Studies on the volatile compounds in fish sauce

(魚醤の香気成分に関する研究)

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Contents

Introduction

Fish sauce is a brown, liquid seasoning commonly used in Asian countries. It has various names according to countries producing: patis in Philippine, shotturu in Japan, budu in Malaysia, nampla in Thailand, nouc-mam in Vietnam, ketjap-ikan or bakasang in Indonesia, yu lu in China and ngapi in Myanmar. Fish sauce is commonly used in local dishes as a flavoring agent and as a supplement of amino acids, peptides, vitamins and minerals. It is now widely used in many prepared foods and sauces in Japanese and Western markets due to its favorable taste, an amino acid balance and high quantities of peptides. In Japan, fish sauce of about 6,000 Mt was consumed in 2003, among which about a half was imported from Southeast Asian countries. Fish sauce sales in 2003 were estimated to be about 25 billion yen in Japan and are presumed to increase further. Many seasonings in Japan are composed of hydrolyzed animal and vegetable proteins, but these seem to have some problems in the viewpoint of food safety due to the possible presence of carcinogenic byproducts. Consumers tend to require natural seasonings like fish sauce that is made from only fish and salt without any additives or suspicious byproducts. However, the consumption of fish sauce as the table additives for dishes at home or restaurant has been limited in Japan and Europe due to a distinctive odor.

Fish sauce contains 20 g/L nitrogen, 16 g of which is in the form of amino acids and oligopeptides; thus, it is considered to be a good source of protein (Uyenco, *et al.*, 1952). The production of fish sauce is simple and cheap, but the process varies according to producers or species of fish used (Mabesa, *et al.*, 1983). It is basically produced from a mixture of fish and salt (3:1, w/w) that has been allowed to ferment for a period over 6

months at 30-35°C. Briefly, fish and salt are put into a tank made of wood or concrete and the mixture occasionally mixed gently. During fermentation of fish sauce, oil and residues are separated by decantation several times and qualities of fish sauce evaluated by salt and nitrogen concentrations are controlled during fermentation along with sanitation. The final products of fish sauce thus prepared are shipped after being sterilized by heating or filtration. The products have a distinctive odor and flavor, which develops progressively as the fermentation progresses.

A considerable number of studies concerning the flavor have been carried out on fish sauce over the past decades (Vialard-Goudou, 1942; Nguyen-An-Cu and Vialard-Goudou, 1953; Van-Chom, 1957; Saisithi *et al.*, 1966; Dougan and Haward, 1975; Nonaka *et al.*, 1975; McIver *et al.*, 1982; Sanceda *et al.*, 1986a, b; Ijong and Ohta, 1996; Peralta *et al.*, 1996; Shimoda *et al.*, 1996). Dougan and Haward (1975) reported that three distinctive notes contributed to the odor of fish sauce, those being ammoniacal, cheesy, and meaty notes. They are derived from protein hydrolysate and lipid oxidation products by either autolytic or microbial activity (Saisithi, *et al.*, 1966; Beddows, *et al.*, 1980; McIver, *et al.*, 1982).

The ammoniacal note is attributed to ammonia, amines, and other basic nitrogen-containing compounds (Van-Chom, 1957; Yurkwski, 1965a, b; Saisithi *et al.*, 1966; Dougan and Haward, 1975). The cheesy note is mainly due to low molecular weight volatile fatty acids (Nguyen-An-Cu and Vialard-Goudou, 1953; Van-Chom, 1957; Yanagihara *et al.*, 1963; Saisithi *et al.*, 1966; Dougan and Haward, 1975; Beddows *et al.*, 1980; Sanceda *et al.*, 1986a, b; Peralta *et al.*, 1996) and methylketone (Van Veen, 1953). Shimoda *et al.* (1996) reported 2-methylpropanoic acid as the major contributor to cheesy

and stinking notes. The meaty note is much more complicated, but it is believed that it could be produced by atmospheric oxidation of precursors that are still present in mature fish sauces (Dougan and Haward, 1975).

Although a number of studies have already been conducted on fish sauce, the work on the volatile compounds is still incomplete and possible contributors to its distinctive odor have not been clarified. Therefore, it is necessary to show clearly which odors contribute to unpleasant notes of fish sauce and how these unpleasant odors are removed from fish sauce so that fish sauce can be used widely for dishes in the world.

From these backgrounds, the present study was carried out as follows. First, the distinctive odor of fish sauce was investigated in detail using aroma extract dilution analysis (AEDA), which has not been used before. Also, the effect of the volatile compounds added to a deodorized fish sauce was determined by sensory evaluation on eight descriptors, employing quantitative description analysis (QDA). Second, for elimination of the distinctive odor in fish sauce, microorganisms were tried to be isolated from the fish sauce mush with less distinctive odor made from frigate mackerel and soy sauce koji. The idea was that there is the possibility that microorganisms in the fish sauce mush can remove an unpleasant odor in fish sauce. The bacteria isolated were examined on their ability to eliminate a distinctive odor of fish sauce made in Thailand. The relationship of the distinctive odor in fish sauce determined by AEDA with those to be eliminated by the bacteria isolated was also clarified. Third, the identification and distribution of the bacteria were studied and the possibility to use the bacteria in the production of a preferable fish sauce was discussed.

Chapter I deals with the specification of a distinctive odor of fish sauce using the purge

and trap technique for extraction of volatile compounds which are considered to be changed during extraction by conventional methods. First, the correlation between the purge time and amount of adsorption was investigated for volatile compounds from fish sauce in order to evaluate the AEDA method, resulting in good results. Thus, the volatile compounds in fish sauce were identified using the AEDA method, revealing that 7 compounds, i.e. 2-methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal and 3-methylbutanoic acid, were principal contributors to the distinctive odor of fish sauce. These volatile compounds had odor characters of burnt, strong burnt, fruity, grassy, fishy, grassy, and rancid, respectively.

Second, it was examined how these volatile compounds would participate in fish sauce odor. The contribution of each volatile compound to fish sauce odor became clear by comparing the fish sauce with that deodorized by alkali processing. 2-Methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide decreased on deodorization of fish sauce. Then, these four volatile compounds were added to fish sauce deodorized and the resultant odor was compared with that of deodorized fish sauce by sensory evaluation using QDA for eight descriptors described above. As a result, 2-ethylpyridine and dimethyl trisulfide contributed to cheesy and fishy notes, respectively, and to rancid note cooperatively. 2-Methylbutanal, 2-methylpropanal and 2-ethylpyridine enhanced meaty note cooperatively. These four volatiles also contributed to sweaty, fecal, and rancid notes.

Many species belonging to *Bacillus, Micrococcus, Staphylococcus, Streptococcus* and *Pediococcus* as well as other halophilic bacteria producing lactic acid are found in fish sauce including nampla, shotturu, bakasang and nouc-mam (Saisithi *et al.*, 1966; Taing *et*

al., 1982; Tanasupawat and Daengsubha, 1983; Choorit and Prasertsan, 1992; Tanasupawat and Komagata, 1995; Ijong and Ohta, 1996; Mura *et al.*, 2000). However, it remains uncertain how these bacteria act on the production of characteristic taste and odor of fish sauce during fermentation. A little information is available on proteolytic activity of these bacteria in fish sauce processing (Taing *et al.*, 1982; Mura *et al.*, 2000). However, there are very few studies devoted to odor changes caused by the bacteria contained in fish sauce.

Chapter II deals with the isolation of the bacteria that would have an ability to eliminate a distinctive odor of fish sauce. The isolation was attempted from the fish sauce made from soy sauce koji, salt and frigate mackerel in materials (moromi = fish sauce mush), since a less unpleasant odor was conceived from this fish sauce compared with one made in Thailand (Funatsu, 2001). It was assumed that some bacteria would exist in fish sauce mush (moromi) which could control volatile compounds with an unpleasant odor.

Six strains were isolated from the mush on the plate of GYP (glucose, yeast extract, and pepton) medium containing 22% NaCl. Accordingly, two bacterial strains designated R4Nu and R5G were isolated, which were able to improve the odor of fish sauce. Further examination was carried out using strain R4Nu because the two strains had the same morphorogical and biochemical properties except color.

Sensory evaluation with QDA showed that scores of unfavorable notes in fish sauce such as fishy, sweaty, rancid, and fecal ones, were declined by the treatment with the bacterium after incubation at 32°C for 24 days. The preference test for the bacteria-treated fish sauce using scores from dislike -2 to like +2 in comparison with non-treated fish sauce revealed that the average scores were +0.143 and +0.857 by sniffing and tasting in mouth, respectively.

