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Characterization of Biogenic Amine-Producing Microorganisms Isolated from Myeolchi-Jeot, Korean Salted and Fermented Anchovy

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Abstract The changes of physicochemical and microbiological states of Myeolchi-jeot, a Korean salted and fermented anchovy, were investigated during 20 days of storage at 4°C, 15°C, and 30°C. A total of 314 bacterial strains isolated from Myeolchi-jeot samples at different time intervals were identified, and their abilities to produce biogenic amines were determined by both decarboxylating agar media and HPLC analysis. The salinity and water activity of Myeolchi-jeot changed little, while the pH increased slightly over 20 days at the tested temperatures. A significant increase of total plate count was observed in the sample stored at 30°C. *Staphylococcus* spp. were dominant in Myeolchi-jeot, and its amine productivity was very weak. *Bacillus* spp. appeared increasingly with the progress of storage at all temperatures tested, and the increase was considerably dependent on the increase of storage temperature. Also, 58–73%, 83–90%, 81–90%, and 83–93% of *Bacillus* strains had the ability to produce histamine, tyramine, putrescine, and cadaverine, respectively. Therefore, the main amine producer in Myeolchi-jeot stored for a long period seems to be the genus *Bacillus*, especially *B. licheniformis*.

Key words: Myeolchi-jeot, Korean salted and fermented anchovy, storage, biogenic amines, HPLC, *Staphylococcus*, *Bacillus*

Biogenic amines, including tyramine, histamine, putrescine, cadaverine, spermine, and spermidine, are produced as a result of microbial decarboxylation of dietary amino acids in animals, plants, and microorganisms [8, 11], and are reported to be toxic to humans. Common toxic symptoms by biogenic amines in humans are nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, a bright red rash, oral burning, and hypertension as well as hypotension [25]. The frequently observed foodborne intoxications among biogenic amines are caused by histamine [11]. A hazard level of histamine for human health has been suggested as 500 mg/kg [4]. On the other hand, 100–800 mg/kg of tyramine and 30 mg/kg of phenylethylamine in foods are also toxic [8]. Putrescine and cadaverine inhibit the activities of intestinal diamine oxidase and histamine-*N*-methyltransferase that metabolize histamine, so they can also increase histamine toxicity [31]. Biogenic polyamines have also been recognized as precursors for carcinogenic nitroso compounds [30]. Like polyamines related to tumorigenesis in human [29], putrescine [7, 35], cadaverine [35], spermidine [7, 30], spermine, and agmatine [30] are reported to be potentially carcinogenic by converting to nitrosamine. Tyramine has been identified as a major mutagenic precursor [24]. Most biogenic amines are pharmacologically active, but oral administration generally does not provoke adverse reactions, because amine oxidases in the intestine rapidly detoxify these compounds [3]. However, food intoxication may occur [14] if the amine-metabolizing capacity of the human body is saturated due to ingesting high doses and/or the metabolic activity is impaired by specific inhibitors [33].

Jeotkals are Korean traditional salted and fermented fish products, and are popularly taken not only as side dishes, but also as ingredients of kimchi in Korea. For preparing Jeotkal, salt should be added at 5–20% to raw fish containing most fishery wastes, and then fermented for a long period to develop taste. The fermentation period varies depending on the salt concentration and fermentation temperature; 2 months for most Jeotkals with low salt levels (6–18%), and a few years for Myeolchi-jeot with high salt (over 20%) [16]. Thus, they contain relatively high concentrations of amino acids degraded from fish protein, the source for

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biogenic amine formation. Amine formation during the ripening of salted fish such as sardines [18], salted anchovies [12], and Jeotkals [20] has been reported in different countries. Therefore, the biogenic amine intoxication resulting from Jeotkal intake may be of more local interest to Korean specialities. The presence of histidine decarboxylase activity has been described in different microbial groups, such as pseudomonads [18], vibrios [23], sporulated microorganisms [25, 32], and lactic acid bacteria [14, 21]. The microflora in Jeotkals include *Achromobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Halobacterium*, *Leuconostoc*, *Micrococcus*, *Pediococcus*, *Pseudomonas*, *Staphylococcus*, *Sarcina*, *Saccharomyces*, and *Torulopsis* [22, 34]. Nevertheless, no information is available on the biogenic amine-producing microorganisms and their growth in Jeotkals.

