



Isolation of halophilic lactic acid bacteria possessing aspartate decarboxylase and application to fish sauce fermentation starter

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ABSTRACT

The aims of this study were to isolate halophilic lactic acid bacteria possessing aspartate decarboxylase and elucidate the property of the isolates as starter cultures for fish sauce fermentation. Seventy-four strains were isolated from fermented fish foods on aspartate indicator broth containing bromocresol purple, and all isolates were identified as *Tetragenococcus halophilus* and confirmed to possess the aspartate decarboxylase gene (*aspD*) by PCR amplification. The isolates were classified into 14 groups based on their *aspD*-encoding plasmid construction. Strains selected from each group and a control strain incapable of aspartate decarboxylation were inoculated into fish sauce mash as starter cultures. Isolated strains possessing *aspD* converted aspartate into alanine almost completely in the fish sauce mash. In addition, the strains prevented the accumulation of biogenic amines, as did the control strain, whereas various amines were accumulated in fish sauce mash without starter cultures. Sensory evaluation tests indicated that converting the sour amino acid aspartate into the sweet amino acid alanine made the fish sauce taste milder. In conclusion, the use of *T. halophilus* possessing aspartate decarboxylase as a fish sauce fermentation starter causes the conversion of aspartate to alanine, accompanied by taste alteration, and prevents biogenic amine accumulation in fish sauce products.

1. Introduction

Fish sauce is a common, traditional seasoning produced by the fermentation of fish, widely used in Southeast and East Asia. During the fermentation process, fish protein is hydrolyzed by the digestive enzymes of fish, resulting in peptides and amino acids, which give a strong umami and unique complex taste to fish sauce. Fish sauce also contains high concentrations of NaCl, which prevents rotting and allows halophilic bacteria to reside. As fish sauce production solely depends on a natural fermentation process, its quality is seriously affected by microbes in the fish sauce mash.

Halophilic lactic acid bacteria, mainly *Tetragenococcus* spp., were isolated from the fish sauce and other fermented sea foods as the dominant microbe (Kobayashi et al., 2000; Sato et al., 1995; Satomi et al., 1997; Taira et al., 2007; Thongsanit et al., 2002). Lactic acid produced by lactic acid bacteria reduces the pH and gives a moderate sour taste to fish sauce. However, certain strains of *Tetragenococcus* spp. degrade particular amino acids, such as histidine (Kimura et al., 2001; Satomi et al., 2008), tyrosine (Satomi et al., 2014), arginine (Iituka and Goan, 1973), serine, threonine, phenylalanine (Uchida, 1982) and

aspartate (Higuchi et al., 1998). Although the degradation pathways of these amino acids are not fully clarified, some of them become amines by decarboxylation. Previous reports demonstrated that histidine is transformed into histamine by histidine decarboxylase, encoded by *hdcA* on plasmids of *Tetragenococcus halophilus* (Satomi et al., 2011) and *Tetragenococcus muraticus* (Satomi et al., 2012) and that tyrosine is converted to tyramine by tyrosine decarboxylase, encoded by *tdcA* on the plasmids of *T. halophilus* (Satomi et al., 2014).

Histamine is known as a causative agent of food related intoxication, participating in allergic and inflammatory responses, whereas tyramine is related to food-induced migraines and hypertensive crisis (Rice et al., 1976; Santos, 1996; Taylor and Eitenmiller, 1986). Tyramine and other biogenic amines, such as phenethylamine, cadaverine, putrescine, spermidine and spermine, can enhance the toxicity of histamine by inhibiting histamine-metabolizing enzymes in the small intestine (Prester, 2011; Stratton et al., 1991). Furthermore, these amines potentially react with nitrous acid and make carcinogenic nitrosamines (Nagahara et al., 1986; Sen et al., 1990; Shalaby, 1996). In addition to their potential toxicity, biogenic amines are used for the evaluation of the hygienic quality of a marine species (Prester, 2011). Therefore,

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amino acid-decarboxylating bacteria are generally not preferred for fish sauce fermentation. To prevent the accumulation of biogenic amines, selected strains of *Tetragenococcus* incapable of amine production are often used as starter cultures for fermented fish foods, including fish sauce (Kimura et al., 2015; Kuda et al., 2012; Udomsil et al., 2011).

It is also known that aspartate is transformed into another amino acid, alanine, via decarboxylation by *T. halophilus* possessing aspartate decarboxylase encoded by *aspD* on plasmids (Higuchi et al., 1998). The conversion of aspartate to alanine potentially makes the taste milder by combination of a decrease in the amount of the sour amino acid aspartate and an increase in the amount of the sweet amino acid alanine (Uchida, 2000). This reaction is highly similar to malolactic fermentation, which is often performed for red wine brewing. The reaction is conducted by lactic acid bacteria, such as *Oenococcus oeni*, which transforms malic acid into lactic acid by decarboxylation (Nielsen and Richelieu, 1999). The conversion of malic acid, a dicarboxylic acid, to lactic acid, a monocarboxylic acid, deacidifies wine, resulting in a milder taste. Hence, for fish sauce fermentation, aspartate decarboxylating bacteria can possibly be considered as a desirable starter. However, while a few strains of *T. halophilus* possessing aspartate decarboxylase were isolated from soy sauce mash (Higuchi et al., 1998; Uchida, 1982), such strains have never been isolated from fish sauce or any other sea foods, nor applied to fish sauce fermentation so far.