GC-MS analysis showed that treatment of Thai fish sauce with strain R4Nu at 32°C for 24 days reduced dimethyl disulfide to about one half, 2-ethylpyridine to about one half, dimethyl trisulfide to about one half, and butanoic acid to about one third, whereas 3-methyl-1-butanol and 2,6-dimethylpyrazine increased to about 10 and 80 folds, respectively.

Then, a model solution containing 22% NaCl and the above-mentioned several volatile compounds, was treated with strain R4Nu at 32°C and pH 5.4, resulting in the decrease of 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, 3-(methylthio)propanal, and dimethyl disulfide. The reduction of 2-ethylpyridine and dimethyl disulfide in the model solution by strain R4Nu was consistent with that of volatile compounds in the bacteria-treated fish sauce. However, the reduction of 2-methylpropanal, 2-methylbutanal and 3-methylbutanoic acid was specific to the model solution. 2-Methylpropanal, 2-methylbutanal, 3-methylbutanoic acid, 3-methyl-1-butanol, and 2,6-dimethylpyrazine were considered to be produced from amino acids in the bacteria-treated fish sauce and volatile compounds in the model solution by strain R4Nu. Therefore, strain R4Nu was added into a solution at pH 5.4 containing amino acids and NaCl in the same amounts found in the fish sauce and incubated at 32°C for 15 days. 2-Methylpropanal and 2-methylbutanal were produced from valine and isoleucine, respectively, and 3-methylbutanoic acid was metabolized from leucine via 3-methyl-1-butanol.

The above results are summarized as follows: (1) strain R4Nu isolated from fish sauce mush has an ability to metabolize 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, 3-(methylthio)propanal, and dimethyl disulfide; (2) 2-methylpropanal and 2-methylbutanal are produced from valine and isoleucine, respectively, by strain R4Nu, whereas

3-methyl-1-butanol and 3-methylbuanoic acid from leucine.

It was not clear why 3-(methylthio)propanal was not changed in the bacteria-treated fish sauce, even though it was reduced in the model solution containing volatile compounds by action of strain R4Nu. 3-(Methylthio)propanal was not produced by strain R4Nu in a solution at pH 5.4 containing amino acids and NaCl with the same contents as found in fish sauce. It was also not apparent which components in fish sauce were metabolized to 2,6-dimethylpyrazine by strain R4Nu, because it was not produced in the model solution containing amino acids added with strain R4Nu.

Chapter III is devoted to identification of the strains isolated from fish sauce mush and to determination of their distribution to various fish sauce and soy sauce malt. To identify the two strains, morphorogical and biochemical properties were examined. Molecular biological approaches such as taxonomic studies using the sequences of 16S rRNA and *rpoB* and the DNA-DNA hybridization experiments were also employed. The two strains were both identified as *Staphylococcus nepalensis* which had been previously isolated from the respiratory tract of goats in the Himalayan region (Spergser *et al.*, 2003). However, several differences were observed in the phenotype between *S. nepalensis* reported previously and that found in the present study.

Furthermore, the present strain was examined for its possible distribution to 19 fish sauces from Asian countries and to malt for soy sauce made in Toyama Prefecture, Japan. PCR primers specific to the strain were designed for *rpoB*. The soy sauce malt was investigated because it is used to make fish sauce as a starter. Isolated from these samples were 37 colonies which were obtained by inoculating samples on the nutrient broth plate containing 18% NaCl and subsequent incubation at 32°C. The amplified DNA fragment

bearing the gene of *S. nepalensis* was detected from 15 colonies isolated from the soy sauce malt, but no positive reaction was obtained from 19 fish sauce samples. These results suggest that *S. nepalensis* was originated from the fish sauce starter malt. This is the first discovery to the author's knowledge for *S. nepalensis* to be present in food. It seems that the bacterium has lived in the factory of soy sauce as a contaminant in soy sauce malt for a long time, possibly controlling the flavor of soy sauce.

General discussion deals with summary of the present results and discussion on future development.

The contents of the present study have been published as follows:

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

2. Fukami K., Funatsu Y., Kawasaki K., Watabe S. (2004) Improvement of fish sauce odor by treatment with bacteria isolated from the fish sauce mush (moromi) made from frigate mackerel. *J. Food Sci.* **69**, FMS45-49.

3. Fukami K., Satomi M., Funatsu Y., Kawasaki K., Watabe S. (2004) Characterization and distribution of *Staphylococcus* sp. implicated for improvement of fish sauce odor. *Fish. Sci.* **70**, 916-923.

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Abbreviations

AEDA: Aroma extract dilution analysis

AraT: Aromatic aminotransferase

BacT: Branched chain aminotransferase

cfu: Colony foming unit

DDBJ: DNA Database of Japan

DNA: Deoxyribonucleic acid

EMBL: European Molecular Biology Laboratory

EPA: The U.S. Environmental Protection Agency

FD: Flavor dilution

FID: Flame ionization detector

GC: Gas chromatography

GC-MS: Gas chromatography-mass spectrometry

GYP: Glucose, yeast extract and peptone

I.D.: Internal diameter

KMBA: α-keto-γ-methylthiobutyrate

NA: Nutrient agar

NIH: The National Institute of Health

NIST: National Institute of Standards and Technology

P&T: Purge and trap

PCR: Polymerase chain reaction

PDMS: Polydimethylsiloxane

QDA: Quantitative descriptive analysis

RI: Retention index

rRNA: Ribosomal ribonucleic acid

SE: Staphylococcal enterotoxin

SPME: Solid phase microextraction

TE: Tris-EDTA

TTE: Tris-EDTA supplemented with Triton X-100

Chapter I

Identification of distinctive volatile compounds in fish sauce

Fish sauce is a brown, liquid seasoning commonly used in most parts of Southeast Asia. It imparts good taste to local food preparation and supplementary protein in the diet. Recently, it has been commonly used in several prepared foods and sauces in the Japanese and European markets due to its characteristic taste. However, due to its distinctive odor, there is a limit to its usage for not only home-cooked foods but also prepared foods, although fish sauce is a good source of protein (Uyenco *et al.*, 1952). The production of fish sauce is simple and cheap, but the process varies according to producers and species of fish to be used (Mabesa *et al.*, 1983). It is basically produced from a mixture of fish and salt (3:1, w/w) that is allowed to ferment for a period over 6 months at 30-35 °C. The resulting product has a distinctive odor and flavor, which develops progressively as the fermentation progresses.

A number of studies have been carried out on fish sauce over the past decades (Vialard-Goudou, 1942; Van-Chom, 1957; Saisithi *et al.*, 1966; Dougan and Haward, 1975; Nonaka *et al.*, 1975; McIver *et al.*, 1982; Sanceda *et al.*, 1986a, b; Ijong and Ohta, 1996; Peralta *et al.*, 1996; Shimoda *et al.*, 1996). Dougan and Haward (1975) reported that three distinctive notes contributed to the odor of fish sauce, those being ammoniacal, cheesy, and meaty notes. They are derived from protein hydrolysates and lipid oxidation products by autolytic and microbial activities (Saisithi *et al.*, 1966; Beddows *et al.*, 1980; McIver *et al.*, 1982).

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nitrogen-containing compounds (Van-Chom, 1957; Yurkwski, 1965a, b; Saisithi *et al.*, 1966; Dougan and Haward, 1975). The cheesy note is mainly due to low molecular weight volatile fatty acids (Nguyen-An-Cu and Vialard-Goudou, 1953; Van-Chom, 1957; Yanagihara *et al.*, 1963; Saisithi *et al.*, 1966; Dougan and Haward, 1975; Beddows *et al.*, 1980; Sanceda *et al.*, 1986a, b; Peralta *et al.*, 1996) and methyl ketone (Van Veen, 1953). Shimoda *et al.* (1996) defined 2-methylpropanoic acid as the major contributor to cheesy and stinking notes.

The meaty note is much more complicated, but it is believed that it could be produced by atmospheric oxidation of precursors that still exist in mature fish sauces (Dougan and Haward, 1975).

Although a number of studies have already been carried out on fish sauce, those on the volatile compounds are still incomplete and possible contributors to its distinctive odor have not been clarified.

The objective of this chapter was to identify some possible contributors to the distinctive odor of fish sauce by an addition test using QDA.

