Acceleration of Thai Fish Sauce Fermentation Using Proteinases and Bacterial Starter Cultures

J. YONGSAWATDIGUL, S. RODTONG, AND N. RAKSAKULTHAI

ABSTRACT: A means to accelerate fish sauce fermentation without adversely affecting fish sauce quality was investigated. Starter cultures prepared from *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5 were added separately to anchovy that was hydrolyzed by 0.25% Alcalase at 60 °C for 2 h followed by 0.5% Flavourzyme at 50 °C for 4 h. The mixtures were then adjusted to contain 25% solar salt and incubated at 35 °C for 4 mo. α-Amino contents of all inoculated samples were higher than the control (without the addition of starter culture) during the course of fermentation. After 4-mo fermentation, the samples inoculated with *Staphylococcus* sp. SK1-1-5 contained the highest α-amino content of 733.37 ± 13.89 mM while that of the control was 682.67 ± 3.33 mM. Amino acid profiles of inoculated samples showed similar patterns to that of commercial product fermented for 12 mo, with glutamic, aspartic, and lysine being predominant amino acids. *Virgibacillus* sp. SK33 appeared to decrease histamine content of fish sauce by 50% when compared to the control. Volatile compounds analyzed by GC–MS of all inoculated samples fermented for 4 mo exhibited a similar pattern to those of the 12-mo-old commercial product. Samples inoculated with *Staphylococcus* sp. SK1-1-5 produced higher levels of volatile fatty acids and showed similar sensory characteristics to the commercial fish sauce fermented for 12 mo. *Staphylococcus* sp. SK1-1-5 is a potential strain that can be applied to produce fish sauce with overall sensory characteristics of traditional fish sauce in shorter time.

Keywords: amino acids, anchovy, biogenic amines, fish sauce, *Staphylococcus*, *Virgibacillus*, volatile compounds

Introduction

Thai fish sauce or "nam-pla" is a clear amber liquid with distinct flavor and aroma. Nam-pla is produced through a natural fermentation of anchovy (*Stolephorus* sp.) at high salt content (approximately 28% to 30%). Fermentation is usually carried out in a cement tank at ambient temperature. Protein hydrolysis during fermentation mainly relies on the action of endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Saisithi 1994; Gildberg and Thongthai 2001). Typically, no control measure is implemented during the fermentation process, which sometimes results in inconsistent product qualities. More importantly, complete fermentation to yield a premium quality fish sauce takes about 12 to 18 mo. For this reason, the growth of the fish sauce industry is rather limited by high capital investment of land and extremely long fermentation time.

Acceleration of the fish sauce fermentation process has been investigated worldwide. Gildberg and others (1984) produced fish sauce from anchovy (*Stolephorus* sp.) within 2 mo by adjusting to pH 4 using acetic and hydrochloric acid and lowering salt content to 5% to 15%. Increasing fermentation temperature of Pacific whiting fish sauce to 50 °C also resulted in a product containing total nitrogen equivalent to commercial fish sauce within 15 d (Lopetcharat and Park 2002). This was mainly because of an increase in proteolytic activity of endogenous cathepsins at the optimal condition (55 °C). Capelin harvested during summer could be used to produce fish sauce with a shorter fermentation period of 9 mo due to the high activity of digestive enzymes (Hjalmarsson and others 2007). Addition of 5% to 10% enzyme-rich cod pyloric cecum resulted in a good recovery of capelin fish sauce after 6 mo of storage (Gildberg 2001). These studies demonstrated the role of endogenous proteinases and/or proteinases from by-product of digestive tracts in accelerating protein solubilization during fish sauce fermentation. However, proteinase activity from fish declined gradually during fish sauce fermentation due to high salt content (25% NaCl) and acidic pH (5.5) (Siringan and others 2006). Therefore, addition of viscera as a source of proteinase might not be an effective measure to promote protein solubilization. Another important source of proteinases is microflora found in fish sauce fermentation. Uchida and others (2004) isolated *Bacillus subtilis* CN2 from a Vietnamese fish sauce that produced alkaline proteinase. *Filobacillus* sp. RF2-5 isolated from nam-pla produced a serine proteinase that was activated and stable at high NaCl content (15% to 25%) (Hiraga and others 2005). Therefore, the addition of proteinase-producing halophilic bacteria as a starter culture could be an alternative means to accelerate protein hydrolysis during fish sauce fermentation. This approach has not been thoroughly investigated.

Various volatile compounds, including acids, carbonyls, nitrogen-containing compounds, and sulfur-containing compounds, are formed during fermentation and thought to be responsible for the distinct aroma of fish sauce (Peralta and others 1996; Fukami and others 2002). These compounds are formed through various reactions, including lipolysis, Maillard browning reaction, and Strecker degradation (Shimoda and others 1996). In addition, they can be derived from the action of indigenous microorganisms. *Staphylococcus xylosus* isolated from fish sauce mash played a significant role in producing desirable notes of an odor in fish sauce (Fukami and others 2004). The addition of proteinase-producing halophilic bacteria might not only increase the rate of protein solubilization, but also contribute to flavor development. Consequently, a fish sauce with the desirable amino content and flavor/aroma characteristics could be obtained in a

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shorter period. This approach would definitely revolutionize the traditional fish sauce fermentation process. However, the suitable halophilic bacteria have not been obtained and their effects on protein hydrolysis and flavor/aroma development of fish sauce have not yet been determined.

Recently, we isolated and selected 3 strains of potential proteinase-producing bacteria, 2 Gram-positive endospore-forming aerobic bacterial and 1 Gram-positive coccus strains, from 1-mo-old fish sauce. The bacteria were identified as *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5, based on their morphological and some physiological characteristics, and their 16S rRNA gene sequences with accession nr DQ910838, DQ910840, and DQ910844, respectively. The objectives of this study were to accelerate fish sauce fermentation using the combination of commercial proteinases and bacterial starter cultures prepared from the 3 selected strains of halophilic bacteria. In addition, we investigated the effect of these starter cultures on the changes of important chemical properties, namely, amino acids, biogenic amines, and volatile compounds, as well as sensory characteristics of the fish sauce samples.

