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Full Paper

Isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: A traditional fermented seafood product in Indonesia

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Lactic acid bacteria from "terasi" shrimp paste, a highly popular fermented seafood in Indonesia were isolated and characterized. Viable cell counts were 10^4 to 10^6 cfu/g on MRS medium. All the isolates were catalase-negative, gram-positive cocci and were able to grow at 15% NaCl. Numerical phenotypic analysis showed that the isolates clustered into one group. However, they could be classified into two types: the *Tetragenococcus halophilus* group and the *T. muriaticus* group as revealed by a restriction fragment length polymorphism (RFLP) analysis and sequencing of the 16S rRNA gene. This study is the first to show that both species of *Tetragenococcus* are distributed in Indonesian fermented foods.

Key Words——lactic acid bacteria; "terasi" shrimp paste; *Tetragenococcus*

Introduction

In Indonesia, there are many types of traditional fermented food made of various agricultural products such as rice, soybean and cassava (Aryanta et al., 2000). Moreover, many marine products are also processed as various traditional fermented seafood products, among which "terasi" shrimp paste is highly popular. "Terasi" shrimp paste is either dark brown, gray or red, and has a distinct taste and strong aroma. It is consumed widely by the Indonesian people as a spice in various dishes. The method of manufacturing "terasi" shrimp paste may differ depending on the factory. For example, small salted shrimps mixed with salt are dried under the sun for 2 days, then ground into fine paste and allowed to ferment for 2 days. After this, the paste is ground again and processed into balls, which are then fermented for several weeks, then cut into small pieces of various sizes.

Previously, several microbiological investigations of "terasi" shrimp paste have been reported. Surano and Hosono (1994a, b) described the microbiological analysis of "terasi" shrimp paste. They reported a considerable number of strains of bacilli; pseudomonas, micrococci, kurthia and sporolactobacilli were isolated from this product. However, the strains were only isolated under aerobic conditions and identified at the genus level by phenotypic characterization. Lactic acid bacteria are one of the most important and commonly used bacteria in the manufacture of traditional fermented food products; in the manufacture of "terasi" shrimp paste, pH decreases temporally to 4.5 due to lactic acid production (Jennie and Muchtadi, 1978).

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Despite these studies, information on the lactic acid bacteria in this product is still limited. In the present study, we isolated the lactic acid bacteria from "terasi" shrimp paste, and characterized them phenotypically and genotypically.

Materials and Methods

Sample. Three samples (samples A, B, C) of "terasi" shrimp paste were obtained from a local home-based factory in Cirebon City, West Java in June 2001. They were transported to Japan by air and used for isolation of lactic acid bacteria.

Isolation of lactic acid bacteria. Viable bacterial cells in "terasi" shrimp paste were enumerated by the ordinary counting method. MRS agar medium (Merck, Darmstadt, Germany) was used for enumeration of lactic acid bacteria. After incubation at 30°C for 1 week under anaerobic conditions using an AnaeroPack system (Mitsubishi Gas Chemical Co., Tokyo, Japan), the colonies that appeared on the plates were counted. The same agar medium supplemented with 10% NaCl was also used. Colonies were streaked on the same medium and individual colonies were picked up for further studies. Twenty strains and 18 strains were isolated from sample A using MRS agar medium and MRS agar medium supplemented with 10% NaCl, respectively. Ten strains each were also isolated from sample B using MRS agar medium and MRS agar medium supplemented with 10% NaCl, respectively. Moreover, from sample C ten strains each were isolated using the above two media, respectively. All isolates were stab-cultured into MRS medium supplemented with 0.5% $CaCO_3$ and preserved at 5°C.

Phenotypic characteristics. The general characteristics of all the isolates from sample A were observed as described previously (Kobayashi et al., 2000). Gram stain, cell form, and motility were observed by light microscopy. Nitrate reduction was tested by the method of Davis (1955). To measure growth at different salt concentrations and pH values, MRS broth (De Man et al., 1960) was used. The carbohydrate fermentation test was performed using a broth medium supplemented with various substrates at a final concentration of 1% (Uchida, 1982). The salt tolerance tests were carried out with MRS broth medium containing 0%, 4%, 15%, and 18% NaCl. The range of growth temperature was assayed at 35°C, 40°C, and 45°C in MRS broth, respectively. Lactic acid produced in the broth medium was analyzed using enzymatic kits from Boehringer Mannheim (Mannheim, Germany) to determine its optical rotation. Histamine concentration in the broth cultures was also determined using HPLC equipped with a fluorescence detector (Yamanaka and Matsumoto, 1989).