I-1 Materials and methods

I-1-1 Materials

Fish sauce sterilized by filtration was imported from the Thai Fish Sauce Factory Co., Ltd. and used as a research sample. It was made only from fresh anchovies without any additives such as sugars and satisfied the quality of the first grade fish sauce containing over 2.0% (w/w) total nitrogen in Thailand. A Tenax TA column (20-35 mesh) was purchased from GL Sciences, Tokyo, Japan. 2-Methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, 3-methylbutanoic acid, and 3-(methylthio)propanal were from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Dimethyl trisulfide was synthesized by Shiratori Pharmaceutical Co., Ltd., Chiba, Japan.

I-1-2 Isolation of volatiles by the purge and trap (P&T) method

Fifty grams of fish sauce, to which 3 μL of 1% cyclohexanol had been added as an internal standard, were purged for 16 min with helium at 50 mL/min in a water bath at 40 °C. The volatile compounds were concentrated onto a Tenax column, which was maintained at 40 °C. The column consisted of a glass tube (15.8 cm x 3.0 mm I.D.) (GL Sciences) packed with Tenax TA (70 mg, 20-35 mesh).

I-1-3 Capillary gas chromatography (GC)

Separation of volatile compounds was performed on a Hitachi G-3900 gas chromatograph equipped with a flame ionization detector (FID). GC was connected to a thermal desorption cold trap injector CP4020 TCT (GL Sciences). Volatile compounds, which were thermally discharged from the Tenax trap at 200 °C and concentrated in a cold trap at -130 °C, were introduced into a capillary column. Separation was achieved on a fused silica capillary column (60 m x 0.25 mm I.D.) coated with cross-linked polyethylene

glycol (20M) at a film thickness of 0.25 μm (TC-Wax; GL Sciences). The oven temperature was programmed from 50 to 230 °C at 3 °C/min. The injector and detector temperatures were set at 200 and 250 °C, respectively. The flow rate of helium used as a carrier gas was 0.7 mL/min. An outlet splitter system (OSS-2; GL Sciences) was used for sniffing GC effluents.

I-1-4 Capillary gas chromatography-mass spectrometry (GC-MS)

A JMS-DX303 mass spectrometer connected to a GC-06 gas chromatograph (Japan Electron Optics Laboratory, Tokyo, Japan) was used for identification in mass spectrometry. Volatile compounds concentrated on the Tenax trap were thermally desorbed at 220 °C for 30 s and introduced into a capillary column, using an injection and focusing instrument (thermal desorption unit; Supelco, Bellefonte, USA). Separation was carried out on the same capillary column as that used in the GC analysis. The oven temperature was programmed from 50 to 230 °C at a rate of 2 °C/min. The flow rate of helium was 0.7 mL/min. The mass spectra were obtained by electron impact ionization at 70 eV. Retention indices (RI) were calculated by using a modified Kovats index. Seven authentic compounds in materials were identified using mass spectral databases (EPA/NIH Mass Spectral Database, 1978, National Bureau of Standards, Washington, DC) and a computer DA-5000 (Japan Electron Optics Laboratory). Thus, seven compounds were completely identified, and other compounds were tentatively identified.

I-1-5 GC sniffing (aroma extract dilution analysis)

According to the principle of AEDA (Grosch, 1993), the headspace gas prepared from fish sauce was absorbed by each volume of purged helium gas: 1600 mL, 800 mL (1/2), 400 mL (1/4), 200 mL (1/8), 100 mL (1/16), 50 mL (1/32), 25 mL (1/64), and 12.5 mL

(1/128). Effluents from the splitter system were sniffed by three assessors with respect to each odor concentrate on the Tenax trap. Odor acceptance was decided by two of three assessors.

I-1-6 Preparation of deodorized fish sauce

Shimoda *at el.* (1996) reported that the alkalization of fish sauce enhanced the concentration of nitrogenous and sulfurous volatile compounds in the headspace gas. Therefore, fish sauce was deodorized by alkalization at pH 9.5 with 20% NaOH and subsequent evaporation under reduced pressure at 30 °C for 4 h. After the evaporation, pH and the contents of total nitrogen, moisture and NaCl were adjusted to the same as those before alkalization.

I-1-7 Quantification of principal odorants

Various amounts of principal odorants including 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide dissolved in ethanol were added to non-treated fish sauce for quantification by the standard addition method (Shimoda *at el.*, 1996).

I-1-8 Sensory evaluation methods

The major contributing fish sauce odors are described as ammoniacal, cheesy, and meaty notes (Dougan and Haward, 1975). These three sensory attributes were further divided into eight attributes as the descriptors for QDA (Stone *et al.*, 1974), burnt, fishy, sweaty, fecal, rancid, cheesy, meaty, and ammoniacal notes.

Panelists were selected from employees of Japan Tobacco Inc. on the basis of interest, time available, and a personal acceptance of the taste of fish sauce: 4 females and 10 males, with ages ranging from 29 to 52 years. As different people might have different ideas on the odor descriptors of fish sauce, the odor attributes of each descriptor were explained in

detail, and then the typical fish sauce odor was repeatedly evaluated. The odor descriptors were identified by an odor profiling method with fish sauce as a sample (Keane, 1992). During the training, the panelists were asked to identity and define the odor descriptors of fish sauce. The panelists were presented with a non-treated fish sauce sample, a deodorized fish sauce, and a deodorized fish sauce added with some of four odorants including 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide at 370.7, 38.5, 1.4, and 7.5 ng/mL, respectively. A blank consisted of the same volume of ethanol in the deodorized fish sauce. All of the samples were kept at 30 °C before the presentation.

Sensory evaluation was carried out in individual booths with incandescent light. The eight descriptors in deodorized and addition samples were compared to those of the non-treated sample with scores of -2 to +2 scales: -2, very weak; -1, weak; 0, same; +1, strong; +2, very strong. The significances in QDA were assessed by Student's *t*-test.

I-2 Results and discussion

I-2-1 Identification of the odorants in fish sauce and their flavor dilution (FD) factors

For determination of potent distinctive odorants in fish sauce, AEDA was adopted in combination with the P&T method. The FD factors were determined from 1 to 1/128 by 1/2 stepwise. Table I-1 lists the odor characteristics and FD values of 43 odorants in the headspace gas. Twenty-three compounds with high FD factors were identified by GC-MS analysis. 2-Methylpropanal (peak 2), 2-methylbutanal (peak 5), 2-pentanone (peak 7), 2-ethylpyridine (peak 21), dimethyl trisulfide (peak 26), 3-(methylthio)propanal (peak 31),

and 3-methylbutanoic acid (peak 39) had FD factors over 64, and their odor characteristics were burnt, burnt, fruity, grassy, fishy, grassy, and rancid, respectively. In the present study, trimethylamine had a small FD factor. Many types of fish sauce are known to contain various concentrations of trimethylamine (Saisithi *et al.*, 1966; Dougan and Howard, 1975).

The linearity between the peak area of GC analysis and the purge gas volume was confirmed (Figure I-1). A good linearity was obtained between the two factors except for 2-methylpropanal and 2-methylbutanal. These two compounds broke through the Tenax trap with the purge gas over 200 mL. This breakthrough, however, did not give any problems with AEDA, because the FD factors of these aldehydes were determined in the range within their linearity.

I-2-2 Quantification of distinctive odorants in fish sauce

The gas chromatogram of non-treated fish sauce is shown in Figure I-2. Relative concentrations of odorants in the headspace for non-treated and deodorized sauce are listed in Table I-1. Decreased significantly by deodorization were 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide, which were used for the following addition tests. On the other hand, 2-pentanone and 3-(methylthio)propanal in addition to benzaldehyde (RI 1526), *n*-octanol (RI 1568), benzonitrile (RI 1613), and acetophenone (RI 1650) increased in the deodorized fish sauce.

It is likely that the volatile compounds which decreased in the deodorized fish sauce would be principal contributors to the distinctive odor of fish sauce. By the standard addition method, the concentrations of 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide in fish sauce were estimated to be 370.7, 38.5, 1.4, and 7.5 ng/mL,

respectively.

