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# Use of *Tetragenococcus halophilus* as a Starter Culture for Flavor Improvement in Fish Sauce Fermentation

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**ABSTRACT:** The potential of *Tetragenococcus halophilus* as a starter culture for flavor improvement in fish sauce fermentation was elucidated. Four strains of *T. halophilus* isolated from fish sauce mashes were inoculated to anchovy mixed with 25% NaCl with an approximate cell count of $10^6$ CFU/mL. The α-amino content of 6-month-old fish sauce samples inoculated with *T. halophilus* was 780−784 mM. The addition of *T. halophilus* MRC10-1-3 and *T. halophilus* MCD10-5-10 resulted in a reduction of histamine ($P < 0.05$). Fish sauce inoculated with *T. halophilus* showed high contents of total amino acids with predominantly high glutamic acid. Major volatile compounds in fish sauce were 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and benzaldehyde. *T. halophilus*-inoculated fish sauce samples demonstrated the ability to reduce dimethyl disulfide, a compound contributing to a fecal note. The use of *T. halophilus* for fish sauce fermentation improves amino acid profiles and volatile compounds as well as reduces biogenic amine content of a fish sauce product.

**KEYWORDS:** *Tetragenococcus halophilus*, amino acids, biogenic amines, volatile compounds, fish sauce

## INTRODUCTION

Thai fish sauce, known as *nam pla*, is an important seasoning consumed widely in Southeast Asia and is gaining popularity worldwide. *Nam pla* is produced by mixing anchovy (*Stolephorus* sp.) with salt at a ratio of 3:1 and fermenting in a cement tank for 12−18 months.[1] Fish proteins are hydrolyzed by enzymes from fish and halotolerant/halophile microorganisms, resulting in peptides and amino acids. Ripened fish sauce is a clear brown liquid with a salty and umami taste with distinctive odor. The unique flavor of fish sauce is one of the important quality parameters resulting from protein hydrolysis and lipid oxidation during fermentation.[2] As fish sauce production solely relies on a natural fermentation process, control of the flavor/aroma quality is rather limited.

*Tetragenococcus halophilus* is one of the halophilic lactic acid bacteria found in fish sauce fermentation[3] and other fermented foods, such as soy sauce,[4] shrimp paste,[5] and fermented black beans.[6] The bacterial species can grow in a high-salt environment and is tolerant to 18% NaCl or greater.[7] Udomsil et al.[8] reported that *T. halophilus* could play an important role in aroma formation of fish sauce as they produced numerous volatile compounds, including 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and benzaldehyde, in anchovy broth containing 25% NaCl. In addition, lactic acid bacteria (LAB) exhibited intracellular aminopeptidase activities that convert peptides and/or oligopeptides to amino acids which, in turn, serve as precursors of volatile compounds.[8,9] 3-Methylbutanal and 2-methylpropanal are important aldehydes contributing to meaty and fishy notes, respectively.[9] The use of *T. halophilus* as a starter culture for fish sauce fermentation could be a means to improve the flavor characteristic of fish sauce. However, the role of *T. halophilus* on flavor improvement of fish sauce has not been systematically investigated.

The use of bacterial starter culture has been reported to improve the aroma characteristics of fish sauce as well as shorten the fermentation process. Fukami et al.[4] reported that fish sauce mash inoculated with *Staphylococcus xylosus* contained lower amounts of dimethyl disulfide and dimethyl trisulfide than did the control (without inoculation). Moreover, the inoculated sample had higher contents of 2-methylpropanal and 2-methylbutanal responsible for meaty note. The use of proteinase-producing bacteria, *Virgibacillus* sp. SK 33 and SK37 and *Staphylococcus* sp. SK1-1-5, as a starter culture reduced the fermentation time to 4 months and increased the desirable volatile compounds of fish sauce.[10] Free amino acids responsible for the unique taste of fish sauce also increased with addition of such bacterial starter cultures. Gildberg and Thongthai[11] demonstrated that the addition of *T. halophilus* to the fish sauce fermented at a reduced salt level of 20% resulted in lactic acid production. However, the role of *T. halophilus* as a starter culture on fish sauce quality pertaining to volatile compounds has not been systematically elucidated. Our objectives were to investigate the use of *T. halophilus* as a starter culture to improve flavor characteristics, particularly volatile compounds and amino acid profiles, of Thai fish sauce.

