DOI:10.1021/jf904548u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Effect of Halotolerant Starter Microorganisms on Chemical Characteristics of Fermented Chum Salmon (*Oncorhynchus keta*) Sauce

Shuji Yoshikawa,*,† Hideyuki Kurihara,‡ Yuji Kawai,‡ Koji Yamazaki,‡ Akira Tanaka,† Takafumi Nishikiori,† and Tomoki Ohta†

†Hokkaido Food Processing Research Center, 589-4, Bunkyodai-Midorimachi Ebetsu 069-0836, Japan, and ‡Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1, Minato-cho, Hakodate, Hokkaido 041-8611, Japan

Chum salmon sauce mash was inoculated with barley koji (barley steamed and molded with *Aspergillus oryzae*) and halotolerant microorganisms (HTMs), *Zygosaccharomyces rouxii*, *Candida versatilis*, and *Tetragenococcus halophilus*, in nine different combinations under non-aseptic conditions similar to the industrial fish sauce production and fermented at 35 ± 2.5 °C for 84 days. The changes in the chemical components, color, and sensory properties during fermentation were investigated. Free amino acid content was increased, and the browning of fish sauce was enhanced by the usage of barley koji during fermentation. The halotolerant yeast (HTY) produced ethanol and repressed the browning by consumption of reducing sugar. Inoculated *Z. rouxii* in the fish sauce mash produced 2-phenylethanol (2-PE) and 4-hydoxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF), and *C. versatilis* in the fish sauce mash produced 4-ethylguaiacol (4-EG), known as characteristic flavor compounds in soy sauce, adding soy-sauce-like flavor to the fish sauce. Thus, inoculation of HTMs and barley koji was effective for conferring the soy-sauce-like flavor and increasing free amino acid and ethanol contents in fish sauce product.

KEYWORDS: Fish sauce; halotolerant microorganisms; taste; flavor; *Zygosaccharomyces rouxii*; *Candida versatilis*; *Tetragenococcus halophilus*

INTRODUCTION

Fish sauce is a fermented seasoning commonly produced in inshore countries of southeast and east Asia (*1*, *2*). Generally, a long term for more than 1 year is required to complete the fermentation of fish sauce because of its higher salt concentration to prevent microbial deterioration. In many cases, off-odor is derived from short-chain fatty acids and amines, such as trimethylamine, during the long fermentation period (*3*−*5*). The fermenting process with koji could lead to the enrichment with free amino acid in fish sauce and alleviation of the fishy odor by the repression of short-chain fatty acid production (*4*, *6*). However, the addition of koji to fish sauce mash also enhances the browning of the product through the Maillard reaction (*7*), because koji could supply reducing sugar.

In a previous paper, we showed that the concurrent addition of barley koji (barley steamed and molded with *Aspergillus oryzae*) and halotolerant microorganisms (HTMs), *Zygosaccharomyces rouxii*, *Candida versatilis*, and *Tetragenococcus halophilus*, at the beginning of the fermentation in chum salmon sauce production could enrich free amino acid and severely mitigate fishy odor without browning of the product (*8*). However, the time course of flavor formation, mechanisms of browning suppression, and effects of HTM inoculation on the chemical components have not been elucidated.

Z. rouxii and *C. versatilis* have been known as flavor-producing yeast in soy sauce fermentation (*9*−*12*). *T. halophilus* produces lactic acid as the main organic acid in soy sauce (*13*). These three HTMs have been used as commercial starters in soy sauce fermentation to enhance the flavor and taste.

The objectives in this paper are to elucidate the effects of koji and HTM inoculation on the sensory quality, color diversity, and chemical components of fermented chum salmon sauce (FCSS). Also the generation of some flavor compounds, typical in FCSS fermented with halotolerant yeast (HTY), was investigated.

MATERIALS AND METHODS

Koji and HTMs. The concentrated starter cultures of HTMs, *T. halophilus*, *Z. rouxii*, and *C. versatilis*, were purchased from Bioc Co. (Toyohashi, Japan). Barley koji (barley steamed and molded with *A. oryzae*) was also supplied from the same company.

Preparation of FCSS. Chum salmon (*Oncorhynchus keta*) were caught off of the northern coast of Japan and kept frozen at −25 °C until use. After the chum salmon were thawed at 4 °C for 16 h, they were gutted and ovarian tissues were removed. They were then cut and minced with a meat chopper (82 mm grinder, Higashimoto Co., Nara, Japan). A total of 800 g of commercial salt and 800 g of barley koji rehydrated according to the instructions of the manufacturer were mixed into 4000 g of the minced salmon. Then, variously combined HTMs (**Table 1**) were suspended in

*To whom correspondence should be addressed. E-mail: yoshikawa-syuji@hro.or.jp.

 Published on Web 04/21/2010

Table 1. Combinations of the starter microorganisms inoculated in the FCSS mash

mash number	barley koji	*Z. rouxii*	*C. versatilus*	*T. halophilus*
1	−	−	−	−
2	+	−	−	−
3	+	+	−	−
4	+	−	+	−
5	+	−	−	+
6	+	+	+	−
7	+	+	−	+
8	+	−	+	+
9	+	+	+	+

200 mL of 5% (w/v) salt solution and inoculated in the mash. The viable cell counts of each microbe in the salt solution and in the mash were 2.9×10^7 colony forming unit (CFU)/mL and 1.0×10^6 CFU/g, respectively. The FCSS mashes were fermented at 35 ± 2.5 °C for 84 days. The preparation and fermentation of FCSS mashes were carried out under non-aseptic conditions similar to industrial fish sauce production.

During the fermentation, parts of FCSS mashes were periodically picked up and centrifuged at 6800*g* for 30 min at 4 °C. The top fat layer was carefully removed, and the remaining supernatant was heated at 85 °C for 30 min in a water bath. The supernatant was mixed with 0.5% diatomaceous earth and then allowed to stand for 3 days at room temperature. Finally, the product was obtained by filtration through a layer of 5C filter paper (Advantec, Tokyo, Japan) with suction.

Analysis of Chemical Components of FCSS. Mash did not liberate FCSS in the early stage of fermentation. After 28 days, part of the mash was drawn and FCSS was expressed at 14 day intervals to determine some chemical characteristics and flavor compounds.

The total nitrogen and reducing sugar were determined by the Kjeldahl method (*14*) and the Somogyi−Nelson method using glucose as a standard (*15*), respectively.