The main objective of this study was to investigate the role of microbial flora for the biogenic amine formation in Myeolchi-jeot. Therefore, physicochemical and microbial analyses in Myeolchi-jeot during storage were carried out. Bacteria isolated from Myeolchi-jeot were also identified and their ability to produce biogenic amines was determine.

MATERIALS AND METHODS

Materials and Sampling

Myeolchi-jeot, Korean salted and fermented anchovy, was purchased at a local store in Seoul, in November 2000, and transported in ice to the laboratory. The sample was stored at three different temperatures, 4°C, 15°C, and 30°C. Then, the physicochemical and microbial states of samples were analyzed every 5 days during 20 days of storage.

Physicochemical and Microbial Measurements

Several physicochemical properties of Myeolchi-jeot were measured. pH was measured with a pH meter (pH meter 340, Corning Inc., NY, U.S.A.), the salinity by the AOAC method [2], and water activity by a thermoconstanter (Novasina, Switzerland). Total plate count was made by plate count agar (PCA, Difco Laboratories, Detroit, U.S.A.) with 3% NaCl added. The number of colonies that appeared after 24 h at 30°C and 37°C were counted.

Test Microorganisms

Three-hundred-fourteen bacterial strains were isolated from Myeolchi-jeot samples stored at 4°C, 15°C, and 30°C. Stock cultures were maintained on nutrient (Difco) agar slants at 4°C.

Detection of Potential Biogenic Amine Producer

Biogenic amine formation was tested by inoculating each strain on two decarboxylating agar media as described by Niven *et al.* [23] and Mah *et al.* [19]. Each medium was supplemented with 0.5% (w/v) different amino acids (L-histidine hydrochloride monohydrate, L-tyrosine, L-ornithine hydrochloride, or L-lysine hydrochloride) (all from Sigma Chemical Co., St. Louis, MO, U.S.A.).

Colonies were picked, seeded directly onto each media, and incubated at 30°C for 24/48 h, and the plates were then observed. The presence of red colonies surrounded by a purple or deep red halo on a yellowish background indicated a positive reaction.

Biogenic Amine-Producing Activity of Individual Producer

All strains were investigated for their abilities to produce biogenic amines. Strains were seeded in tryptic soy agar (TSA, Difco) plates enriched with 0.1% L-histidine hydrochloride, L-tyrosine, L-ornithine hydrochloride, and L-lysine hydrochloride, and incubated at 30°C for 24 h. A loopful from the culture was then inoculated in 9 ml of tryptic soy broth (TSB, Difco) with 0.5% L-histidine, L-tyrosine, L-ornithine hydrochloride, and L-lysine hydrochloride (pH 5.8) supplemented with 0.0005% pyridoxal-HCl (Sigma). One-milliliter aliquots of these cultures were transferred to new tubes containing 9 ml of the same broth and the inoculated broths were incubated. Five milliliters of these broth cultures were taken with a sterile syringe, filtered through a 0.2 μm membrane (Millipore Co., Bedford, MA, U.S.A.), and kept at -25°C until assayed by HPLC.

Determination of Biogenic Amine by HPLC

Biogenic amines in the filtered broth cultures were determined by HPLC according to the procedure developed by Eerola *et al.* [10] and modified by Ben-Gigirey *et al.* [5, 6].

Preparation of Amine Standard Solutions. Amine standard solutions were prepared as follows. Stock solutions of histamine, tyramine, putrescine, and cadaverine (all from Sigma) were separately prepared in Milli-Q water at a concentration of 10,000 ppm. Working solutions of amines at the concentrations of 100 or 1,000 ppm were prepared by diluting 100 μl or 1,000 μl of each stock solution in Milli-Q water to bring to a final volume of 10 ml.

Preparation of Culture Extracts. Biogenic amine extracts were prepared by adding 9 ml of 0.4 M perchloric acid (Merck KGaA, Darmstadt, Germany) to 1 ml of the filtered broth culture, and the mixture was homogenized by a vortex mixer. After centrifugation of the homogenate at 3,000 ×g for 10 min, the supernatant was filtered through Whatman paper No. 1.