The aims of this study were to isolate halophilic lactic acid bacteria possessing aspartate decarboxylase from fermented fish foods, to apply to starter cultures for fish sauce fermentation and to elucidate the effects of the isolates on taste improvement and biogenic amine reduction.

2. Materials and methods

2.1. Samples

Samples used for this study are shown in Table 1. All fish sauces were obtained from a fish sauce manufacturing company in Japan, and fish *nukazuke* (salted and fermented fish with rice bran) were collected from markets in Japan.

2.2. Isolation of aspartate decarboxylating bacteria

Aspartate decarboxylating bacteria were isolated in aspartate indicator broth containing bromocresol purple (Higuchi et al., 1998). pH reduction by lactic acid production makes the color of this medium yellow, but when aspartate is decarboxylated, the color remains purple. Samples were properly diluted with 10% saline, inoculated in the broth and incubated at 30 °C for 6 days with an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) in a hermetically sealed box.

2.3. Detection of aspartate decarboxylase and other genes

Specific detection of *aspD*, *hdcA* and *tdcA* was performed using PCR

Table 1

List of the sources from which aspartate decarboxylating bacteria were isolated.

Sample number	Sample	Fish material	The number of the isolates
1 ^a	Fish sauce mash	Deep-sea smelt	14
2 ^a	Fish sauce mash	Deep-sea smelt	12
3 ^a	Fish sauce mash	Deep-sea smelt	15
4 to 12 ^a	Fish sauce mash	Deep-sea smelt	0
13 to 17	Fish <i>Nukazuke</i>	Mackerel	0
18	Fish <i>Nukazuke</i>	Sardine	1
19 to 20	Fish <i>Nukazuke</i>	Sardine	0
21	Fish <i>Nukazuke</i>	Sardine	13
22	Fish <i>Nukazuke</i>	Sardine	19

^a Same manufacturer but different lots.

Table 2

Primers list.

Primer name	Sequence (5' → 3')
aspF	AATGTTTTCCCTACTGAAGG
aspR	CCTGTTCACCCATAAAGTTT
hdcF	ACTTGGGGTTGACCCGTATCTCAGTGAGTC
hdcR	TCTTCGTTAGGAGTCTCCCAAACACCAGC
tdcF	CGTGGTGGCATGGATCTTTC
tdcR	GCACCCCTCTCAGTTGAACCAG
Th67F	ACGCTGCTTAAGAAGAACTTCGG
Th800R	TGGACTACCAGGGTATCTAATCC
aspInvF	CATTGGCTAACCAACCAGCTG
aspInvR	GACATACCTTTTAATTGTTCAATATCTAATGATTCC
repInvF	GCAAAAAGAAGACGAACGCAAC
repInvR	TTTTTGATTATGGCAATTAATAAATCC
pinInvF	GATTGAACGAAAAACAGGCATTAGTG
pinInvR	TTGATCTGTGTACTTACCCTAGCATAACC

amplification with oligonucleotide primer sets aspF-aspR, hdcF-hdcR and tdcF-tdcR, respectively (Table 2). A brief identification of *T. halophilus* or not was performed using PCR amplification with *T. halophilus* species-specific oligonucleotide primer Th67F and Th800R, which amplifies the 16S rRNA gene (Table 2).

Each PCR reaction mixture (50 µl) consisted of 25 µl of SapphireAmp (Takara Bio, Kusatsu, Japan), 25 pmol of each primer and only a few cells. PCR was performed for 30 cycles after preincubation at 94 °C for 1 min using the following conditions: denaturation at 98 °C for 5 s; annealing at 54 °C for 5 s; and extension at 72 °C for 10 s. The PCR products were visualized by electrophoresis with 0.9% agarose gel (Nippon Gene, Tokyo, Japan), which was stained with ethidium bromide. Strains decarboxylating aspartate, histidine or tyrosine were used as positive controls for PCR detection of *aspD*, *hdcA* and *tdcA* (Table 3).

The 16S rRNA gene amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), and their DNA sequences were determined by commercial DNA sequencing service (Fasmac, Atsugi, Japan) and analyzed using the Blast program.

2.4. Plasmid typing

For plasmid typing, specific oligonucleotide primer sets aspInvF-aspInvR, repInvF-repInvR and pinInvF-pinInvR were designed at *aspD*, *repA* (encoding rolling-circle replication type plasmid replication initiation protein) and *pinR* (encoding site-specific recombinase), respectively (Table 2). These three primer sets were mixed, and direct colony PCR was performed on all isolates using KOD FX Neo (Toyobo, Osaka, Japan) with 25 pmol of each primer. PCR was performed for 35 cycles after pre-incubation at 94 °C for 2 min using the following conditions: denaturation 98 °C for 10 s; annealing at 67 °C for 30 s; and extension at 68 °C for 40 min. The PCR products were detected with 0.7% agarose gel (Nippon Gene), and the isolates were classified based on their amplified DNA fragment patterns.