Materials and Methods

Raw materials

Indian anchovy (*Stolephorus indicus*) were caught off the Gulf of Thailand at Chonburi province. The samples were kept in ice on board and transported to Suranaree Univ. laboratory within 4 h after catch. Fish (1.5-kg block) were immediately frozen in a blast freezer (–20 °C) upon arrival and stored for 2 wk in a freezer before fermentation was prepared. Solar salt used in the fish sauce industry was collected from a fish sauce plant and used throughout the study. Alcalase 2.4L and Flavourzyme 500L were a gift from by Novozymes A/S (Denmark). Fish sauce samples fermented for 12 mo were obtained from Rayong Fish Sauce Industry Co. (Rayong, Thailand) and used as the "commercial sample."

Starter culture preparation

From our preliminary studies, 3 bacterial strains, *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5, grew well in fish broth prepared by homogenizing 1 part of anchovy (*Stolephorus indicus*) with 9 parts of distilled water, filtering, adjusting to pH 7.0, and autoclaving at 121 °C for 15 min. Optimum NaCl concentrations for growth of SK33, SK37, and SK1-1-5 in fish broth were 18%, 20%, and 15%, respectively. The inocula were prepared in a 500-mL Erlenmeyer flask containing 100-mL fish broth containing the optimum NaCl concentration of each bacterial strain at the initial pH 7.0 and incubated at 35 °C. *Staphylococcus* sp. SK1-1-5 was cultured for 24 h, while *Virgibacillus* sp. SK33 and SK37 were cultured for 3 d with a shaking speed of 100 rpm (INNOVA™ 4340, Incubator Shaker, New Brunswick Scientific Co. Inc., N.J., U.S.A.) to attain an approximate cell count of 6 log CFU/mL.

Preparation of fish sauce fermentation

Four fermentation treatments (control without starter culture, samples separately added either *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, or *Staphylococcus* sp. SK1-1-5) were prepared in 1 kg each in duplicate. Frozen anchovies were thawed in a running tap water (25 °C) for 1 h. The thawed fish were heated until the core temperature reached 65 °C and were packed in a glass jar (8.7-cm dia × 17-cm height). Alcalase 2.4L was added at 0.25% (w/w) and the samples were incubated in a 65 °C water bath for 2 h. Subsequently, the samples were cooled to 50 °C and 0.5% Flavourzyme 500L was added. The samples were hydrolyzed at 50 °C for another 4 h. The amount of commercial proteinases and hydrolysis conditions were determined from the preliminary studies to be the optimum for anchovy hydrolysis. When the time was attained, the hydrolysates were adjusted to contain 25% solar salt (w/w) and cooled to room temperature (28 °C). Each bacterial starter culture was then added at 10% inoculum size (w/w). The control was added with 10% fish broth. The mixture of all treatments occupied about 90% of jar volume. Fermentation was carried out in a 35 °C incubator (Hotpack an SP Industries Co., Philadelphia, Pa., U.S.A.) for 4 mo. Fermentation at 35 °C was selected in this study because it represented the average temperature used in the industry where the fermentation was carried out outdoors in the cement tank buried in the ground. Samples (10 g) were taken at each time interval and centrifuged at 8000 rpm (PK 121R, ALC Intl. Srl, Italy) at 4 °C for 10 min. The supernatant was collected and analyzed for α-amino content. When the fermentation time of 4 mo was attained, the mixtures were filtered through cheesecloth and filter paper (Whatman nr 1). The filtrates were collected and analyzed for biogenic amines, total nitrogen (TN), ammonical nitrogen (AN), amino acid profiles, volatile compounds, and sensory evaluation.

Microbiological analysis

The samples (10 g) were taken aseptically from the fermentation jar at each time interval, and halophilic bacteria were enumerated using the spread-plate technique on agar plates of JCM medium nr 168 (composed of [per L] 200 g NaCl, 5 g casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g tri-sodium citrate, 20 g $MgSO_4.7H_2O$, 36 mg $FeCl_2.4H_2O$, 0·36 mg $MnCl_2.4H_2O$, 20 g agar; pH 7.2) with incubating at 35 °C for 7 to 14 d (Namwong and others 2005).

α-Amino content

Soluble peptides of anchovy, anchovy hydrolysate, and fish sauce collected at each time interval were measured as α-amino content by the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen 1979). Liquid obtained after centrifugation at each time interval was diluted with 1% sodium dodecyl sulfate (SDS) about 50 to 200 times, depending on the extent of hydrolysis. Absorbance was measured at 420 nm using leucine as a standard. α-Amino content was expressed as mM of leucine.

Amino acid profiles

Total and free amino acid profiles of all samples including the commercial fish sauce were analyzed. To determine total amino acids, samples were hydrolyzed in 6 N HCl at 110 to 115 °C for 16 h using an autoclave. Hydrolyzed samples as well as standard amino acid mixture aliquots were dried by rotary evaporation under vacuum. The residue was then dissolved in citrate buffer (pH 2.2). Samples for cysteine determination were oxidized with performic acid at 0 °C overnight before hydrolysis. Free amino acids were determined by directly diluting all samples with sodium citrate buffer. Amino acids were quantified using an amino acid analyzer (Model 8500L, Hitachi High-Technologies, Tokyo, Japan) equipped with an ion exchange column (Hitachi amino acid column, 4.6 × 60 mm) with postcolumn ninhydrin derivatization. Sodium citrate buffers at pH 3.2 to 4.9 were used as a mobile phase. The standard amino acid for physiological fluid was analyzed in the same condition to identify retention time. The amount of amino acids was expressed as milligrams of amino acid per 100 mL of fish sauce. Difference between total and free amino acids was defined as oligopeptide content of the sample.

Biogenic amine analysis

Determinations of histamine, cadaverine, tyramine, putrescine, spermidine, and spermine were carried out by high-performance liquid chromatography (HPLC) by the method of Mietz and Karmas (1977) with slight modifications. Internal standard solution (1000 mg/L) of 1,7-diaminoheptane (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used. Biogenic amines of anchovy tissue and hydrolysate were extracted by adding 15 mL of 0.4 M perchloric acid to 5 g of homogenized fish sample, followed by the addition of 125 μL of internal standard solution (Eerola and others 1993). Biogenic amines of fish sauce samples were determined directly without extraction. Fish sauce samples were diluted with 0.4 M perchloric acid and mixed with internal standard to contain a final concentration of 1 mg/L. Recoveries of individual biogenic amines were determined by adding working standard solutions at 10 to 100 mg/L to fish or fish sauce samples before extraction.