Derivatization of Extracts and Standards. One milliliter of biogenic amines extract was mixed with 200 μl of 2 N sodium hydroxide and 300 μl of saturated sodium bicarbonate. Two milliliters of a dansyl chloride (Sigma) solution (10 mg/ml) prepared in acetone were added to the mixture, which was then incubated at 40°C for 45 min. Residual dansyl chloride was removed by adding 100 μl of 25% ammonium

hydroxide. After 30 min incubation at room temperature, the volume of extracts was adjusted with acetonitrile to 5 ml. Finally, the mixture was centrifuged at 2,500 ×g for 5 min, and the supernatant was filtered through 0.2 μm-pore-size filters (Millipore).

Chromatographic Separations. An HPLC unit (Waters 2690) equipped with a Waters 996 photodiode array detector and Millennium 2010 software was employed. A Nova-Pak C_{18}, 4 μm, 150×3.9 mm column (Waters) was used, with ammonium acetate (0.1 M; Merck; solvent A) and acetonitrile (Merck; solvent B) as the mobile phases at the flow rate of 1 ml/min. The program was set for a linear gradient starting from 50% of solvent B to reach 90% of the solvent at 19 min. The sample volume injected was 20 μl and the sample was monitored at 254 nm.

Identification of Strains

Morphological, physiological, and cultural properties were examined to identify the strain with biogenic amine-producing activity. These tests, including cell morphology, Gram stain, spore formation, motility, biochemical test, growth of isolate at different temperatures, pH, and concentrations of NaCl, were performed according to Bergey's Manual [9, 13].

Fatty acid methyl ester was prepared and extracted according to the standard protocol of the Microbial Identification System (Microbial ID, Inc., Newark, DE, U.S.A.). The extract was analyzed by a Hewlett-Packard model HP 6890 gas chromatograph equipped with a 25 m ×0.2 mm methyl phenyl silicone fused silica capillary column (HP 19091B-102).

RESULTS

Physicochemical and Microbial Changes of Myeolchi-jeot

As shown in Fig. 1(a-c), physicochemical properties of Myeolchi-jeot showed little change after 20 days of storage at 4°C, 15°C, and 30°C. The pH of Myeolchi-jeot samples was 6.14 at the initial phase and gradually increased up to its maximum pH (approximately, pH 6.25) after 10 days of storage. The water activities of the samples showing little change were determined at the range of 0.71 to 0.73. Myeolchi-jeot samples showed high salt concentrations, ranging from approximately 18 to 20%.

Microbiological change was analyzed to evaluate the effect of the growth of the microflora in Myeolchi-jeot stored at 4°C, 15°C, and 30°C, as shown in Fig. 1(d). At the initial period, the total plate count of Myeolchi-jeot sample was detected in 2.03×10^4 cfu/g. The total plate counts decreased slightly to 1.17×10^4 and 1.16×10^4 cfu/g during storage at 4°C and 15°C, respectively. The sample stored at 30°C, however, showed the total plate count increased to 3.61×10^4 cfu/g.

