Characterization of Lactic Acid Bacteria That Have Ability to Improved Number of Carnosine on Bakasang Based on I6S rRNA Gene

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Abstract—**Diversity of Lactic acid bacteria (LAB) that have ability to improved number of carnosine on bakasang were determind by application of molecular approach. Molecular characterization of LAB was based on 16S rRNA gene sequencing. The result showed that one isolate lactic acid bacteria (Pediococcus sp.B.5.1) among 9 isolates LAB exhibited the highest ability to improved number of carnosine. The molecular characterization based on 16S rRNA gene sequence showed that the Pediococcus B.5.1 isolate clearly belonged to members of species Pediococcus acidilactici. Therefore, lactic acid bacteria with the high ability to improved number of carnosine could be found from fermented fish bakasang and make bakasang as a functional food.**

Index Terms—**LAB; Molecular; Carnosine; Bakasang; 16S rRNA.**

I. Introduction

Lactic acid bacteria (LAB) commonly utilized in the production and preservation of various fermented foods for example *bakasang*, an Indonesian traditional fermented fish sauce. *Bakasang* is fermented fish products traditionally made from the guts of big fish (*Katsuwonus pelamis* L.), small fish and egg fish which is the typical food of North Sulawesi (Manado) [8],[15]. *Bakasang* generally have high in protein and amino acid compounds [8] besides its potential source of wide variety of LAB species because low-pH product with an added carbohydrate source, will support the predominance of LAB [15],[17]. Some of the genera that play an important role in fermentation foods commonly are *Lactobacillus*, *Pediococcus, Enterococcus*, *Leuconostoc*, and *Weissella* [3],[5],[6],[20].

Utilization of fermented foods has great potential in developing functional foods. This is very promising especially foods that contain high protein such as fish. Functional foods that are being developed at this time are foods that contain bioactive peptides that have the body's functional ability to maintain physiological performance and minimize the risk of degenerative diseases such as carnosine [1],[11],[12].

Carnosine is a dipeptide which composed of two amino acids, namely β-alanine-L-histidine which is abundant in fish protein [4]. In this research it was suspected that lactic acid bacteria play a role in increasing the number of carnosine. Carnosine has many benefits for the body's metabolic system [4],[7] including functioning as an antioxidant and having the ability as a buffer capacity that can maintain the acidity of the intracellular environment due to muscle activity that produces lactic acid. Carnosine can also inhibit lipid oxidation and is able to protect cell membranes [4].

Accurate identification of BAL species is very important, especially for security and legal issues namely i.e., labelling and patents. However, routine identification of LAB in most laboratories still depends on traditional techniques. During the past two decades, molecular means especially those relied on rRNA gene sequence have been shown to be a powerful tool for identification and these techniques are not only simple and rapid but have also successfully overcome the limitations of the traditional methods [19]. It is therefore not surprising that a number of articles have been reported on molecular identification and characterization of LAB [2]; [13]–[16], [19], [23]. Isolation and characterization of LAB from *bakasang* is scarce still.

The phenotypic method is not enough to provide clear information in distinguishing intra-and inter-species strains, so molecular methods are needed to obtain more accurate information. Molecular methods are more consistent, fast, reliable, and reproducible and can even distinguish microbes at species level when compared to phenotypic methods, [23]

The methods used for the current study of LAB such as 16S rRNA sequencing, a method that is universally suitable for the LAB with a high resolving power both on the species and intraspecies level. The aimed of the present work was to identify the predominant lactic acid bacteria that have ability to improved number of carnosine in the traditional fermented fish product (bakasang) on the basis of molecular characteristics including 16S rRNA gene sequencing.

II. Methods and Materials

A. *Isolation of Lactic Acid Bacteria*

Guts of Big fish, meat 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 was added and mix thoroughly. The mixture was packed into bottles, corked and then incubated at 37ºC for 7 days. LAB were isolated from sample of bakasang [14]-[16]. Ten gram 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 Syclo-

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hexamide. All plates were incubated at 37°C for 48 hours. 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 [20].

Cell morphology, Gram staining and catalase test, motility, non-spore forming was performed as a preliminary screening for lactic acid bacteria. The selected lactic acid bacteria were maintained as stock cultures at -80 °C in 10% skim milk and 20% glycerol [15].