## MATERIALS AND METHODS

**Preparation of Starter Culture.** Four strains of halophilic lactic acid bacteria, namely, *T. halophilus* MS33, *T. halophilus* MRC10-1-3,

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*T. halophilus* MCD10-5-10, and *T. halophilus* MRC 10-7-8, accession no. FJ715465, FJ715466, FJ715468, and FJ715470, respectively (http://www.ncbi.nlm.nih.gov/genbank), were isolated from fish sauce fermentation and identified as detailed by Udomsil et al.[8] These isolates were cultured in 100 mL of fish broth containing 25% NaCl, pH 7.0, at 30 °C for 7−10 days under anaerobic condition to obtain approximate cell counts of $10^6$ CFU/mL after inoculation in fish sauce mash. Fish broth was prepared by boiling 1 part of Indian anchovy (*Stolephorus indicus*) with 2 parts of distilled water for 20 min. Then, the fish extract was filtered and NaCl was added to 25%. The pH was adjusted to 7.0. The extract was autoclaved at 121 °C for 15 min.[10]

**Fish Sauce Fermentation.** Indian anchovy (*Stolephorus indicus*) was obtained from the Gulf of Thailand at Chonburi province and kept in ice for up to 24 h before the fermentation experiment was begun. One kilogram of anchovy contained in a glass jar (9 cm diameter × 17 cm height) was incubated in a 65 °C water bath until the temperature at the center of the sample reached 65 °C. Subsequently, 0.25% of Alcalase 2.4 L (Novozyme Nordisk, Bagsvaerd, Denmark) was added, and the samples were further incubated for 2 h. The samples were then cooled to 50 °C, 0.5% Flavouzyme 500 L (Novozyme Nordisk, Bagsvaerd, Denmark) was added, and the mixture was further incubated at 50 °C for 4 h.[10] The samples were left at room temperature until the temperature attained 35 °C, when 25% (w/w) solar salt and 10% (v/w) LAB starter cultures were added. Fish broth without LAB was added to the control in the same volume that was added into the samples. All treatments were incubated in a 35 °C incubator for 6 months. Microbiological changes and α-amino content were monitored at 0, 14, 30, 60, 90, 120, 150, and 180 days of fermentation. Volatile compounds of the liquid fraction and other physicochemical properties, including salt, pH, and total nitrogen content, were determined when fermentation was completed at 180 days.[12]

**Microbiological Changes.** Fish sauce mashes (10 g) were aseptically taken at various fermentation intervals and enumerated for LAB population by spread plate method using De Man, Rogosa, and Sharpe (MRS) agar containing 18% NaCl and 0.5% $CaCO_3$ and incubated at 30 °C for 5−7 days under anaerobic condition. The population of haloplilic bacteria was also monitored using modified JCM168 medium containing 18% instead of 20% NaCl and incubated at 35 °C for 5−7 days under aerobic condition.

**Chemical Analyses.** *α-Amino Content.* The α-amino content of samples was followed using trinitrobenzenesulfonic acid (TNBS) with L-leucine as a standard.[13] One hundred microliters of fish sauce was added to 1 mL of 0.2125 M phosphate, pH 8.2. Subsequently, 1 mL of 0.05% TNBS was added, and the mixture was incubated at 50 °C for 1 h. After incubation, the reaction was stopped by the addition of 0.1 N HCl and left at room temperature for 30 min. Absorbance was measured at 420 nm using a spectrophotometer (GBC UV−vis 916; GBC Scientific Equipment Pty, Ltd., Australia).

*Amino Acid Profiles.* Fish sauce samples fermented for 6 months were analyzed for total and free amino acid profiles according to the method of Tungkawachara et al.[14] Standard amino acids were analyzed at the same condition and used for peak identification based on retention time. The amounts of amino acids were expressed as milligrams per 100 mL of fish sauce.

*Biogenic Amine Content.* Biogenic amines of fish sauce samples were analyzed according to the method of Yongsawatdigul et al.[15] Derivatization was carried out using dansyl chloride. Biogenic amines were separated using a mobile phase consisting of a mixture of acetonitrile and 0.02 M acetic acid (1:9) as a solvent A and a mixture of 0.02 M acetic acid, acetonitrile, and methanol (1:4.5:4.5) as solvent B at a flow rate of 1 mL/min on a Hypersil BDS column $C_{18}$ (3 μm, 100 × 4 mm). An HPLC (HP 1100, Agilent Technologies Inc., Palo Alto, CA) equipped with a diode array detector was used with the detection wavelength at 254 nm and the reference wavelength at 550 nm. The gradient elution was run as described by Udomsil et al.[8]