2.5. Preparation of starter cultures

Starter strains were cultured in fish sauce medium, which contains fish sauce (BlessingFavour, Kumamoto, Japan) with the same composition as that of soy sauce medium for halophilic lactic acid bacteria (Noda et al., 1982). Concretely, fish sauce was diluted so that the total nitrogen concentration becomes 0.4%, added NaCl to make the final concentration 10%, and added 1% glucose. The pH was adjusted to 8.0 with NaOH before sterilization by autoclave. Cultures were incubated at 30 °C for 5 days without stirring, additional pH adjustment or making anaerobic conditions.

Table 3
Strains used in this study.

Strain	Relevant features	Source or reference
<i>Tetragenococcus halophilus</i> C1	Not aspartate-, histidine- or tyrosine-decarboxylating strain	Isolated from fish sauce mash. Kept in our laboratory.
<i>Tetragenococcus halophilus</i> A-24	Aspartate-decarboxylating strain	Isolated from soy sauce mash. Kept in our laboratory.
<i>Tetragenococcus halophilus</i> H	Histidine-decarboxylating strain	Satomi et al., 2008
<i>Tetragenococcus halophilus</i> TyrA	Tyrosine-decarboxylating strain	Satomi et al., 2014

2.6. Fish sauce manufacturing

Minced anchovy (*Engraulis japonicus*) was mixed with NaCl (final concentration 17%) and glucose (final concentration 0.5%). The mixture, fish sauce mash, was stored at 5 °C until use. In small-scale fermentation, 2 ml of starter cultures prepared in fish sauce medium were added to 200 g of fish sauce mash. In large-scale fermentation, 30 ml of starter cultures prepared in fish sauce medium were added to 20 kg of fish sauce mash and fermented at 30 °C for 28 days.

As the fermentation indicator strain, *T. halophilus* C1 was used. C1 was previously confirmed not to produce gas in Durham tube with aspartate, histidine nor tyrosine (Table 3).

2.7. Chemical analysis

The fish sauce mash was filtered through filter paper (No. 2; Toyo Roshi, Tokyo, Japan), and the pH of the filtrate was determined using pH meter F-52 (Horiba, Kyoto, Japan). Amino acid composition was analyzed by high-performance liquid chromatography (HPLC) with ninhydrin detection. The HPLC system used was Hitachi ELITE LaChrom (Hitachi High-Technologies, Tokyo, Japan). The concentration of biogenic amines was also determined by HPLC with post-column *o*-phthalaldehyde derivatization and fluorescence detection. The HPLC system used was SCL-10A_{VP} (Shimadzu, Kyoto, Japan). Total nitrogen content was measured by the Dumas combustion method using SUMI-GRAPH NC-220F (Sumika Chemical Analysis Service, Osaka, Japan). The NaCl concentration was determined from chlorine content measured by potentiometric titration using an automatic titration apparatus COM-1700 (Hiranuma, Mito, Japan). Reducing sugar concentrations were assayed using the ferricyanide method (Hulme and Narain, 1931). Lactic acid and acetic acid content was determined using Shodex OA (Shodex, Tokyo, Japan) following the instructions of the manufacturer.

2.8. Bacterial count

The population of halophilic lactic acid bacteria was monitored using LA13 medium (Yamasato and Iizuka, 1959) with a slight modification, containing 1% polypeptone, 0.4% yeast extract, 1% KH₂PO₄, 12% NaCl, 0.5% glucose, 5% soy sauce (Yamasa, Choshi, Japan) and 1.5% agar. Starter cultures or fish sauce mashes taken at various fermentation periods were diluted with 10% saline, spread on the LA13 medium and incubated at 30 °C for 5 days with an AnaeroPack (Mitsubishi Gas Chemical) in a hermetically sealed box.

2.9. Sensory evaluation

After 28 days fermentation, fish sauce mash was filtered through a filter paper (No. 2; Toyo Roshi), and the filtrate was heated till it reached 90 °C. Then, the resulting fish sauce was dialyzed by the electro dialysis system Micro Acilyzer S3 (Astom Corp., Tokyo, Japan) and adjusted to total nitrogen 1.5% and NaCl 1.5%. The fish sauce fermented with control strain C1 and with the isolated strain 1-1 or 21-11 were scored in 5-point scales to characterize the quality (salty, umami, sweet, bitter and sour). Fish sauce was served to panelists at room temperature in a random order. All nine panelists (men 24–38 years old) were the members of Yamasa Corporation.

2.10. Statistical analysis

The data were analyzed using SPSS software (SPSS Inc., Chicago, USA). Comparison of the two groups was performed using an unpaired *t*-test. The data involving more than two groups were assessed using a one-way ANOVA followed by post hoc Dunnett's or Bonferroni's multiple comparison test. Statistical significance was considered at *p* < 0.05. All experiments were carried out in triplicate. Exceptional sensory evaluation tests were carried out using fish sauce obtained by mixing three samples inoculated with the same strains.