I-2-3 Sensory evaluation of fish sauces

The addition of individual odorants to the deodorized fish sauce could not restore the distinctive odor of fish sauce, so the method of combination addition was employed. The effects of burnt, grassy, and fishy notes on the restoration of fish sauce odor were determined by using reconstituted fish sauces, which were prepared by the addition of four distinctive odorants decreased in the deodorized fish sauce in various combinations. The combinations of odorants added were as follows: (A) all four volatiles (2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide), (B) three volatiles (2-methylpropanal, 2-methylbutanal, and dimethyl trisulfide), (C) three volatiles (2-methylpropanal, 2-methylbutanal, and 2-ethylpyridine), (D) two volatiles (2-ethylpyridine and dimethyl trisulfide), and (E) two volatiles (2-methylpropanal and 2-methylbutanal).

QDA was performed to examine the contributors to the distinctive odor of fish sauce (Figure I-3). Sample A showed that four volatiles cooperatively contributed to the fishy, sweaty, rancid, cheesy, and ammoniacal notes. In samples A-D, the fishy note was well restored but it was decreased in sample E. Therefore, 2-ethylpyridine and dimethyl trisulfide could contribute to the fishy note. Although 2-ethylpyridine was described as grassy by GC-sniffing (Table I-1), this compound was found to contribute to the fishy note of fish sauce by sensory evaluation. The sweaty note was restored in every reconstituted sample, indicating that every odorant is involved in this note. The sensory evaluation revealed that 2-ethylpyridine and dimethyl trisulfide were essential to the fecal note, because it was significantly decreased by the removal of 2-ethylpyridine and dimethyl

trisulfide (sample D). The two compounds had grassy and fishy notes, respectively, but they contributed to the fecal note in the fish sauce probably as a result of olfactory interaction.

The rancid note was restored only in sample A, indicating that the four volatiles were essential to the development of this note. The cheesy note was restored in samples A and C, and partially restored in sample D with an average score. However, it completely disappeared in sample B. 2-Pentanone and volatile fatty acids have been considered to be responsible for the cheesy note (Devos *et al.*, 1995; Shimoda *et al.*, 1996). In the present experiments, 2-pentanone increased over 4 times after the alkali treatment, although no difference was obtained in volatile fatty acid contents before and after the treatment (data not shown).In the present study, it was considered that 2-ethylpyridine along with 2-pentanone and volatile fatty acids was an essential factor for the development of the cheesy note.

Shimoda *et al.* (1996) reported that nitrogen-containing compounds together with aldehydes were responsible for the meaty note of fish sauce. The results of sample C indicated that 2-ethylpyridine together with 2-methylpropanal and 2-methylbutanal could be responsible for the meaty note. The ammoniacal note was restored only in sample A despite the absence of ammonia and volatile amines. The burnt note was restored in samples B, D, and E, but decreased in samples A and C. These results did not show any contributors to burnt odor.

In this study, the contribution of volatile fatty acids could not be investigated because no difference in the their contents was observed before and after the alkali treatment. Further study is needed to investigate contributions of volatile fatty acids.

Aldehydes, which are considered to cause unpleasant oxidation flavor in foods (Heath and Reineccius, 1986), are produced through lipid oxidation during fermentation and from branched amino acids by transamination and decarboxylation (Massey *et al.*, 1976; Masson *et al.*, 1999). These compounds could certainly contribute to the overall odor due to their low odor threshold values. Aroma conferred by methyl aldehydes such as 2-methylpropanal and 2-methylbutanal has been defined as malty off-flavor in Cheddar cheese (Morgan, 1976). These aldehydes form the major part of the volatile fraction in several cheeses such as Cheddar, Camembert, Emmental, and Parmesan (Barbieri *et al.*, 1994; Yvon and Rijinen, 2001; Thierry and Maillard, 2002), suggesting that the overall acceptance of these compounds depends on the final balance of volatiles. The addition of 2-methylpropanal, 2-methylbutanal, and 2-ethylpyridine to reconstituted fish sauce enhanced fishy, cheesy, and meaty notes. Moreover, the addition of 2-methylpropanal, 2-methylbutanal, and dimethyl trisulfide restored the fishy note. These results indicate that aldehydes bear unpleasant notes in fish sauce. It is recommended to reduce these for improving fish sauce odor. The two methods seem possible to reduce these aldehydes in fish sauce, one to make it under limited oxidation employing anaerobic fermentation and the other to convert aldehydes to alcohols by reduction. Sanceda *et al.* (1992) claimed that fermentation under anaerobic conditions during the manufacturing process underwent changes in the aroma quality of fish sauce, yielding an acceptable product. Aldehydes are easily converted to alcohols by enzymes from microorganisms such as yeast (Ashida *et al.*, 1987).

This is the first time to define 2-ethylpyridine as a compound to produce distinctive odor in foods. Hidalgo and Zamora (2004) reported that it was produced from

4,5(E)-epoxy-2(E)-heptenal and phenylalanine by strecker-type degradation. The preparation of fish sauce at about 30 °C for 1-2 years would produce 2-ethylpyridine from amino acids by streker degradation. This volatile has a low odor threshold value of the grassy note (Devos *et al.*, 1995). Although 2-ethylpyridine was found to be responsible for cheesy, meaty, and fishy notes in fish sauce in this study, it might mainly contribute to the fishy note in fish sauce finding from the comments from the panelist in QDA of fish sauce. The fishy note produces an unpleasant odor of fish sauce and thus 2-ethylpyridine might be a key compound to be removed from fish sauce for producing an acceptable one.

Sulfur compounds produce a key flavor in many food products such as cognac, whiskey, wine, beer (Nedjima and Hoffmann, 1996), yogurt (Ott *et al.*, 1997) and ripened cheese (Gill *et al.*, 1966). Dimethyl trisulfide especially has a low odor threshold value, e.g. 0.1 ppb in beer (Peppard, 1978). The contents of dimethyl trisulfide in aged beer stored at 45°C for 4 days increased to 2.5 ppb, which was enough to impart a characteristic off-flavor (Williams and Gracey, 1982). The sulfur compounds such as 3-(methylthio)propanal and dimethyl disulfide were also detected in baked potato aroma and cooked cabbage flavor (Mandin *et al.*, 1999). A proposed pathway to produce sulfur compounds from methionine is shown in Figure I-4. Ballance (1961) showed that the strecker degradation of methionine first gave rise to 3-(methylthio)propanal, which was subsequently decomposed into methanthiol and acrolein. Dimethyl sulfide was also found in the condensates in the cold trap due to the strecker degradation of methionine. 3-(Methylthio)propanal was considered to be as a potential precusor of dimethyl trisulfide in aged beer (Gijs *et al.*, 2000). Furthermore, 3-(methylthio)propanal was found to be more easily produced by the degradation of methionine, whereas dimethyl polysulfides,

especially dimethyl disulfide and dimethyl trisulfide, were more easily formed from the thermal degradation of methionine sulfoxide (Yu and Ho, 1995). Methionine is easily oxidized to methionine sulfoxide, which is often detected in meat, fish protein, and casein (Slump and Schreuder, 1973; Sjoberg and Bostrom, 1977). 3-(Methylthio)propanal, dimethyl disulfide, and dimethyl trisulfide in fish sauce volatiles would be also derived from the strecker degradation of methionine during the production of fish sauce at about 35 °C for 1-2 years.

Bacterial reaction also produces these sulfur compounds from methionine. The origin of many sulfur compounds in cheese is methanthiol produced from methionine by bacteria which are used in the preparation of cheese. The direct metabolic pathway responsible for the production of methanthiol involves the bacterial degradation of methionine. Bacterial enzymes involved include methionine-γ-lyase, cystathionine-ß-lyase and cystathionine-γ-lyase, which play important roles in the development of cheese flavor (Dias and Weimer, 1998; Berger *et al.*, 1999). Methionine-γ-lyase is though to have the major role in the catabolism of methionine and generation of methanthiol from methionine in several bacteria. Other enzymes that are capable of producing methanthiol from methionine include cystathionine-ß-lyase and cystathionine-γ-lyase in lactic acid bacteria. After deamination of methionine by bacterial enzymes, α-keto-γ-methylthiobutylate produced is converted to methylthioacetaldehyde in the presence of manganese (II) (Figure I-4). In cognac brandies, the reaction of methanthiol with hydrogen sulfide produced by yeast in the presence of copper (II) leads to formation of dimethyl disulfide and dimethyl trisulfide (Nedjma and Hoffmann, 1996).