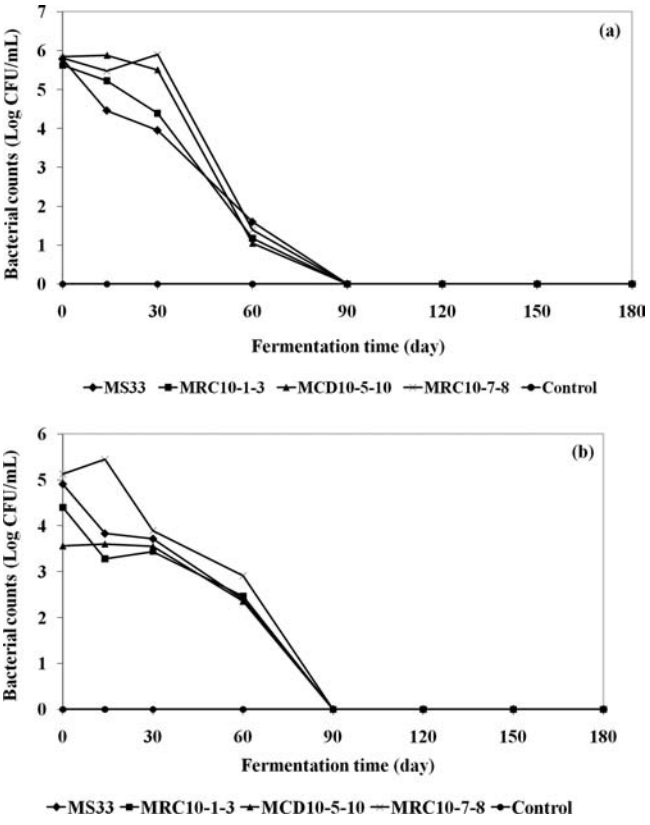


**Figure 1.** Changes in LAB (a) and total halophilic bacteria counts (b) of fish sauce samples inoculated with bacterial starter cultures and incubated at 35 °C for 6 months. Each value is the mean of four determinations ($n$ = 4).

**Analysis of Volatile Compounds.** Ten milliliters of fish sauce was added with cyclohexanol as an internal standard to a final concentration of 1 mg/L. Volatile compounds of fish sauce samples were analyzed using purge and trap (Texmar velocity XPT, Teledyne Tekmar, Mason, OH) as described by Udomsil et al.[8] Separation of the desorbed volatiles were achieved by gas chromatography−mass spectrometry (Varian Inc., Walnut Creek, CA) equipped with a capillary column (DB-WAX, 60 m × 0.25 mm × 0.25 μm Agilent Technologies, Redwood, CA) and a quadrupole mass detector. The oven temperature was increased from 25 to 200 °C at 15 °C/min. The mass spectra of volatile compounds were obtained by electron ionization (EI) at 70 eV. Identification of volatile compounds was performed by comparing retention time and mass spectral data with mass spectral libraries (National Institute of Standards; NIST data). The Kovats index of each compound was reported. The content of volatile compound was calculated from peak area relative to the internal standard cyclohexanol.

**Statistical Analyses.** The fermentation experiment was independently repeated twice using two different lots of fish. Each chemical analysis was carried out in duplicate. Analysis of variance (ANOVA) was used to determine significant differences between samples using Statistical Analysis System (SAS Inst. Inc., Cary, NC). Mean differences were determined by Duncan's multiple-range test (DMRT) at $P < 0.05$.

## RESULTS AND DISCUSSION

**Microbiological Changes.** The initial bacterial counts on MRS agar of all inoculated fish sauce mashes were $10^5$ CFU/mL

**Table 1. α-Amino Content of Fish Sauce Samples Inoculated with LAB Bacterial Starter Cultures and Incubated at 35 °C for 6 Months[a]**

| | α-amino content (mM) | | | | |
|---|---|---|---|---|---|
| fermentation time (days) | MS33 | MRC10-1-3 | MCD10-5-10 | MRC10-7-8 | control |
| 0 | 445.87 ± 38.58 | 462.92 ± 4.82 | 458.65 ± 42.20 | 487.64 ± 4.82 | 436.49 ± 24.11 |
| 14 | 636.12 ± 32.76ab | 643.48 ± 20.26ab | 691.74 ± 51.14a | 657.00 ± 38.17ab | 584.08 ± 28.09b |
| 30 | 684.14 ± 23.51 | 648.08 ± 52.08 | 676.95 ± 27.80 | 687.65 ± 24.58 | 644.89 ± 40.45 |
| 60 | 679.52 ± 69.84 | 672.03 ± 80.43 | 711.51 ± 27.00 | 701.15 ± 87.47 | 611.60 ± 85.11 |
| 90 | 726.92 ± 40.20 | 672.91 ± 67.14 | 744.95 ± 38.79 | 730.49 ± 52.01 | 676.13 ± 15.55 |
| 120 | 704.50 ± 8.52 | 727.01 ± 32.38 | 740.35 ± 79.42 | 744.61 ± 69.28 | 658.64 ± 1.57 |
| 150 | 724.17 ± 7.94 | 760.58 ± 15.03 | 786.15 ± 45.81 | 796.73 ± 78.26 | 691.46 ± 12.75 |
| 180 | 783.96 ± 19.18a | 783.38 ± 13.28a | 779.65 ± 32.58a | 782.41 ± 10.92a | 707.46 ± 3.58b |

[a] Different letters within a row indicate significant differences ($P < 0.05$).

(Figure 1a), whereas those on the modified JCM 168 agar ranged from approximately $10^3$ to $10^5$ CFU/mL (Figure 1b). Bacteria were not detected on either medium (MRS and the JCM 168 agar) throughout the course of fermentation (Figure 1). This could be because anchovies used in this study were subjected to enzymatic digestion at 50−60 °C for 6 h, leading to thermal inactivation of bacterial microflora of the fish. In addition, LAB and halophilic bacteria were not found in the solar salt used in this study. These results implied that the microbial population observed on MRS and the modified JCM 168 at day 0 was likely to be the inoculated *T. halophilus*. Because the LAB starter cultures grow under facultatively anaerobic to microaerophilic conditions, they could grow on the modified JCM 168 agar. Leisner et al.[16] indicated that bacteria on plate count agar (PCA) of acid-fermented condiment (tempoyak) were LAB.