3. Results and discussion

3.1. Isolation and identification of aspartate decarboxylating bacteria

Seventy-four candidates of aspartate decarboxylating halophilic bacteria were isolated as purple colonies on aspartate indicator broth, 41 from fish sauce and 33 from fish *nukazuke* (Table 1). All of the candidates were confirmed to possess *aspD*, but not *hdcA* or *tdcA*, and were estimated as *T. halophilus* by PCR amplification using gene or species-specific primer sets (data not shown). The strains were named by connecting the sample numbers from which they were isolated and using consecutive numbers with hyphens (Table 4).

3.2. Classification of the isolated strains

Because the isolates from same samples may be genetically identical to each other, we tried to classify these isolates. First, to classify the isolates based on their plasmid construction, PCR from three genes *aspD*, *repA* and *pinR* was conducted. *AspD* is a structural gene of aspartate decarboxylase and confirmed to be possessed by all of the isolates in a preceding experiment. *RepA* exists on plasmids of *T. halophilus* encoding *hdcA* and *tdcA* with slight exceptions, and *pinR* exists on those plasmids with no exception so far (Satomi et al., 2011, 2014). Thus, this PCR from three genes was expected to generate three DNA fragments. And considering the length of plasmids encoding *hdcA* (21–37 kb) and *tdcA* (27–29 kb), the total length of the three fragments was expected to

Table 4
Classification of isolated strains based on PCR.

PCR	Strains
A	<u>1-1</u> , 1-7, 1-9, 1-10, 1-14, 2-1, 2-5, 3-15
B	<u>1-2</u> , 1-3, 1-5, 1-6, 2-2, 2-6, 2-7, 2-8, 2-10, 3-4, 3-6, 3-8, 3-9, 3-14
C	<u>1-4</u> , 1-12
D	<u>1-8</u> , 3-2, 3-5
E	<u>1-11</u> , 2-3, 2-4, 2-11, 2-12, 3-3, 3-7, 3-10, 3-11, 3-12, 3-13
F	<u>1-13</u>
G	<u>2-9</u>
H	<u>3-1</u>
I	<u>18-1</u> , 21-2, 21-6, 21-8, 22-14, 22-18
J	<u>21-1</u> , 22-2
K	<u>21-3</u> , 21-5, 21-7, 21-9, 21-10, 21-12, 22-4, 22-5, 22-6, 22-8, 22-9, 22-10, 22-11, 22-12, 22-13, 22-15, 22-16, 22-17, 22-19
L	<u>21-4</u> , 22-1, 22-7
M	<u>21-11</u> , 22-3
N	<u>21-13</u>

Underlined strains were selected for subsequent experiments.