For free amino acid composition analysis, 1 mL of 5-fold diluted FCSS was vigorously mixed with 4 mL of 99.5% ethanol and centrifuged at 2100*g* for 15 min. A total of 300 μL of the supernatant was vacuum-dried, redissolved in 1 mL of 0.02 N hydrochloric acid, and subjected to an automatic amino acid analyzer (L-8800, Hitachi, Tokyo, Japan).

Concentrations of formic, citric, and pyrubic acids were measured with F-kit for formic, L-citric, and pyrubic acids, respectively, according to the instructions of the manufacturer (Roch Diagnostics, Basel, Switzerland). Concentrations of L-lactic, L-malic, pyroglutamic, succinic, and acetic acids were determined with high-performance liquid chromatography (HPLC). FCSS samples were diluted 5-fold with water, filtrated through a 0.45 μm cellulose acetate filter, and then analyzed using a HPLC system (system 8010, Tosoh, Tokyo, Japan) with the following conditions: columns, a TSK-gel OApak-P (6.0 × 40 mm) and two TSK-gel OApak-A (7.8 × 300 mm) (Tosoh, Tokyo, Japan) catenated in tandem; mobile phase, 0.75 mM sulfuric acid (pH 2.8); flow rate, 0.8 mL/min; reaction mixture, 0.2 mM bromothymol blue (BTB)−15 mM disodium phosphate (pH 8.6); column temperature, 60 °C; detection, absorbance at 450 nm.

The ethanol content was determined by gas chromatography (GC) (*16*). FCSS samples were mixed with 0.25 volume of acetone as an internal standard and then analyzed using a 263-70 gas chromatograph (Hitachi, Tokyo, Japan) with the following conditions: column, 5% polyethylene glycol 1000, 3 mm × 2.1 m glass (GL Sciences, Tokyo, Japan); column temperature, 75 °C; detector, flame ionization detector (FID); injector/detector temperature, 230 °C; carrier gas, nitrogen; flow rate, 40 mL/min.

Flavor compounds were also detected with GC analysis equipped with a capillary column. Three typical flavor compounds in soy sauce, 2-phenyl ethanol (2-PE), 4-ethyl guaiacol (4-EG), and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF), were extracted from FCSS with the same modified solid extraction method as Suezawa (*17*). Sep-Pak C18 plus cartridge (Waters, Milford, MA) was conditioned with successive, 10 mL each of methanol and water. Before the solid extraction, FCSS was saturated with sodium chloride. A total of 5 mL of FCSS saturated with sodium chloride was vigorously mixed with 50 μL of 1 mg/mL 1-decanol as an internal standard and then loaded onto the conditioned cartridge. After loading the sample, flavor compounds were extracted with 0.5−0.8 mL of methyl acetate. The capillary GC analysis for 2-EP, 4-EG, and HEMF in the extracts was performed as described by Owari et al. (*18*) with some modification using a GC16A system (Shimadzu, Kyoto, Japan) under the following conditions: column, TC-WAX (0.25 mm × 30 m, 0.25 μm film, GL Sciences, Tokyo, Japan); column temperature, initial at 40 °C for 3 min, then raised to 210 °C at the rate of 4 °C/min, and finally held at 210 °C for 6 min; detector, FID; injector/detector temperature, 250 °C; carrier gas, helium; flow rate, 0.5 mL/min.

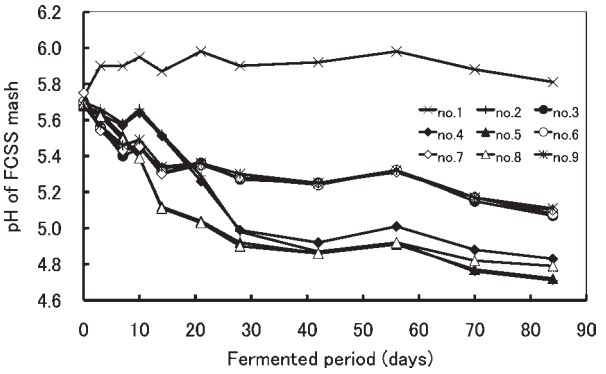

Figure 1. Changes in pH of FCSS mashes during fermentation. The minced chum salmon was inoculated with various combinations of microbial starters and fermented at 35 °C. Combinations of starter microorganisms for FCSS mashes 1−9 are shown in **Table 1**.

Measurement of the Browning Degree of FCSS. The browning degree of FCSS was evaluated as the optical density (OD) of FCSS at 550 nm measured using a spectrophotometer (UV-1200, Shimadzu, Kyoto, Japan).

Sensory Evaluation. A taste panel was composed of well-trained six members (four men and two women) aged 25−45 who have been previously evaluated soup and seasonings. FCSS samples were diluted 5-fold with water and served to panels. Eight FCSS samples were compared to the control FCSS fermented only with koji for the following seven descriptors of quality: soy-sauce-like flavor, umami, tartness, saltiness, sweetness, lightness of the color, and total favorableness. For four descriptors, except for lightness of the color, FCSS samples were scored on 1−5 scales: 1, very weak (particularly unfavorable); 2, strong (unfavorable); 3, same as the control; 4, strong (favorable); 5, very strong (particularly favorable). Lightness of the color was scored on 0−6 scales: 0, particularly dark; 1, dark; 2, relatively dark; 3, same as the control; 4, relatively light; 5, light; 6, particularly light. The general results for the whole panel were consolidated. Scores were analyzed using two-way analysis of variation (ANOVA) (*19*).

RESULTS

Changes in pH Value of FCSS Mash during Fermentation. The starter microorganism mixtures inoculated in the FCSS mash were listed in **Table 1**. The time course of pH change of the FCSS mashes during the fermentation was shown in **Figure 1**. The pH of mash 1, without inoculation of HTM or koji, was almost constant in the range between 5.7 and 6.0 throughout the fermentation. In contrast, pH values of other FCSS mashes containing koji were lowered during the fermentation. pH values of mashes 2, 4, 5, and 8 were remarkably lowered in the first 28 days of fermentation and then slightly lowered. In the other FCSS mashes inoculated with HTM containing *Z. rouxii*, pH values were quickly lowered only in the first 14 days of fermentation and then reduced slightly.