Although all strains of *T. halophilus* used in this study were isolated from industrial scale fish sauce fermentation, halophilic LAB counts were not detected in the control. As mentioned earlier, heating fish samples was part of the reason. In addition, industrial fermentation could be regarded as a back-slopping fermentation in which microflora accumulated in the nonsterile tank serve as a natural starter culture of the new batch. The diversity and quantity of the microbial population would be greater than those found in the clean glass jars used in this experiment.

Fish sauce samples inoculated with *T. halophilus* MS33 and MRC10-1-3 showed a slight decrease of counts to $10^4$ CFU/mL at day 30 of fermentation, whereas the counts of those inoculated with *T. halophilus* MCD10-5-10 and MRC10-7-8 remained approximately $10^6$ CFU/mL (Figure 1). These results indicated that the latter strains showed higher survival rates in the fermentation system than did the former at the first month of fermentation. It should also be noted that the counts on the modified JCM 168 of *T. halophilus* MCD10-5-10 and MRC10-7-8 were approximately 100 times lower than those on MRS agar. This could be because these two strains could not grow well on modified JCM 168 and/or their growth suppressed other halophilic bacteria in the fermentation system. At day 60, LAB counts of all samples decreased to approximately $10^2$ CFU/mL, and approximately 100 times greater counts were observed on the modified JCM 168. Growth of *T. halophilus* starter cultures might activate the microbial succession. Some halophilic bacteria also could recover from injury resulting from thermal treatment during the enzymatic digestion of fish at 50−60 °C for 6 h. LAB and halophilic aerobic bacteria were not found at 90 days of fermentation and thereafter. Although these *T. halophilus* strains are halophilic LAB, they appeared to show limited growth in fish sauce fermentation containing 25% NaCl.

**Table 2. Total Amino Acid Contents of Fish Sauce Samples Inoculated with *T. halophilus* and Fermented for 6 Months[a]**

| | total amino acid (mg/100 mL) | | | | |
|---|---|---|---|---|---|
| amino acid | MS33 | MRC10-1-3 | MCD10-5-10 | MRC10-7-8 | control |
| aspartic acid | 695.00a | 631.54b | 688.05a | 673.71a | 669.20a |
| threonine | 447.28 | 429.97 | 445.62 | 438.64 | 443.05 |
| serine | 353.65 | 369.51 | 316.32 | 325.60 | 389.92 |
| glutamic acid | 1350.71a | 997.63b | 986.55b | 978.58b | 926.77b |
| proline | 729.74 | 572.95 | 575.88 | 564.45 | 570.06 |
| glycine | 371.60 | 366.68 | 383.79 | 379.09 | 381.08 |
| alanine | 486.01 | 473.96 | 493.93 | 489.94 | 487.07 |
| valine | 489.23 | 444.55 | 448.60 | 447.44 | 443.92 |
| methionine | 331.69 | 301.62 | 309.26 | 307.54 | 306.26 |
| isoleucine | 449.31 | 424.39 | 422.94 | 422.91 | 423.46 |
| leucine | 523.57a | 470.37b | 454.59b | 453.00b | 460.74b |
| tyrosine | 169.44a | 111.22b | 91.30b | 96.19b | 97.06b |
| phenylalanine | 572.80a | 459.70b | 460.13b | 444.55b | 430.05b |
| histidine | 479.92 | 463.35 | 461.55 | 460.50 | 457.86 |
| lysine | 752.16 | 663.72 | 738.23 | 739.86 | 607.13 |
| arginine | 457.09b | 581.30a | 414.52b | 384.67b | 645.01a |
| **total** | **8659.68a** | **7762.45b** | **7691.25a** | **7606.66a** | **7738.64a** |

[a] Different letters within a row indicate significant differences ($P < 0.05$).

**Protein Hydrolysis and Amino Acid Profiles.** The α-amino content of all fish sauce samples increased during the course of fermentation (Table 1). An increase in α-amino content of the control was mainly contributed by residual activity of fish endogenous proteinases and added exogenous proteinases. Samples inoculated with *T. halophilus* rapidly increased to 636−691 mM after 14 days, which was higher than the control ($P < 0.05$). This was concomitant with high cell counts of halophilic LAB at day 14 (Figure 1a). It could be speculated that an increase in α-amino content was partly attributed to the proteolytic activity of *T. halophilus*. The α-amino contents of samples inoculated with *T. halophilus* were gradually increased to 779−784 mM after 6 months of fermentation and remained higher than the control ($P < 0.05$). Although the numbers of *T. halophilus* counts did not increase during fermentation, the α-amino content continually increased in all samples during fermentation. Besides residual proteolytic activity from fish and added proteinases, addition of *T. halophilus* starter cultures

