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Characterization of Protein Hydrolysis and Odor-Active Compounds of Fish Sauce Inoculated with *Virgibacillus* sp. SK37 under Reduced Salt Content

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ABSTRACT: The effect of *Virgibacillus* sp. SK37, together with reduced salt content, on fish sauce quality, particularly free amino acids and odor-active compounds, was investigated. *Virgibacillus* sp. SK37 was inoculated with an approximate viable count of 5 log CFU/mL in samples with varied amounts of solar salt, for example, 10, 15, and 20% of total weight. Eighteen selected odorants were quantitated by stable isotope dilution assays (SIDA), and their odor activity values (OAVs) were calculated. Samples prepared using 10% salt underwent spoilage after 7 days of fermentation. The viable count of *Virgibacillus* sp. SK37 was found over 3 months in the samples containing 15 and 20% salt. However, acceleration of protein hydrolysis was not pronounced in inoculated samples at both 15 and 20% salt. *Virgibacillus* sp. SK37, together with salt contents reduced to 15−20%, appeared to increase the content of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, acetic acid, and 2-methylpropanoic acid. However, only aldehydes were found to have an effect on the overall aroma of fish sauce based on high OAVs, suggesting that the inoculation of samples with *Virgibacillus* sp. SK37 under reduced salt contents of 15−20% likely contributed to stronger malty or dark chocolate notes.

KEYWORDS: *Virgibacillus sp. SK37, fish sauce, salt reduction, odor-active compound, odor activity value*

INTRODUCTION

Thai fish sauce, or *nam pla*, is a widely consumed seasoning in Southeast Asia and has been increasingly recognized worldwide. Generally, Thai fish sauce is produced by mixing anchovy (*Stolephorus* spp.) with solar salt at a ratio of 2:1 or 3:1, depending on the area of production.[1] After 12−18 months of fermentation in a cement tank at an ambient temperature of 32−40 °C, the liquid is drained off and ripened for another 2−12 weeks before bottling. Because of the high salt concentration, proteolysis occurs slowly, leading to an extremely long fermentation time of 12−18 months. Several moderately halophilic bacteria have been proposed to act as potential starter cultures that could shorten fermentation time and improve the aroma characteristics of fish sauce. Fukami et al.[2] reported that fish sauce inoculated with *Staphylococcus xylosus* contained lower amounts of compounds that contribute to an undesirable fecal note, specifically dimethyl disulfide, 2-ethylpyridine, dimethyl trisulfide, and butanoic acid, compared with the control (without inoculation). Sensory evaluation has shown that fishy, sweaty, fecal, and rancid notes were significantly reduced by this strain. *Tetragenococcus halophilus* has also been shown to reduce dimethyl disulfide and to improve the amino acid content, particularly of glutamic acid.[3]

Recently, a moderately halophilic bacterium, *Virgibacillus* sp. SK37 (GenBank/NCBI no. DQ910840), was isolated from 1-month-old Thai fish sauce mashes. The strain has been proven to reduce fermentation time by 50%.[4] It has also been shown to produce Na^+-activated and Na^+-stable extracellular proteinases and to possess cell-associated proteinases,[5,6] which render an increase in protein hydrolysis during high-salt fermentation. However, bacterial counts of this strain drastically decreased during fermentation containing 25% solar salt. High salt content at fermentation (25−28%) would suppress the growth and production of *Virgibacillus* sp. proteinases.

The level of salt in the fermentation has a large impact on microbial populations and their metabolites, as well as the extent of proteolysis. Most studies of fish sauce starter cultures have been performed under Na^+-saturated conditions at a fish-to-salt ratio of 3:1. The growth of moderately halophilic starter cultures is typically limited as these organisms reach optimal growth at 3−15% NaCl.[7] Reduced salt content would extend the survival of inoculated cultures. In addition, reduced salt content would increase the endogenous activities of fish and microbial proteinases, resulting in increased protein hydrolysis. The formation of volatile compounds, which are derived from peptides and/or amino acid precursors or metabolic pathways of microorganisms in fermentation, is expected to vary with salt content. However, the effect of *Virgibacillus* sp. starter culture at varied salt contents on volatile compounds and fish sauce quality has not been systematically investigated. Understanding such a

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relationship would further the development of starter culture technologies for fish sauce fermentation.

The extraction and quantitation of volatile compounds in fish sauce are typically performed by purge and trap or solid-phase microextraction (SPME) using regular internal standards. According to different physicochemical properties of various compounds, the quantitative determination of odor-active compounds in fish sauce requires selective and sensitive analytical methods. It has been reported that the use of an isotopically labeled analogue as an internal standard for quantitation provides highly accurate data because the physicochemical properties of both the internal standard and analyte are very similar.[8] Combining the precision of a stable isotope dilution assay (SIDA) with an appropriate sampling technique for each compound extraction should be taken into account. The objectives of this study were to investigate the effect of *Virgibacillus* sp. SK37, together with reduced salt content, on fish sauce quality, particularly odor-active compounds.

MATERIALS AND METHODS

Chemicals. Odorants obtained from commercial sources include *o*-aminoacetophenone, butanoic acid, dimethyl sulfide, dimethyl trisulfide, 2-ethyl-3,5(or6)-dimethylpyrazine, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, methanethiol, 2- and 3-methylbutanal, 3-methylbutanoic acid, 2-methylpropanal, 2-methylpropanoic acid, 3-(methylthio)propanal, 3-(methylthio)propanol, phenylacetaldehyde, 2-phenylacetic acid, 3-phenylpropionic acid, propanoic acid, and trimethylamine (Sigma-Aldrich, Inc., St. Louis, MO, USA); acetic acid (Fisher Scientific, Pittsburgh, PA, USA); and 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (TCI America, Portland, OR, USA). 2-Acetyl-1-pyrroline was synthesized using the procedure described by Fuganti et al.[9]

Isotropically Labeled Compounds. The following labeled compounds were obtained from commercial sources: [$^2H_3$]-acetic acid (IS-8), [$^2H_2$]-3-methylbutanal (IS-6), [$^2H_9$]-trimethylamine (IS-1) (CDN, Pointe-Claire, Quebec, Canada); [$^2H_6$]-dimethyl sulfide (IS-3), [$^2H_6$]-dimethyl trisulfide (IS-7), [$^2H_2$]-2-methylpropanal (IS-4) (Sigma-Aldrich, Inc., St. Louis, MO, USA); [$^2H_5$]-propanoic acid (IS-10) (Cambridge Isotope Laboratories, MA, USA); and [$^{13}C_2$]-2-phenylacetic acid (IS-17) (Isotec, Miamisburg, OH, USA). The following labeled compounds were synthesized according to the literature cited: [$^2H_2$]-butanoic acid (IS-12);[10] [$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (IS-16);[11] [$^2H_3$]-methanethiol (IS-2);[12] [$^2H_2$]-3-methylbutanoic acid (IS-14);[13] [$^2H_2$]-2-methylpropanoic acid (IS-11);[14] [$^2H_3$]-3-(methylthio)propanal (IS-9);[15] [$^2H_3$]-3-(methylthio)propanol (IS-15);[15] [$^{13}C_2$]-phenylacetaldehyde (IS-13);[16] and [$^2H_2$]-3-phenylpropionic acid (IS-18).[17] Odorless deionized−distilled water was prepared by boiling glass-distilled water in an open flask until its volume was reduced by one-fourth of the original volume.