One milliliter of diluted extract was mixed with 200 μL of 2 N sodium hydroxide and 300 μL of saturated sodium bicarbonate. Two milliliters of dansyl chloride solution (10 mg/mL) prepared in acetone were added to the mixture, and then were incubated at 40 °C for 45 min. Residual dansyl chloride was removed by the addition of 300 μL of 30% ammonia. After 30 min at room temperature, the extracts were adjusted to 5 mL with acetonitrile. The solution was filtered through a 0.45 μm regenerated cellulose membrane filter (Agilent Technologies Inc., Palo Alto, Calif., U.S.A.).

An HPLC unit (HP 1100, Agilent Technologies Inc.) equipped with a photodiode array detector and HP ChemStation software (Rev.A.09.03) was employed. A Hypersil BDS C_{18} (100 × 4 mm I.D., 3 μm, 100 Å) reverse phase column fitted with a Hypersil BDS C_{18} (4 × 4 mm I.D., 5 μm, 100 Å) guard column was used. The mobile phase consisted of the mixture of acetonitrile and 0.02 M acetic acid (1:9) as a solvent A and the mixture of 0.02 M acetic acid, acetonitrile and methanol (1:4.5:4.5) as a solvent B. The flow rate was set at 1 mL/min. Isocratic elution was initiated with 50% solvent B for 5 min; subsequently the gradient elution was started and ended at 90% solvent B in 25 min. The column was equilibrated with 50% solvent A and B for 10 min before the next injection. The column was kept at 40 °C in a column compartment. The sample volume injected was 10 μL and the dansylated amines were detected at 254 nm with 550 nm as reference.

Physicochemical properties

Fish sauce samples fermented for 4 mo were analyzed for total nitrogen, ammonical nitrogen, and salt content (AOAC 1995). Degree of browning was monitored by diluting fish sauce filtrate with distilled water at a ratio of 1 to 4 and measuring absorbance at 440 nm. The pH was measured by inserting the cleaned pH electrode to the fish sauce samples.

Dynamic headspace and capillary gas chromatography–mass spectrometry (GC–MS)

Dynamic headspace was carried according to Wanakhachornkrai and Lertsiri (2003) with slight modifications. Five milliliters of fish sauce were added to 2-methyl-3-heptanone at a final concentration of 76.3 ng/mL as an internal standard and purged for 20 min with helium at 40 °C. The volatile compounds were concentrated on a Tenax TA trap and thermally discharged at 220 °C for 2 min. The desorbed volatiles were directly introduced onto GC-MS via electric pressure-control volatile interface with split ratio of 25:1. Separation of volatile compounds was carried out using an Agilent 6890 GC coupled to a mass-selective detection (Agilent 5973 MSD) with HP-FFAP capillary column (25 m × 0.32-mm inner dia × 0.5-μm film thickness, Agilent Technologies Inc.). The oven temperature was programmed from 45 to 240 °C at the rate of 15 °C/min. The initial holding time was 2 min. The inlet temperature was set at 220 °C and the flow rate of helium as a carrier gas was 1.5 mL/min. Mass spectra were obtained by electron impact ionization at 70 eV. The volatile compounds were determined with retention indices (RI) and the Wiley 275L mass spectra database.

Sensory evaluation

The panel consisted of 18 individuals who work at 3 different fish sauce plants at Rayong province, Thailand. These panelists deal with fish sauce tasting on the regular basis as a quality control or production operator. The panelists were asked to give acceptance scores for 4 attributes: color, odor, flavor, and overall acceptance, using the 7-point hedonic scale. Four fish sauce samples (SK1-1-5, SK33, SK37, commercial fish sauce) were presented to each panelist in a random order. Samples inoculated with starter culture were fermented for 4 mo in the laboratory and the commercial product was 12-mo-old fish sauce collected from the fish sauce plant (Rayong Fish Sauce Industry, Thailand). No sugar or any other additives were added in any sample. The samples fermented for 4 mo without starter culture were not tested because they showed the sensory characteristics of unripe fish sauce with fishy odor and light yellow color.

Ten milliliters of samples were filled into a 15-mL glass cup with approximately 2-cm headspace. The sample cups were covered with lids and left at room temperature (approximately 28 °C) for 30 min before sensory evaluation. The panelists compared odor by opening the lid of the container and sniffing. Flavor preference was assessed by tasting approximately 0.5 mL of fish sauce samples using a plastic spoon. The panelists were asked to use drinking water and plain cracker for rinsing their mouth before tasting the next sample.

Statistical analysis

All chemical analysis was carried out at least in duplicate. Differences between periods of fermentation and type of starter culture were determined by analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine differences between mean at $P < 0.05$ (SAS Inst. Inc., Cary, N.C., U.S.A.).

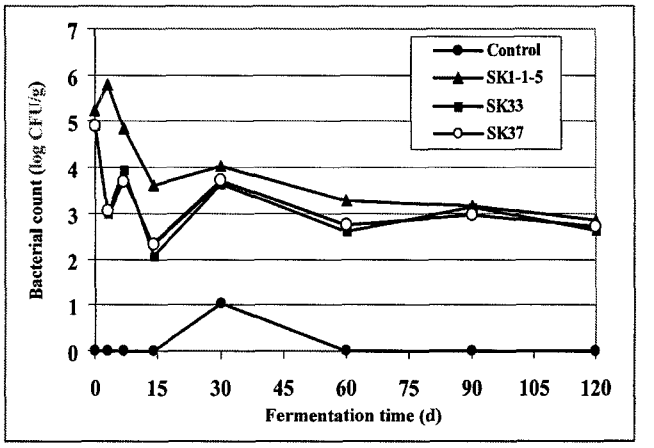

Figure 1 – Changes in halophilic bacterial counts of fish sauce samples prepared from anchovy hydrolysate inoculated with bacterial starter cultures and incubated at 35 °C for 4 mo.

Results and Discussion

Microbiological changes

The initial microbial load of samples separately inoculated with 3 strains of starter cultures was approximately 5 log CFU/g and gradually declined to about 3 log CFU/g at day 60 (Figure 1). It remained constant at 3 log CFU/g until day 120. For the control (without inoculation), only a small number of halophilic bacteria (1 log CFU/g) were observed at day 30 (Figure 1). Since anchovies were exposed to relatively high temperature (50 to 60 °C) for 6 h during enzymatic hydrolysis, halophilic bacteria naturally presented in the fish could be thermally inactivated. These results implied that the microbial population observed in the culture added samples was most likely the inoculated strains themselves. Although the inoculated cultures did not increase their numbers, they appeared to endure high salt environment for at least up to 4 mo. Our results also indicated that in fact all bacterial strains remained in the system up to 8 mo (data not shown). The high survival rate of all strains at high salt content (25% NaCl) is one of important characteristics of starter culture for fish sauce fermentation.