Changes in Chemical Characteristics of FCSS during Fermentation. The time course of free amino acid contents in the FCSS fermented with various combinations of HTMs was shown in **Figure 2**. Free amino acid contents of all FCSS samples were increased during the fermentation and became stated at the 84th day, when the fermentation was finished. The free amino acid content of sample 1 was the least of all samples during the fermentation period. The addition of koji was effective on the increase in free amino acid of the FCSS. FCSS samples fermented

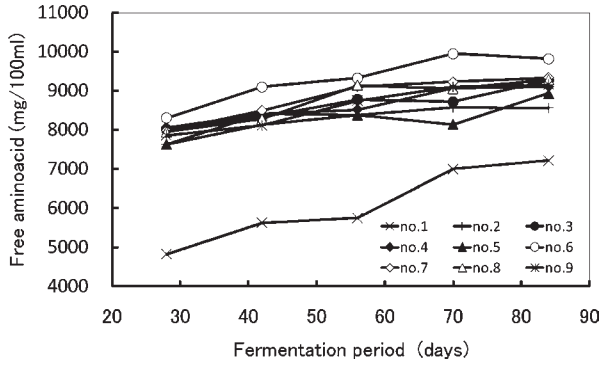

Figure 2. Changes in free amino acid content in FCSS samples inoculated with combined microbial starters during fermentation at 35 °C.

Table 2. Free amino acid contents of FCSS samples fermented for 84 days with various combinations of the HTMs (mg/100 mL)[a]

amino acid	1	2	3	4	5	6	7	8	9
alanine	532	676	710	689	674	750	698	704	667
arginine	27	410	697	523	165	758	697	276	670
asparagine	89	77	nd	58	81	30	49	78	nd
aspartic acid	336	636	566	589	654	600	613	609	604
glutamic acid	613	715	821	801	806	890	795	826	844
glutamine	nd	nd	nd	9	15	nd	14	5	nd
glycine	543	451	479	475	451	520	461	466	475
histidine	82	127	158	93	150	174	136	164	154
isoleucine	223	395	428	434	406	439	429	436	446
leucine	433	732	731	794	754	730	788	810	822
lysine	695	732	921	864	813	1010	845	881	912
serine	18	381	414	398	382	445	401	392	412
methionine	189	250	281	282	269	302	269	284	302
phenylalanine	176	314	381	373	332	401	358	361	379
proline	190	423	410	502	385	432	391	407	392
threonine	251	372	416	399	374	443	395	396	408
tryptophan	25	16	40	14	11	37	42	12	35
tyrosine	316	244	196	92	223	203	186	218	192
valine	312	511	581	565	533	608	565	565	569
β-alanine	nd	183	118	137	164	94	159	128	115
α-amino-*n*-butyric acid	nd	18	21	26	27	nd	23	29	nd
γ-aminobutyric acid	2	54	31	32	23	26	38	22	nd
β-aminoisobutyric acid	nd	37	nd	8	68	nd	nd	46	29
anserine	950	523	640	608	611	692	617	636	625
carnosine	24	7	8	5	13	8	10	7	8
cystathionine	nd	nd	nd	3	28	nd	21	27	nd
hydroxylysine	nd	nd	nd	2	nd	nd	nd	nd	nd
hydroxyproline	35	36	34	46	31	37	33	32	34
1-methyl histidine	0	0	0	3	nd	nd	nd	nd	nd
3-methyl histidine	3	2	2	nd	nd	2	2	nd	2
ornithine	408	108	6	137	336	7	7	306	9
phosphoserine	8	37	20	25	31	17	26	27	21
sarcosine	nd	nd	13	17	24	16	22	25	13
total	6633	8565	9271	9101	8933	9818	9157	9279	9335

[a] Combinations of starter microorganisms for FCSS samples 1−9 are listed in **Table 1**. nd = not detected. All values are means of duplicate measurements.

with HTM and koji had higher free amino acid than sample 2 fermented only with koji.

Free amino acid composition of FCSS samples fermented for 84 days are shown in **Table 2**. Aspartic acid, alanine, glutamic acid, glycine, lysine, and anserine were mainly detected from all FCSS samples. Arginine and serine in FCSS 1 were less than in the other FCSS samples inoculated with HTM or koji. On the other hand, glycine, tyrosine, anserine, and ornithine contents of FCSS 1 showed a higher value than those of other FCSS samples. There was no characteristic feature about other amino acid contents among all of the FCSS samples.

Table 3. Organic acid contents of FCSS samples fermented for 84 days with various combinations of the HTMs (mg/100 mL)[a]

organic acid	1	2	3	4	5	6	7	8	9
malic acid	93	102	171	123	111	183	163	112	165
pyroglutamic acid	420	653	662	637	637	675	626	617	628
lactic acid	2870	3642	1365	2959	4245	1333	1324	3880	1352
acetic acid	914	489	701	621	536	227	591	425	431
succinic acid	nd	nd	415	113	nd	443	273	64	412
pyruvic acid	2	10	4	4	5	3	3	7	5
formic acid	38	20	12	3	1	4	10	2	5
citric acid	5	nd	47	16	10	41	36	13	35
total	4341	4916	3377	4476	5546	2910	3026	5121	3032

[a] Combinations of starter microorganisms for FCSS samples 1−9 are listed in **Table 1**. nd = not detected. All values are means of duplicate measurements.

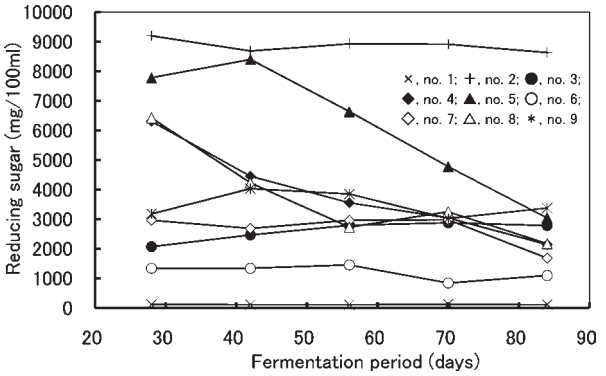

Figure 3. Changes in reducing sugar content in FCSS samples inoculated with combined microbial starters during fermentation at 35 °C. Reducing sugar was calculated as glucose.