Starter Culture Preparation. Starter culture was prepared by inoculating a loopful of *Virgibacillus* sp. SK37 into 100 mL of modified halophilic medium or Y-medium (1% yeast extract, 0.3% trisodium citrate, 0.2% KCl, and 2.5% $MgSO_4 \cdot 7H_2O$) containing 15% NaCl, pH 7, at 35 °C.[6] *Virgibacillus* sp. SK37 was cultured for 3 days at a shaking speed of 100 rpm (New Brunswick Scientific Co. Inc., Edison, NJ, USA) to attain an approximate cell count of 7 log CFU/mL. The cultured broth was centrifuged, and cell pellets were collected and washed with sterile 15% NaCl solution three times and then resuspended in 100 mL of 15% NaCl solution before being added to the fermentation.

Fish Sauce Fermentation. Indian anchovies (*Stolephorus indicus*) were caught off the Gulf of Thailand and kept on ice during transportation to the Suranaree University of Technology laboratory. Solar salt was collected from a fish sauce factory. One kilogram of anchovy was mixed with various amounts of solar salt, for example, 10, 15, and 20% of total weight and packed in glass jars (9 cm diameter × 17 cm height). Cell suspensions of *Virgibacillus* sp. SK37 were added at 10% (v/w) of the mixtures of fish and salt and then homogenously mixed. The controls without starter culture at each salt content were also prepared by adding sterile 15% NaCl at 10% (v/w). The sample representing the conventional process was also prepared by mixing anchovies with solar salt concentrations of 25%. All samples were incubated in a 35 °C incubator for 90 days. Changes in microbial population and oligopeptide and α-amino contents were monitored at 0, 7, 14, 30, 45, 60, 75, and 90 days of fermentation. Amino acid profiles, sodium (Na) content, volatile compounds, and other physicochemical properties, including total nitrogen, ammoniacal nitrogen content, degree of browning, and pH, were determined in the finished products after a 3 month fermentation.

Microbiological Analyses. Fish sauce mashes (10 g) were taken aseptically from the fermentation jar at each time interval. Total plate counts of all samples were performed on plate count agar (PCA, Merck KGaA, Darmstadt, Germany) without NaCl addition and incubated at 35 °C for 2−3 days under aerobic conditions. Halophilic bacteria were enumerated using the spread plate technique using halobacterium medium on modified JCM 168 agar containing 15% NaCl and incubated at 35 °C for 4−5 days under aerobic conditions.

Chemical Analyses. *Oligopeptide Content.* Fish sauce mashes (3 g) were added to 27 mL of 5% (w/v) cold trichloroacetic acid (TCA) solution, after which the mixture was homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) and centrifuged at 10000*g* for 15 min at 4 °C. The supernatant was analyzed for oligopeptide content using Lowry's assay method[18] with tyrosine as a standard. Oligopeptide content was expressed as micromoles of tyrosine per gram of fish sauce mash.

α-Amino Content. The α-amino content was determined using trinitrobenzenesulfonic acid (TNBS) with leucine as a standard.[19] Fifty microliters of the filtered fish sauce was mixed with 0.5 mL of 0.2125 M phosphate buffer (pH 8.2) and 0.5 mL of 0.05% TNBS reagent. The mixture was incubated at 50 °C for 1 h in a water bath. After incubation, the reaction was stopped by the addition of 1 mL of 0.1 N HCl and left at room temperature for 30 min. Absorbance was measured at 420 nm using a spectrophotometer. α-Amino content was expressed as millimolar leucine.

Amino Acid Profiles. Free amino acids were determined by directly diluting all samples with sodium citrate buffer (pH 2.2). All samples were derivatized with propyl chloroformate.[20] The quantitative analysis of amino acids was achieved by a gas chromatography−mass spectrometry (GC-MS) system equipped with an Agilent 6890NGC/5973 mass selective detector (MSD; Agilent Technologies, Inc., Darmstadt, Germany). The separations were performed on a ZB-AAA column (10 m × 0.25 mm i.d.; 0.25 μm film, Phenomenex, Torrance, CA, USA). The amino acid content was expressed as milligrams per 100 g of fish sauce.

Sodium (Na) Content. All samples (50 mL) were digested with 10 mL of concentrated HNO_3 for approximately 3 h. After cooling, the digested sample was adjusted to 100 mL with deionized water. Na content was determined by the air−acetylene flame atomization technique using atomic absorption spectroscopy (AAS) (model AAnalyst 100, Perkin-Elmer Corp., Norwalk, CT, USA).

Biogenic Amine Content. Biogenic amines of fish sauce samples were analyzed according to the method of Udomsil et al.[3] Derivatization was carried out using dansyl chloride. Biogenic amines were separated using a mobile phase consisting of the mixture of acetonitrile and 0.02 M acetic acid (1:9) as solvent A. The mixture of solvent B was prepared from 0.02 M acetic acid, acetonitrile, and methanol (1:4.5:4.5), and the flow rate of mobile phase was set at 1 mL/min. A Hypersil BDS column C18 (3 μm, 100 × 4 mm, Agilent Technologies Inc., Palo Alto, CA, USA) equipped with HPLC (HP 1100, Agilent Technologies Inc.) was used, and detection was carried out using a diode array detector set at 254 nm with the reference wavelength at 550 nm.

Other Chemical Parameters. Fish sauce samples fermented for 3 months were analyzed for total nitrogen and ammoniacal nitrogen.[21] The degree of browning was monitored by diluting the sample with distilled water at a ratio of 1:4 and measuring the absorbance at 440 nm.[4]

Identification of Odor-Active Compounds. *Sample Preparation.* Volatile compounds were extracted by direct solvent extraction−

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solvent-assisted flavor evaporation (DSE-SAFE). Commercial Thai fish sauce (20 g) premium grade with total nitrogen of 2.58% (w/w) was diluted and mixed well with 100 mL of odorless-distilled water and then spiked with 25 μL of internal standards (1.15 μg/μL of 2-ethylbutyric acid in methanol as acidic fraction internal standard; 1.32 μg/μL of 2-methyl-3-heptanone and 1.13 μg/μL of 2,4,6-trimethylpyridine in methanol as neutral/basic fraction internal standard). The pH was adjusted to ~2.0 using 2 M HCl and extracted three times with diethyl ether (100 mL total volume). After the third extraction, the pH of the sample was raised to ~9.0 using 2 M NaOH and extracted three times with diethyl ether (100 mL total volume). The pooled solvent extract (200 mL) was evaporated to 50 mL using a Vigreux column in a 43 °C water bath and further separated from the nonvolatile material by SAFE distillation[22] at 43 °C for 2 h under vacuum (~10^{-5} Torr). Then, acidic compounds were separated from the neutral/basic compounds by washing three times with 0.1 M NaOH (3 × 20 mL), and the upper layer (diethyl ether) containing the neutral/basic volatiles fraction was collected. The bottom layer (aqueous layer) was then acidified to pH ~2.0 with 4 M HCl, saturated with NaCl, and extracted three times with diethyl ether (3 × 20 mL). The upper layer containing the acidic volatiles fraction was collected. Both fractions were washed twice with 15 mL of saturated NaCl solution and then concentrated to 10 mL using a Vigreux column, dried over 2 g of anhydrous Na_2SO_4, and further concentrated to 900 μL of acidic fraction and 100 μL of neutral/basic fraction under nitrogen gas stream. Samples were kept at −70 °C until analysis.