Numerical analysis. Traits were coded 1 for positive and 0 for negative. The matrix contained 38 isolates from sample A. For comparison, the phenotypic profiles of 14 *Tetragenococcus* strains (8 strains isolated from Japanese fermented pufferfish, 4 strains isolated from Japanese soy sauce, and 2 strains isolated from Japanese squid liver sauce) were cited from a previous report (Kobayashi et al., 2000). A computer cluster analysis of 34 profiles was carried out by agglomerative hierarchical clustering and a phenogram was constructed by the UPGMA method using the S-Plus computer program.

16S rRNA sequencing and restriction fragment length polymorphism (RFLP) analysis of isolates. Deoxyribonuleic acids (DNAs) were extracted and purified by the method of Marmur (1961). Sequencing of 16S rDNA was carried out using an Applied Biosystems commercial PCR Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The reaction mixture was analyzed with an Applied Biosystems 310 DNA sequencer as described previously (Kobayashi et al., 2000). Sequence data were compiled from overlapping sequence data using the GENETYX computer program. For RFLP analysis, a large fragment of the 16S rRNA gene was also amplified from chromosomal DNA by polymerase chain reaction (PCR). The products were digested with the restriction enzymes *Afa*I and *Mbo*I, and electrophoresed in 2% agarose gels according to a previous report (Kobayashi et al., 2000).

Results

The bacterial counts of "terasi" shrimp paste on various media are shown in Table 1. Viable cell counts were 10^4 to 10^6 cfu/g on both MRS agar medium and 10% NaCl-MRS agar medium. Similar bacterial counts were obtained in TSA agar medium and 10% NaCl-TSA agar medium, which were slightly higher than that obtained in MRS medium.

The phenotypic characteristics of the isolates from sample A are shown in Table 2. Not all the isolates could be classified up to the genus level based on only

Table 1. Viable cell counts of bacteria of "terasi" shrimp paste (cfu/g).

Medium	NaCl conc. (%)	Sample		
		A	B	C
MRS	0	1.8×10^6	1.0×10^5	2.1×10^5
	10	9.7×10^5	1.4×10^5	1.2×10^4
TSA	0	7.0×10^6	3.9×10^5	1.1×10^5
	10	2.2×10^6	3.0×10^5	8.8×10^4

general phenotypic characteristics. They were homofermentative, gram-positive, catalase-negative cocci that were able to grow at pH 8.5 but not at pH 4.2. No isolates produced ammonia from arginine. Nitrate reductions were not observed. They could grow even in 18% NaCl but not at 45°C. These results indicate that all the isolates should be classified as halophilic lactic acid bacteria, genus *Tetragenococcus* by the identification protocols for lactic acid bacteria (Kobayashi et al., 2000; Kozaki et al., 1992; Nakagawa and Kitahara, 1959).

The species of the genus *Tetragenococcus* are widely distributed in various fermented foods containing high salt. To date, two species, namely, *T. halophilus* and *T. muriaticus*, have been identified by phenotypic and genotypic typing techniques. In this study, in order to compare our isolates with these two *Tetragenococcus* species, we used the phenotypic characteristics data cited from a previous report (Kobayashi et al., 2000).