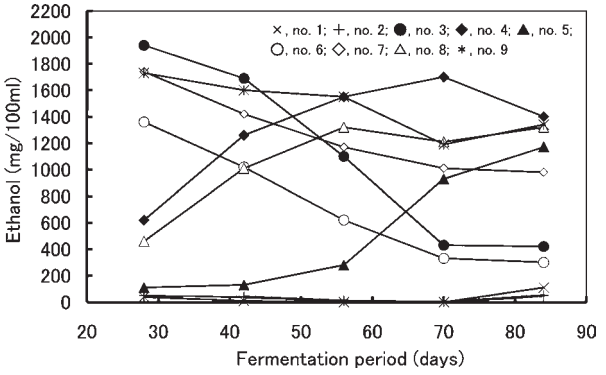

Figure 4. Changes in the ethanol content in FCSS samples inoculated with combined microbial starters during fermentation at 35 °C.

Organic acid composition of FCSS samples fermented for 84 days are shown in **Table 3**. The total organic acid contents in FCSS inoculated with the starter containing *Z. rouxii*, samples 3, 6, 7, and 9, were less than those in the other FCSS samples. FCSS samples 5 and 8, inoculated with *T. halophilus*, showed higher organic acid content. Lactic, acetic, and pyroglutamic acids were mainly detected in all FCSS samples. Succinic acid was detected only from yeast-inoculated FCSS samples, and especially FCSS samples 3, 6, 7, and 9 inoculated with *Z. rouxii* had a higher content of succinic acid and a lower content of lactic acid.

Changes in reducing sugar content of FCSS samples during fermentation are shown in **Figure 3**. The reducing sugar content was the least in sample 1 and the highest in sample 2 of all FCSS samples tested during fermentation. The reducing sugar of sample 5 was decreased remarkably after 42 days of fermentation. In

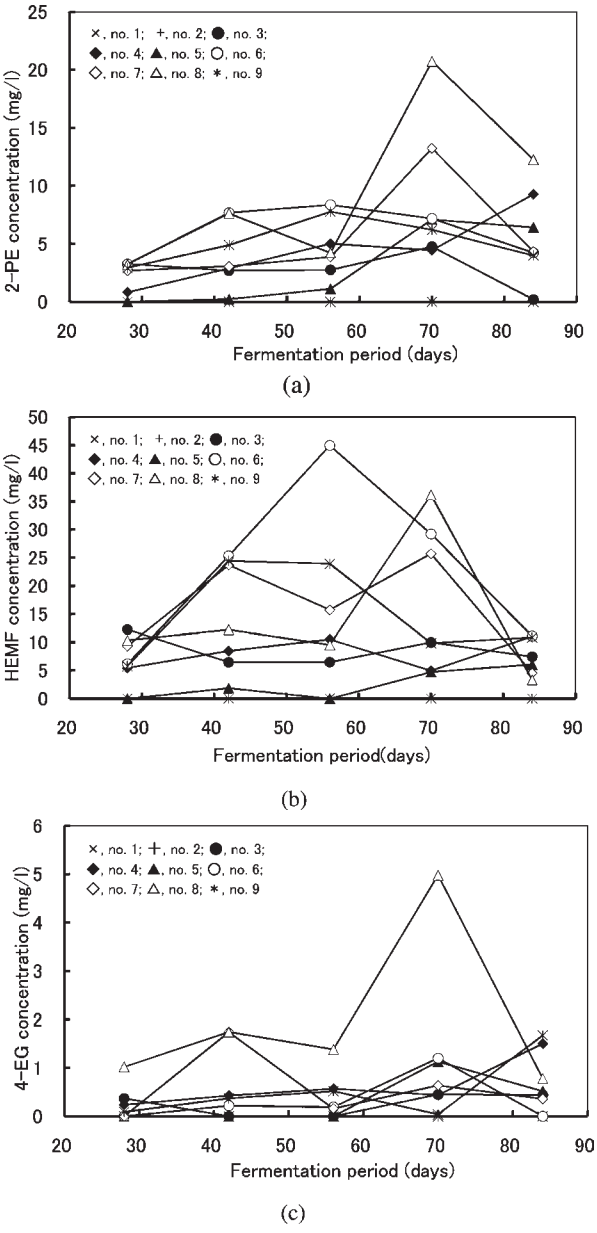

Figure 5. Changes in the content of flavor compounds in FCSS samples inoculated with combined microbial starters during fermentation at 35 °C. Flavor compounds: (a) 2-PE, (b) HEMF, and (c) 4-EG. These compounds were not detected in samples 1 and 2 during the fermentation.

FCSS samples 4 and 8, a decreased rate of sugar was stalled after 56 days of fermentation. Reducing sugar contents of the other FCSS samples inoculated with HTMs hardly changed.

Changes in the ethanol content of FCSS samples during fermentation are shown in **Figure 4**. Ethanol was produced in FCSS samples inoculated with yeasts. In FCSS samples 3, 6, 7 and 9, inoculated with *Z. rouxii*, ethanol was greatly produced by the first 28 days of fermentation, and then samples 3 and 6 showed a remarkable decrease in the ethanol content. Ethanol in FCSS samples 7 and 9 was also decreased, but those decrease rates were lower than samples 3 and 6. In contrast, the ethanol contents of samples 4 and 8, inoculated with *C. versatilis*, were increased after 28 days of fermentation. Although HTY was not inoculated in sample 5 mash, ethanol in it was continuously increased after 56 days of fermentation. In the later period of the fermentation of sample 5 mash, the growth of contaminated yeast was confirmed by culture-dependent and -independent methods from a previous

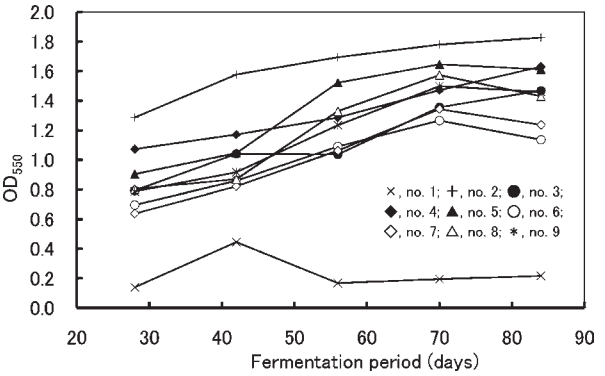

Figure 6. Changes in brownness (OD_{550}) of FCSS samples inoculated with combined microbial starters during fermentation at 35 °C.

paper (*20*). Therefore, the ethanol production of sample 5 might be due to the growth of contaminated yeast.