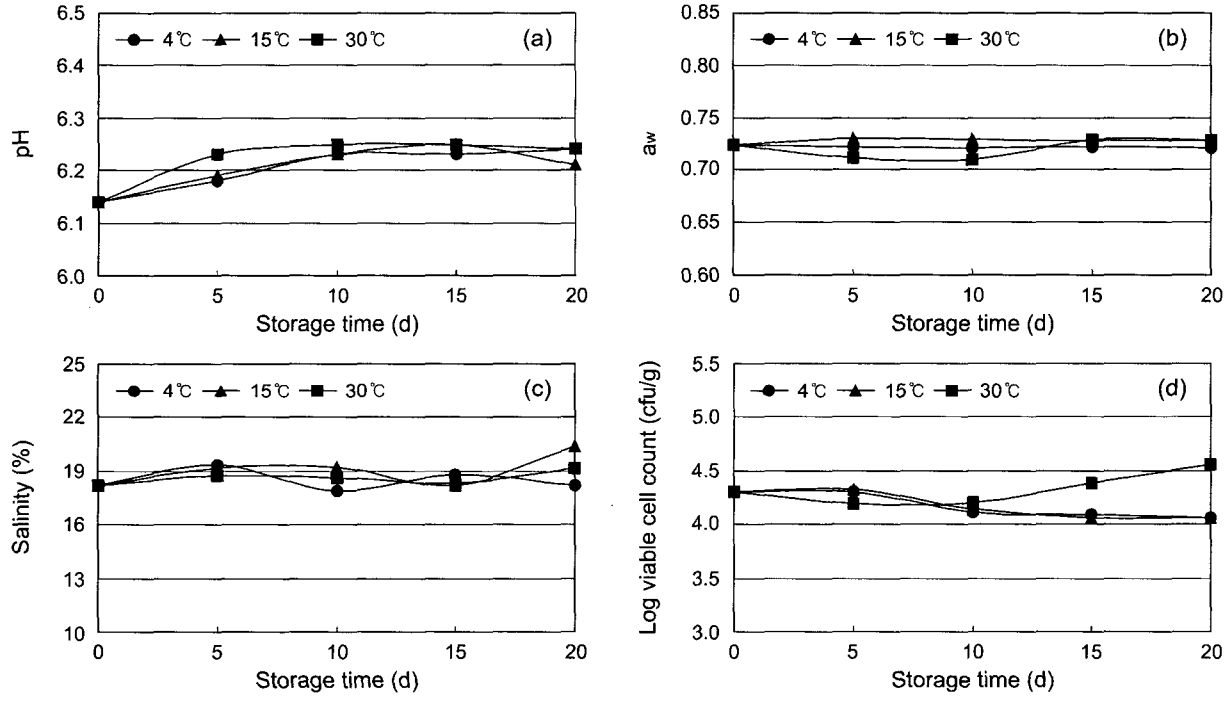

Fig. 1. Physicochemical and microbial changes in Myeolchi-jeot, a Korean salted and fermented anchovy, stored at 4 °C, 15°C, and 30°C.

(a) pH, (b) a_w, (c) salinity, (d) total plate count.

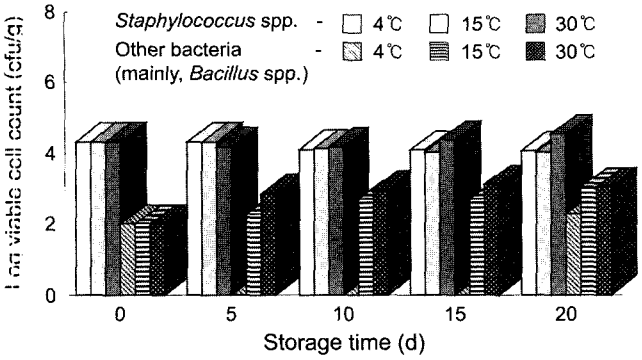

Fig. 2. Microfloral changes in Myeolchi-jeot, a Korean salted and fermented anchovy, stored at 4°C, 15°C, and 30°C.

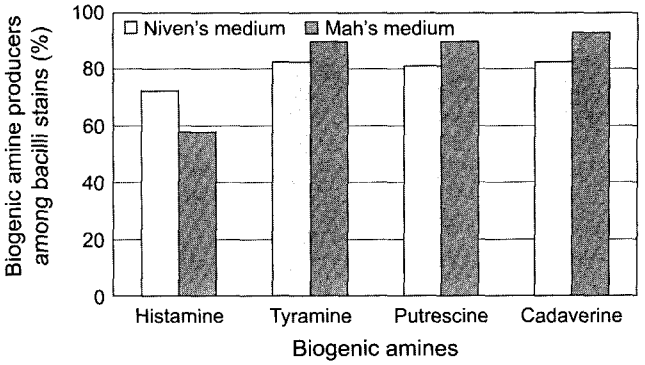

Fig. 3. Occurrence of biogenic amine-producing bacilli strains in Myeolchi-jeot, a Korean salted and fermented anchovy, stored at 4°C, 15°C, and 30°C.