B. Screening of Lactic Acid Bacteria (LAB) for Ability to Improved Number of Carnosine

The selected LAB isolates were LAB isolates which had the highest ability to increase the number of carnosine during the fermentation process. Carnosine measurements were carried out using a spectrophotometer according to the Parker Method [18]. The first step is to make a standard curve, which is to make a pure stock of carnosine and then make a series of dilutions at λ430 nm. Making the stock of carnosine is weighing 1000 mg of carnosine in 1 L of distilled water (1000 ppm). Extraction of carnosine in the sample is taking 0.3-0,7 g of bakasang solution and then put it into a screw cap after dissolving it with 5 ml of distilled water. The sample heat at 100^0C for 15 minutes, then cool to room temperature. The sample was centrifuge at 3500 rpm for 15 minutes, after that, the supernatant is taking as much as 2 ml, add 1 ml of aquades and 0.8 ml buffer phyrophosphate 0.2 M at pH 9, then add 0.2 ml of FDMB (fluorodynitrobenzene) 63 mM in ethanol 95 %, and then heat at 55^0C for 60 minutes. When heating for the first 30 minutes, 1 ml of 10 M NaOH is added, then heating is continued for up to 60 minutes. After that, add 1 ml of 10 M H2SO4 and 3 ml of toulene : ethyl acetate with a ratio of 4 : 1. After that, centrifuge at 3500 rpm for 15 minutes, and then measure using spectrophotometer at λ430 nm. The final step, enter the data in the equation so the content of carnosine will be obtained.

C. Identification of Isolate Lactic Acid Bacteria

The isolated LAB showing the highest ability to improved number of carnosine were identified based on genotypic characterization. Genotypic characterization was conducted by 16S rDNA sequence comparison.

1) Genomic DNA Extraction

Extraction of genomic DNA were done with using Wizard® Genomic DNA Purification Kit (Promega). The prosedure was add 1 ml of an overnight culture to a 1,5 ml microcentrifuge tube. Centrifuge at 13,000-16,000 x *g* for 2 minutes to the pellet cells. Remove the supernatant. Resuspend the cells thoroughly in 480µl of 50 mM EDTA. Add the appropriate lytic enzyme(s) to the resuspended cells pellet in atotal volume of 120µl, and gently pipet to mix. The purpose of this pretreatment is to weaken the cell wall so that efficient cells lysis can take place. Incubate the sample at 37^0C for 30-60 minutes. Centrifuge for 2 minutes at 13,000-16,000 x *g* at remove the supernatant. Add 600 µl nuclei lysis solution. Gently pipet until the cells are resuspended. Incubate at 80^0C for 5 minutes to lyse the cell; then cool to room temperature. Add 3 µl of Rnase solution to the cells lysate. Invert the tube 2-5 times to mix. Incubate at 37^0C for 15-60 minutes. Cool the sample to room temperature. Add 200 µl of protein precipitation solution to the Rnase-treated cell lysate. Vortex vigorously at high speed for 20 seconds to mix the protein presipitation solution with the lysate. Incubate the sample on ice for 5 minutes. Centrifuge at 13,000-16,000 x *g* for 3 minutes. Transfer the supernatant containing the DNA to a clean 1,5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. Gently mix by inversion until the thread-like strands of DNA form a visible mass. Centrifuge at 13,000- 16,000 x *g* for 2 minutes. Carefully aspirate the ethanol. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10-15 minutes. Add 100 µl of DNA rehydration solution to the tube and rehydrate the DNA by incubating at 65^0C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4^0C. Store the DNA at 2-8^0C. The purify of DNA solution was resuspended with TE buffer and checked spectro-photometrically at λ_{260} and λ_{280} nm.

2) 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'-AGAGTTTAGT CCTGGCTCAG-3') and 1492r (5'-GGTTACCTT GTTACGACTT-3') for 16S rDNA [24]. The condition of amplified gene fragment : pre-denaturation of the target DNA at 96ºC for 4 min followed by 30 cycles at 94ºC for 1 min, primer annealing at 51,5ºC for 1 min and 30 s and primer extension at 68ºC 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 [13]-[15].

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).

3) Phylogenetic analysis

For phylogenetic analysis, sequences 16S rRNA gene of LAB isolates that have ability to improved number of carnosine selected were aligned by using CLUSTAL X software [25]. The phylogenetic tree of the 16S rDNA sequences was constructed by the neighbor-joining algoritm [21]. The root position on the unrooted tree was estimated by using *Bacillus subtilis* DSM 10 as the outgroup strain [14]-[16].