Typically, aerobic plate counts of fish sauce decreased as fermentation progressed because high salt content (25% to 28% NaCl) inhibits the majority of microorganisms except for halophilic/halotolerant bacteria (Saisithi 1994). From our preliminary study, halophilic bacterial counts of samples collected from various fish sauce plants were relatively low (< 30 CFU/g) during the fermentation period of 3 to 12 mo. Therefore, protein hydrolysis relies mainly on the action of endogenous proteinases associated with fish muscle and digestive tract. These enzymes are likely to be inactivated at high salt environment. This is the main reason why fish sauce fermentation is an extremely long process. The addition of these 3 studied strains significantly increased halophilic/halotolerant bacterial counts during fish sauce fermentation when compared to the commercial process.

Changes of α-amino content

An increase in α-amino content indicated the extent of proteolysis during fish sauce fermentation as it reflected the formation of oligopeptides and/or amino acids (Adler-Nissen 1979). α-Amino content of the control increased during fermentation due to proteolytic activity of digestive enzymes and added commercial proteinases (Table 1). Generally, samples inoculated starter culture showed higher α-amino content than the control ($P < 0.05$, Table 1). These results suggested that all 3 starter cultures could produce proteinases hydrolyzing anchovy proteins at high salt content. Sinsuwan and others (2007) reported that *Virgibacillus* sp. SK37 secreted extracellular proteinases exhibiting subtilisin-like characteristic with activity at 25% NaCl. Our results suggested that bacterial proteinase was one of critical factors that contributed to protein hydrolysis during fish sauce fermentation. This is the 1st study demonstrating that starter culture prepared from either *Virgibacillus* sp. or *Staphylococcus* sp. accelerates protein solubilization during fish sauce fermentation.

In the conventional fermentation process, an increase of soluble peptides extensively took place during the initial stage of fermentation, especially within the first 13 wk of fermentation and became minimal thereafter (Yongsawatdigul and others 2004). Typically, α-amino content of liquid drained from 3-d fermentation of anchovy was less than 200 mM (Yongsawatdigul and others 2004). High initial α-amino content observed in this study at 0 d was attributed to the action of commercial proteinases. α-Amino content of 12-mo-old fish sauce was approximately 860 to 878 mM. Amino contents obtained from this study were relatively lower than that of the commercial product. This could be because the fermentation time was only 4 mo in our study. More importantly, addition of 10% inoculum could have a dilution effect on α-amino content. Nevertheless, it was evident that addition of starter culture resulted in higher α-amino content than the control.

Total and free amino acid profiles

Amino acid profiles of all treatments were similar to that of the commercial product (Table 2). Free amino acid contents of all treatments ranged from 6785 to 7024 mg/100 mL, while that of the commercial product fermented for 12 mo was 8722 mg/100 mL. Total amino acid contents of samples inoculated with *Virgibacillus* sp. SK33 and SK37 and the 12-mo-old fish sauce were in the same range of 9000 to 9500 mg/100 mL (Table 2). These results suggested that both strains accelerated protein hydrolysis during fermentation. Extracellular proteinases of these bacteria could play a vital role in peptide and amino acid formation. Aspartic acid, glutamic acid, and lysine were predominant amino acids in all samples (Table 2). These results were in agreement with the amino acid profiles of fish sauce reported by Park and others (2001). Glutamic acid and alanine were reported to be the taste-active components providing characteristic taste of fish sauce along with threonine, tyrosine, histidine, and methionine (Park and others 2002). The majority of alanine in all samples appeared to exist in free amino acid (Table 2). Threonine, tyrosine, histidine, and methionine contents were comparable in all samples. It should be noted that total tyrosine and methionine contents of some samples were lower than those of free form. Since these amino acids were prone to oxidation, they could be oxidized during sample preparation by acid hydrolysis. Total glutamic content of samples inoculated with starter cultures appeared to be greater

Table 1 — α-Amino acid contents of fish sauce prepared from anchovy hydrolysate inoculated with bacterial starter cultures and fermented at 35 °C for 4 mo.

Fermentation time (d)	Fish sauce sample			
	Control	SK1-1-5	SK33	SK37
0	500.93 ± 14.27^{a}	527.60 ± 17.32^{a}	525.44 ± 5.10^{a}	510.66 ± 0.51^{a}
3	578.05 ± 9.17^{a}	582.73 ± 4.58^{a}	585.97 ± 3.06^{a}	585.61 ± 12.74^{a}
7	620.99 ± 13.50^{b}	666.87 ± 5.71^{a}	669.07 ± 9.86^{a}	658.06 ± 1.56^{a}
14	574.18 ± 4.99^{b}	602.42 ± 4.99^{ab}	606.31 ± 8.49^{a}	612.66 ± 17.47^{a}
30	580.54 ± 0.00^{c}	671.26 ± 1.49^{a}	652.90 ± 17.47^{a}	614.42 ± 14.97^{b}
60	631.88 ± 22.80^{b}	683.32 ± 19.54^{a}	698.67 ± 8.68^{a}	686.39 ± 1.08^{a}
90	665.66 ± 7.59^{b}	708.65 ± 8.68^{a}	691.38 ± 5.98^{a}	686.39 ± 1.08^{ab}
120	682.67 ± 3.33^{b}	733.37 ± 13.89^{a}	714.11 ± 6.67^{ab}	713.33 ± 10.01^{ab}

Different superscripts within a row indicate significant differences ($P < 0.05$).
SK1-1-5 = *Staphylococcus* sp. SK1-1-5.
SK33 = *Virgibacillus* sp. SK33.
SK37 = *Virgibacillus* sp. SK37.

than the control and comparable to the commercial product fermented for 12 mo (Figure 2). However, free glutamic content of the inoculated samples was lower than the commercial product. These results indicated that protein hydrolysis of the inoculated samples mainly resulted from the action of endoproteinases rather than exoproteinases. When samples collected from various fish sauce plants were analyzed for amino acid profiles, they showed the average free glutamic content of 343.8, 645.8, and 797.1 mg/100 mL at the 1st, 3rd, and 5th month of fermentation, respectively. Free glutamic content increased to about 1199.3 mg/100 mL after 7 mo and reached 1394.5 mg/100 mL at the 12th month. Our results demonstrated that free glutamic content of starter culture-inoculated samples increased to about 1000 mg/100 mL within 4 mo of fermentation, which was equivalent to that of sample conventionally fermented for 7 mo. Based on free glutamic content, addition of starter culture of these 3 selected strains could shorten the fermentation time by 40%.