For manufacturing pleasant fish sauce, it is important to prevent the production of the

sulfur volatile compounds by reducing oxidation as much as possible using the nitrogen replacement method and low temperature fermentation. Such treatment would regulate the conversion of methanthiol to dimethyl disulfide and dimethyl trisulfide. It seems also effective to prevent contamination of bacteria having enzymes which catalize deamination of methionine during the process of fish sauce production. Another possible method is to remove cations such as copper (II) and manganese (II), which would react as catalysts.

I-3 Conclusion

2-Methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, and 3-methylbutanoic acid were principal contributors to the distinctive odor of fish sauce. 2-Ethylpyridine and dimethyl trisulfide were found to contribute to the fishy note. The sweaty note could be attributed to all four volatiles. 2-Ethylpyridine and dimethyl trisulfide were essential to the fecal note. All four volatiles were essential to the development of the rancid note. 2-Ethylpyridine along with 2-pentanone and volatile fatty acids were essential to the cheesy note. 2-Ethylpyridine together with 2-methylpropanal and 2-methylbutanal was responsible for the meaty note. The burnt note was developed in the presence of 2-ethylpyridine, dimethyl trisulfide, 2-methylpropanal, and 2-methylbutanal.

Chapter II

Improvement of fish sauce odor by treatment with bacteria isolated from the fish sauce mush (moromi) made from frigate mackerel

Fish sauce is basically made from fish and salt in a weight ratio of 3:1, the mixture of which is allowed to ferment at 30-35°C for a period over 6 months. During this period, fish proteins are hydrolyzed both by endogenous proteases and exogenous ones of microbial origin. The procedure to make fish sauce is simple, but the resulting products have a distinctive odor and various flavors according to fish species used and the process of fermentation (Uyenco *et al.*, 1952). It is assumed that the volatiles of fish sauce are produced by non-enzymatic reactions of various components (i.e. amino acids, lipids and sugars) and enzymatic reactions by endogenous enzymes of fish origin and those of microorganisms surviving during fermentation.

Many species belonging to Bacillus, Micrococcus, Staphylococcus, Streptococcus, and Pediococcus as well as other halophilic bacteria producing lactic acid are found in fish sauce including nampla, shotturu, bakasang, and nouc-mam (Saisithi *et al.*, 1966; Taing *et al.*, 1982; Tanasuoawat and Daengsubha, 1983; Itoh *et al.*, 1985; Platt, 1987; Choorit and Prasertsan, 1992; Tanasuoawat and Komagata, 1995; Ijong and Ohta, 1996; Mura *et al.*, 2000). However, it remains uncertain how these bacteria act on the production of characteristic taste and odor of fish sauce during fermentation. Because fish sauces made in several factories had different odor and taste, bacteria may be associated with such changes of the fish sauce odor. A few information is available on proteolytic activity of bacteria concerned in fish sauce processing (Taing *et al.*, 1982; Mura *et al.*, 2000).

However, very few studies have been devoted to changes in the odor caused by existing bacteria.

In the previous chapter, it was found that seven volatile compounds were principal contributors to the distinctive odor of fish sauce. To reduce such odor, these volatile compounds are to be decreased.

Chapter II deals with the isolation of the bacteria, which had an ability to reduce the distinctive odor of fish sauce. The change of the odor in fish sauce was also investigated when treated with the bacteria. The bacterial isolation was carried out from the fish sauce made from soy sauce koji, salt and frigate mackerel in materials, since it has fewer unpleasant odor compared with fish sauce made in Thailand (Funatsu, 2001).

II-1 Materials and methods

II-1-1 Materials

Fish sauce mash (moromi) from which bacteria were isolated was prepared by fermenting the mixture of the waste of frigate mackerel (*Auxis thazard*) following surimi processing, soy sauce koji and 20% NaCl for one year at room temperature in Toyama Prefectural Food Institute (Funatsu, 2001). Alternatively, the fish sauce purchased from Thai Fish Sauce Factory Co., Ltd., which contained 22% NaCl and 1.7% of total nitrogen, was used for the deodorization test. 2-Methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, 3-(methylthio)propanal, 3-methylbutanoic acid, dimethyl disulfide, 3-methyl-1-butanol, 2,6-dimethylpyrazine, and butanoic acid used as authentic volatile compounds were purchased from Tokyo Kasei Kogyo Company, Ltd. Dimethyl trisulfide was synthesized by Shiratori Pharmaceutical Co., Ltd., Chiba, Japan. Taurine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, cysteine, methonine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine, proline, and tryptophan were from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

II-1-2 Isolation of bacteria from fish sauce mush

Fish sauce mush was diluted 10 folds with sterilized distilled water and incubated at 32°C for 3-4 days on the plates of GYP consisting of 2% glucose (Wako Pure Chemical Industry, Ltd.), 1% yeast extract (Becton, Dickinson and Company, FranklinLakes, USA), 1% peptone (Becton, Dickinson and Company), 0.5% Tween 80 (Wako Pure Chemical Industry), 1% $CaCO_3$ and 1.5% agar in the presence of 12% NaCl and 1.2% $MgSO_4$, or those of nutrient agar (NA) (Becton, Dickinson and Company) containing 12% NaCl and

1.2% $MgSO_4$ (Onishi *et al.*, 1980). After incubation, bacteria were transferred to and isolated from new plates containing the same NA. After confirming viability on the plate of NA containing 18% NaCl, the purified bacteria were maintained on the plate of NA containing 18% NaCl, 10% Thai fish sauce and 1.8% $MgSO_4$.

II-1-3 Screening procedure of bacteria from fish sauce mush

Purified bacteria were incubated aerobically at 32°C for two days in a 100mL nutrient broth containing 18% NaCl, 10% Thai fish sauce, and 1.8% $MgSO_4$. Subsequently, bacteria were collected and washed twice with 20 mL of sterile 18% saline and suspended in 5 mL of the same solution. To 50 mL of Thai fish sauce were added 5 mL of the suspended bacteria. The mixture was incubated at 32°C for 12 days under stirring and defined as the bacteria-treated fish sauce. The bacteria-treated fish sauce during incubation was evaluated with three persons by sniffing in comparison with the fish sauce treated under the same condition but without bacteria.

II-1-4 Preparation of the bacteria-treated fish sauce

Isolated bacteria were cultured at 32°C for 48 h in a 100 mL nutrient broth (Becton, Dickinson and Company) containing 18% NaCl, 1.8% $MgSO_4$, and 10% fish sauce by shaking. Bacteria cultured were collected by centrifugation and suspended in 10 mL of 18% NaCl. Fish sauce was passed through a 0.2 μm filter (Advantec Mfs, Inc. Tokyo, Japan) to avoid any contaminating microorganisms that may influence subsequent odor examination. For deodorizing examination, to 550 mL of the fish sauce filtrated were added 5 mL of the bacterial solution concerned, and the mixture was incubated at 32°C for several days under gentle stirring. After incubation, fish sauce was filtrated through 0.45μm filter.

II-1-5 Isolation of volatiles by the P&T method

Volatiles were isolated as described in Section I-1-2 of Chapter I.

II-1-6 Capillary gas chromatography (GC) I

Separation of volatile compounds was carried out as described in Section I-1-3 of Chapter I. The quantification of 3-methyl-1-butanol and 2,6-dimethylpyrazine was carried out by the standard addition method (Shimoda *et al.,* 1996), employing the above GC analytical system.

II-1-7 Capillary gas chromatography-mass spectrometry (GC-MS) I

Volatiles were identified as described in Section I-1-4 of Chapter I. RI was calculated by the modified Kovats Index using n-alkanes including n-hexane, n-heptane, n-octane, n-nonane, n-decane, n-undecane, n-dodecane, n-tridecane, n-tetradecane, n-pentadecane, n-hexadecane, n-heptadecane, n-octadecane, n-nonadecane, and n-eicosane (GL Sicence). The volatile compounds in fish sauce were determined with RI, using authentic compounds described in Materials and the mass-spectra database (EPA/NIH mass spectral database, 1978, National Bureau of Standards, Washington, DC, USA).

II-1-8 Sensory evaluation methods

Two types of sensory evaluation were carried out for the bacteria-treated fish sauce, one by sniffing and the other in mouth. The odor of fish sauce was divided into 8 attributes as the descriptors of QDA (Fukami *et al.,* 2002). The odor descriptors were identified by the odor profiling method with Thai fish sauce as a sample (Keane, 1992). They were burnt, fishy, sweaty, fecal, rancid, cheesy, meaty, and ammoniacal notes.

