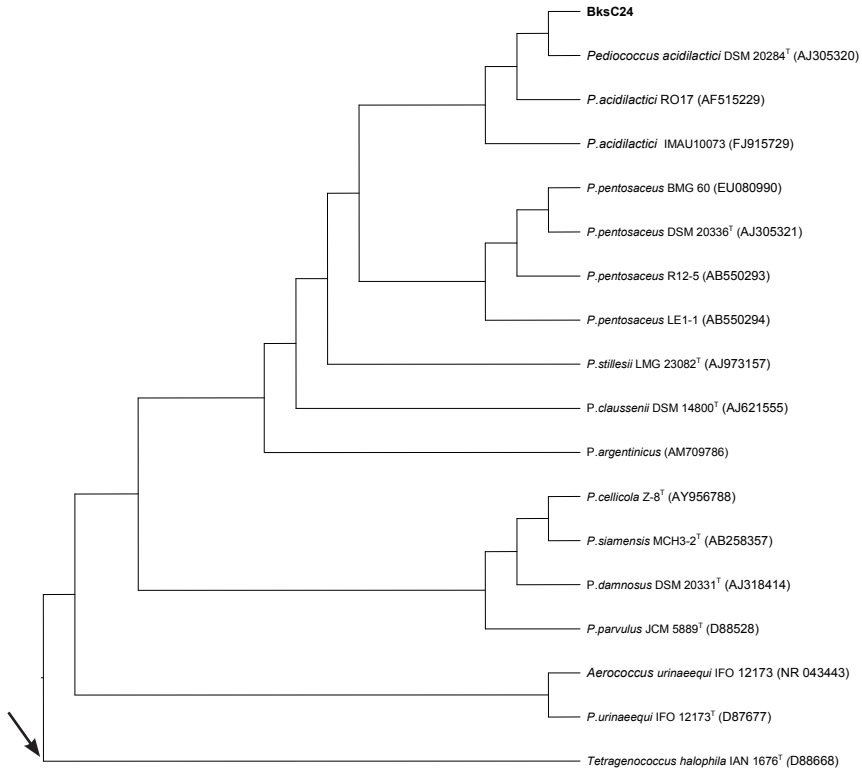

Figure 1. Phylogenetic tree showing the relationship between strain BksC24 and representatives of the genus *Pediococcus* based on 16S rDNA sequences. The scale bar indicates 10 nucleotide substitution per 100 nucleotides in 16S rRNA gene sequences.

Identification of Strain BksC24

Identification of strain BksC24 which has highest antimicrobial activity was done based on phenotypic and genotypic characterization. The phenotypic properties of strain BksC24 is summarized in Table 3. From the morphological examination, BksC24 strain which has highest antimicrobial activity was Gram positive and coccus (tetrad). The isolate was catalase negative, facultative anaerobes, negative to the production of gas from glucose, non endospora forming, and non motil. Based on these characteristics, the isolate was phenotypically identified as member of Genus *Pediococcus* (Holt *et al.,* 1994). Genotypic characterization was carried out to identify strain BksC24 based on 16S rRNA gene sequence comparison (Petti *et al*., 2005). Phylogenetic analysis demonstrated that BksC24 isolate aligned most closely with *Pediococcus acidilactici* DSM 20284 (GeneBank) (Figure 1). The 16S rRNA gene sequencing identified the isolate BksC24 as *Pediococcus acidilactici* (98.79% similarity compared to the type strain of species *Pediococcus acidilactici* DSM 20284 in GenBank).

In conclusion, a new strain, *Pediococcus acidilactici* BksC24 which was isolated from Bakasang had the highest ability to produce antimicrobial compound inhibiting the growth of pathogenic bacteria and spoilage bacteria.

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