To increase free amino acid content to the similar level of conventionally fermented sample, the fermentation by starter culture should be extended beyond 4 mo. The action of exoproteinases, namely, aminopeptidase and carboxypeptidases, is responsible for formation of free amino acids during fish sauce fermentation. Vo-Van and others (1984) reported that aminopeptidase purified from 1-mo-old fish sauce showed similar biochemical characteristics to those of sardine aminopeptidase. Addition of dipeptidyl aminopeptidase (cathepsin C) from squid hepatopancrease to fish sauce fermentation of capelin resulted in higher free amino content (Raksakulthai and others 1986). Lactic acid bacteria isolated from various types of cheese have also been reported to be a potential source of aminopeptidases and dipeptidyl peptidases (Magboul and McSweeney 1999; Macedo and others 2000; Herrerose and others 2003). In addition, γ-glutamyltranspeptidases and glutaminases that catalyze the hydrolysis of γ-glutamyl compounds to glutamic acid are important enzymes responsible for free

Table 2—Total and free amino acid contents of fish sauce prepared from anchovy hydrolysate inoculated with bacterial starter cultures and fermented at 35 °C for 4 mo.

	Amino acid content (mg/100 mL)									
	Commercial		Control		SK1-1-5		SK33		SK37	
Amino acid	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free
Trp	193	71	201	85	223	87	213	79	214	85
Thr	461	516	415	412	453	415	467	420	488	418
Ile	312	410	364	410	384	471	416	475	440	408
Leu	396	491	494	532	517	525	536	535	548	521
Lys	932	986	847	725	912	740	941	758	968	745
Met	218	262	228	259	244	274	258	276	275	256
Cys	66	63	78	85	77	76	83	73	73	88
Phe	310	426	327	420	349	393	359	418	372	437
Tyr	62	129	88	171	89	150	100	166	91	165
Val	515	635	485	577	535	590	559	592	587	557
Arg	266	277	490	471	81	29	394	325	74	29
His	446	398	421	353	413	339	443	370	453	370
Ala	595	675	580	585	650	633	673	623	719	616
Asp	998	825	911	539	1006	572	1023	578	1090	576
Glu	1856	1402	1542	789	1796	1007	1791	926	1932	955
Gly	561	344	520	152	542	196	563	180	580	201
Pro	415	222	373	120	381	126	375	134	399	131
Ser	366	423	300	273	208	154	295	238	162	91
Taurine	71	167	65	84	68	82	87	84	48	136
Total	9039	8722	8729	7042	8928	6859	9576	7250	9513	6785

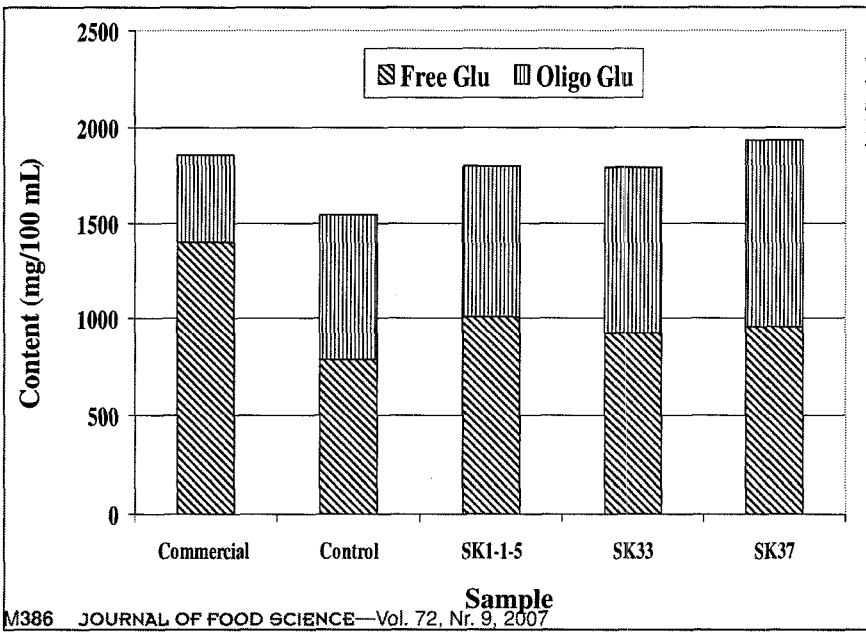

Figure 2—Glutamic acid contents in the free and oligopeptide forms of fish sauce samples prepared from anchovy hydrolysate inoculated with bacterial starter cultures and fermented at 35 °C for 4 mo.

glutamic formation and flavor enhancement during soy sauce fermentation (Weingand Ziadé and others 2003). The enzymes were widely distributed in *Micrococcus luteus*, *Lactobacillus rhamnosus*, and various species of *Bacillus* (Nandakumar and others 2003). These enzymes could be important for increasing free amino, particularly glutamic acid, in fish sauce fermentation. However, bacteria producing these enzymes in fish sauce have not to date been characterized.