Panelists selected from employees of Japan Tobacco Inc. on the basis of interest and time available were 3 females and 5 males with ages ranging from 29 to 52 years who well

knew sensory evaluation methods and did not dislike fish sauce. As different people might have different ideas on the descriptors of fish sauce, 8 attributes of fish sauce were explained in detail to and evaluated repeatedly during training by panelists. All 50 g of the samples were kept at 30°C before presentation in a 100 mL bottle. Sensory evaluation was carried out in individual booths with incandescent light. The 8 descriptors for the bacteria-treated fish sauce compared to non-treated sample were scored on -2 - +2 scales: -2 very weak, -1 weak, 0 same, +1 strong, and +2 very strong.

The preference test was performed by 11 panelists, who were selected openly from employees of Japan Tobacco Inc. The preference of the bacteria-treated fish sauce to non-treated one was scored on -2 - +2 scales: -2 very dislike, -1 dislike, 0 same, +1 like, and +2 very like.

II-1-9 Preparation of the model solution containing volatile compounds

The bacterium (R4Nu) was cultured at 32 °C for 48 h by shaking in 100 mL of nutrient broth (Becton, Dickinson and Company) containing 18% NaCl, 1.8% $MgSO_4$, and 10% fish sauce. The bacterial cells cultured were collected by centrifugation and suspended in 10 mL of 18% NaCl (bacteria solution).

Thirty five mL of the reaction mixture containing 22% (w/v) NaCl, 0.05M phosphate buffer (pH5.4), 350 μL diluted volatile compounds mixture, and 1 mL of the bacterial solution or 1 mL of inactivated bacterial solution as a negative control, were incubated at 32 °C for 5h with gentle stirring. Inactivation was performed by heating the bacterial solution at 80 °C for 30 min. Five mL of the reaction mixture were filtered through a 0.25 μm membrane (Toyo Roshi Kaisha Ltd., Tokyo, Japan), put into a 15 mL screw vial (21 mm I.D.× 70 mm) (Sigma-Aldrich Co., St. Louis, USA), and added with 200 μL of 1% (w/v)

cyclohexanol as the internal standard. Each diluted volatile compounds, namely, 100μL 2-methylbutanal, 50 μL 2-methylpropanal, 20 μL 2-pentanone, 50 μL 2-ethylpyridine, 5 μL dimethyl trisulfide, 100 μL 3-(methylthio)propanal, 20 μL 3-methylbutanoic acid, 50 μL dimethyl disulfide and 20 μL butanoic acid, was dissolved in 5 mL ethanol for dilution.

A solid phase microextraction (SPME) fiber (75 μm Carboxen/PDMS, Sigma-Aldrich Co.) was inserted into the vial so that volatile compounds were absorbed on the fiber at 40 °C for 1 h.

II-1-10 Preparation of synthetic fish sauce and amino acid solutions

A synthetic fish sauce was made with amino acids in 0.05M sodium phosphate (pH 5.4) containing 22% NaCl (NaCl solution) and passed through a 0.25 μm membrane filter (Toyo Roshi Kaisha, Ltd.) for sterilization. Amino acid concentrations of synthetic fish sauce were the same as those determined by amino acid analysis in fish sauce made in Thailand. Synthetic fish sauce contained amino acids as follows: taurine 100 mg, aspartic acid 600 mg, threonine 300 mg, serine 300 mg, glutamic acid 1000 mg, glycine 300 mg, alanine 600 mg, valine 500 mg, cysteine 10 mg, methionine 200 mg, isoleucine 300 mg, leucine 300 mg, tyrosine 50 mg, phenylalanine 300 mg, lysine 500 mg, histidine 200 mg, arginine 15 mg, proline 200 mg, and tryptophan 100 mg per 100g of the NaCl solution. The amino acid solution contained 500 mg valine, 200 mg methionine, 300 mg isoleucine, and 300 mg leucine per 100 g of the NaCl solution, respectively.

For determination of volatile compounds produced from amino acids, 2 mL of the bacterial solution mentioned above was added to 100 mL of the synthetic fish sauce or amino acid solution, and the mixture was incubated at 32 °C with gentle stirring in an incubator. GC analysis was performed with 5 mL of the reaction mixture which had been

passed through a 0.25 μm filter, put into 15 mL screwed vials, and added with 4.5 μL of 1% cyclohexanol as an internal standard.

II-1-11 Capillary gas chromatography (GC) II

The SPME fiber after absorption of volatile compounds was injected into a gas chromatograph. Separation of volatile compounds was achieved in a fused silica capillary column (60 m × 0.25 mm I.D.) coated with cross-linked polyethylene glycol (20M) at a film thickness of 0.25 μm (TC-WAX, GL Sciences) on a gas chromatograph (GC353B, GL Sciences) equipped with a FID. The oven temperature was programmed from 50 °C to 230 °C at 3 °C/ min. The injector and detector temperatures were set at 200 and 250 °C, respectively. The flow rate of helium used as a carrier gas was 0.7 mL/ min.

II-1-12 Capillary CG-mass spectrometry (GC-MS) II

An HP5973 mass spectrometer connected to an HP6890 gas chromatograph (Hewlett Packard Co. Ltd., CA, USA) was used for identification of volatile compounds. The SPME fiber (75 μm Carboxen/PDMS) was inserted into the vial so that volatile compounds were absorbed on the fiber at 40°C for 1 h. After absorption of volatile compounds, the SPME fiber was inserted into a gas chromatograph, and the volatile compounds were discharged at 250 °C and introduced into a fused silica packed capillary column (30 m× 0.32 mm I.D.) coated with (5%-phenyl)-methylpolysiloxane at a film thickness of 1.5 μm (PTA-5, Spelco) with split-less. The detector temperature was set at 230 °C. Separation was performed with a program in which the column temperature was maintained at 40 °C for 2 min, increased from 40 °C to 230 °C at a linear rate of 10 °C/ min, and maintained at 230 °C for 23 min, using helium gas at a flow rate of 1.5 mL/min. Mass spectra were obtained with an electron-impact ionization at 70 eV. The peaks appeared were identified

using the standard National Institute of Standards and Technology (NIST) mass spectrum database and by comparing their RIs with those of the authentic compounds.

II-1-13 Statistical analysis

Volatile compounds in samples were quantified from three different experiments. Resulting data were given by means ± standard deviations and subjected to one way analysis of variance. The significant differences in QDA and preference tests between different samples were analyzed by Student's *t* -test.

II-2 Results and discussion

II-2-1 Isolation of bacteria from fish sauce mush (moromi) made from frigate mackerel

The fish sauce mush made from frigate mackerel was inoculated on the selective GYP and nutrient plates containing 12% NaCl and 1.2% $MgSO_4$. Bacteria grown on the selective plates were separated according to colony color and bacterial shape. Four strains were obtained from the GYP plates and named as strains R2G, R3G, R4G, and R5G, whereas two strains R3Nu and R4Nu from the nutrient plates. Each strain was cultured in the nutrient broth containing 18% NaCl, 10% fish sauce, and 1.8% $MgSO_4$. A bottle containing 50 mL of Thai fish sauce was added with 5 mL of the suspended bacteria in 18% NaCl from each strain and incubated at 32°C for 12 days to examine the capability of changing the distinctive odor of fish sauce. As shown in Table II-1, strains R5G and R4Nu remarkably decreased the distinctive odor of fish sauce. The other bacteria-treated fish sauces strengthened fish sauce odor like a burnt note. Strains R4Nu and R5G showed

the same morphological and biochemical properties as described below except colony color.

II-2-2 Volatile compounds in the bacteria-treated fish sauce

The fish sauce treated with strain R4Nu was analyzed for volatile compounds in comparison with the non-treated fish sauce by the P&T method as described in Materials and methods (Table II-2).

As described in Chapter I, a high FD factor was obtained with 7 volatiles including 2-methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, and 3-methylbutanoic acid in fish sauce, amongst which 4 volatiles including 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide contributed cooperatively to fishy, fecal, rancid and sweaty notes of fish sauce. Besides these 7 compounds, it has been indicated that dimethyl disulfide and butanoic acid have an important role in the development of fish sauce odor (Nguyen-An-Cu and Vialard-Gougou, 1953; Van-Chom, 1957; Saisithi *et al.*, 1966; Dougan and Haward, 1975; Shimoda *et al.*, 1996).