III. Result and Discussion

A total of 15 lactic acid bacteria (LAB) in which production clear zone around their colonies were obtained from *bakasang*. The clear zone appearance is due to the dissolution of $CaCO_3$ on MRS medium by acid agent [17]. Among the 15 isolates were rearrange and confirmed as LAB in amount of 9 isolates. All these isolates were gram positive, rods or cocci, appeared singly, in pair, chain,

tetrad, non-motile, non sporing, and they gave negative reaction for catalase. These strains were classified into genus level using profile matching method. Based on the profile matching method (see Table I) showed that 9 isolates were represented as cocci (tetrad) homofermentative which were identified as genus *Pediococcus*. It was concluded that lactic acid bacteria isolated from *bakasang* are dominated by *Pediococcus*.

Table II shows the number of carnosine during the fermentation process inoculated with LAB *(Pediococcus* genera). Three (3) isolates of LAB were able to improve the number of carnosine compared to controls (without inoculation of lactic acid bacteria).

TABLE I: IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATES INTO GENERA LEVEL BY PROFILE MATCHING METHOD

Characteristic	*Lactobacillus*	*Pediococcus*	*Leuconostoc*	*Enterococcus/*	*Streptococcus*	I[a]
Number of Isolates						9
Cell shape Rod	+	-	-	-	-	-
Cell shape Cocci	-	+	+	+	+	+
Cell arrangement tetrad	-	+	-	-	-	+
Cell arrangement pair/chain	+	-	+	+	+	-
Gram stain	+	+	+	+	+	+
Spore Formation	-	-	-	-	-	-
Catalace	-	-	-	-	-	-
Gas Production	+/-	-	+	-	-	-
Motility	-	-	-	-	-	-
Homofermentatif	+/-	+	-	+	+	+

TABLE II: INCRETION NUMBER OF CARNOSINE DURING THE FERMENTATION PROCESS BAKASANG

	Isolates	Carnosine (mg/100gr) Increation
1.	*Pediococcus* sp. B5.1	9,16 x
2.	*Pediococcus* sp. B1.0	8,92 x
3.	*Pediococcus* sp. B4.3	8,73 x
4.	Control	Constan

Control = without inoculation of LAB

The data on Table II, shows that *Pediococcus* sp.B5.1 isolate has a highest ability to increase the number of carnosine. An increase in the number of carnosine in LAB isolates (*Pediococcus* sp.B5.1.), showed that LAB isolate had proteolytic abilities. This was supported by research conducted by [26], [27] which combined the fermentation of Mackarel with protease enzymes from *Aspergillus oryzae* BRCC 30118 and type of S and L of *Pediococcus pentosaceus*. The number of carnosine increased by 25x, whereas for fermentation using the S type of Pediococcus pentosaceous there was an increase in carnosine by 17x and an increase of carnosine by 18x in Oyster fermentation [9]. Several studies have been conducted to prove that bioactive peptides have health effects.

Characterization and Identification of LAB Based on 16S rDNA gene Sequencing

Molecular characterization and identification were carried out based on 16S rRNA gene sequence analysis. 16S rRNA gene of LAB isolates was amplified using universal primers. Furthermore, the 16S rRNA gene sequences of LAB isolate was analyzed along with 16S rRNA gene sequences of reference strains belonging to the genus Pediococcus to find familial relationships among them. This familial relationship is visualized in the form of a phylogeny tree.

The phylogeny tree shown in Fig. 1 shows that *Pediococcus* sp.B5.1 isolate obtained during the fermentation process, had the closest relationship with *P. acidilactici* DSM 20284^T compared to the type of strain of other *Pediococcus* species included in *Pediococcus* genus group. This shows that isolate *Pediococcus* B5.1 has close familial relationship with *P. acidilactici* DSM 20284^T species compared to other species in *Pediococcus* genus.