**Table 3. Free Amino Acid and Amino Derivative Contents of Fish Sauce Samples Inoculated with *T. halophilus* and Fermented for 6 Months[a]**

| | free amino acid and amino derivative contents (mg/100 mL) | | | | |
|---|---|---|---|---|---|
| | MS33 | MRC10-1-3 | MCD10-5-10 | MRC10-7-8 | control |
| **amino acid** | | | | | |
| aspartic | 593.83 | 571.29 | 593.95 | 595.94 | 551.40 |
| threonine | 571.75 | 553.97 | 564.04 | 572.84 | 528.47 |
| serine | 303.49b | 397.83a | 285.19b | 312.43b | 418.84a |
| glutamic | 982.24 | 943.16 | 971.82 | 981.57 | 907.84 |
| proline | 988.08 | 967.47 | 924.43 | 980.42 | 846.64 |
| glycine | 236.00 | 217.07 | 236.53 | 237.43 | 212.19 |
| alanine | 715.09 | 676.20 | 706.27 | 720.80 | 662.02 |
| valine | 757.30a | 743.05a | 745.84a | 759.39a | 702.49b |
| methionine | 263.73 | 284.15 | 268.29 | 267.23 | 255.75 |
| isoleucine | 434.16 | 431.34 | 416.53 | 423.73 | 406.10 |
| leucine | 514.39 | 515.32 | 483.14 | 488.85 | 479.90 |
| tyrosine | 96.22 | 97.46 | 86.42 | 95.28 | 89.33 |
| phenylalanine | 428.10a | 416.71a | 418.18a | 423.04a | 399.21b |
| histidine | 385.73 | 386.76 | 386.61 | 395.51 | 362.23 |
| lysine | 990.38 | 957.99 | 975.21 | 992.87 | 912.27 |
| arginine | 406.29c | 602.12b | 422.46c | 404.50c | 697.37a |
| **total** | **8666.78** | **8761.90** | **8484.91** | **8651.85** | **8133.34** |
| **amino derivatives** | | | | | |
| cystine | 59.35 | 75.24 | 81.85 | 73.84 | 61.25 |
| ammonia | 491.16a | 361.04b | 481.49a | 485.05a | 292.09c |
| taurine | 100.91 | 101.04 | 101.89 | 100.99 | 97.57 |
| hydroxyproline | 700.68 | 663.80 | 661.33 | 688.33 | 630.02 |
| ornithine | 199.95a | 33.71b | 186.10a | 196.47a | 7.03c |
| citrulline | 163.11a | 152.34a | 149.53a | 161.57a | 49.94b |
| **total** | **1715.16a** | **1387.17b** | **1662.20a** | **1706.25a** | **1137.91c** |

[a] Different letters within a row indicate significant differences ($P < 0.05$).

appeared to contribute to protein hydrolysis during fish sauce fermentation. This implied the possession of extracellular and/or cell-associated proteinases of *T. halophilus*.

Total nitrogen (TN), a prime quality parameter for fish sauce, of all inoculated samples ranged from 2.05 to 2.16%., whereas the control contained 2.03% TN. It is observed that TN values of inoculated fish sauce samples were comparable to that of the control. TN content reflects both amino nitrogen and nonprotein nitrogen, particularly ammonia. Our results demonstrated that TN does not truly reflect the extent of proteolysis in fish sauce and does not truly correlate with the amino acid content of fish sauce.

Total and free amino acid contents are important quality parameters of fish sauce. Total amino acid content of fish sauce inoculated with *T. halophilus* MS33 was highest ($P < 0.05$, Table 2), confirming that this strain was able to hydrolyze fish protein at a high salt content of 25%. Fish sauce with added *T. halophilus* MS33 also showed the highest contents of total glutamic, leucine, tyrosine, and phenylalanine ($P < 0.05$, Table 2). Branched-chain amino acids (leucine, isoleucine, and valine) are precursors for branched aldehydes, such as 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, via amino acid catabolism pathway.[17] Therefore, these results indicated that *T. halophilus* MS33 could increase amino acids, serving as a precursor of important aldehydes in fish sauce.

The sum of free amino acid content of inoculated fish sauce was comparable to the control ($P > 0.05$, Table 3). Free glutamic acid, proline, and lysine were major amino acids found in fish sauce. Free amino acids are important for the flavor and taste of fish sauce. As the amounts of free glutamic were comparable to those of total glutamic of respective samples, glutamic acids in these samples were likely to be in the form of free amino acid, rather than peptides. This would contribute to the strong umami taste. Udomsil et al.[8] reported that *T. haphilus* strains (MS33, MCD10-1-3, MCD10-5-10, and MRC10-7-8) showed high intracellular aminopeptidase activity toward glutamic acid. This could in part contribute to high glutamic acid in all LAB-inoculated samples. Free proline and lysine contents of LAB-inoculated samples were comparable to the control ($P > 0.05$, Table 3) and appeared to be higher than the respective contents obtained from total amino acid analysis (Table 2). In fact, free amino contents of alanine, valine, and leucine were also higher than those obtained from total amino content. For total amino acid determination, fish sauce samples were hydrolyzed by HCl, leading to oxidation and degradation of these amino acids. Park et al.[18] reported that proline and alanine contributed to sweetness and were responsible for a flavor characteristic of sea urchin roe. LAB-inoculated fish sauce samples also showed higher free phenylalanine than the control ($P < 0.05$). Both phenylalanine and tyrosine have been reported to enhance the umami taste of