Totally 11 volatile compounds including 3-methyl-1-butanol and 2,6-dimethylpyrazine were analyzed in this study and compared between the bacteria-treated and non-treated fish sauces (Table II-2). As a result, the quantities of 2-methylpropanal, 2-methylbutanal, 2-pentanone, and 3-(methylthio)propanal were not significantly different between the two groups. On the other hand, the quantities of 2-ethylpyridine, dimethyl trisulfide, dimethyl disulfide, and butanoic acid, which are all predicted to contribute to the development of fish sauce order as described above, were less in the bacteria-treated than non-treated fish sauce.

The contents of 3-methyl-1-butanol and 2,6-dimethylpyrazine were markedly increased,

whereas the content of 3-methylbutanoic acid was slightly increased. It was noted that the content of 3-methyl-1-butanol was about 10 folds higher in the bacteria-treated than non-treated fish sauce. This compound would be possibly derived from leucine in fish sauce through precursors of α -ketoisocaproate and isovaleraldehyde by the action of strain R4Nu (Masson *et al.*, 1999). It has been reported that 3-methyl-1-butanol has much higher threshold value of 30 ppm in water/ethanol (90/10, w/w) (Goth, 1997). The concentration of this compound in this study was determined by the standard addition method to be 1.81 ppm in the bacteria-treated fish sauce even after 24 days. Therefore, its contribution to the odor of the bacteria-treated fish sauce is questionable. The content of 2,6-dimethylpyrazine was also increased 35-79 folds by the treatment of fish sauce with the bacterium. However, the FD factor of this compound was determined by AEDA to be 32 and below 16 in the bacteria-treated and non-treated fish sauce, respectively. These results suggest that the contribution of 2,6-dimethylpyrazine to the odor of the bacteria-treated fish sauce would be also negligible.

No additional volatile compounds with a high FD value (≥64) were found by AEDA in the fish sauce treated with strain R4Nu. Taken together, it was indicated that reduction of the distinctive odor of fish sauce by treatment of strain R4Nu was possibly derived from reduction of 2-ethylpyridine and dimethyl trisulfide.

The bacterial counts before and after treatment of the fish sauce with strain R4Nu for 24 days were 2.0×10^7 and 2.3×10^7 pfu/mL, respectively, indicating no change in the viability of the bacterium during incubation.

II-2-3 Sensory evaluation of the bacteria-treated fish sauce

Two types of sensory evaluation were performed for the bacteria-treated and

non-treated fish sauce, in mouth and by sniffing. In QDA, the sensory evaluation in mouth roughly coincided with evaluation by sniffing (Figure II-1). Fishy, sweaty, fecal and rancid notes of fish sauce were significantly reduced by bacterial treatment. The results obtained in Chapter I indicated that 2-ethylpyridine and dimethyl trisulfide contirbuted to fishy and sweaty notes and further were essential to the fecal note. The addition of the two compounds to the alkali-treated fish sauce demonstrated that they recovered fishy, sweaty and fecal notes. These two compounds in fish sauce were also decreased in their quantities after bacterial treatment (see Table II-2). Therefore, it is likely that the reduction of these three notes in the bacteria-treated fish sauce was attributed to the decrease of the two compounds. Chapter I also demonstrated that the addition of 2-methylpropanal and 2-methylbutanal to the alkali-treated fish sauce recovered burnt, sweaty and rancid notes. Although the two compounds gave almost the same peak concentration between the bacteria-treated and non-treated fish sauce in the present study (see Table II-2), the rancid note was significantly reduced by bacterial treatment in sensory evaluation. As described above, the decrease of 2-ethylpridine and dimethyl trisulfide contributed only to the decline of fishy, sweaty, and fecal notes, but not rancid one. Therefore, the decline of the rancid note in the bacteria-treated fish sauce might be caused by the decrease of other volatile compounds such as dimethyl disulfide and butanoic acid. Shimoda *et al.* (1996) reported dimethyl disulfide contributed to an unfavorable odor of fish sauce. Further study is needed to define clear contribution of various volatile compounds to the odor of fish sauce.

A preference test was then performed on the bacteria-treated fish sauce. The averages of score were +0.143 and +0.857 in mouth and by sniffing, respectively. There was no

significant difference in the score for evaluation in mouth between the bacteria-treated and non-treated fish sauce. However, the score by sniffing gave a significant difference between the two fish sauces ($p<0.05$), demonstrating that the fish sauce treated with strain R4Nu could reduce the unfavorable odor of fish sauce. It is, however, to be taken into consideration that high NaCl concentration of 22% in fish sauce might have disturbed a preference sense in mouth.

There is the possibility that the existence of the bacterium concerned with the odor of fish sauce depends on the maker and place, because many bacteria, i.e. *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., and *Streptococcus* sp., have been detected in the Vietnamese and Malaysian fish sauces which have reputation to be the most favorable ones (Mura *et al.*, 2000; Chihara *et al.*, 2002).

II-2-4 Catabolism in a model solution of volatile compounds responsible for the distinctive odor of fish sauce

The results of bacterial reaction on volatile compounds are shown in Figure II-2. Nine volatile compounds including 2-methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, 3-methylbutanoic acid, dimethyl disulfide, and butanoic acid were reacted in the presence of strain R4Nu in a model solution containing volatile compounds in 0.05M phosphate buffer (pH 5.4) containing 22% NaCl. Six volatile compounds including 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, and dimethyl disulfide, were decreased in the model solution. However, the contents of 2-methylbutanal, dimethyl trisulfide, and dimethyl disulfide were also decreased even in the inactivated bacterial solution and in 0.05M phosphate buffer (pH 5.4) containing 22% NaCl

only (data not shown). However, strain R4Nu significantly enhanced the decreasing rate of contents of 2-methylbutanal and dimethyl disulfide. On the other hand, the rate of decrease in the content of dimethyl trisulfide was almost the same between the solutions in the presence and absence of strain R4Nu. No changes in the contents of 2-pentanone and 3-methylbutanoic acid were observed in the presence of the bacterium.

The changes in volatile compounds in the fish sauce and the model solution treated with strain R4Nu in comparison with those not treated with the bacterium are summarized in Table II-3.

As 2-ethylpyridine and dimethyl disulfide were decreased both in the bacteria-treated fish sauce and in the model solution, it was clear that the bacterium had an ability to metabolize these compounds to other ones. However, secondary metabolites of these compunds were not detected in the bacteria-treated model solution. 2-Pentanone showed no change both in the bacteria-treated fish sauce and the model solution, suggesting that the bacteria could not metabolize this compound.

While 2-methylpropanal and 2-methylbutanal were decreased in the bacteria-treated model solution, no change was observed in the bacteria-treated fish sauce. Furthermore, 3-methylbutanoic acid was increased in the bacteria-treated fish sauce, but showed no change in the bacteria-treated model solution. It was thought that 2-methylpropanal and 2-methylbutanal would be increased in the bacteria-treated fish sauce, because they are synthesized from amino acids with a strain of *Staphylococcus* sp. (Masson *et al.*, 1999; Sanceda *et al.*, 2001).

3-(Methylthio)propanal was decreased in the model solution, though not changed in the bacteria-treated fish sauce. Dimethyl trisulfide was decreased in the bacteria-treated fish

sauce, but not changed in the model solution. Gijs *et al.* (2000) showed that 3-(methylthio)propanal and its reduced form, 3-(methylthio)propanol, were metabolized to dimethyl trisulfide in aged beer. Furthermore, the formation of 3-(methylthio)propanal has been reported to be more easily produced by the thermal degradation of methionine, whereas dimethyl polysulfides, especially dimethyl disulfide and dimethyl trisulfide, are formed from the thermal degradation of methionine sulfoxide (Yu and Ho, 1995). It is thus speculated that 3-(methylthio)propanal was not decreased in the fish sauce reacted with strain R4Nu, because it is produced from methionine in the fish sauce by chemical reaction (Ballance, 1961), though it is expected to be further converted to dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide via methanthiol. The proposed pathway of methionine is summarized in Figure I-4.

Dimethyl trisulfide and butanoic acid were not changed in the model solution, but decreased in the bacteria-treated fish sauce. Although it is possible that the metabolic reactions related to bacterial enzymes could be changed in the fish sauce, further study is required for the changes in dimethyl trisulfide and butanoic acid in fish sauce.