Production of Flavor Compounds by HTYs in FCSS during Fermentation. Changes in concentrations of three flavor compounds, 2-PE, HEMF, and 4-EG, in FCSS mash during fermentation are shown in **Figure 5**. None of these flavor compounds was detected in FCSS samples 1 and 2, not inoculated with HTYs throughout the fermentation. Although FCSS sample 5 was inoculated only with *T. halophilus*, these flavor compounds that should be specific in products fermented with yeasts were detected after 70 days of fermentation.

Concentration of 2-PE was less than 10 ppm in most of the FCSS samples inoculated with HTM. However, it reached 20 ppm in FCSS sample 8 at the 70th day. HEMF was produced less than 15 ppm in FCSS samples 3, 4, and 5, while it reached over 20 ppm in samples 6, 7, 8, and 9. FCSS sample 6 at the 56th day of fermentation contained the highest concentration (45 ppm) of HEMF among the samples. FCSS sample 8 had the highest concentration of 4-EG among the samples in every sampling time, and its maximum concentration reached 5 ppm in the mash.

Browning of FCSS during Fermentation. The optical density at 550 nm was measured to estimate the degree of browning of the FCSS (**Figure 6**). Sample 1 without inoculation of koji and HTMs showed the lowest browning of all FCSS samples throughout the fermentation period. Koji used samples without HTM inoculation, sample 2, showed the highest increase in brownness during the fermentation. FCSS samples inoculated with HTM showed lower browning than sample 2, regardless of the addition of koji, which might suggest that HTMs could suppress browning of FCSS during the fermentation.

Sensory Properties of FCSS Samples. Among the seven descriptors, a significant difference was observed in soy-sauce-like flavor, lightness of the color, umami, and total favorableness (**Figure 7**). Soy-sauce-like flavor was only given by the addition of both koji and HTM inoculation. FCSS samples inoculated with only *Z. rouxii* or two of three HTMs showed a significantly higher umami score than FCSS samples not inoculated with either koji or HTY. While lightness of the color was decreased by koji addition, FCSS samples inoculated with HTY were not darkened, owing to koji addition. Koji-added FCSS samples showed the higher score in total favorableness.

DISCUSSION

The pH of sample 1 mash without the starter microorganisms remained almost constant during the fermentation, and in contrast, pH values of the other mashes with the addition of koji have fallen from the beginning of fermentation (**Figure 1**). Generally,

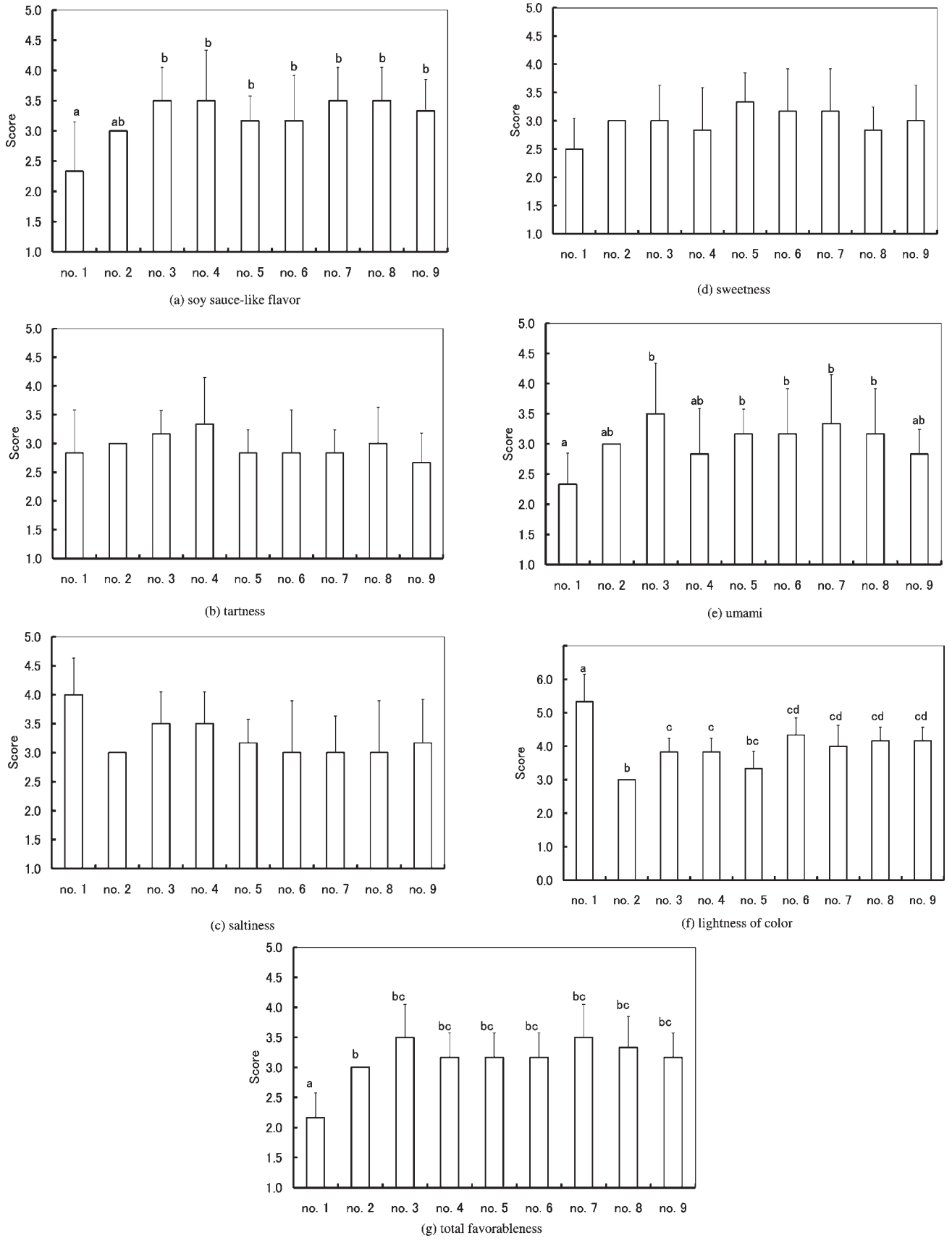

Figure 7. Sensory properties of FCSS samples inoculated with various combined microbial starters. Eight FCSS samples were compared to the FCSS sample fermented only with koji (sample 2 in **Table 1** used as a control) with seven descriptors of quality; soy-sauce-like flavor, tartness, saltiness, sweetness, umami, lightness of color and total favorableness. For six descriptors except for lightness of the color, FCSS samples were scored on 1−5 scales: 1, very weak (particularly unfavorable); 2, strong (unfavorable); 3, same as the control; 4, strong (favorable); 5, very strong (particularly favorable). Lightness of the color was scored on 0−6 scales: 0, particularly dark; 1, dark; 2, relatively dark; 3, same as the control; 4, relatively light; 5, light; 6, particularly light. The general results for the whole panel were consolidated and analyzed using two-way analysis of variation (ANOVA) (*19*). (∗) $p < 0.05$.