Biogenic amines content

Recovery of histamine, putrescine, cadaverine, and tyramine was 86.8%, 88.0%, 85.6%, and 78.7%, respectively. High recovery of tryptamine was obtained at 90.8%. Recovery of spermidine and spermine was relatively poor at 68.2% and 56.3%, respectively. However, the latter 2 compounds are usually minor biogenic amines found in fish sauce and various fermented fish products (Stratton and others 1991; Veciana-Nogués and others 1996; Mah and others 2002; Yongsawatdigul and others 2004). The major biogenic amines in fresh anchovy were histamine, cadaverine, and tyramine (Table 3). When anchovies undergo spoilage, some biogenic amines, namely, putrescine, histamine, cadaverine, and tyramine, greatly increase (Veciana-Nogués and others 1996). This is mainly caused by contamination of bacteria exhibiting amino-decarboxylase activity. Histamine and cadaverine level of anchovy increased after enzymatic hydrolysis while other biogenic amines were not affected (Table 3). Typically, biogenic amine accumulation in fish mainly results from metabolism of microorganisms (Brink and others 1990). As protein was hydrolyzed by fish endogenous and exogenous proteinases, free amino acids were formed. These amino acids, particularly histidine and lysine, served as substrates for histidine- and lysine-decarboxylases produced by microflora. Fujii and others (1994) reported that histidine decarboxylase activity was found in the cell suspension of *Photobacterium histamium* in spite of complete loss of viability. Moreover, pH optimum of histidine decarboxylase from *Klebsiella planticola, Enterobacter aerogenes*, and *Morganella morganii* AM-15 was 6.5 (Tanas and others 1985; Guirard and Snell 1987), which was close to pH 6.3 to 6.8 of raw fish and fish hydrolysate. The release of histidine decarboxylase by microflora might occur in the early stage of fish handling and react with amino acids formed during protein hydrolysis process. Biogenic amine contents would have been higher if the spoiled fish had been employed. Spoiled fish contain higher amino decarboxylase acivities associated with biogenic amine-forming bacteria, resulting in higher extent of amino acid decarboxylation. Therefore, freshness quality of raw material is a critical parameter that should be strictly controlled so that low biogenic amine fish sauce could be obtained.

Arginine is normally converted to ornithine, which is further decarboxylated to putrescine by bacteria (Silla Santos 1996). Low level of putrescine, spermidine, and spermine in anchovy hydrolysate implied that ornithine decarboxylase activity was limited. This was probably due to low population of bacteria producing ornithine decarboxylase. In addition, ornithine content could be minimal in anchovy with high freshness quality. Putrescine was prevalent in anchovy stored at abused temperature (Yongsawatdigul and others 2004), and fermented anchovy products (Rodriguez-Jerez and others 1994). Thus, the use of decomposed anchovy is likely to increase putrescine level in the hydrolysate and the finished product.

Histamine, cadaverine, and tyramine were the major biogenic amines in 4-mo-old fish sauce (Table 3). Histamine level of the control was slightly higher than the maximum limit of 20 mg/100 mL imposed by Canadian Food Inspection Agency (CFIA 2003) and was comparable to samples inoculated with *Virgibacillus* sp. SK37 and *Staphylococcus* SK1-1-5 ($P > 0.05$). Fish sauce samples inoculated with *Virgibacillus* sp. SK33 showed the lowest histamine content ($P < 0.05$), but the highest tyramine content ($P < 0.05$, Table 3). It should be noted that tyramine content detected in our study was lower than that reported in literatures. Kirschbaum and others (2000) found high level of tyramine of 33.7 to 73.9 mg/100 mL in anchovy fish sauce. Yongsawatdigul and others (2004) found high level of tyramine (20 mg/100 mL) in anchovy fish sauce prepared from temperature abused raw material and low level (< 0.5 mg/100 mL) in the sample prepared from fresh anchovy. Cadaverine content of all samples was similar and considered to be in the lower range, particularly when compared to those previously reported in fish sauce (29.1 to 68.6 mg/100 mL, Yongsawatdigul and others 2004). The lower levels of tyramine and cadaverine observed in this study could be mainly attributed to the high freshness quality of raw material used. Our results indicated that *Virgibacillus* sp. SK-33 was able to reduce histamine level more than 50% during the course of 4-mo fermentation. In addition, *Virgibacillus* sp. SK-37 and *Staphylococcus* sp. SK1-1-5 did not increase biogenic amine content during fish sauce fermentation. Based on the biogenic amine levels, all 3 selected strains exhibited the desirable feature of starter culture since they did not increase biogenic amines. In addition, application of *Virgibacillus* sp. SK-33 could be an effective measure to control histamine level in fish sauce fermentation.

Some strains of *Brevibacterium linens*, *Micrococcus varians*, and coryneform bacteria were reported to degrade histamine and tyramine (Leuschner and others 1998). Diamine oxidase located in the bacterial cytoplasm was responsible for the amine degradation. The optimum condition of tyramine oxidase from *Micrococcus varians* was at 37 to 40 °C and pH 7 to 8. *Staphylococcus carnosus* did not degrade histamine and tyramine (Leuschner and others 1998). This corresponded with our results indicating that *Staphylococcus* sp. SK1-1-5 had no effect on biogenic amine reduction. Enes Dapkevicius and others (2000) also reported that some strains of

Table 3—Biogenic amine contents of raw materials and fish sauce samples inoculated with starter cultures and fermented at 35 °C for 4 mo.

Sample	Cadaverine	Histamine	Tyramine	Tryptamine	Putrescine	Spermine	Spermidine
Biogenic amine of raw material(mg/100 g)							
Fresh anchovy	1.91 ± 0.39^{a}	6.37 ± 0.50^{a}	3.27 ± 0.02^{b}	ND	ND	ND	ND
Hydrolyzed anchovy	5.37 ± 0.40^{b}	25.64 ± 1.56^{b}	1.19 ± 0.09^{a}	ND	ND	ND	ND
Biogenic amine of fish sauce (mg/100 mL)							
Control	4.42 ± 1.72	21.53 ± 4.41^{y}	4.96 ± 0.93^{x}	ND	ND	ND	ND
SK1-1-5	6.58 ± 1.59	20.65 ± 1.62^{y}	2.21 ± 0.41^{x}	ND	ND	ND	ND
SK33	6.23 ± 0.24	11.76 ± 0.07^{x}	9.06 ± 1.45^{y}	ND	ND	ND	ND
SK37	4.61 ± 2.11	15.42 ± 2.88^{xy}	4.56 ± 0.94^{x}	ND	ND	ND	ND

Superscripts a, b and x, y indicate significant differences ($P < 0.05$) in column within raw material and fish sauce, respectively.
ND = not detected.

Lactobacillus sakei isolated from fish paste degraded histamine more than 50%. Our study is the 1st report describing proteolytic activity and histamine reduction ability of *Virgibacillus* sp. isolated from Thai fish sauce. Further study is needed to clarify the effect of environmental factors, namely, pH, NaCl content, and temperature, on histamine degradation of *Virgibacillus* sp.