Aroma Extract Dilution Analysis (AEDA). Acidic and neutral/basic fractions were diluted in stepwise (1:3; 1 part aroma extract to 2 parts solvent) using diethyl ether as the solvent. Sniffing of dilution was continued until no odorant was detectable. The flavor dilution (FD) factor of each compound is the last dilution that was detectable. The FD factors were performed and averaged by two panelists differing by not more than two FD factors. The gas chromatography−olfactometry (GC-O) system consisted of an HP6890 GC (Agilent Technologies, Inc.) equipped with a flame ionization detector (FID) and olfactory detection port (DATU Technology Transfer, Geneva, NY, USA). The sample (2 μL) was injected using a direct cool-on-column mode (3 °C temperature tracking mode) into a column (Rtx-Wax, 15 m × 0.32 mm i.d.; 0.5 μm film; Restek, Bellefonte, PA, USA). Column effluent was split 1:1 between the FID and the sniffing port using deactivated fused silica tubing (1 m × 0.25 mm i.d.; Restek). The GC oven temperature was programmed from 40 to 225 °C at a rate of 10 °C/min with initial and final holding times of 5 and 20 min, respectively. The FID and sniffing port were maintained at a temperature of 250 °C.

Static Headspace Dilution Analysis (SHDA). GC-O was conducted on an HP6890 GC (Agilent Technologies, Inc.) equipped with flame ionization detector and olfactory detector port (ODP2, Gerstel). Commercial Thai fish sauce (5 g) was placed in a 250 mL round-bottom flask connecting with a Wheaton connecting adapter and sealed with PTFE-lined septum. The flask was equilibrated at 40 °C for 30 min with agitation. Each headspace volume (25, 5, 1, 0.2, or 0.04 mL) was injected via a heated (50 °C) gastight syringe into a CIS-4 cooled injection system (Gerstel) operating in the splitless mode [initial temperature, −120 °C (0.10 min hold); programming rate, 12 °C/s; final temperature, 260 °C (10 min hold)]. Separations were performed on Rtx-Wax (15 m × 0.53 mm i.d.; 1 μm film; Restek). Column effluent was split 1:1 between the FID and ODP2 using deactivated fused silica tubing. The FID and ODP2 temperatures were maintained at 250 °C. The GC oven temperature was programmed from 35 to 225 °C at a rate of 6 °C/min with initial and final holding times of 5 and 10 min, respectively.

Compound Identification. Volatile compounds were identified on the basis of comparison of their mass spectra with those in a database. Retention indices (RIs) of compounds were calculated using a homologous series of *n*-alkanes according to the method of van den Dool and Kratz.[23] The odor property of each volatile compound was matched with those of published data sources. Each compound was further confirmed by comparing its mass spectra, RIs, and odor properties with those obtained for authentic standards.

Quantitation of the Selected Odor-Active Compounds. Volatile compounds that are identified and considered as odor-active compounds in commercial Thai fish sauce were selected for quantitation. Odor-active compounds in the fish sauce samples fermented for 3 months were quantitated using stable isotope dilution assays (SIDA). In brief, highly volatile compounds were quantitated by headspace−solid phase microextraction (H-SPME). Fish sauce sample (1 g) was placed in a 22 mL headspace vial and spiked with isotope internal standards. The sample was preincubated at 40 °C for 10 min with agitation. Then, a SPME fiber (50/30 μm DVB/carboxen/polydimethylsiloxane fiber; Supelco, Bellefonte, PA, USA) was exposed to the vial headspace for 20 min. The fiber was desorbed by splitless injection (injector temperature, 260 °C; splitless time, 4 min; vent flow, 50 mL/min) into a GC-MS system at the same settings as described below. The GC oven temperature was programmed from 35 to 225 °C at a rate of 6 °C/min with initial and final holding times of 5 and 20 min, respectively.

Intermediate/low volatility with low-abundance compounds and highly abundant compounds was quantitated by direct solvent extraction−solvent-assisted flavor evaporation (DSE-SAFE) and direct solvent extraction (DSE), respectively. Sample preparation for DSE-SAFE (50 g) and DSE (5 g) was spiked with isotope internal standards and prepared as described above. Each sample was prepared in duplicate and kept at −70 °C until analysis by GC-MS.

The response factor and the area of selected ions of each compound were used for compound quantitation. The compound concentration was expressed in micrograms per kilogram (ppb). Odor-activity values (OAVs) were calculated by dividing the concentrations by the respective odor thresholds in water from the literature.

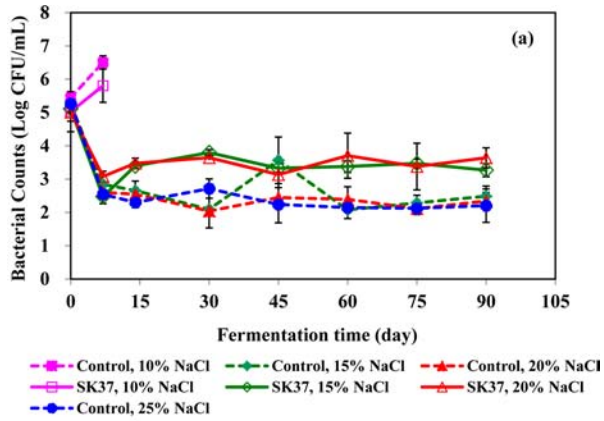
Gas Chromatography−Mass Spectrometry (GC-MS). The GC-MS system consisted of an HP6890GC/5973 mass selective detector (MSD; Agilent Technologies, Inc.). Each aroma extract (1 μL) was injected into a CIS-4 cooled injection system (Gerstel) operating in the splitless mode [initial temperature, −50 °C (0.10 min hold); ramp rate, 12 °C/s; final temperature, 260 °C (10 min hold)]. The separations were performed on a Stabilwax (30 m × 0.25 mm i.d.; 0.25 μm film; Restek) and Sac-5 (30 m × 0.25 mm i.d.; 0.25 μm film; Supleco). Helium was used as carrier gas at a constant rate of 1.0 mL/min. The GC oven temperature was programmed from 35 to 225 °C at a rate of 4 °C/min with initial and final holding times of 5 and 20 min, respectively. The MSD conditions are listed as follows: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 35−350 amu; electron multiplier voltage (Autotune +200 V); scan rate, 5.2 scans/s.

Statistical Analyses. The fermentation experiment was prepared in duplicate. All chemical analysis was performed in duplicate, and the mean values are presented. Analysis of variance (ANOVA) was carried out using the SPSS program (SPSS version 14, Windows version). The differences among mean values were established using Duncan's multiple-range test (DMRT) at $P < 0.05$.

RESULTS AND DISCUSSION

Microbiological Changes. The initial bacterial counts on PCA of all samples were approximately 5 log CFU/mL, whereas the inoculated samples showed a count >5 log CFU/mL on JCM 168 agar (Figure 1). This result demonstrated that *Virgibacillus* sp. SK37 grew in JCM 168 agar better than in PCA. The samples prepared using 10% salt underwent spoilage after 7 days with total viable counts of 6−7 log CFU/mL on both PCA and JCM 168 agar. The use of *Virgibacillus* sp. SK37 as a starter culture at this salt level could not prevent microbial spoilage. Thus, these samples were not further investigated.

The viable counts on JCM agar of samples inoculated with starter cultures were 4.4−4.8 log CFU/mL at day 7 and remained approximately 5.0 log CFU/mL throughout the course of fermentation. In contrast, the controls without starter cultures showed a drastic decrease of microbial population in the first 15 days of fermentation and relatively low bacterial counts of 2.0−



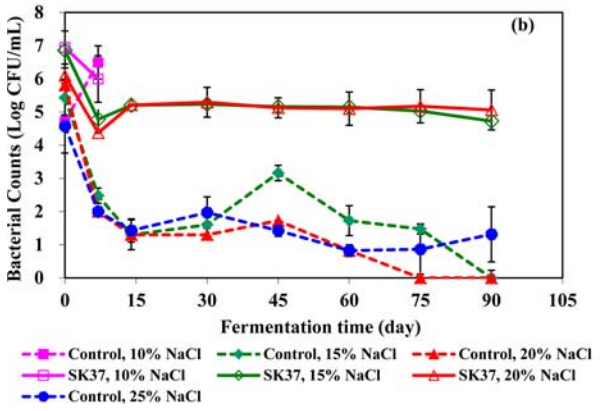


Figure 1. Changes in the bacterial counts of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and incubated at 35 °C for 3 months on plate count agar without NaCl (a) and JCM 168 agar medium containing 15% NaCl (b).