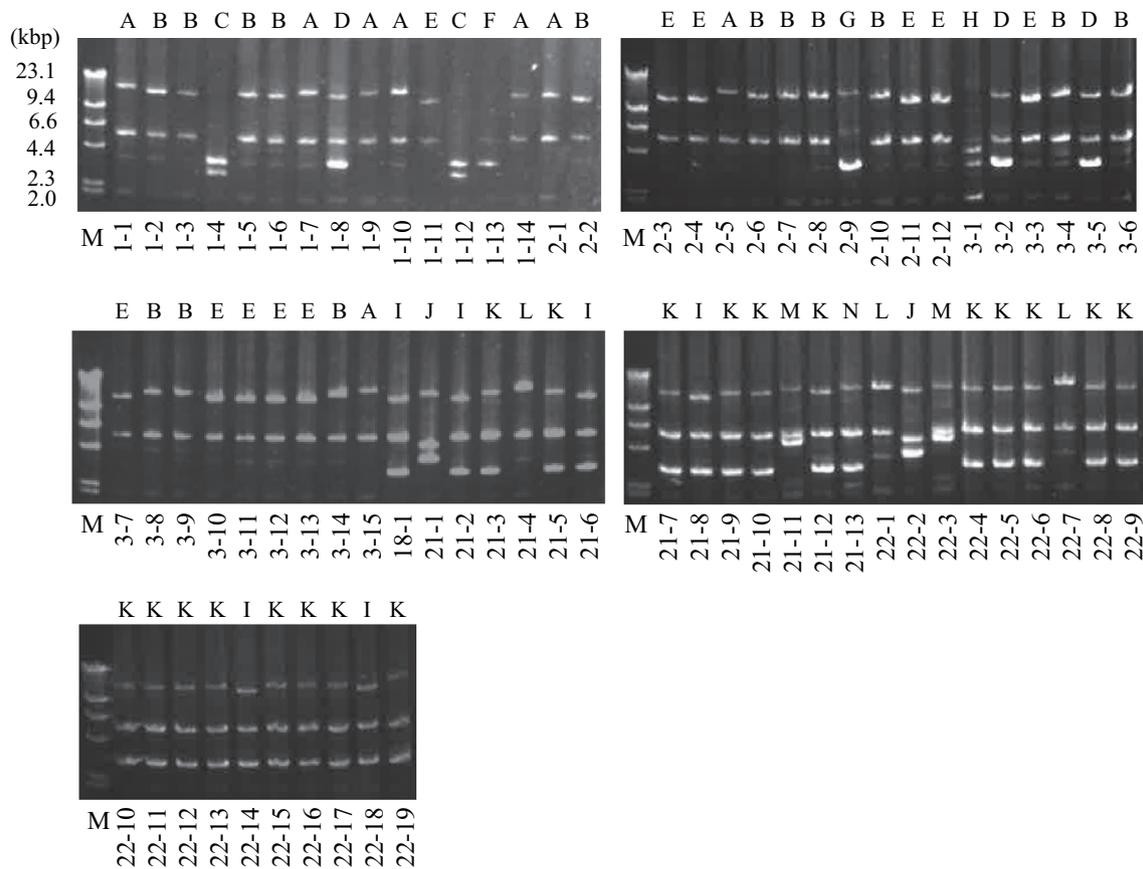


Fig. 1. Amplified DNA fragment patterns of PCR for classification of the isolated strains. Strain names are shown under the lanes (M: Molecular weight marker Lambda *Hind*III). The upper letters indicate groups corresponding to Table 4.

appear within this range. Although not all isolates made clear three fragments, their fragment patterns could be typed into 14 groups (Fig. 1, Table 4).

Eight different types of strains were isolated from fish sauces, and six types of strains were isolated from fish *nukazuke*. While multiple strains were isolated from five samples, no strain was isolated from 16 samples. This result suggests the possibility that the residence of halophilic bacteria possessing aspartate decarboxylase depends on the property of the samples. For example, shortage of glucose would provide an advantage to aspartate decarboxylating bacteria because the aspartate alanine conversion cycle contributes to ATP synthesis (Abe et al., 1996). This cycle is also involved in acid tolerance by extruding protons from cells (Abe et al., 2002). In addition, starter cultures naturally outcompete wild strains. The samples from which the isolates were obtained in this study might have met some of these conditions.

3.3. Amino acid conversion and biogenic amines accumulation

A total of 14 strains were selected, generally one from each group (Table 4). Their 16 rDNA sequences were determined, all of which showed > 99.8% identity to that of *T. halophilus* (DDBJ accession numbers LC430618 - LC430631). To evaluate the property of *T. halophilus* possessing aspartate decarboxylase as a fish sauce fermentation starter, we performed small scale fermentation. The selected 14 strains and strain C1, incapable of aspartate, histidine or tyrosine decarboxylation (Table 2), were cultured in fish sauce medium and inoculated in fish sauce mash. After 28 days fermentation, the amino acid content and biogenic amines accumulated in the fish sauce mash were analyzed. All 14 strains possessing aspartate decarboxylase decreased aspartate by 28.0–31.6 mM and increased alanine by 28.2–37.2 mM compared to that of the control strain C1 (Table 5).

Strains 1–11 diminished arginine levels in addition to aspartate levels, probably by the arginine deiminase pathway, which converts arginine to ornithine. It has been known that most strains of *T. halophilus* possess an arginine deiminase system, but some strains do not (Iitaka and Goan, 1973; Uchida, 1982; Udomsil et al., 2011). In contrast, 13 out of 14 isolates used in this experiment did not decrease the arginine levels. Arginine deimination and aspartate decarboxylation are both used to complement the pH reduction occurring with lactic acid fermentation. Considering that arginine deimination and histidine decarboxylation are reciprocally regulated in *Lactobacillus hilgardii* (Lamberti et al., 2011), possessing both arginine deimination and amino acid decarboxylation systems may not be so beneficial for lactic acid bacteria. *T. halophilus* strains decarboxylating multiple amino acids have never been found, even though they could possess a multitude of plasmids (Higuchi et al., 1998). It may be the survival strategy of *T. halophilus* that each of the strains utilizes different amino acids to adapt to various environments.

In the fish sauce mash without starters, arginine and histidine levels significantly decreased, and various biogenic amines were remarkably accumulated (Table 5). Compared to this result, the accumulation of biogenic amines was entirely suppressed in fish sauce mash with starters, indicating that they were most probably produced by a contaminated wild microbe. Tyramine, histamine, phenylethylamine, tryptamine and spermidine levels tended to increase with certain strains, such as 1–4, 21–1 and 21–3. Given that these amines were also produced by wild microbes, such strains should have allowed slight growth for some reason. Not much is known about the way these amines accumulate at high NaCl concentration, except for tyramine and histamine. In this study, we did not determine which bacteria participated in producing these biogenic amines, but further research is necessary from the point of food hygiene.

Table 5
Amino acid and biogenic amine content in the small-scale fermentation of fish sauce.