Other physicochemical properties

Total nitrogen, which is currently used as a quality index in the fish sauce industry, of all samples was comparable ($P > 0.05$, Table 4). Ammonical nitrogen of samples inoculated with *Staphylococcus* sp. SK1-1-5 and *Virgibacillus* sp. SK37 was greater than others ($P < 0.05$), suggesting that degradation of protein to amino acids and ammonia occurred to a greater extent by these 2 species. This also resulted in higher pH ($P < 0.05$). Liquid obtained from samples inoculated with *Staphylococcus* sp. SK1-1-5 also showed greater extent of browning ($P < 0.05$). A greater extent of protein hydrolysis induced by proteolytic activity of *Staphylococcus* sp. SK1-1-5 could result in higher content of amino acids and peptides (Table 1), which were subsequently served as substrates for Maillard browning reaction. Addition of bacterial starter cultures did not affect the salt content of the finished products.

Table 4—Physicochemical properties of fish sauce samples prepared from anchovy hydrolysate fermented with various strains of bacterial starter cultures at 35 °C for 4 mo.

Fish sauce sample	pH	Abs @ 440 nm	Total nitrogen (%)	Ammonical nitrogen (%)	NaCl (%)
Control	5.46 ± 0.01[c]	0.302 ± 0.001[b]	1.88 ± 0.01	0.13 ± 0.004[b]	27.06 ± 0.62
SK1-1-5	5.71 ± 0.00[a]	0.345 ± 0.090[a]	1.87 ± 0.01	0.21 ± 0.008[a]	26.33 ± 0.41
SK33	5.49 ± 0.00[b]	0.310 ± 0.001[b]	1.87 ± 0.01	0.14 ± 0.002[b]	26.62 ± 0.00
SK37	5.71 ± 0.01[a]	0.311 ± 0.001[a]	1.88 ± 0.01	0.22 ± 0.003[a]	26.47 ± 0.21

Different superscripts within a column indicate significant differences ($P < 0.05$).

Table 5—Volatile compounds of fish sauce fermented conventionally and samples inoculated with bacterial starter cultures and fermented at 35 °C for 4 mo.

			Relative peak area[a]				
Nr	RI[b]	Compounds	Com[c]	Control	SK1-1-5	SK33	SK37
		Acids					
25	1470	Acetic acid	0.220	0.251	0.352	0.167	0.206
28	1590	2-Methyl propanoic acid	0.023	0.028	0.038	ND[d]	ND
29	1657	Butanoic acid	0.115	0.102	0.156	0.050	0.033
31	1698	3-Methyl butanoic acid	0.045	0.068	0.135	0.056	0.067
		Alcohols					
8	931	2-Propanol	0.048	ND	ND	ND	ND
9	940	Ethanol	0.278	0.193	2.361	1.468	1.811
11	1047	n-Propanol	0.066	0.029	0.069	0.073	0.041
13	1100	2-Methyl-1-propanol	0.038	ND	0.021	0.132	0.114
14	1155	n-Butanol	0.015	0.025	0.026	0.035	0.037
15	1170	1-Penten-3-ol	ND	0.051	0.120	0.162	0.152
17	1215	3-Methyl-1-butanol	0.106	0.021	0.556	0.276	0.276
18	1259	Isobutenylcarbinol	ND	ND	0.027	0.032	ND
19	1260	n-Pentanol	ND	0.018	ND	ND	0.022
22	1331	cis-2-Pentenol	ND	0.013	0.021	0.027	ND
30	1692	2-Furanmethanol	0.013	0.042	0.023	0.039	0.014
		Aldehydes					
6	921	2-Methylbutanal	0.324	0.152	0.179	0.229	0.212
7	924	3-Methylbutanal	0.197	0.101	0.151	0.142	0.144
26	1476	3-(Methylthio) propanal	0.106	0.021	ND	ND	ND
27	1560	Benzaldehyde	0.096	0.121	0.130	0.089	0.077
		Esters					
4	902	Ethyl acetate	ND	0.014	0.019	ND	0.026
		Ketones					
2	828	2-Propanone	1.360	0.564	0.918	1.079	0.925
5	913	2-Butanone	0.249	0.185	0.303	0.326	0.331
10	992	2,3-Butanedione	ND	ND	0.031	ND	ND
21	1300	3-Hydroxy-2-butanone	ND	ND	0.072	0.127	0.020
		Nitrogen-containing compounds					
20	1286	Methylpyrazine	0.030	ND	ND	ND	ND
23	1343	2,5-Dimethyl pyrazine	ND	ND	0.014	ND	ND
24	1344	2,6-Dimethyl pyrazine	ND	ND	ND	0.014	ND
		Furans and sulfur-containing compounds					
1	<828	Dimethyl sulfide	0.040	0.026	0.030	0.048	0.049
3	874	Tetrahydrofuran	0.024	0.008	ND	ND	ND
12	1088	Dimethyl disulfide	0.023	0.011	0.025	0.018	0.024

[a]The values represent the ratios of the peak area in fish sauce to that of the internal standard (3-methyl-3-heptanone).
[b]Retention indices calculated for HP-FFAP column using n-alkanes as standards.
[c]Commercial fish sauce fermented for 12 mo without any additives added.
[d]ND = not detected.

Volatile compounds

A total of 30 volatile compounds were analyzed by dynamic headspace and compared between commercial product and starter cultures added samples (Table 5). Volatile fatty acids detected were acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid. Acetic acid appeared to be a predominant acid found in all samples, which was in agreement with the findings of Park and others (2001). Fukami and others (2002) reported that 3-methylbutanoic acid was one of potent distinctive volatile fatty acids detected in nam-pla and contributed to a rancid note. Volatile fatty acids were also associated with cheesy odor and had relatively low threshold value in the range of 2.45 to 7.10 ppb, indicating that the odor of these acids could be easily recognized (Peralta and others 1996). Only the samples inoculated with *Staphylococcus* sp. SK1-1-5 appeared to have higher levels of volatile fatty acids, particularly butanoic and 3-methyl butanoic acid, than others. *Staphylococcus* sp. SK1-1-5 was the only starter culture showing lipase activity, which could subsequently promote lipolytic degradation during fermentation. Polyunsaturated fatty acids resulted from lipolysis would, in turn, undergo oxidation to form volatile fatty acids. These volatile fatty acids could also be formed through amino acid metabolism of starter cultures.