2.5 CFU/mL during the 3 month period. These results demonstrated that *Virgibacillus* sp. SK37 grew equally well in the fermentation containing 15 and 20% salt. In this study, a higher count of *Virgibacillus* sp. SK37 was observed compared with those previously reported in fermentation containing 25% NaCl, which was approximately 3 log CFU/mL at 3 months.[5] This finding suggests that reducing salt content to 15−20% could extend the viable count of inoculated cultures.

Chemical Changes. *Oligopeptide Content.* The oligopeptide content of all fish sauce samples increased over the course of fermentation (Figure 2). The oligopeptide content of samples fermented at 10% salt rapidly increased to the highest level of 21−22 μmol/g sample at day 7. The growth of spoilage microorganisms contributed to extensive proteolysis. In addition, lowering the salt content is likely to increase the activity of fish and microflora proteinases. Samples prepared at 15% salt with the addition of starter culture showed the highest oligopeptide content up to 45 days of fermentation ($P < 0.05$). At day 60, the oligopeptide content of the inoculated samples was comparable to the controls ($P > 0.05$). These results demonstrated that the addition of *Virgibacillus* sp. SK37 increased protein hydrolysis only at the early stage of fermentation. This finding suggests that the proteolytic activity of *Virgibacillus* sp. SK37 was rather limited at the later stages of fermentation. Proteinase is a primary metabolite that is synthesized parallel to microbial growth.[24] As fermentation progressed, the counts of *Virgibacillus* sp. SK37 did not continually increase but rather stabilized, leading to limited proteinase secretion. Oligopeptide contents of fish sauce

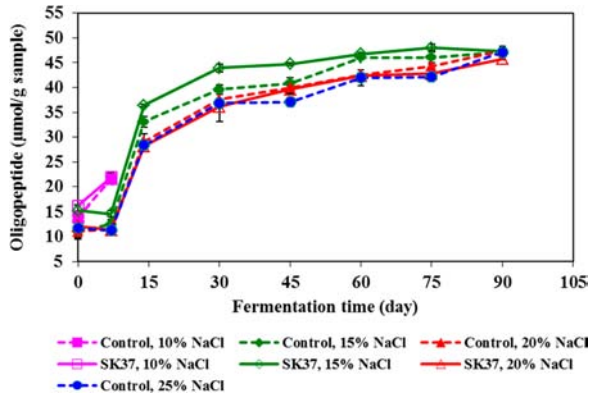


Figure 2. Changes in oligopeptides of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and incubated at 35 °C for 3 months.

inoculated with *Virgibacillus* sp. SK37 with the addition of 20% salt were comparable to the respective controls during the course of fermentation ($P > 0.05$). This result indicated that the addition of starter culture at 20% salt did not help to accelerate protein hydrolysis.

α-Amino Content. The α-amino content of all fish sauce samples increased during fermentation in a pattern similar to that of oligopeptide content (Table 1). An increase in α-amino content of the control samples resulted from the proteolytic activity of microflora and endogenous enzymes in fish. The fish sauce samples inoculated with *Virgibacillus* sp. SK37 starter culture, together with a reduced salt content of 15%, showed the highest α-amino content at day 30 ($P < 0.05$), corresponding to the highest oligopeptide content (Figure 2). However, when fermentation progressed to 90 days, the α-amino content of the samples with added starter culture was comparable to that of the respective controls ($P > 0.05$). This result confirmed that the addition of *Virgibacillus* sp. SK37 increased protein hydrolysis only at the early stage of fermentation but not at the later stage. When the amount of added salt increased to 20%, the α-amino content of the inoculated samples was comparable to the control during the course of fermentation ($P > 0.05$). It was observed that the controls (without starter culture) with reduced salt contents at 15 and 20% showed higher α-amino content than the sample prepared at 25% salt added at 90 days of fermentation ($P < 0.05$).

Other Chemical Parameters. The total nitrogen content of all inoculated samples was lower than that of their respective controls ($P < 0.05$, Table 2). At 3 months of fermentation, the total nitrogen of all fish sauce samples was >2%, which is classified as first-grade fish sauce.[1] The ammoniacal nitrogen of all samples was comparable ($P > 0.05$). A high number of inoculated cultures (5 log CFU/g) during fermentation might lead to an increase in N-source utilization because C-sources were very limited in the system. As a result, total nitrogen of inoculated samples was lower. This was in agreement with a lower browning index of inoculated samples as the main substrates, N-compounds, for Maillard browning were relatively lower than respective controls. It should be mentioned that the browning index of controls with salt reduction to 15 and 20% was greater than the traditionally fermented sample containing 25% NaCl. Histamine was the predominant biogenic amine found in all samples. Samples without starter culture showed comparable histamine contents, regardless of salt ratio used in the fermentation ($P > 0.05$, Table 2). The histamine level of samples inoculated with *Virgibacillus* sp. SK37 at 15 or 20% NaCl

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Table 1. α-Amino Content of Fish Sauce Samples Inoculated with *Virgibacillus* sp. SK37 and Fermented at 35 °C for 3 Months

	α-amino content[a] (mM)				
	15% NaCl		20% NaCl		25% NaCl
fermentation time (days)	control	SK37	control	SK37	control
30	710.65 ± 12.10 c	841.05 ± 26.20 a	719.14 ± 15.09 bc	754.63 ± 12.20 b	730.71 ± 14.23 bc
45	865.52 ± 22.43 ab	899.92 ± 8.03 a	844.23 ± 10.32 bc	810.65 ± 34.73 c	827.03 ± 6.92 bc
60	874.22 ± 4.70 ab	901.93 ± 5.91 a	875.90 ± 9.50 ab	838.96 ± 19.45 b	848.19 ± 23.90 ab
75	946.62 ± 10.90 a	958.60 ± 3.60 a	915.83 ± 35.10 ab	878.19 ± 10.98 b	917.54 ± 6.10 ab
90	975.25 ± 2.30 a	938.70 ± 23.50 abc	962.79 ± 38.80 ab	911.30 ± 10.33 bc	895.51 ± 14.23 c

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

Table 2. Physicochemical Properties of Fish Sauce Samples Inoculated with *Virgibacillus* sp. SK37 and Fermented at 35 °C for 3 Months

	fish sauce sample[a]				
	15% NaCl		20% NaCl		25% NaCl
parameter	control	SK37[b]	control	SK37	control
total nitrogen (%)	2.26 ± 0.01 a	2.06 ± 0.03 c	2.20 ± 0.02 ab	2.01 ± 0.03 c	2.14 ± 0.04 b
ammonical nitrogen (%)	0.12 ± 0.004	0.13 ± 0.008	0.11 ± 0.003	0.13 ± 0.006	0.11 ± 0.002
color (Abs at 440 nm)	0.446 ± 0.003 a	0.338 ± 0.001 c	0.419 ± 0.002 b	0.326 ± 0.001 d	0.319 ± 0.001 e
Na (g/100 mL)	9.57 ± 0.20 b	9.44 ± 0.08 b	9.95 ± 0.21 ab	9.93 ± 0.31 ab	10.27 ± 0.11 a
pH	5.46 ± 0.01 b	5.50 ± 0.01 a	5.45 ± 0.01 b	5.45 ± 0.01 b	5.43 ± 0.01 c
histamine (mg/L)	238.49 ± 28.24 a	130.05 ± 14.41 b	245.06 ± 4.54 a	156.23 ± 21.58 b	225.21 ± 27.18 a