	C1	1-1	1-2	1-4	1-8	1-11	1-13	2-9	3-1	18-1	21-1	21-3	21-4	21-11	21-13	NS
Amino acid (mM)																
Aspartic acid	31.6	0.1*	0.2*	0.1*	0.0*	2.1*	3.6*	1.0*	1.2*	1.9*	1.7*	1.8*	0.1*	0.3*	1.4*	31.7
Threonine	19.7	19.3	19.9	20.4	20.1	20.7	20.9	20.1	19.9	19.2	20.3	20.4	18.9	19.3	19.6	21.1
Serine	19.8	19.6	19.8	20.4	20.4	20.1	20.2	20.3	19.7	18.9	19.6	19.9	19.1	19.6	19.4	17.5
Glutamic acid	34.3	33.6	34.6	35.4	35.1	34.9	35.3	34.9	34.2	32.9	34.6	34.8	33.5	35.0	34.1	35.1
Glycine	14.8	14.7	15.5	15.2	15.3	15.4	15.4	15.2	14.9	14.3	14.9	14.8	14.6	15.2	14.6	16.0
Alanine	35.8	69.0*	70.2*	71.5*	73.0*	68.2*	67.0*	68.9*	67.5*	64.0*	68.5*	68.8*	67.0*	69.8*	66.7*	39.1
Cysteine	0.4	0.4	0.4	0.4	0.3	0.5	0.5	0.3	0.3	0.6	0.5	0.4	0.5	0.4	0.6	0.6
Valine	22.0	21.5	22.0	22.5	22.2	22.5	22.7	22.1	21.9	21.0	22.4	22.4	21.2	22.4	21.8	23.3
Methionine	11.4	11.6	11.8	12.1	11.9	12.1	12.1	12.0	11.7	10.9	11.7	12.0	11.2	11.6	11.3	12.1
Isoleucine	16.3	15.9	16.3	16.6	16.5	16.6	16.7	16.5	16.2	15.5	16.4	16.4	15.7	16.6	16.2	16.9
Leucine	31.6	30.8	31.3	32.4	31.9	32.1	32.3	32.0	31.5	30.3	31.8	32.0	30.2	31.9	31.5	32.6
Tyrosine	1.3	1.1	1.2	1.2	1.2	1.1	1.3	1.1	1.0	1.0	1.3	1.2	1.1	1.1	1.0	0.4
Phenylalanine	10.6	10.2	10.2	10.8	10.3	10.8	10.6	10.4	10.4	9.9	10.3	10.7	9.8	10.8	10.5	9.5
Lysine	29.4	30.4	31.2	31.6	31.6	31.7	31.6	31.4	30.9	29.8	31.5	31.3	30.0	31.6	31.0	27.4
Histidine	14.3	14.4	15.1	14.5	15.1	15.1	14.7	15.2	15.1	13.9	13.7	13.4	14.7	15.0	13.8	2.6*
Arginine	15.9	16.2	16.8	15.4	17.1	0.2*	16.1	16.8	16.6	15.7	14.9	14.4	16.2	16.8	15.5	0.0*
Biogenic amine (ppm)																
Tyramine	21	5	4	42	4	4	26	8	4	12	41	46	4	6	21	1172*
Putrescine	125	22	22	110	17	78	345	21	12	31	153	185	18	23	64	2888*
Cadaverine	215	16*	15*	50*	18*	15*	49*	15*	14*	21*	51*	61*	16*	18*	35*	770*
Histamine	73	16	14	105	12	10	71	20	11	43	159	190	28	30	78	1510*
Agmatine	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Phenethylamine	5	5	2	9	6	1	9	1	2	1	14	15	1	1	5	209*
Tryptamine	154	20*	4*	275*	8*	7*	239*	37*	9*	68*	261*	270*	20*	46*	172	419*
Spermidine	13	13	12	13	14	10	15	10	111	13	25	28*	13	10	15	57*

Values are expressed as the mean (n = 3).

Values with an asterisk are significantly different from samples fermented with C1 at $p < 0.05$ by Dunnett's multiple test.

NS: Fermented without starter cultures.

The cadaverine content increased in fish sauce mash inoculated with C1 more than that in other strains. Udomsil et al. (2011) also reported that using certain strains of *T. halophilus* increased the cadaverine content in fish sauce. C1 and such strains may possess lysine decarboxylase activity.

3.4. Fish sauce manufacturing

To elucidate the effects of aspartate decarboxylation in more detail, larger-scale fish sauce manufacturing was carried out using strains C1, 1-1 and 21-11. In the small-scale experiment mentioned earlier, it was confirmed that 1-1 and 21-11 converted aspartate to alanine almost completely and that C1 did not. Although C1 slightly changed some amine content compared to that of the others, it did not change any amino acid content other than that of aspartate and alanine (Table 5). Therefore, we decided to use C1 as a control strain to assess the effects of aspartate decarboxylation on sensory characteristics.

The bacterial counts of C1, 1-1 and 21-11 in fish sauce medium just before inoculation in fish sauce mash were 1.3×10^7 , 1.4×10^8 and 1.0×10^8 CFU/ml, respectively. The counts of fish sauce mash samples inoculated with 1-1 and 21-11 were higher than those inoculated with C1 throughout the fermentation, which probably resulted in higher organic acid and lower reducing sugar levels (Fig. 2a, Table 6). This result should be due to the acid tolerance caused by aspartate decarboxylation of 1-1 and 21-11. Aspartate decarboxylation eliminates protons from cells and generates the more alkaline alanine while simultaneously producing carbonic acid that would be released into the air. This effect can also explain the higher pH in fish sauce mash inoculated with 1-1 and 21-11, even though their organic acid content increased (Fig. 2b).