A total of 314 bacterial strains were isolated from Myeolchi-jeot samples. The dominant, Gram-positive and oxidase-negative cocci with yellowish pigment, were characterized as the genus *Staphylococcus*, according to their morphological, physiological, and cultural properties. As shown in Fig. 2, the appearance of nonstaphylococcal strains increased depending on the increment of time and temperature of storage. Most of the strains, ranging from 10^2 to 10^3 cfu/g, were endospore-forming, Gram-positive, catalase-positive, and motile rods, indicating that these strains are the genus *Bacillus*.

Detection of Biogenic Amine-Producing Bacteria in Myeolchi-jeot by Decarboxylating Agar Media

The amine formation abilities of the bacterial strains, isolated from the Myeolchi-jeot samples were tested by two decarboxylating agar media [19, 23]. For the staphylococcal strains, amine productivity and growth on the decarboxylating agar media were very weak (the production ability of each amine determined by HPLC were below 4-6 μg/ml). On the other hand, most of the *Bacillus* strains grown on the media were strong producers of biogenic amines, although they were not dominant in Myeolchi-jeot (Table 1). As shown in Fig. 3, 58-73%, 83-90%, 81-90%, and 83-93% of the strains characterized as *Bacillus* spp. showed the ability to produce histamine, tyramine, putrescine, and cadaverine, respectively. Therefore, the increments of biogenic amines in Myeolchi-jeot storage were most likely due to biogenic amine-producing *Bacillus* strains rather than staphylococcal strains.

Determination of Biogenic Amine-Producing Activity of Individual Producer

To confirm the correlation between biogenic amine contents and the microbial flora, 10 strains of strong biogenic amines producers and 7 strains of weak producers were selected from the Myeolchi-jeot isolates, based on the amine productivities detected by the decarboxylating agar media. Isolates No. 0037, 1043, 1056, 1059, 1060, 1542, 1543, 1544, 1553, and 2036 were classified as strong producers, and isolates No. 0042, 0538, 2028, 0038, 0549, 1039, and 1051 were weak producers. Their abilities to produce biogenic amines were tested by HPLC, as shown in Table 2. The classification based on the amine produced on the decarboxylating agar media corresponded to the amine contents determined by HPLC.

Each amine-producing group with strong or weak productivity confirmed by HPLC analysis was identified according to Bergey's Manual, which is supported by the

Table 1. Biogenic amine productivity of *Bacillus* strains isolated from Myeolchi-jeot samples stored at 4°C, 15°C, and 30°C.

No. of isolate	Decarboxylating agar media							
	Niven's medium				Mah's medium			
	Histamine	Tyramine	Putrescine	Cadaverine	Histamine	Tyramine	Putrescine	Cadaverine
Positive (percent)	50 (72.5)	57 (82.6)	56 (81.2)	57 (82.6)	40 (58.0)	62 (89.9)	62 (89.9)	64 (92.8)
Negative (percent)	19 (27.5)	12 (17.4)	13 (18.8)	12 (17.4)	29 (42.0)	7 (10.1)	7 (10.1)	5 (7.2)

Niven's medium consists of 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $CaCO_3$, 0.006% bromocresol purple, 2% agar, and 2.7% of corresponding amino acid (only histidine·2HCl was established in original article), adjusted to pH 5.3, to make the amine producer a purple color (colony with strong intensity of positive reaction is surrounded by a purple halo on a yellowish background).

Mah's medium consist of 0.125% tryptone, 0.125% yeast extract, 0.75% $(NH_4)_2SO_4$, 0.5% NaCl, 0.1% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$, 0.004% $FeSO_4 \cdot 7H_2O$, 0.05% Tween 80, 0.02% cresol red, 3% agar, and 2% of corresponding amino acid, adjusted to pH 5.3, to make the amine producer a deep red color (colony with strong intensity of positive reaction is surrounded by a red halo on a yellowish background).

Table 2. Classification of significant strains depending on biogenic amine-producing activity determined by HPLC.