[a]Different letters within a row indicate significant differences ($P < 0.05$). [b]SK37, *Virgibacillus* sp. SK37.

decreased by 36−46% as compared to their respective nonstarter culture counterparts ($P < 0.05$, Table 2). The histamine levels of all samples were lower than the allowable limit of 400 mg/L, imposed by Codex.[25] This study confirmed the histamine reduction ability of *Virgibacillus* sp. SK37 as previously reported.[4]

Although the additions of salt content varied in the fermentation, the Na content of the liquid drained from the mash showed a similar Na content of 9.4−10.3 g/100 mL, which was equivalent to 24.0−26.1% NaCl. The samples prepared at 15% salt showed lower salt contents than those prepared at 25% salt ($P < 0.05$) but were comparable to those prepared with 20% salt added ($P > 0.05$). During salting of fish, fish are surrounded by granular salt, which is initially dissolved by the surface moisture of the fish. Then, water is removed from the fish, and salt ions penetrate into the fish during osmotic dehydration.[26] It has been reported that the rate of salt penetration into the muscle varies with the fat content, freshness of the fish, surface/volume ratio of the flesh, and temperature.[27] The moisture content of the fresh anchovies used in this study was approximately 65%. Limited water flux from the fish resulted in an almost saturated salt content in the drained liquid. Although reducing the salt added to the fermentation yielded a greater extent of proteolysis, the salt content of the finished product did not deviate from the standard of fish sauce, which was set to be not less than 20%.[25]

Amino Acid Profiles. The taste and flavor precursor of fish sauce is derived from several free amino acids. Thus, this study focused only on free amino acids. Compared with the traditional salt addition (25%), the reductions of salt content to 15 and 20% increased the total amount of free amino acids (Table 3). It was also observed that salt reduction yielded fish sauce with higher contents of free alanine, aspartic acid, glutamic acid, methionine, phenylalanine, and serine. Lysine, histidine, glutamic acid, and aspartic acid were the predominant amino acids in all samples.

The free glutamic acid content of inoculated samples was comparable to that of the corresponding controls. Glutamic acid and alanine were reported to be the taste-active components of fish sauce, along with threonine, tyrosine, histidine, valine, proline, cystine, and methionine.[28] Inoculated samples at both levels of salt addition showed higher free isoleucine and leucine contents than the corresponding controls. Branched-chain amino acids (leucine, isoleucine, and valine) are reported as precursors for major volatile compounds in fish sauce, including

Table 3. Free Amino Acid Contents of Fish Sauce Samples Inoculated with *Virgibacillus* sp. SK37 and Fermented at 35 °C for 3 Months

	free amino acid (mg/100 g)				
	15% NaCl		20% NaCl		25% NaCl
amino acid	control	SK37	control	SK37	control
alanine	493	452	502	449	446
arginine	95	84	101	102	91
aspartic acid	766	731	885	642	675
cysteine	289	280	225	247	216
glutamic acid	1323	1335	1248	1255	1085
glycine	307	296	221	318	300
histidine	1236	1400	1235	1308	1480
hydroxylysine	168	23	165	35	17
hydroxyproline	50	49	31	14	29
isoleucine	421	474	377	481	462
leucine	553	578	448	608	632
lysine	2359	2330	2308	2383	2389
methionine	294	288	270	273	266
phenylalanine	651	359	563	397	345
proline	317	361	245	338	321
serine	516	459	603	449	438
threonine	635	666	458	460	556
tryptophan	205	295	232	266	247
tyrosine	219	227	198	210	255
valine	486	474	503	435	488
total	11383	11161	10818	10670	10738

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Table 4. Odor-Active Compounds in Commercial Thai Fish Sauce Characterized by Aroma Extract Dilution Analysis (AEDA) (FD Factor > 3) and Static Headspace Dilution Analysis (SHDA) (FD Factor > 5)

			FD factor[a]		
RI[b]	compound	odor	AEDA	SHDA	identification[c]
625	methanethiol	rotten, sulfurous, cabbage	–[d]	25	RI, Std, MS
804	2-methylpropanal	malty, dark chocolate	–	25	RI, Std, MS
904	2-methylbutanal	malty, dark chocolate	9	25	RI, Std, MS
916	3-methylbutanal	malty, dark chocolate	27	25	RI, Std, MS
1320	2-acetyl-1-pyrroline	popcorn	9	–	RI, Std
1371	dimethyl trisulfide	rotten, sulfurous, cabbage	–	25	RI, Std, MS
1433	acetic acid	sour	729	–	RI, Std, MS
1452	3-(methylthio)propanal	cooked potato	729	25	RI, Std, MS
1484	3,6-dimethyl-2-ethylpyrazine	roasted potato	9	–	RI, Std
1514	propanoic acid	cheesy, sweaty	81	–	RI, Std, MS
1550	2-methylpropanoic acid	cheesy, Swiss cheese	81	–	RI, Std, MS
1611	butanoic acid	cheesy	6561	–	RI, Std, MS
1640	phenylacetaldehyde	rosy, sweet	81	–	RI, Std, MS
1654	3-methylbutanoic acid	cheesy, sweaty	2187	–	RI, Std, MS
2005	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	burnt sugar	729	–	RI, Std, MS
2056	4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone	burnt sugar	2187	–	RI, Std
2077	4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone	burnt sugar	9	–	RI, Std
2173	3-hydroxy-4,5-dimethyl-2(5H)-furanone	curry, spicy	27	–	RI, Std
2202	*o*-aminoacetophenone	musky, grape, stale	81	–	RI, Std
2515	2-phenylacetic acid	rosy, plastic	6561	–	RI, Std, MS
2590	3-phenylpropanoic acid	rosy	9	–	RI, Std, MS

[a]Flavor dilution (FD) factors of AEDA and SHDA were determined from stepwise dilution by diethyl ether and various injected headspace volumes, respectively, as described under Materials and Methods. [b]Rentention index (RI) was based on Rtx-Wax column. [c]Identification was confirmed with RI reported, authentic standard references (Std), and mass spectra (MS) in the database. [d]Not detected.

aldehydes (3-methylbutanal, 2-methylbutanal, and 2-methylpropanal), alcohols (3-methylbutanol, 2-methylbutanol, and 2-methylpropanol), and acids (3-methylbutanoic acid, 2-methylbutanoic acid, and 2-methylpropanoic acid).[29]

The free phenylalanine content of inoculated samples appeared to be lower than that of the controls at both levels of salt addition. The addition of *T. halophilus* starter cultures increased free phenylalanine content.[3] Phenylalanine has been reported to be a precursor of phenylacetaldehyde, 2-phenylethanol, and 2-phenylacetic acid via amino acid catabolism by aminotransferase.[29] Free phenylalanine was not judged as a taste-active component of fish sauce.[28] Thus, reducing the content of phenylalanine by *Virgibacillus* sp. SK37 might not affect the taste of fish sauce.