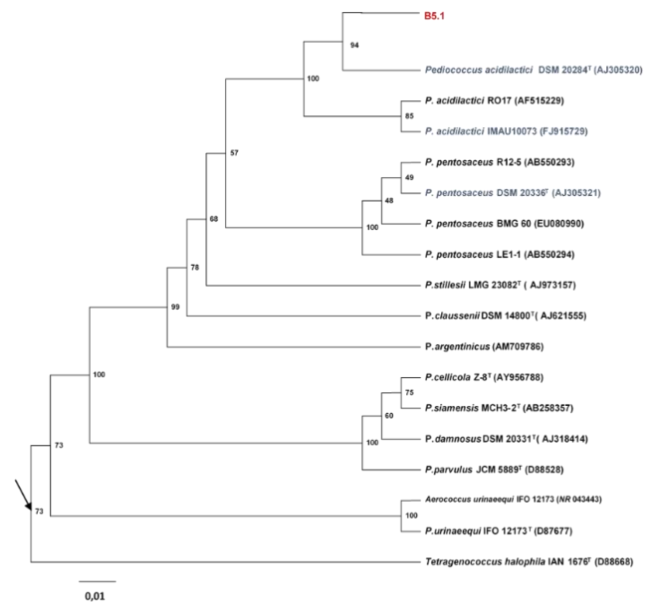

Fig. 1. Neighbor-joining tree (21) showing relationship between representative isolates lactic acid bacteria that have ability to improved number of carnosine and the reference strain representing the *Pediococcus* genus based on 16S rRNA sequences.

Familial relationship of *Pediococcus* B5.1 isolate with reference strains belonging to the *Pediococcus* genus obtained from the phylogeny tree was supported by similarity index and different number of nucleotides between 16S rRNA gene sequences compared. The similarity index and different number of nucleotides that differ between 16S rRNA gene sequences of bacterial strains incorporated in the *Pediococcus* genus group are shown in Table III.

Pediococcus B5.1 isolates had the highest nucleotide similarity with *P. acidilactici* DSM 20284T which was 99.18% compared to *P. pentosaceus* DSM 20336^T (97.6%). Thus, based on 16S rRNA gene sequence analysis, *Pediococcus* B5.1 isolates isolated during the fermentation process had high nucleotide similarities and close filogenetic relationship with *P. acidilactici* DSM 20284^T species.

TABLE III: SIMILARITY INDEX (%) AND NUMBER OF DIFFERENT NUCLEITIDES IN SEQUENCE OF 16S RRNA BETWEEN PEDIOCOCCUS B5.1 ISOLATE AND TYPE STRAIN OF PEDIOCOCCUS ACIDILACTICI DSM 20284^T DAN P. PENTOSAEUS DSM 20336^T

Strain Code	*Pediococcus* B5.1	*Pediococcus acidilactici* DSM 20284 ^{T}	*P.pentosaceus* DSM 20336 ^{T}
Pediococcus B5.1	---	12/1460	35/1460
Pediococcus acidilactici DSM 20284^T	99.18	---	33/1566
P.pentosaceus DSM 20336^T	97.6	97.89	---

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Analysis of the 16S rRNA gene sequence is a standard procedure for determining phylogenetic) relationships between bacterial strains. Identification of bacterial strains based on 16S rRNA gene sequence analysis is more accurate, objective and even possible to find out in one species the differences between strains [19],[22]. That bacterial strains were identified as one species when the sequence similarity value of the 16S rRNA gene with type strain was at least 99% [10].

Table III shows that *Pediococcus* B5.1 isolate with *P. acidilactici* DSM 20284^T had 12 nucleotides different of 1460 nucleotides compared. This shows that the number of different nucleotides from strains *Pediococcus* B5.1 is still below or less than (<) the number of nucleotides which is between the type of *P. acidilactici* DSM 20284^T strain and Type of *P. pentosaceus* DSM 20336^T strain so that *Pediococus* B5.1 isolate is probably a novel strain of a member of the *P. acidilactici* species. The identification results were supported by the results of identification based on phenotypic characters which showed that *Pediococcus* B5.1 was similar to *P. acidilactici* DSM 20284^T.

IV. Conclusion

Isolate of LAB *Pediococcus* B5.1 has ability to improve number of carnosine as much as 9,16x during fermentation process of bakasang. Characterization based on 16s rRNA gene show that *Pediococcus* B5.1 isolate as a species within the genus *Pediococcus* and as a strain belonged to members of species *P. acidilactici.* Isolate of LAB *Pediococcus* B5.1 has potential to be applied to traditional fermented foods which might be expected to make a traditional fermented food into health food or functional food.

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