Isolate			Biogenic amines determined by HPLC				
Activity	Type	Isolate No.	Histamine	Tyramine	Putrescine	Cadaverine	Total amount
Strong producers	I	0037	-	-	+	+	+
	I	1043	+	-	+	+	+
	I	1056	+	-	+	+	+
	I	1059	+	-	+	+	+
	I	1060	-	-	+	(+)	+
	I	1542	(+)	-	+	(+)	+
	I	1543	-	-	+	-	+
	I	1544	-	(+)	+	-	+
	I	1553	(+)	(+)	(+)	-	(+)
	I	2036	-	-	+	-	+
Weak producers	II	0038	-	+	(+)	-	(+)
	II	0549	-	(+)	(+)	(+)	(+)
	II	1039	-	-	(+)	-	(+)
	II	1051	-	+	(+)	+	(+)
	III	0042	-	-	(+)	-	(+)
	III	0538	-	(+)	(+)	-	(+)
	IV	2028	-	(+)	(+)	-	(+)

+ indicates the level of productivity over the mean of amine content produced by each producer, (+) indicates the level of productivity up to the mean of that, - indicates the level of productivity below the detection limit of HPLC.

pattern of fatty acid profile by the Microbial Identification System: The endospore-forming, Gram-positive, catalase-positive, and motile rods with strong abilities to produce biogenic amines were identified to be *B. licheniformis*, as shown in Table 3 (SI=0.323-0.464). Various properties of 4 *Bacillus* strains with weak abilities to produce biogenic amines are also shown in Table 3. The result corresponded to *B. coagulans* (SI=0.507-0.807). The other weak amine producers were identified as *Staphylococcus xylosus* (2 strains) and *Micrococcus luteus* (1 strain). Therefore, the majority of biogenic amines in Myeolchi-jeot stored for a long time might have been due to the biogenic amine-producing *Bacillus* strains, especially *B. licheniformis*.

DISCUSSION

Myeolchi-jeot samples had sufficiently high concentrations of NaCl, ranging from 18 to 20%, to prevent the growth of pathogenic bacteria, therefore, the water activities of the samples would be detected below 0.75. As the samples had very low water activities along with high NaCl concentrations, the total plate counts of Myeolchi-jeot samples stored at 4°C, 15°C, and 30°C were maintained at a level of around 10^4 cfu/g. However, the amine contents in Myeolchi-jeot during storage apparently increased, depending on both time and temperature, as reported previously [20], even though it had low water activity and total plate count. Therefore, the amine contents in Myeolchi-jeot might be related to microbial flora.

Under all the conditions tested in this study, staphylococci were dominant in Myeolchi-jeot. *Staphylococcus* spp. isolated from Japanese fermented and salted fish were also reported by Yatsunami and Echigo [36] as strong histamine producers in a broth medium with 12% NaCl. In this study, the production of biogenic amines by *Staphylococcus* spp. in Myeolchi-jeot was unexpectedly weak. Nevertheless, staphylococci contributed to the amine contents in Myeolchi-jeot which was fermented for a long period, and then the produced amines gradually accumulated in Myeolchi-jeot.

The presence of amino acid decarboxylase has been described in the genus *Bacillus* [19, 26, 32] that is known to be a microbial origin of useful enzymes [17]. In this study, similar to the changes of the amine contents in Myeolchi-jeot during storage [20], the endospore-forming bacteria *Bacillus* spp. increased, dependent on both time and temperature of storage. In addition, all the *B. licheniformis* isolated from Myeolchi-jeot samples were strong producers of biogenic amines. Considering the increase in cell count of bacilli, *B. licheniformis* possibly increased in the Myeolchi-jeot sample stored at an ambient temperature. Therefore, *B. licheniformis* might have increased the amine contents during the storage (particularly, at an ambient temperature).

Hernández-Herrero *et al.* [12] reported that NaCl concentrations in the range of 0.5 to 10% had a stimulatory effect on the amine formation. It was also reported that biogenic amine-producing enzymes retained the amine formation activity in Myeolchi-jeot, which had a

Table 3. Physiological and cultural characteristics of strains classified as strong and weak producers of biogenic amines.