As shown in Table 2, they were classified into 16 groups (A to P) on the basis of the fermentation patterns. All the isolates produced acid from D-glucose, D-ribose, maltose, sucrose, glycerol, arbutin, and α-methylglucoside but not rhamnose, dextrin, or starch. There was a variety in the ability to ferment the other 9 carbohydrates. The isolates except for one strain (group C or J) fermented maltotriose and trehalose. Twenty-eight strains fermented D-melezitose (64% of the total number of isolates), 18 strains (46% of the total number of isolates) did D-mannitol and 12 strains (31% of the total number of isolates) did L-arabinose. Five strains produced acid from D-xylose, and 4 strains did lactose and D-sorbitol. The most predominant group, A (7 strains, 18% of the total number of isolates), and the fourth predominant group, D (4 strains, 11% of the total number of isolates), were identified as *T. halophilus* except for only one trait, namely, L-arabinose or D-mannitol fermentation ability. The physiological properties of the second predominant group, B (6 strains, 16% of the total number of isolates), was also identified as *T. halophilus* except for L-arabinose and D-mannitol fermentation ability. The phenotypic characteristic of the third predominant group, G (5 strains, 13% of the total number of isolates), was identified as that of *T. halophilus* except for L-arabinose and D-melezitose fermentation ability.

The dendrogram obtained from numerical analysis by the UPGMA method is shown in Fig. 1. These isolates were divided into two groups (I and II). The dendrogram placed the two groups at 80% similarity level. Each strain of group I included four strains of *T. halophilus* from the IAM culture collection and three strains of *T. halophilus* isolated from Japanese puffer fish ovaries in a previous study (Kobayashi et al., 2000). All the 38 strains isolated from "terasi" shrimp were included in group I. Group II was designated as the *T. muriaticus* group. This cluster consists of two strains of *T. muriaticus* from the JCM culture collection and three strains of *T. muriaticus* obtained in a previous study (Kobayashi et al., 2000).

The digestions of the 16S rRNA gene with the restriction enzymes *Afa*I and *Mbo*I yielded several fragments. Thirty-eight strains exhibited the same digestion pattern, while one exhibited a different one. The typical digestion patterns obtained from the RFLP analysis of the 16S rRNA gene are shown in Fig. 2. The fragment pattern of 38 strains coincided with that of type strain of *T. halophilus*. The pattern obtained from one strain (a9) of group J coincided with that of type strain of *T. muriaticus*. In Fig. 2, the results of two representative isolates are shown. Strains belonging to 15 groups, i.e, A to P except for J, shown in Table 1 were identified as *T. halophilus*. A strain (a9) of group J, which was the most outer strain of the *T. halophilus* group in the dendrogram, was identified as *T. muriaticus*. The results of the RFLP analysis presented here indicate that tetragenococci in "terasi" shrimp paste could be genotypically classified into two groups. The 37 predominant strains were identified as *T. halophilus* and the one minor strain as *T. muriaticus*.

The phylogenetic position regarding these isolates was determined by sequence analysis of the 16S rRNA gene (1,376 bp). The sequence of strain a9 (group J) exhibited a high level of similarity (99.7%) with that of *T. muriaticus* JCM 10006^T (database ac-

Table 2. Characteristics of strains isolated from "terasi" shrimp paste.

Characteristics	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	*T. halophilus* IAM 1676^T	*T. muriaticus* JCM 10006^T
Shape	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore forming	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NO_3^- to NO_2^-	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NH_3 from arginine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gas from D-glucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:																		
L-Arabinose	−	−	−	+	−	+	−	−	−	−	+	+	+	+	+	+	+	−
D-Xylose	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	−	−
Lactose	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−
D-Melezitose	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	+	+	−
Trehalose	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
Maltotriose	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
D-Mannitol	−	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	+
D-Sorbitol	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−
Growth at:																		
35°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45°C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pH 4.2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pH 7.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl 0%	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
NaCl 4%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl 18%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate formed	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
No. of isolates from 0% NaCl-MRS medium	5	3	0	2	1	1	5	0	1	0	0	0	0	0	1	1		
No. of isolates from 10% NaCl-MRS medium	2	3	1	2	1	0	0	3	0	1	2	1	1	1	0	0		
Total isolates	7	6	1	4	2	1	5	3	1	1	2	1	1	1	1	1		

+, positive; −, negative; C, coccus.

All the strains were able to ferment D-glucose, D-ribose, maltose, sucrose, glycerol, arbutin and α-methylglucoside, but not able to ferment L-rhamnose, dextrin or starch.