**Table 4. Biogenic Amine Contents of Fish Sauce Samples Inoculated with *T. halophilus* and Fermented for 6 Months[a]**

| | biogenic amine contents (mg/100 mL) | | | | |
|---|---|---|---|---|---|
| biogenic amine | MS33 | MRC10-1-3 | MCD10-5-10 | MRC10-7-8 | control |
| tryptamine | 14.39 | 15.66 | 12.90 | 13.70 | 16.03 |
| putrescine | nd[b] | nd | 2.33 | nd | nd |
| cadaverine | nd | 2.48b | 6.12a | 2.10b | 2.40b |
| histamine | 6.06ab | 4.50b | 4.50b | 7.34ab | 10.24a |

[a] Different letters within a row indicate significant differences ($P < 0.05$). [b] Not detected.

glutamic acid in soy sauce.[19] Our results suggested that the addition of *T. halophilus* starter culture increased important free amino acids contributing to the umami taste and sweetness of fish sauce. Free valine contents of fish sauce sample inoculated starter culture were higher than the control ($P < 0.05$, Table 3), implying that these LAB strains could increase branched-chain amino acids serving as precursors for volatile compounds, such as 3-methylbutanal, via amino acid catabolism pathway.[9]

Amino derivatives including ammonia, ornithine, and citrulline of fish sauce added starter cultures were higher than control ($P < 0.05$, Table 3). These compounds contributed to the ammonical note of fish sauce.[2] However, no distinct ammonia-like odor was found in *T. halophilus*-inoculated samples as compared to the control, based on a sensory evaluation by trained panelists. An arginine degradation system commonly found in bacteria results in a product of ammonia by the action of arginine deiminase (ADI) pathway.[20,21] In addition, ornithine, citrulline, and $CO_2$ were generated from this pathway.[20] Deamidation of arginine via ADI yields citrulline, which is subsequently converted to ornithine by ornithine transcarbamylase.[20,22] Therefore, free arginine was expected to be lower, whereas degradation products (ammonia, ornithine, and citrulline) were higher in inoculated fish sauce samples. Our results implied that *T. halophilus* might metabolize arginine via the ADI pathway.

**Biogenic Amine Contents.** Tryptamine, putrescine, cadaverine, and histamine were biogenic amines found in all fish sauce samples, whereas tyramine, spermine, and spermidine were not detected (Table 4). Biogenic amines resulted from amino decarboxylase acitivity produced by bacteria and have been reported in some lactobacilli.[22] Several LAB such as *Lactobacillus curvatus*, *Lactobacillus buchneri*, *Lactobacillus brevis*, *Leuconostoc* spp., and *Pediococcus* spp. are known to produce decarboxylase enzymes, resulting in the formation of histamine, tryptamine, and tyramine.[23] As high contents of ornithine and low contents of putrescine were evident in samples inoculated with *T. halophilus* (Tables 3 and 4), it is suggested that ornithine decarboxylase activity of these selected LAB strains was minimal. Histamine contents of four fish sauce samples inoculated with *T. halophilus* MS33, MRC10-1-3, MCD10-5-10, and MRC10-7-8 were lower than that of the control ($P < 0.05$). In general, the cadaverine content of all samples was relatively low with the MCD10-5-10-inoculated sample showing the highest (Table 4). It is likely that this particular strain exhibited higher activity of lysine decarboxylase. These results demonstrated that these *T. halophilus* strains are not regarded as potent biogenic-forming strains. In addition, *T. halophilus* MRC10-1-3 and MCD10-5-10 showed the ability to decrease the histamine content in fish sauce ($P < 0.05$, Table 4).

There are several studies reporting reductions of histamine, tyramine, and cadaverine by bacteria. Leuschner et al.[24] reported that diamine oxidases located in the cytoplasm of *Micrococcus varians*, *Brevibacterium linens*, and coryneform bacteria were responsible for the degradation of histamine and tyramine. Moreover, *P. acidilactici* showed the ability to reduce histamine.[24] Similarly, Latorre-Moratalla et al.[25] reported that *Lactobacillus sakei* was a decarboxylase-negative starter culture responsible for the reduction of tyramine and histamine in Greek traditional sausage (*aerosthasou*). The use of mixed starter cultures *Lb. sakei* and *Staphylococcus equorum* also reduced cadaverine in traditional fermented sausage. Satomi et al.[26] analyzed the 30 kbp plasmid encoding histidine decarboxylase gene (*hdc*) of *T. halophilus* and found that the *hdc* gene was conserved among several LAB. *T. halophilus* is typically reported to produce histamine, particularly in histidine broth (HB) containing 1.0% L-histidine.[26] It produced histamine as high as 300 mg/100 mL in HB.[26] Udomsil et al.[8] reported that these *T. halophilus* strains were moderate histamine producers in the range of 6−22 mg/100 mL in mGYP medium fortified with 0.25% L-histidine. However, formation of biogenic amines, particularly histamine in fish sauce fermentation system, seems to be restricted. This is the first report demonstrating the histamine reduction ability of *T. halophilus* in a fish sauce fermentation system. Probably, *T. halophilus* might possess diamine oxidase or histamine *N*-methyltrasferase that is able to degrade biogenic amines. Further study is needed to clarify the biogenic amine reduction ability of these *T. halophilus* strains.