Although alcohols were commonly found in fish sauce, they typically did not influence overall odor of fish sauce because of their relatively high threshold values (Devos and others 1995). However, all inoculated samples contained higher amount of various alcohols than the control and the conventionally fermented sample (Table 5). Ethanol, 2-methyl-1-propanol, and 3-methyl-1-butanol were predominant alcohols in the starter culture added fish sauce samples. Ethanol content of all inoculated samples was approximately 10 times greater than the conventionally fermented sample. High level of ethanol was hardly reported in fish sauce, but it was typically detected in soy sauce produced from yeast fermentation (Lee and others 2006). It should be noted that yeast was not detected in all samples during the course of fermentation. Ethanol observed in our study could be formed via primary metabolic activity of starter cultures under limited oxygen condition. Bulthuis and others (1991) reported that ethanol and acetaldehyde were among volatile compounds formed via glycolysis and pentose phosphate pathway during the anaerobic growth of *Bacillus licheniformis* in batch culture. An approximately 5-fold increase in 3-methyl-1-butanol content was also noted in the sample inoculated with *Staphylococcus* sp. SK-1-1-5 when compared to the 12-mo-old fish sauce. This compound was responsible for the burnt note (Fukami and others 2002) and it would possibly be derived from leucine in fish sauce through precursors of α-ketoisocaproate and isovaleradehyde by the action of the inoculated *Staphylococcus* sp. (Masson and others 1999). When nam-pla was inoculated by *Staphylococcus xylosus* strain R4Nu, the content of 3-methyl-1-butanol also increased about 10 times after 24 d compared to the untreated sample (Fukami and others 2004).

2-Methylbutanal was reported to be one of distinctive volatile compounds of nam-pla and responsible for a meaty note (Fukami and others 2002). The samples conventionally fermented for 12 mo contained the highest amount of 2-methylbutanal. The samples fermented for 4 mo appeared to contain lesser amounts of 1-methylbutanal, but those added starter cultures tended to have higher amount than the control. These results suggested that these 3 selected bacteria could contribute to the formation of a distinctive volatile compound of fish sauce.

The major ketone found in conventionally fermented fish sauce was 2-propanone. Shimoda and others (1996) reported that high concentration of 2-butanone, 3-methyl-2-butanone, 2-pentanone, and 3-methyl-2-pentanone was detected in fish sauce. These compounds were assumed to be responsible for a cheesy note, but might not contribute to fish sauce odor due to their high odor threshold value (Devos and others 1995). It should be noted that starter culture added samples contained comparable amounts of 2-propanone to the 12-mo-old fish sauce, while the control contained only 50%. These results seemed to suggest that the selected starter cultures could play a part in formation of these ketones.

Pyrazines were not detected in these fish sauce samples. This was in agreement with Peralta and others (1996). However, Fukami and others (2004) reported that inoculation of *Staphylococcus* sp. R4Nu resulted in an increase in 2,6-dimethylpyrazine content of fish sauce, but its contribution to overall odor of fish sauce was negligible. Dimethyl disulfide and dimethyl trisulfide were potent compounds contributing to the distinctive fish sauce odor (Peralta and others 1996). Fukami and others (2002) demonstrated that dimethyl trisulfide and 2-ethylpyridine contributed to fishy, sweaty, and fecal notes. Dimethyl trisulfide was not detected in our study, agreeing with the reports of Shimoda and others (1996) and Peralta and others (1996). Samples inoculated by starter cultures showed comparable dimethyl sulfide content to the conventionally fermented sample. Thus, inoculation of 3 selected starter cultures could result in odor characteristics similar to the long conventionally fermented product with a shorter fermentation time.

Table 6 — Mean score of color, odor, flavor, and overall acceptance of fish sauce samples inoculated with various strains of bacterial starter cultures.

	Attributes			
Samples	Color	Odor	Flavor	Overall acceptance
Commercial	4.18 ± 1.47^{b}	4.24 ± 1.35	4.94 ± 0.97^{a}	4.82 ± 1.07^{a}
SK1-1-5	5.29 ± 0.59^{a}	4.24 ± 1.15	3.53 ± 1.07^{b}	4.24 ± 1.09^{ab}
SK33	4.53 ± 1.12^{b}	4.24 ± 0.75	3.94 ± 0.83^{b}	3.94 ± 0.83^{b}
SK37	4.35 ± 1.17^{b}	3.59 ± 0.87	3.71 ± 1.16^{b}	3.82 ± 0.95^{b}

Acceptance score: 7 = extremely like; 4 = neither like nor dislike; 1 = extremely dislike.
Different superscripts within a column indicate significant differences ($P < 0.05$).

Sensory acceptability

The panelists were considered to be a group of experts in fish sauce tasting as they had at least 1-y experience in product tasting at their respective plants. The sample inoculated by *Staphyloccoccus* sp. SK1-1-5 having the greatest brownish color (Table 4) showed the highest hedonic score in color ($P < 0.05$, Table 6). Although volatile compounds analyzed by GC–MS exhibited different profiles, odor quality perceived by the panelists was comparable in all samples ($P > 0.05$). It is important to note that odor quality of 4-mo-old fish sauce samples added with various starter cultures was equivalent to that of sample fermented for 12 mo by the traditional process. Flavor preference of all inoculated samples was lower than that of the commercial product ($P < 0.05$). Amino acids and peptides were likely to contribute to flavor characteristics. Lower free amino acid content of starter culture added samples would result in lower flavor intensity. However, overall preference of the sample inoculated by *Staphylococcus* sp. SK1-1-5 was comparable to the commercial product ($P > 0.05$). Therefore, the fish sauce sample with comparable sensory qualities to the traditional product could be obtained within 4 mo by addition of proteinases and starter culture prepared from *Staphylococcus* sp. SK1-1-5.

Conclusions

The addition of starter cultures prepared from *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and *Stapyloccoccus* sp. SK1-1-5 to the anchovy hydrolysate increased the degree of hydrolysis during 4-mo fermentation. Total amino acid profiles of fish sauce inoculated by *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and fermented for 4 mo were comparable with those of sample traditionally fermented for 12 mo. *Virgibacillus* sp. SK33 also showed histamine-reducing potential during fish sauce fermentation. Volatile compounds detected from the samples inoculated with starter cultures were similar to those of the sample conventionally fermented for 12 mo. Sample inoculated by *Staphylococcus* sp. SK1-1-5 was rated to have comparable sensory properties to the commercial fish sauce. *Staphylococcus* sp. SK1-1-5 could be a potential strain applied to accelerate fish sauce fermentation without adversely affecting the sensory characteristics.

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