The addition of *Virgibacillus* sp. SK37 as a starter culture did not increase the free methionine content at either salt addition level compared with the controls. Methionine has been reported to contribute to umami and the overall taste of fish sauce.[28] In addition, methionine is a precursor for methanethiol, 3-(methylthio)propanal, 3-(methylthio)propanol, 3-(methylthio)-propanoic acid, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide.[30] Some of these compounds are responsible for the aroma characteristics of Thai fish sauce.[31]

Odor-Active Compounds. Twenty-one compounds with FD factors of >3 and >5 were detected by AEDA and SHDA, respectively (Table 4). Compounds showing the highest FD factor, which were absolutely identified by MS, RI, and standards, were butanoic acid (cheesy), 2-phenylacetic acid (rosy, plastic), 3-methylbutanoic acid (cheesy, sweaty), and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (burnt sugar). Six compounds were not detected by GC-MS but found in GC-O and confirmed by standards and RI (Table 4). In addition, a compound contributing to fishy odor, trimethylamine, and metabolites of cysteine and methionine (dimethyl sulfide and 3-(methylthio)-propanol), which were found in GC-MS, were selected for quantitation. Thus, a total of 18 compounds were quantitated using stable isotope of the analytes as internal standards (Table 5). The highest abundant compound in all fish sauce samples was acetic acid, followed by butanoic acid, 3-methylbutanoic acid, and propanoic acid. Wichaphon et al.[31] also showed that organic acids, including acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid, contributed to the aroma characteristics of Thai fish sauce sample based on a combination score performed by dynamic headspace dilution analysis.

The inoculated samples appeared to have a higher content of volatile acids, particularly acetic acid and 2-methylpropanoic acid ($P < 0.05$), compared with the corresponding controls. A salt content reduction to 15−20% did not affect these volatile acids ($P > 0.05$). Acetic acid and 2-methylpropanoic acid contribute to sour and cheesy notes, respectively (Table 4). Acetic acid can be formed via the oxidation of ethanol and acetaldehyde. Bulthuis et al.[32] reported that ethanol and acetaldehyde were among the volatile compounds formed via glycolysis and the pentose phosphate pathway during the anaerobic growth of *Bacillus licheniformis* in batch culture. 2-Methylpropanoic acid is formed by the oxidation of 3-methylpropanal, which is derived from valine via transamination and decarboxylation pathways.[30] Shimoda et al.[33] suggested that volatile fatty acids, especially 2-methylpropanoic acid, could be major contributors to the cheesy and stinging odor of fish sauce, on the basis of their quantitative and odor threshold values.

Inoculation with *Virgibacillus* sp. SK37 increased butanoic acid and 3-methylbutanoic acid contents at salt addition of 15% ($P < 0.05$) but not 20% ($P > 0.05$). 3-Methylbutanoic acid is derived from leucine via transamination and decarboxylation pathways.[30]

Table 5. Selected Ions (m/z) and Response Factors Used in Quantitation by Stable Isotope Dilution Assay and Concentrations of Odor-Active Compounds in Fish Sauce Samples Inoculated with *Virgibacillus* sp. SK37 and Fermented at 35 °C for 3 Months

					concentration[a] (μg/kg)				
					15% NaCl		20% NaCl		25% NaCl
no.[b]	compound[c]	IS no.[d]	selected ion (UL/L)[e]	Rf[f]	control	SK37	control	SK37	control
1	trimethylamine	IS-1	58/66	0.57	1.38 (±5.15) b	1.65 (±1.45) a	1.27 (±1.56) c	1.31 (±5.80) bc	1.29 (±1.36) bc
2	methanethiol	IS-2	48/51	0.96	41 (±16.57) a	40 (±14.78) a	36 (±14.10) ab	37 (±9.97) ab	31 (±6.68) b
3	dimethyl sulfide	IS-3	62/68	4.27	17 (±9.78)	15 (±15.25)	16 (±13.83)	15 (±4.46)	14 (±14.13)
4	2-methylpropanal	IS-4	72/74	2.13	340 (±5.48) b	355 (±3.21) ab	286 (±6.64) c	383 (±3.04) a	299 (±10.17) c
5	2-methylbutanal	IS-6	86/88	0.95	209 (±9.78) e	539 (±6.73) a	396 (±7.83) c	463 (±3.24) b	331 (±7.04) d
6	3-methylbutanal	IS-6	86/88	1.45	373 (±3.36) c	823 (±7.65) a	566 (±11.74) b	849 (±8.85) a	628 (±5.85) b
7	dimethyl trisulfide	IS-7	126/132	1.14	5.03 (±12.25)	4.78 (±5.62)	4.76 (±9.13)	4.71 (±9.56)	4.5 (±13.93)
8	acetic acid	IS-8	60/63	0.99	482452 (±7.82) c	735923 (±4.83) a	484265 (±6.34) c	549590 (±3.04) b	514783 (±5.87) bc
9	3-(methylthio)propanal	IS-9	104/107	1.70	810 (±12.22) a	714 (±14.35) ab	598 (±10.42) b	651 (±13.95) b	609 (±9.48) b
10	propanoic acid	IS-10	74/79	1.28	3409 (±4.81) b	3835 (±7.48) ab	3913 (±8.62) ab	4193 (±9.63) a	3730 (±16.58) ab
11	2-methylpropanoic acid	IS-11	73/75	0.35	1391 (±12.95) b	2347 (±9.37) a	1468 (±7.15) b	2184 (±6.99) a	1453 (±7.20) b
12	butanoic acid	IS-12	88/90	1.20	12461 (±12.10) bc	25905 (±3.86) a	13575 (±17.93) bc	14252 (±5.53) b	11392 (±11.96) c
13	phenylacetaldehyde	IS-13	120/122	2.03	79 (±11.46) a	35 (±13.03) c	51 (±14.05) b	27 (±13.58) c	57 (±12.23) b
14	3-methylbutanoic acid	IS-14	87/89	0.50	5333 (±11.27) b	8405 (±17.85) a	5906 (±16.84) b	6613 (±7.31) b	6171 (±13.29) b
15	3-(methylthio)propanol	IS-15	106/109	1.07	103 (±11.77) b	307 (±12.96) a	81 (±14.07) bc	71 (±6.85) c	57 (±9.22) c
16	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	IS-16	128/130	0.95	1061 (±8.63)a	831 (±4.70) bc	920 (±6.60) b	730 (±16.10) cd	689 (±6.59) d
17	2-phenylacetic acid	IS-17	136/138	0.76	1605 (±7.19) a	1200 (±11.13) b	1619 (±6.28) a	1083 (±15.38) b	1613 (±5.32) a
18	3-phenylpropanoic acid	IS-18	150/152	0.96	41 (±8.73) a	34 (±14.57) bc	35 (±15.98) ab	28 (±10.04) c	27 (±9.23) c

[a]Different letters within a row indicate significant differences ($P < 0.05$). Average of four determinations ($n = 4$) (± percent relative standard deviation). [b]Highly volatile ompounds (**1**–**6**), intermediate/low volatility with low abundance compounds (**7**, **9**, **13**, and **15**–**18**), and highly abundant compounds (**8**, **10**–**12**, and **14**) were quantitated by H-SPME, DSE-SAFE, and DSE, respectively. [c]All compounds were identified on the basis of mass spectra (MS) in the database, retention index (RI), and comparison of MS and RI of authentic standard references. [d]IS no., isotopically labeled compounds used as internal standards are indicated under Materials and Methods. [e]UL, unlabeled compound; L, labeled compound. [f]Rf, response factor between analyzed compound (unlabeled compound) and its internal standard (labeled compound).