the glucide content of marine products is relatively less than that of agro-products (*21*) and, therefore, should not be sufficient for microorganisms to produce organic acid to drop pH. In this experiment, starch in koji was glycolysed by *A. oryzae* and then starter or HTMs from the environment could produce organic acid from glucide supplied from the koji. The pH decrease rate of mash (sample 8) inoculated with *T. halophilus* and *C. versatilis* showed the same rate as that of mash (sample 5) inoculated with *T. halophilus* alone. In the soy sauce fermentation, it has been known that *C. versatilis* does not influence the growth of *T. halophilus* (*22*).

In soy sauce fermentation, when the growth of *Z. rouxii* predominated over that of *T. halophilus*, it has been known that the pH of mash would tend to be higher because of the repression of *T. halophilus* growth (*23*−*25*). The organic acid concentration of FCSS samples inoculated with starters containing *Z. rouxii* was lower than others (samples 3, 6, 7, and 9), as shown in **Table 3**.

Succinic acid was detected in FCSS samples inoculated with yeast starters. It is known that succinic acid could be produced by autolysis of HTY in soy sauce fermentation (*26*). Succinic acid in the FCSS samples might be produced by the same mechanism as in soy sauce. The organic acid concentration of 1 and 2 samples in **Table 3** indicates that lactic acid should increase using glucide supplied from koji. Then, lactic acid was decreased because of the inhibition of *T. halophilus* growth or the competitive consumption of glucide by *Z. rouxii* (samples 3, 6, 7, and 9).

As for the amino acid composition of FCSS products, the arginine content of FCSS samples (samples 5 and 8) inoculated with *T. halophilus*, except with *Z. rouxii* at the same time, was the least of all samples. Decomposition of arginine by the *T. halophilus* strain was reported by Iituka et al. (*27*). It is known that ornithine and citrulline were formed from arginine by lactic acid bacteria or koji (*A. oryzae*) (*28*). This information seems to explain the higher ornithine content and lower arginine content of samples 5 and 8. In the case of samples 7 and 9, *Z. rouxii* and *T. halophilus* were concurrently inoculated and *Z. rouxii* is presumed to inhibit the conversion of arginine to ornithine by *T. halophilus*. It was also reported that the strain that had an arginine degrading activity or an amino acid decarboxylating activity, repressed the acidification of mash (*13*).

Park et al. (*29*) reported that alanine, aspartic acid, cystine, glutamic acid, histidine, methionlne, proline, tyrosine, valine, and pyroglutamic acid were taste-active components in Vietnamese fish sauce. Percentages of the taste-active compounds to the amount of free amino acid and organic acid in FCSS samples 1−9 were 21.7, 30.7, 31.8, 30.8, 31.4, 33.6, 31.7, 31.8, and 30.8, respectively. This suggested that the addition of koji did not only increase the free amino acid by proteolysis but was also effective at enriching the taste of FCSS. Furthermore, FCSS samples with koji added had a stronger aftertaste and foretaste and less bitterness than that fermented without koji and HTMs. This might be due to the difference of the composition of taste-active compounds.

The reducing sugar content in sample 1, fermented without koji and HTMs addition, was at a remarkably low level, because of the lack of a great sugar source (**Figure 3**). Sample 2, added only with koji, showed the greatest sugar content among the samples, and the other FCSS samples (3−9) inoculated with HTMs contained less sugar. HTY counts of the FCSS mash of this experiment were reported in detail in a previous paper (*20*). From 2 mash, HTY as a reducing sugar consumer had not been detected throughout fermentation. In mashes 3, 6, 7, and 9, the first growth peak of HTY was observed within 10 days and reducing sugar would be almost consumed by the starter HTY in the early period of fermentation. Reducing sugar contents of FCSS samples 4 and 8, in which HTY counts were less than the initial count until the 14th day, were higher than those of other HTY-inoculated FCSS samples. The fluctuation of the sugar content in each FCSS might reflect the growth pattern of HTY. In mash 5, the maximum growth (about 10^8 CFU/g of mash) of halotolerant lactic acid bacteria was observed from the 10th to 28th day and then the growth of wild yeasts was observed after 42 days of fermentation. The reducing sugar content of FCSS sample 5 was likely to be affected by the yeast rather than lactic acid bacteria (HTL). Thus, the decrease in sugar during fermentation might be closely related to the function of HTY. The reducing sugar content of FCSS should be characterized by the supply from koji and consumption by HTY.

Z. rouxii produced a greater amount of ethanol than *C. versatilis* (**Figure 4**). The ethanol content of FCSS inoculated with HTM starters including *Z. rouxii* (samples 3, 6, 7, and 9) was the highest at the 28th day and then decreased during fermentation. On the other hand, the maximum ethanol content was observed at the 70th and 56th days in FCSS sample 4 inoculated with *C. versatilis* alone and in FCSS sample 8 inoculated with *C. versatilis* and *T. halophilus*, respectively. These suggest that the ethanol formation in FCSS at the earlier and later period of the fermentation depended upon *Z. rouxii* and *C. versatilis*, respectively. Thus, the ethanol content should also be closely related to HTY growth. A decrease in the ethanol content produced from sugar by HTY in the FCSS samples during fermentation might be due to consumption to produce other compounds, such as ethyl ester.

Some flavor compounds that have been typical in soy sauce fermented with HTYs were detected in FCSS with HTY of this study. Furanone is one of the characteristic flavor compounds in soy sauce, and HEMF is especially known as a flavor compound produced by yeast (*30*, *31*). However, HEMF had not been detected in fish sauce previously (*3*−*5*). 4-EG is known as a specific secondary metabolite produced from ferulic acid by *C. versatilis* or *C. etschelsii* in soy sauce fermentation (*9*−*11*). 2-PE, produced mainly by *Z. rouxii*, is also known as a characteristic flavor of soy sauce (*12*). Thus, these flavor compounds in fish sauce fermentation might be produced through inoculation of HTY to the mash. The production of these compounds in fish sauce has not been shown by former experiments (*5*−*7*), and this is the first report to detect these flavor compounds in fish sauce. In this report, HEMF in the fish sauce could be successfully detected by solid-phase extraction after saturation with sodium chloride.