Characteristics	Isolates			
	Strong producers	Weak producers		
	Type I	Type II	Type III	Type IV
Voges-Proskauer test	+	+	+	+
Utilization of citrate	+	-	-	-
propionate	+	-	NG	+
Growth with inorganic N	•	•	-	+
$(NH_4)_2SO_4$	•	•	-	+
Hydrolysis of starch	+	NG	-	-
milk	+	NG	NG	NG
gelatin	+	-	-	-
esculin	+	d	(+)	-
Arginin dihydrolase	+	d	d	-
Nitrate reduction	+	-	+	-
Growth in 2% NaCl	+	+	+	+
5% NaCl	+	+	+	+
7% NaCl	+	+	+	+
10% NaCl	+	(+)	+	(+)
15% NaCl	-	(+)	+	d
20% NaCl	-	-	+	d
Growth at 5°C	-	-	+/-	-
10°C	-	-	•	•
15°C	•	•	+	+
30°C	+	+	+	+
37°C	+	+	+	+
40°C	+	+	+	+
45°C	+	-	(+)	+
50°C	+	-	(+)	(+)
55°C	+	-	-	(+)
Acid from glucose	+	(+)	+	-
fructose	•	•	(+)	•
sucrose	•	•	-/+	•
arabinose	+	(+)	+	•
xylose	(+)	+	+	•
mannitol	+	d	+	•
maltose	•	•	+	•
glycerol	•	•	+	-
mannose	•	•	+	-
cellobiose	•	•	-	•
ribose	•	•	+	•
lactose	•	•	+	-
galactose	•	•	+	-
*Identification result	BL	BC	SX	ML
**SI	0.323 ~0.464	0.507 ~0.807	•	•

Type I: Isolate No. 0037, 1043, 1056, 1059, 1060, 1542, 1543, 1544, 1553 and 2036. Type II: Isolate No. 0038, 0549, 1039, and 1051. Type III: Isolate No. 0042 and 0538. Type IV: Isolate No. 2028.

• Not tested NG: no growth, +: positive, (+): weak positive, d: dubious.

*BL: *Bacillus licheniformis*, BC: *B. coagulans*, SX: *Staphylococcus xylosus*. ML: *Micrococcus luteus*.

**SI: Similarity index gained by the Microbial Identification System.

NaCl concentration as high as around 20% [20], suggesting that a level of 20% NaCl is not sufficient to prevent the amine formation although the growth of amine producers is inhibited at the high salt concentration. Therefore, the amount of more than 20% NaCl may be needed to inhibit both amine-producing activity and growth of amine producers [12], but such a high level of concentration of salts in foods may hardly be practical in foods.

Klausen and Lund [15] reported that amine contents were positively correlated with temperature in both mackerel and herring. Also, it has been reported that the temperature of storage affected the increment of amine contents in Myeolchi-jeot [20]. The reason is that the reactivation rate of amine-producing enzyme, and amino acid decarboxylase corresponding to each amine, can be increased as the temperature of storage increased to the temperature suitable for metabolizing amino acids. In foods, low temperatures below 5°C have been recommended to prevent amine production [28]. However, low storage temperatures are not sufficient to inhibit the formation of toxic histamine [1], and may bring a change in texture and flavor of fermented fish products such as Myeolchi-jeot.

As mentioned above, both high salinity and low temperature are not sufficiently suitable to prevent the amine formation. Therefore, studies on starter culture without any amine-producing activity and/or with amine-degrading activity are needed. Since *B. coagulans*, *S. xylosus*, and *Micrococcus* sp. were isolated from Myeolchi-jeot, and were shown to be weak producers for biogenic amines, they have the potential to be used as starter cultures. The use of the strain without biogenic amine-producing activity as a starter culture may bring a reduction of biogenic amine in fermented foods.

Some reports have described false-positive microorganisms [19, 27]. They might be caused by production of other alkaline bacterial products [6, 12, 27] and/or inhibition of growth of amine producer in the media [27]. In this study, most strains were shown to have good agreement in the amounts of amine obtained by the method using differential media and HPLC. Therefore, the decarboxylating media are suitable to conveniently detect the potential biogenic amine producer, although quantitative assay such as HPLC analysis is required in case of certain false-positive microorganisms.

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