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Table 6. Odor-Activity Values (OAVs) of Compounds in Fish Sauce Samples Inoculated with *Virgibacillus* sp. SK37 and Fermented at 35 °C for 3 Months

				OAV[a]				
				15% NaCl		20% NaCl		25% NaCl
no.	compound	odor threshold[b] (μg/kg)	ref.	control	SK37	control	SK37	control
1	trimethylamine	0.47	37	2.90 b	3.58 a	2.71 c	2.89 bc	2.75 bc
2	methanethiol	0.02	38	2050 a	2000 a	1800 ab	1850 ab	1550 b
3	dimethyl sulfide	0.3	39	57	50	53	50	47
4	2-methylpropanal	1	40	340 b	355 ab	286 c	383 a	299 c
5	2-methylbutanal	3	41	70 e	180 a	132 c	154 b	110 d
6	3-methylbutanal	0.2	41	1865 c	4115 a	2830 b	4245 a	3140 b
7	dimethyl trisulfide	0.01	41	503	478	476	471	450
8	acetic acid	22000	41	22 c	33 a	22 c	25 b	23 bc
9	3-(methylthio)propanal	0.2	41	4050 a	3570 ab	2990 b	3255 b	3045 b
10	propanoic acid	2000	41	1.70 b	1.92 ab	1.96 ab	2.10 a	1.87 ab
11	2-methylpropanoic acid	50	41	28 b	47 a	29 b	44 a	29 b
12	butanoic acid	240	41	52 bc	108 a	57 bc	59 b	47 c
13	phenylacetaldehyde	4	41	20 a	9 c	13 b	7 c	14 b
14	3-methylbutanoic acid	250	41	21 b	34 a	24 b	26 b	25 b
15	3-(methylthio)propanol	250	42	0.41 b	1.23 a	0.32 bc	0.28 c	0.23 c
16	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	31	35	34 a	27 bc	30 b	24 cd	22 d
17	2-phenylacetic acid	1000	43	1.61 a	1.20 b	1.62 a	1.08 b	1.61 a
18	3-phenylpropanoic acid	NA[c]						

[a]Different letters within a row indicate significant differences ($P < 0.05$). OAV = concentration divided by odor detection threshold. [b]Orthonasal odor threshold in water from the literature. [c]NA, not available.

Butanol and 2-methylbutanol could be further converted to butanal and 2-methylbutanal, respectively, by the action of a broad-specificity NADPH-dependent alcohol dehydrogenase, which has been found in the genome of *Virgibacillus* sp. SK37.[34] The oxidation of butanal and 3-methylbutanal also yields butanoic acid and 3-methylbutanoic acid, respectively. It was observed that an increase in 3-methylbutanoic acid content is likely to be related to a high leucine content in inoculated samples.

The free phenylalanine content of the inoculated samples appeared to be lower than that of the corresponding controls, which led to a reduced precursor for phenylacetaldehyde formation. In addition, 2-phenylacetic acid, 3-phenylpropanoic acid, and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone showed lower contents in the inoculated samples than in the corresponding controls ($P < 0.05$). Phenylacetaldehyde and 2-phenylacetic acid are responsible for rosy-like/sweet notes (Table 4). 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone has been found in different types of food. However, the role of this compound in fish sauce flavor has not been reported. It is one of the degradation products of the Amadori compound via 2,3-enolization, elongation by the Strecker aldehydes, and reduction of the resulting acetylformoin-type as intermediates, and it is responsible for a caramel-like odor.[30] It could be speculated that this compound contributes to the flavor of fish sauce because it has a low threshold value of 31 μg/kg.[35]

The 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal contents of the inoculated samples were higher than those of the controls at both levels of salt addition ($P < 0.05$). These compounds were derived from valine, isoleucine, and leucine, respectively, via amino acid catabolism pathways.[29] They contribute to the malty/dark chocolate odors (Table 4). Giri et al.[36] reported that 2-methylpropanal and 2-methylbutanal show high FD factors and contribute principally to the distinct odor of Thai fish sauce. Thus, the addition of *Virgibacillus* sp. SK37, in conjunction with salt content reductions to 15 and 20%, produces more intense volatile aldehydes in fish sauce.

Virgibacillus sp. SK37 and salt reduction had no effect on volatile sulfur compounds, including methanethiol, 3-(methylthio)propanal, dimethyl sulfide, and dimethyl trisulfide ($P > 0.05$). The 3-(methylthio)propanol content of the inoculated samples at 15% salt addition was higher than that of the control ($P < 0.05$). However, its impact is low due to a relatively high threshold as compared to the corresponding aldehyde.

The odor-activity values (OAVs) of 17 odorants in the samples are presented in Table 6. Six compounds, including methanethiol, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, dimethyl trisulfide, and 3-(methylthio)propanal, showed the highest OAVs, suggesting that these compounds have a strong influence on the overall aroma of fish sauce samples because of their high concentrations and low odor threshold values. The rest showed low OAVs, indicating a less important role in the overall aroma of fish sauce. The OAV of 3-phenylproanoic acid was not calculated as its odor threshold has not been reported. It should be noted that highly abundant acid compounds, including acetic acid, propanoic acid, butanoic acid, and 3-methylbutanoic acid, showed low OAVs.

Compared to the typical salt addition of 25%, the reduction of the salt content to 15% increased methanethiol in the finished product, which was likely to cause an undesirable note. The OAVs of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal significantly increased in fish sauce inoculated with *Virgibacillus* sp. SK37 under conditions of reduced salt content ($P < 0.05$). This result suggested that inoculation with *Virgibacillus* sp. SK37, along with salt content reductions to 15 and 20%, provided fish sauce with a more intense malty and/or chocolate-like odor. Salt content and *Virgibacillus* sp. SK37 had no influence on the OAV of dimethyl trisulfide. The relationship of aldehydes, particularly 2-methylpropanal, 2-methylbutanal,

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and 3-methylbutanal, to sensory characteristics of inoculated fish sauce deserves further investigation.

In conclusion, the reduction of salt content to 15−20% increased the rate of protein hydrolysis, resulting in increases in α-amino content, total nitrogen, browning index, pH, and free amino acids in the finished product, as compared to 25% salt treatment. The addition of *Virgibacillus* sp. SK37 at 15% salt content increased protein hydrolysis only at the early stage of fermentation, whereas it did not accelerate protein hydrolysis at 20% salt content at any time during fermentation. Total nitrogen and amino contents of inoculated samples at reduced salt content were lower than those of their respective controls, leading to lower degrees of browning. This is the first report demonstrating that *Virgibacillus* sp. SK37 at 15−20% salt content increases key aroma active compounds, namely, 2-methylbutanal and 3-methylbutanal. Therefore, the flavor of fish sauce could be controlled through application of *Virgibacillus* sp. SK37 starter culture.

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Notes

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REFERENCES

(1) Lopetcharat, K.; Choi, Y. J.; Park, J. W.; Daeschel, M. D. Fish sauce products and manufacturing: a review. *Food Rev. Int.* **2001**, *17*, 65−88.

(2) Fukami, k.; Funatsu, Y.; Kawasaki, K.; Watabe, S. Improvement of fish sauce odor by treatment with bacteria isolated from the fish sauce mush (moromi) made from frigate mackerel. *J. Food Sci.* **2004**, *69*, 45−49.

(3) Udomsil, N.; Rodtong, S.; Choi, Y. J.; Hua, Y.; Yongsawatdigul, J. Use of *Tetragenococcus halophilus* as a starter culture for flavor improvement in fish sauce fermentation. *J. Agric. Food Chem.* **2011**, *59*, 8401−8408.

(4) Yongsawatdigul, J.; Rodtong, S.; Raksakulthai, N. Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. *J. Food Sci.* **2007**, *72*, 382−390.

(5) Sinsuwan, S.; Rodtong, S.; Yongsawatdigul, J. NaCl-activated extracellular proteinase from *Virgibacillus* sp. SK37 isolated from fish sauce fermentation. *J. Food Sci.* **2007**, *72*, 264−269.

(6) Sinsuwan, S.; Rodtong, S.; Yongsawatdigul, J. Hydrolytic activity of *Virgibacillus* sp. SK37, a starter culture of fish sauce fermentation, and its cell-bound proteinases. *World J. Microbiol. Biotechnol.* **2012**, *2*, 2651−2659.

(7) Ventosa, A.; Nieto, J. J.; Oren, A. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 504−544.