Three compounds were not detected from FCSS samples 1 and 2 without HTY. From FCSS sample 5 inoculated only with *T. halophilus*, however, these flavor components were detected after 70 days of fermentation. HTYs, *C. versatilis* and *Pichia guilliermondii*, were detected after 42 days of fermentation (*20*). These flavor components might be produced by yeasts fortuitously contaminated. All FCSS samples inoculated with *Z. rouxii* contained 2-PE. 4-EG was detected from all FCSS samples inoculated with *C. versatilis*. Ethanol produced in FCSS sample 5 at the later period of fermentation is also considered to reflect the action of the wild HTY.

The excess amount of 4-EG has been perceived as an unpleasant chemical flavor of the product. Yokotsuka et al. (*10*) reported that the preferable concentration of 4-EG in high-quality soy sauce ranged from 0.5 to 1 ppm and not over 2 ppm. All HTM starters containing *C. versatilis* produced 4-EG in the favorable range at the 84th day.

HEMF, 2-PE, and 4-EG were detected even in FCSS sample 5 inoculated only with *T. halophilus*. In a previous paper (*20*), HTY was detected after the 42nd day from the mash inoculated only with *T. halophilus* and was identified as *C. versatilis* and

P. guilliermondii. The viable cell count of these yeasts reached ca. 10^7 CFU/g and was almost the same as in FCSS mash inoculated with HTY. The concentrations of these flavor compounds produced in FCSS sample 5, however, were lower than those of HTY-inoculated FCSS samples at the start of fermentation. In FCSS samples 4 and 8, HEMF and 2-PE were formed without *Z. rouxii* inoculation. In those cases, non-starter yeast might temporally grow and produce them. The growth of non-starter yeasts contaminated accidentally would cause the instability of qualities, such as flavors. As mentioned above, the inoculation of yeast starters at the start of fermentation was considered to be effective for enrichment of favorable flavors in FCSS.

As shown in **Figures 3** and **6**, FCSS samples of higher sugar content showed a higher degree of brownness. Because fish meat as the main material of fish sauce contained a limited amount of reducing sugar for the Maillard reaction, the FCSS fermented without koji would show weak browning (sample 1 in **Figure 6**). In a previous paper, we pointed out that the pH falling within the first 7 days of fermentation could prevent FCSS from browning (*8*). FCSS sample 5 showed the highest browning of all FCSS samples inoculated with HTMs, and reducing sugar in FCSS was rapidly consumed for browning after the 42nd day and finally reached the same level as in the other HTM-inoculated FCSS samples.

Sensory evaluation showed that HTM inoculation did not affect saltiness, tartness, and sweetness. However, HTM and koji inoculation had significant quality-improving effects concerning soy-sauce-like flavor, lightness of color, umami, and total favorableness. Panelists recognized more soy-sauce-like flavor from FCSS fermented by HTY and koji than that produced without koji and HTM. Three typical flavor components of soy sauce produced by HTY starter were detected from HTY-inoculated FCSS (samples 3, 4, 6, 7, 8, and 9) and wild-yeast-contaminated FCSS (sample 5). This suggests that the soy-sauce-like flavor was conferred to FCSS by HTY. HTM-inoculated FCSS samples had a less fishy odor in a previous paper (*8*). HTM-inoculated FCSS samples in this experiment also have a less fishy odor. The fishy odor might be masked with soy-sauce-like flavor compounds produced by HTY starter. Although the amount of organic acids was different among the FCSS samples, it did not affect both tartness and total favorableness of the FCSS samples. The lightness score of the color reflected the browning (optical density at 550 nm) of FCSS. It was considered that the combination of the starter and koji (FCSS samples 6−9) was suitable to produce the FCSS with light color. The soy-sauce-like flavor and umami might affect positively the total favorableness because of similarity of the score pattern among them.

In conclusion, inoculation with HTM starters and koji in FCSS mash at the beginning of fermentation was effective to improve color and confer soy-sauce-like flavor and umami on fish sauce. The following combinations of koji and HTM starters are especially suitable for producing an ideal quality of fish sauce: koji, *Z. rouxii*, and *T. halophilus*; koji, *Z. rouxii*, and *C. versatilis*; and koji, *Z. rouxii*, *C. versatilis*, and *T. halophilus*. Thus, microbial starters could be useful for control characteristics and quality of fish sauce.

ACKNOWLEDGMENT

The authors gratefully acknowledge Dr. Tadanobu Nakadai, Japan Soy Sauce Technology Center, and Dr. Tsutomu Abe, Hokkaido Food Processing Research Center, for suggesting the analytical method for the flavor component, Yoshifumi Tamura, Hokkaido Food Processing Research Center, for his assistance on analyses of organic acid composition and ethanol content, and Dr. Makoto Suzuki, Mizkan Nakanosu Co., for sensory evaluation of the FCSS samples.

Note Added after ASAP Publication

There were changes to the text, in the last two paragraphs of the Results section, in the version of this paper published ASAP April 21, 2010; the corrected version published ASAP April 23, 2010.

LITERATURE CITED

(1) Aryanta, W. R. Traditional fermented foods in Indonesia. *Jpn. J. Lactic Acid Bact*. **2000**, *10*, 90–102.

(2) Sanchez, P. C. Microorganisms and technology of Philippine fermented foods. *Jpn. J. Lactic Acid Bact*. **1999**, *10*, 19–28.

(3) Fukami, K.; Ishiyama, S.; Yaguramaki, H.; Masuzawa, T.; Nabeta, Y.; Endo, K.; Shimoda, M. Identification of distinctive volatile compounds in fish sauce. *J. Agric. Food Chem*. **2002**, *50*, 5412–5416.

(4) Funatsu, Y.; Kato, I.; Kawasaki, K.; Konagaya, S.; Usui, K. Determination of volatile compounds in fish sauces prepared from the waste of the frigate mackerel surimi processing and a comparison with those in several Asian fish sauces. *Nippon Suisan Gakkaishi* **2001**, *67*, 489–496.

(5) Funatsu, Y.; Kawasaki, K.; Konagaya, S. A comparison of volatile compounds in fish sauces prepared from frigate mackerel by use of soy sauce koji with those in Japanese fish sauce and soy sauce, with special reference to the flavor. *Nippon Suisan Gakkaishi* **2001**, *67*, 1110–1119.