**Formation of Volatile Compounds.** A total of 20 volatile compounds were identified in fish sauce samples (Table 5). They were separated into six groups: alcohols; aldehydes; ketones; esters; sulfur-containing compounds; and nitrogen-containing compounds. Major alcohols found in samples were ethanol, 1-butanol, and 1-penten-3-ol. 1-Butanol and 1-penten-3-ol appeared to be the predominant alcohols detected in all inoculated samples, similar to the control (Table 5). 1-Butanol contributed to a fragrant and pungent odor, whereas 1-penten-3-ol contributed to a meaty, burnt, grassy, and green odor. Both compounds were also found in fish miso, soy miso, fish sauce, and soy sauce.[27] Methyl-1-butanol in cheese can be formed from the leucine transamination pathway of *Lc. lactis*.[9] However, the pathway of amino acid conversion of *T. halophilus* has not been well characterized thus far. The role of alcohols on overall acceptance in fish sauce has not been reported because their odor threshold was relatively high.[28]

2-Methylpropanal was found in all fish sauce samples inoculated with *T. halophilus* but not detected in the control (Table 5). 2-Methylbutanal and 3-methylbutanal were the major aldehydes found in inoculated fish sauce samples and the control. 2-Methylbutanal, 3-methylbutanal, and 2-methylpropanal have been reported to contribute to a meaty odor of fish sauce.[29,30] *T. halophilus* MRC10-1-3 produced the highest content of 2-methylpropanal ($P < 0.05$). 2-Methylpropanal can be formed via either an enzymatic or a nonenzymatic reaction.[9] *Lc. lactis* can convert valine to α-ketoisovaleric acid (KIVA) by transaminase; subsequently, KIVA is decarboxylated to 2-methylpropanal by decarboxylase.[9] In addition, α-ketoisocaproic acid can be converted to 2-methylpropanal via any chemical reaction, resulting in peroxides, which can further react with the acid or keto carbon atom of a molecule to form unstable dioxylactone or dioxetanol. These compounds generally decompose to 2-methylpropanal and carbon oxides, CO and $CO_2$.[31]

**Table 5. Volatile Compounds of Fish Sauce Samples Inoculated with *T. halophilus* and Fermented for 6 Months**

| | | relative peak area[a] | | | | |
|---|---|---|---|---|---|---|
| RI[b] | compound | MS33 | MRC 10-1-3 | MCD 10-5-10 | MRC 10-7-8 | control |
| | **alcohols** | | | | | |
| 1022 | ethanol | 0.072a | 0.007c | nd[c] | 0.018b | 0.040a |
| 1049 | 1-propanol | 0.006b | nd | nd | 0.001c | 0.011a |
| 1110 | 2-methyl-1-propanol | 0.004 | 0.001 | nd | 0.003 | 0.002 |
| 1185 | 1-butanol | 0.027 | 0.029 | 0.027 | 0.028 | 0.026 |
| 1193 | 1-penten-3-ol | 0.045a | 0.043a | nd | 0.058a | 0.061a |
| 1205 | 3-methyl-1-butanol | 0.010a | nd | nd | 0.002b | 0.001b |
| 1255 | 1-pentanol | 0.004 | 0.005 | 0.003 | 0.007 | 0.007 |
| | **aldehydes** | | | | | |
| 830 | 2-methylpropanal | 0.065b | 0.252a | 0.076b | 0.053b | nd |
| 906 | 2-methylbutanal | 0.245 | 0.247 | 0.227 | 0.234 | 0.220 |
| 911 | 3-methylbutanal | 0.255a | 0.282a | 0.073c | 0.188b | 0.232a |
| 1615 | 3-(methylthio)propanal | 0.019b | 0.026a | 0.016b | 0.018b | 0.015b |
| 1459 | benzaldehyde | 0.023a | 0.024a | 0.028a | 0.030a | 0.014b |
| | **ketones** | | | | | |
| 888 | 2-butanone | 0.270a | 0.241a | 0.009b | 0.282a | 0.312a |
| 929 | 3-methyl-2-butanone | 0.008b | 0.012b | nd | 0.008b | 0.020a |
| 1050 | 2,3-butanedione | 0.003 | 0.003 | nd | 0.007 | 0.008 |
| 1315 | cyclohexanone | 0.043 | 0.042 | 0.046 | 0.048 | 0.046 |
| | **ester** | | | | | |
| 863 | ethyl acetate | nd | 0.013b | 0.121a | 0.134a | 0.006b |
| | **sulfur-contaning compound** | | | | | |
| 1120 | dimethyl disulfide | 0.013b | 0.010b | nd | 0.028a | 0.038a |
| | **nitrogen-containing compounds** | | | | | |
| 1176 | methylpyrazine | 0.003 | 0.003 | 0.005 | 0.005 | 0.005 |
| 1377 | 2,6-dimethylpyrazine | 0.002 | 0.002 | nd | 0.004 | 0.001 |