In fish sauce mash inoculated with 1-1 and 21-11, aspartate levels decreased > 95% and alanine levels increased > 107% compared to those in control samples treated with C1 at 28 days. In fact, at 14 days after inoculation, the aspartate content in fish sauce mash treated with 1-1 and 21-11 was below detection limit (data not shown). Therefore, the aspartate that remained in these products must be released from fish

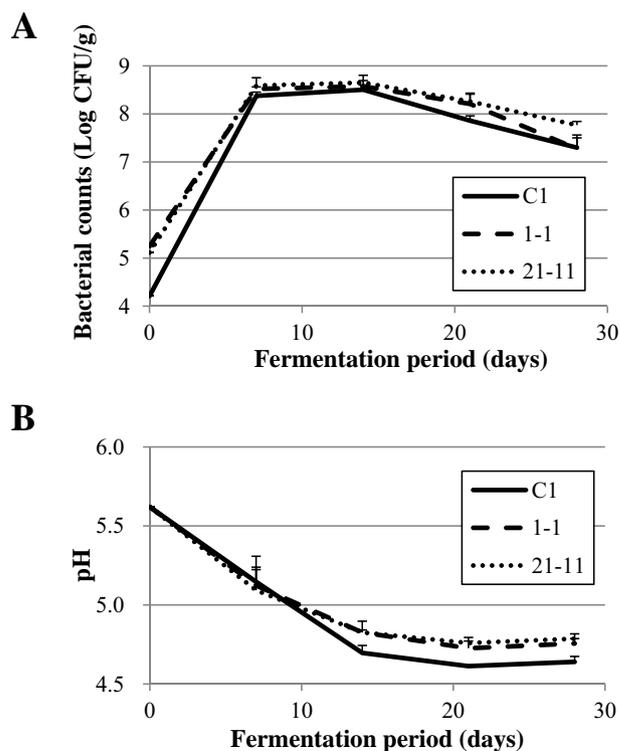


Fig. 2. Bacterial counts (A) and pH (B) evolution of fish sauce mash treated with each strain. Data are expressed as the mean with error bars representing +1 SD (n = 3). Since no bacterium was detected in the fish sauce mash before starter inoculation ($< 5 \times 10^1$ CFU/g), the initial bacterial counts are calculated from the values in the cultures.

Table 6
Profiles of free amino acids, biogenic amines and other components in the large-scale fermentation of fish sauce.

Item	C1	1-1	21-11
Total nitrogen (%)	1.44 ± 0.01	1.39 ± 0.04	1.42 ± 0.01
NaCl (%)	16.5 ± 0.5	16.9 ± 0.4	16.7 ± 0.4
pH	4.64 ± 0.03 ^a	4.76 ± 0.03 ^b	4.79 ± 0.03 ^b
Reducing sugar (%)	0.70 ± 0.11 ^a	0.48 ± 0.03 ^b	0.51 ± 0.03 ^b
Lactic acid (%)	1.19 ± 0.01 ^a	1.29 ± 0.03 ^b	1.30 ± 0.03 ^b
Acetic acid (%)	0.10 ± 0.01	0.11 ± 0.00	0.11 ± 0.00
Amino acid (mM)			
Aspartate	30.7 ± 0.8 ^a	1.4 ± 1.0 ^b	1.4 ± 0.4 ^b
Threonine	18.3 ± 0.5	19.8 ± 2.2	20.0 ± 0.6
Serine	19.4 ± 0.5	20.9 ± 2.1	21.2 ± 0.7
Glutamate	31.1 ± 1.2	33.5 ± 3.5	33.9 ± 1.4
Glycine	14.0 ± 0.3	15.3 ± 1.4	15.3 ± 0.7
Alanine	35.5 ± 0.4 ^a	73.5 ± 6.8 ^b	74.1 ± 3.6 ^b
Cysteine	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0
Valine	21.1 ± 0.7	22.4 ± 2.2	22.6 ± 1.0
Methionine	11.9 ± 0.2	12.8 ± 1.4	12.9 ± 0.5
Isoleucine	15.2 ± 0.4	16.1 ± 1.6	16.2 ± 0.7
Leucine	29.7 ± 0.7	31.8 ± 3.2	31.9 ± 1.3
Tyrosine	0.9 ± 0.1	1.4 ± 0.4	1.1 ± 0.1
Phenylalanine	10.2 ± 0.3	10.7 ± 1.3	10.8 ± 0.4
Lysine	27.7 ± 0.7	31.8 ± 3.1	31.8 ± 1.2
Histidine	12.6 ± 0.3	14.1 ± 1.6	13.8 ± 0.5
Arginine	16.4 ± 0.4	17.5 ± 1.6	18.0 ± 1.2
Total amino acids (mg/ml)	38.7 ± 0.9	40.8 ± 4.2	41.1 ± 1.7
Biogenic amine (ppm)			
Tyramine	90 ± 4	91 ± 3	96 ± 4
Putrescine	26 ± 1 ^a	10 ± 0 ^b	11 ± 1 ^b
Cadaverine	383 ± 43 ^a	48 ± 1 ^b	52 ± 4 ^b
Histamine	3 ± 0 ^a	7 ± 0 ^b	7 ± 0 ^b
Agmatine	21 ± 2	17 ± 1	18 ± 2
Phenethylamine	0 ± 0	7 ± 4	0 ± 0
Tryptamine	14 ± 2 ^a	11 ± 0 ^b	12 ± 1 ^{ab}
Spermidine	18 ± 1	16 ± 1	17 ± 1

Values are expressed as the mean ± SD (*n* = 3).