(6) Dohmoto, N.; Wang, K. C.; Mori, T.; Kimura, I.; Koriyama, T.; Abe, H. Development of new type fish sauce using the soy sauce fermentation method. *Nippon Suisan Gakkaishi* **2001**, *67*, 1103–1109.

(7) Takeshima, F.; Nabeshima, Y.; Funatsu, Y.; Kawasaki, K. Development of fish sauce produced from sexually maturated chum salmon. *Bull. Toyama Food Res. Inst*. **2001**, *4*, 1–8.

(8) Yoshikawa, S.; Tanaka, A.; Nishikiori, T.; Ohta, T. Development of fish sauce from chum salmon by fermentation with barley koji and halotolerant microorganisms. *Nippon Shokuhin Kagaku Kogaku Kaishi* **2006**, *53*, 281–286.

(9) Asao, Y.; Sakai, T.; Yokotsuka, T. Studies on flavorous substances in shoyu. Part XVI: Flavorous components produced by yeast fermentation. *Nippon Nougeikagaku Kaishi* **1967**, *41*, 434–441.

(10) Yokotsuka, T.; Asao, Y.; Sakai, T. Studies on flavorous substances in shoyu. Part XXVII: The production of 4-ethylguaiacol during shoyu fermentation, and its role for shoyu flavor. *Nippon Nougeikagaku Kaishi* **1967**, *41*, 442–447.

(11) Yokotsuka, T.; Sakai, T.; Asao, Y. Studies on flavorous substances in shoyu. Part XV: Flavorous components produced by yeast fermentation. *Nippon Nougeikagaku Kaishi* **1967**, *41*, 428–433.

(12) Suezawa, Y. Bioconversion of ferulic acid and *p*-coumaric acid to volatile phenols by halotolerant yeasts. *Nippon Nougeikagaku Kaishi* **1995**, *69*, 1587–1596.

(13) Uchida, K. Variance of lactic acid bacteria and its application for soy sauce brewing. *J. Brew. Soc. Jpn*. **1982**, *77*, 740–742.

(14) Association of Official Analytical Chemists (AOAC). Protein (crude) determination in animal feed: Copper catalyst Kjeldahl method (984.13). In *Official Methods of Analysis of the Association of Official Analytical Chemists International*, 16th ed.; AOAC International: Arlington, VA, 1990.

(15) Nelson, N. A. Photometric adaptation of Somogyi method for the determination of glucose. *J. Biol. Chem*. **1944**, *153*, 375–380.

(16) Tamura, Y. Manufacture of the vinegar from cheese whey. *Milk Sci*. **2000**, *49*, 15–20.

(17) Suezawa, Y. Rapid analysis of 2-phenylethanol, 4-ethylguaiacol and 4-ethylphenol in soy sauce by the use of solid-phase extraction method. *J. Nihon Soy Sauce Res. Inst*. **1998**, *24*, 21–25.

(18) Owari, K.; Takahashi, K.; Watanabe, T. Relationship between halotolerant yeast in shoyu mash and flavor HEMF. *J. Nihon Soy Sauce Res. Inst*. **2000**, *26*, 191–195.

(19) Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 6th ed.; The Iowa State University Press: Ames, IA, 1967.
(20) Yoshikawa, Y.; Yasokawa, D.; Nagashima, K.; Yamazaki, K.; Kurihara, H.; Tanaka, A.; Nishikiori, T.; Ohta, T.; Kawai, Y. Microbiota during fermentation of chum salmon (*Oncorhynchus keta*) sauce mash inoculated with halotolerant microbial starters: Analyses using the plate count method and PCR-denaturing gradient gel electrophoresis (DGGE). *Food Microbiol*. **2010**, in press.
(21) Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT). *Standard Tables of Food Composition in Japan*, 5th ed.; National Printing Bureau: Tokyo, Japan, 2005.
(22) Fujimoto, H. Microorganisms. In *Science and Technology in Soy Sauce Brewing*, 6th ed.; Tochikura, T., Ed.; Brewing Society of Japan: Tokyo, Japan, 1994; pp 152−227.
(23) Fujimoto, H. Studies on the lactic acid bacterium for soy sauce brewing. *J. Nihon Soy Sauce Res. Inst*. **1982**, *8*, 260–266.
(24) Kusumoto, E.; Inamori, K.; Uchida, I.; Yoshino, H. Studies on microorganisms involved in fermentation of soy sauce mash (Part I). On mixed culture of the lactic acid bacterium and yeast isolated from soy sauce mash. *J. Nihon Soy Sauce Res. Inst*. **1977**, *3*, 283–289.
(25) Matsumoto, I.; Imai, S. Influences of salt concentration in water phase and inoculum size of *Saccharomyces rouxii* on lactic acid fermentation by *Pediococcus halophilus* in salty miso. *Nihon Shokuhin Kogyo Gakkaishi* **1980**, *27*, 112–117.
(26) Nakayama, S.; Takeda, R. The formation of organic acids in the fermentation of amino acid by *Saccharomyces rouxii* sp. Part 1: The formation of succinic acid from L-glutamic acid by washed yeast cells. *Seasoning Sci*. **1969**, *16*, 1–6.
(27) Iituka, K.; Goan, M. Studies on L-arginine decomposition in soy sauce mash. *Seasoning Sci*. **1973**, *20*, 17–24.
(28) Ogasawara, T.; Ito, K.; Abe, N.; Honma, H. Studies on the basic amino acid of the soy sauces and the seasoning liquids: Part II. *Nippon Nougeikagaku Kaishi* **1963**, *37*, 208–213.
(29) Park, J.-N.; Ishida, K.; Watanabe, T.; Endoh, K.; Watanabe, K.; Murakami, M.; Abe, H. Taste-active components in Vietnamese fish sauce. *Fish. Sci*. **2002**, *68*, 913–920.
(30) Nunomura, N.; Sasaki, M.; Asao, Y.; Yokotsuka, T. Isolation and identification of 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone, as a flavor component in shoyu (soy sauce). *Agric. Biol. Chem*. **1976**, *40*, 491–495.
(31) Sasaki, M.; Nunomura, N. Constituents of top note aroma of shoyu. *Nihon Kagaku Kaishi* **1981**, 736–745.

Received for review December 24, 2009. Revised manuscript received March 31, 2010. Accepted April 4, 2010.