[a] Values represent the ratio of the peak area of any compound to that of internal standard (cyclohexanol). Different letters within a row indicate significant differences ($P < 0.05$). [b] Retention indices calculated for DB-WAX column using *n*-alkanes as standards. [c] Not detected.

2-Methylbutanal and 3-methylbutanal were derived from isoleucine and leucine, respectively, via transamination and decarboxylation pathways.[32] In addition, aldehydes can be formed via nonenzymatic reaction including Strecker degradation.[9] Our results indicated that these four selected LAB contributed to the formation of important aldehydes in fish sauce. 2-Methylbutanal, 2-methylpropanal, and 3-methylbutanal contents were also high in fish miso and soy sauce and positively affected the overall flavor of these products due to their low odor threshold values of 1.5, 1.0, and 0.15 $\mu$g/L, respectively.[27] In addition, Steinhaus and Schieberle[33] reported that 3-methylbutanal and 2-methylbutanal are among the most potent odorants in soy sauce and are thought to be essentially produced by bacteria via amino acid biosynthetic pathways of branched-chain amino acids including leucine, valine, and isoleucine. 3-(Methylthio)propanal found in all fish sauce samples was likely to derive from degradation of methionine.[34] This is another important compound contributing to meaty, baked potato, and onion notes. Benzaldehyde, an aromatic compound commonly found in cheese and dairy products, was higher in all inoculated fish sauce samples ($P < 0.05$). Our results indicated that *T. halophilus* isolated from fish sauce may play a significant role in desirable flavor and aroma formation during fermentation. The major ketone found in this study was 2-butanone, which was comparable to the control ($P > 0.05$). 2-Butanone is responsible for a cheesy note, but it is unlikely to be the important compound due to its high threshold value of 1.55−7.76 mg/L.[28] The amounts of 2,3-butanedione and 3-methyl-2-butanone were relatively low in all fish sauce samples.

The only major ester detected in fish sauce samples was ethyl acetate, and it contributed to a fruity and orange note.[27] Ethyl acetate levels of fish sauce with added *T. halophilus* MCD10-5-10 and *T. halophilus* MRC10-7-8 were comparable ($P > 0.05$) and higher than the control ($P < 0.05$). Esters are usually found in fermented seafoods, which are derived from esterification of alcohols with carboxylic acids formed by microbial and enzymatic decomposition of lipids.

Methylpyrazine and 2,6-dimethylpyrazine were negligible in all fish sauce samples. Giri et al.[27] reported that methylpyrazines contributed to fishy, nutty, and ammonical odors, whereas 2,6-dimethylpyrazine contributed to the roasted nuts odor of miso and fish sauce. However, methylpyrazine played a less important role in the overall flavor of miso and fish sauce due to its high odor threshold of 56.56 mg/L.[27]

The sulfur-containing compound identified in fish sauce samples was dimethyl disulfide. Fish sauce samples inoculated with *T. halophilus* MS33 and MRC10-1-3 showed lower contents of dimethyl disulfide than the control ($P < 0.05$). Dimethyl disulfide was not detected in fish sauce sample with added *T. halophilus* MCD10-5-10. Dimethyl disulfide could be formed via catabolism of methionine via transamination.[35] In addition,

demethiolation of methionine results in methanethiol, which subsequently is autoxidized to dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide.[36] Sulfur-containing compounds are considered to be potent odorants because of their low threshold values.[37] These compounds are responsible for a fecal note that is undesirable odor in fish sauce.[4] Fukami et al.[4] found that fish sauce inoculated with *S. xylosus* contained less dimethyl disulfide and dimethyl trisulfide when compared to the control (without *S. xylosus*), implying that *S. xylosus* could reduce undesirable odor. In this study, dimethyl sulfide and dimethyl disulfide were found in premium commercial fish sauce with approximately 3-fold greater intensity than in fish sauce samples with added starter cultures (data not shown). Therefore, the use of four strains of *T. halophilus* as a starter culture for fish sauce fermentation is likely to help eliminate the undesirable odor resulted from sulfur-containing compounds and to promote desirable odor characteristics of fish sauce. This is the first report demonstrating the positive effect of using *T. halophilus* as a starter culture for fish sauce fermentation, particularly in increasing glutamic acid and eliminating undesirable volatile compounds contributing to a fecal note of fish sauce.

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