(8) Schieberle, P.; Grosch, W. Quantitative analysis of aroma compounds in wheat and rye bread crusts using a stable isotope dilution assay. *J. Agric. Food Chem.* **1987**, *35*, 252−257.

(9) Fuganti, C.; Gatti, F. G.; Serra, S. A general method for the synthesis of the most powerful naturally occurring Maillard flavors. *Tetrahedron* **2007**, *63*, 4762−4767.

(10) Schieberle, P.; Gassenmaier, K.; Guth, H.; Sen, A.; Grosch, W. Character impact odour compounds of different kinds of butter. *Lebensm. Wiss. Technol.* **1993**, *26*, 347−356.

(11) Sen, A.; Schieberle, P.; Grosch, W. Quantitative determination of 2,5-dimethyl-4-hydroxy-3-(2*H*)furanone and its methyl ether using a stable isotope dilution assay. *Lebensm. Wiss. Technol.* **1991**, *24*, 364−369.

(12) Guth, H.; Grosch, W. Identification of the character impact odorants of stewed beef juice by instrumental analyses and sensory studies. *J. Agric. Food Chem.* **1994**, *42*, 2862−2866.

(13) Steinhaus, M.; Schieberle, P. Characterization of odorants causing an atypical aroma in white pepper powder (*Piper nigrum* L.) based on quantitative measurements and orthonasal breakthrough thresholds. *J. Agric. Food Chem.* **2005**, *53*, 6049−6055.

(14) Zimmermann, M. Important flavor compounds of bell pepper (*Capsicum annuum* var. *annuum*). Characterization of flavor differences depending on origin and drying procedure. Ph.D. Thesis, Technical University of Munich, Munich, Germany, 2001.

(15) Sen, A.; Grosch, W. Synthesis of six deuterated sulfur containing odorants to be used as internal standards in quantification assays. *Z. Lebensm.-Unters.-Forsch.* **1991**, *192*, 541−547.

(16) Schuh, C.; Schieberle, P. Characterization of the key aroma compounds in the beverage prepared from Darjeeling black tea: quantitative differences between tea leaves and infusion. *J. Agric. Food Chem.* **2006**, *54*, 916−924.

(17) Lin, J.; Welti, D. H.; Vera, F. A.; Fay, L. B.; Blank, I. Synthesis of deuterated volatile lipid degradation products to be used as internal standards in isotope dilution assays. 1. Aldehydes. *J. Agric. Food Chem.* **1999**, *47*, 2813−2821.

(18) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, 264−275.

(19) Field, R. The measurement of amino groups in proteins and peptides. *Biochem. J.* **1971**, *124*, 581−590.

(20) Kaspar, H.; Dettmer, K.; Gronwald, W.; Oefner, P. J. Automated GC−MS analysis of free amino acids in biological fluids. *J. Chromatogr., B* **2008**, *870*, 222−232.

(21) AOAC. *Official Methods of Analysis of the Association of Official Analytical Chemists*; AOAC: Arlington, VA, 1995.

(22) Engel, W.; Bahr, W.; Schieberle, P. Solvent assisted flavour evaporation-a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* **1999**, *209*, 237−241.

(23) van den Dool, H.; Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. *J. Chromatogr.* **1963**, *11*, 463−471.

(24) Yang, S. S.; Lee, C. M. Effect of culture media on protease and oxytetracycline production with mycelium and protoplasts of *Streptomyces rimosus*. *World J. Microbiol. Biotechnol.* **2001**, *17*, 403−410.

(25) Codex Alimentarious official standards. Standard for fish sauce (codex stan 302-2011); pp 1−5.

(26) Hernandez-Herrero, M. M.; Roig-Sagues, A. X.; Lopez-Sabater, E. I.; Rodriguez-Jerez, J. J.; Mora-Ventura, M. T. Influence of raw fish quality on some physicochemical and microbial characteristics as related to ripening of salted anchovies (*Engraulis encrasicholus* L). *J. Food Sci.* **2002**, *67*, 2631−2640.

(27) Narayanaswamy, D.; Narashimha Rao, G. V.; Govidan, J. K. Penetration of sodium chloride during prolonged salting fish. *Fish Technol.* **1980**, *17*, 63−64.

(28) Park, J. N.; Watanabe, T.; Endoh, K. I.; Watanabe, K.; Abe, H. Taste active components in a Vietnamese fish sauce. *Fish. Sci.* **2002**, *68*, 913−920.

(29) Ardo, Y. Flavour formation by amino acid catabolism. *Biotechnol. Adv.* **2006**, 238−242.

(30) Yvon, M.; Rijnen, L. Cheese flavour formation by amino acid catabolism. *Int. Dairy J.* **2001**, *11*, 185−201.

(31) Wichaphon, J.; Thongthai, C.; Assavanig, A.; Lertsiri, S. Volatile aroma compounds of Thai fish sauce in relation to product categorization. *Flavour Fragrance J.* **2012**, *27*, 149−156.

(32) Bulthuis, B. A.; Rommens, C.; Koningstein, G. M.; Stouthamer, A. H.; Verseveld, H. W. Formation of fermentation products and extracellular protease during anaerobic growth of *Bacillus licheniformis*

in chemostat and batch-culture. *Antonie van Leeuwenhoek. Int. J. G.* **1991**, *60*, 351−371.
(33) Shimoda, M.; Peralta, R. R.; Osajima, Y. Headspace gas analysis of fish sauce. *J. Agric. Food Chem.* **1996**, *44*, 3601−3605.
(34) Phrommao, E. Characterization of proteinases, proteinase genes, and genome of *Virgibacillus* sp. SK37 isolated from fish sauce. Ph.D. thesis, Suranaree University of Technology, Nakhon-Ratchasima, 2010.
(35) Buttery, R. C.; Takeoka, G. R.; Krammer, G. E.; Ling, L. C. Furaneol: odor threshold and importance to tomato aroma. *J. Agric. Food Chem.* **1995**, *43*, 1638−1640.
(36) Giri, A.; Osaka, K.; Okamoto, A.; Ohshima, T. Olfactometric characterization of aroma active compounds in fermented fish paste in comparison with fish sauce, fermented soy sauce and sauce products. *Food Res. Int.* **2010**, *43*, 1027−1040.
(37) Amoore, J. E. Specific anosia and the concept of primary odours. *Chem. Senses Flavour* **1977**, *2*, 267−281.
(38) Guadagni, D. G.; Buterry, R. G.; Okano, S. Odour threshold of some organic compounds associated with food flavours. *J. Sci. Food Agric.* **1963**, *14*, 761−765.
(39) Buttery, R. G. Quantitative and sensory aspects of flavor of tomato and other vegetables and fruits. In *Flavor Science − Sensible Principles and Techniques in Flavor Science*; Acree, T., Teranishi, R., Eds.; American Chemical Society: Washington, DC, 1993; pp 259−286.
(40) Guadagni, D. G.; Buttery, R. G.; Turnbaugh, J. G. Odor thresholds and similarity ratings of some potato chip compounds. *J. Sci. Food Agric.* **1972**, *23*, 1435−1444.
(41) Buttery, R. G.; Ling, L. C. Additional studies on flavor component of corn tortilla chips. *J. Agric. Food Chem.* **1998**, *46*, 2764−2769.
(42) Fritsch, H. T.; Schieberle, P. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian pilsner-type Beer. *J. Agric. Food Chem.* **2005**, *53*, 7544−7551.
(43) Carunchia Whetstine, M. E.; Cadwallader, K. R.; Drake, M. A. Characterization of aroma compounds responsible for the rosy/floral flavor in Cheddar cheese. *J. Agric. Food Chem.* **2005**, *53*, 3126−3132.