Values not sharing common superscripts are significantly different at *p* < 0.05 by Bonferroni's multiple test.

protein or peptides after bacterial growth was completed. The amount of increased alanine was slightly larger than the amount of decreased aspartate, as also occurred in the preceding small-scale test, possibly because the activities of aspartate-generating enzymes might be higher when the concentration of aspartate is low. Other amino acid compositions did not significantly differ.

All amines in fish sauce mash inoculated with 1-1 and 21-11 were suppressed to < 100 ppm. The histamine and tryptamine content slightly differs between samples, but this tendency is not consistent with the preceding results. This result might have been caused by a slight difference of microbiota in fish sauce mash samples unrelated to the starter inoculations. C1 increased the levels of putrescine and cadaverine, which also occurred in the small-scale experiments (Table 5). These compounds may have been produced by the same enzyme because it is reported in another species that lysine decarboxylase can also have ornithine decarboxylase activity (Takatsuka et al., 1999).

3.5. Sensory evaluation

Fish sauce samples were dialyzed and adjusted to a total nitrogen of 1.5% and NaCl of 1.5%. The purpose of dialysis was to evaluate a sensory property with low NaCl concentration, avoiding an extreme salty taste. As a matter of fact, the fish sauce before dialysis was so salty that it was not easy to recognize the taste properties of each sample. Concentration by evaporation and removal of the separated salt is actually practiced by Japanese fish sauce manufacturers.

As a result of sensory evaluation, fish sauce treated with 1-1 or 21-11 was sweeter than the control sample treated with C1 was. On the other hand, saltiness, bitterness and sourness tended to be intensified in

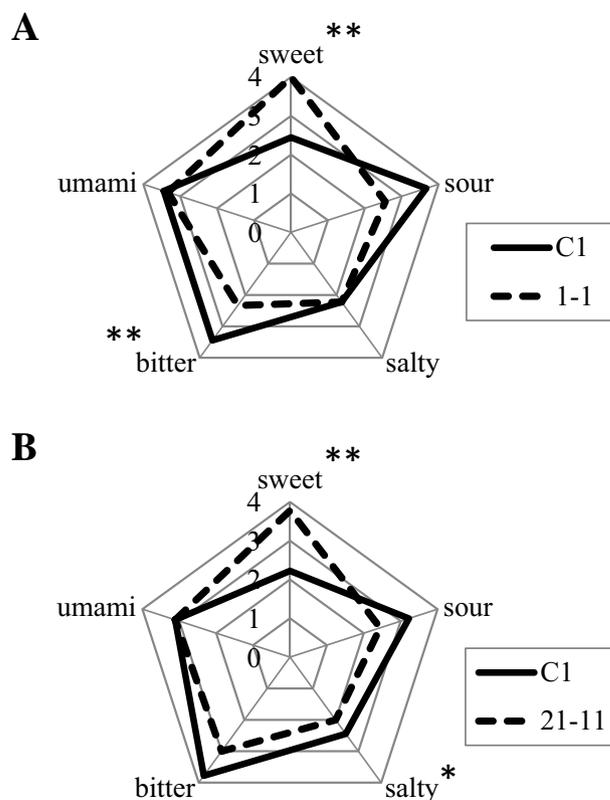


Fig. 3. Sensory evaluation of fish sauce comparing starter 1-1 to C1 (A) and 21-11 to C1 (B). Solid line: C1, Broken line: 1-1 (A) and 21-11 (B). Data are expressed as the mean (nine panelists). Items with asterisks are significantly different (*t*-test; **p* < 0.05, ***p* < 0.01).

the control sample (Fig. 3). The reducing sugar content was higher and the lactic acid and acetic acid content was lower in the control sample, which suggests the attenuated sweetness and intensified saltiness, bitterness and sourness were not caused by differences in these components but by the conversion of aspartate to alanine. Considering the taste qualities and threshold of aspartate (sour, slightly bitter; 0.182–0.226 mM) and alanine (sweet; 6.7–16.2 mM), this result appears to be reasonable (Kirimura et al., 1969; Schiffman et al., 1981).

The sweetness of fish sauce does not necessarily correspond to its quality. Sourness and bitterness are a part of the factors behind the unique and complex taste of the fish sauce. Hence, we do not insist that aspartate-decarboxylating bacteria are superior to others as a fish sauce fermentation starter. Nevertheless, this approach can provide alternatives to modify taste and enrich a variety of fish sauce items. There are various fermented marine food products by country or region, and the effects of use of aspartate-decarboxylating starters should be different in each product.

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