II-2-5 Formation of volatile compounds from amino acids

It was examined by adding the bacterium in the synthetic fish sauce whether or not four volatile compounds, including 2-methylpropanal, 2-methylbutanal, 3-(methylthio)propanal and 3-methylbutanoic acid, were synthesized from amino acids based on the results obtained with the volatile compound model solution and fish sauce in the presence of strain R4Nu. The synthetic fish sauce was made from amino acids with the same contents in Thai fish sauce in 0.05M sodium phosphate (pH 5.4) containing 22% NaCl. The synthetic fish sauce produced aldehydes including 2-methylpropanal, 2-methylbutanal, and

3-methylbutanal, alcohols including 2-methylbutanol and 3-methyl-1-butanol, and volatile fatty acids including 2-mehtylbutanoic acid and 3-methylbutanoic acid in the presence of strain R4Nu (Figure II-3). The bacteria-treated fish sauce contained 2-methylbutanal, 2-methylpropanal, 3-methyl-1-butanol and 3-methylbutanoic acid, as described in the previous section. On the other hand, 3-methylbutanal, 2-methylbutanol, and 2-methylbutanoic acid were not observed in the bacteria-treated fish sauce, suggesting that these compounds might be produced by catabolic reaction with strain R4Nu.

To identify amino acids responsible for the production of these volatile compounds, each amino acid was tested in 0.05M phosphate buffer (pH 5.4) contained 22% NaCl. It was found that 2-methylbutanal, 2-methylbutanol, and 2-methylbutanoic acid were produced from isoleucine (Figure II-4), whereas 3-methylbutanal, 3-methyl-1-butanol, and 3-methylbutanoic acid were from leucine (Figure II-5). It was noted that 2-methylbutanal was observed only at the beginning of the reaction. 2-Methylpropanal were obtained from valine at an early stage of reaction but disappeared after 7 days (Figure II-6). 2-Methybutanal and 2-methypropanal may be easily metabolized to other compounds in the model solution containing isoleucine or valine. Any products were not produced from the model solution containing methionine (data not shown).

The proposed pathway for branched-chain aldehydes and volatile carboxylic acids from branched amino acids is summarized in Figure II-7. 3-Methyl-1-butanol and 3-methylbutanoic acid were synthesized from leucine probably via 3-methylbutanal by strain R4Nu (Figure II-5). There are two pathways to synthesize carboxylic acids from amino acids. It has been reported that α-ketoisocaproic acid is produced from leucine by bacterial deaminases or aminotransferases (Massey *et al.*, 1976). Then, α

-ketoisocapronic acid is degraded into 3-methylbutanoic acid and 3-methylbutanal, by multienzymes complex and by decarboxylase, respectively. In the second pathway, 3-methylbutanoic acid is produced by oxidization of 3-methylbutanal. 3-Methyl-1-butanol is made by the reaction of 3-methylbutanal with aldehyde dehydrogenases (Masson, 1999). A very high production of 3-methyl-1-butanol in fish sauce was observed in the presence of strain R4Nu as described in the previous section. However, this compound hardly affects the odor of fish sauce due to its high threshold. 3-Methylbutanoic acid is attributed to the distinctive odor of fish sauce, because its flavor threshold value is low.

Sulfur compounds are known to produce off-flavors of white wine (Ferreira *et al.*, 2003) and aged beer (Gijs *et al.* 2000). Dimethyl sulfide and polysulfides such as dimethyl disulfide and dimethyl trisulfide are probably derived from methanethiol either by direct oxidation of or through reactions with hydrogen sulfide as described before (Figure I-4). 3-(Methylthio)propanal is generally considered as a precursor of methanthiol. After the strecker degradation of methionine, 3-(methylthio)propanal decomposes to methanthiol and acrolein (Ballance, 1961). Polysulfides, including dimethy disulfide, were not detected even on the treatment with strain R4Nu in the model solution containing methionine, though it was detected when added with *Staphylococcus xylosus* provided from Chr. Hansen A/S (hoersholm, Denmark) (data not shown). Strain R4Nu has an apparent advantage as a starter for culture to reduce the distinctive odor of fish sauce, especially concerning with dimethyl disulfide and dimethyl trisulfide, because it has no ability to produce these sulfur compounds from methionine. Strain R4Nu may rather have an ability to metabolize these sulfur compounds.

2,6-Dimethylpyridine was not detected as well in the synthetic fish sauce inoculated strain R4Nu. It is necessary to investigate the metabolism of this compound in the presence of strain R4Nu.

II-3 Conclusion

Strain R4Nu which improved the fish sauce odor was isolated from fish sauce mush (moromi) made from frigate mackerel. It reduced the contents of volatile compounds such as dimethyl disulfide, 2-ethylpyridine, dimethyl trisulfide, and butanoic acid in Thai fish sauce during incubation at 32°C for 24 days, whereas it increased 3-methylbutanoic acid, 3-methyl-1-butanol, and 2,6-dimethylpyrazine. Concomitantly, unfavorable notes of fish sauce were declined accompanying with the treatment of the bacterium isolated.

It became clear that strain R4Nu has an ability to metabolize 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, 3-(methylthio)propanal, and dimethyl disulfide. A very high production of 3-methyl-1-butanol and 2,6-dimethylpyridine was also observed in fish sauce treated with strain R4Nu. As the result of *in vitro* experiments using a model solution containing various amino acids in the presence of strain R4Nu, it appeared that 2-methylpropnanal and 2-methylbutanal were produced from valine and isoleucine, respectively, whereas 3-methyl-1-butanol and 3-methylbutanoic acid were from leucine. However, the mechanisms involved in the decrease of dimethyl trisulfide and the increase of 2,6-dimethylpyridine in fish sauce inoculated strain R4Nu have remained to be solved.

3-(Methylthio)propanal was metabolized in a model solution containing volatile compounds in the presence of strain R4Nu, although this compound was not decreased significantly in fish sauce inoculated strain R4Nu. There is the possibility that 3-(methylthio)propanal was produced from methionine by heat degradation in the process of removing the distinctive odor of fish sauce added with strain R4Nu.

Chapter III

Characterization and distribution of *Staphylococcus* sp. implicated for improvement of fish sauce odor

In Chapter II, the fish sauce mush that was made from frigate mackerel in Japan with less distinctive odor (Funatsu, 2001) was screened for bacteria that would have the ability of improving the distinctive odor of fish sauce. As a result, two strains were isolated, but these strains are needed to be identified.

Chapter III is devoted to identification of the strains isolated and determination of their distributions to fermented foods using molecular biological approaches. For these purposes, morphological and biochemical properties of the strains were examined and molecular biological approaches such as taxonomic studies using the sequences of 16S rRNA and *rpoB* and the DNA-DNA hybridization experiments were adopted. The two strains isolated from fish sauce mush were both identified as *Staphylococcus nepalensis* (Fukami *et al.*, 2004b), which had been first isolated from the respiratory tract of goat in the Himalayan region (Spergser *et al.*, 2003).

III-1 Materials and methods

III-1-1 Materials

Malt made from fermenting soy beans and barley with *Aspergillus oryzae* (koji) was obtained from a local supplier in Toyama Prefecture, Japan. It is used not only for making soy sauce, but also for making fish sauce as described in our pervious report (Fukami *et al.*, 2004a). Various brands of fish sauce were obtained from local suppliers in Thailand, Vietnam, Philippine, and Japan (Table III-4).

III-1-2 Morphological and biochemical properties

Bacteria screened were identified on the basis of morphological and biochemical properties according to the Bergey's Mannual (Sneath *et al.*, 1984; Holt *et al.*, 1994). The external characteristics including colony morphology, pigmentation, motility and endospore formation were recorded by the methods previously reported (Kloos *et al.*, 1991). Biochemical properties such as catalase activity, oxidase activity, acidification and fermentation (O/F) of glucose were examined according to the Cowan and Steel's Manual (Barrow and Feltham, 1993).

API ID32 Staph (bioMerieux, France) was employed to determine hydrolysis of urea, arginine and ornithine, nitrate reduction, acetoin production, ß-glucosidase activity, arginine arylamidase activity, alkaline phosphatase activity, pyrrolidonyl arylamidase activity, resistancy to novobiocin, ß-glucronidase activity, and acid production from various kinds of sugar and their derivatives (Table III-1).

III-1-3 Bacterial strains

Strains R4Nu and R5G, which could improve fish sauce odor were isolated from fish

sauce mush made from frigate mackerel. The type strains of the genus *Staphylococcus*, *S. cohnii* subsp. *cohnii* JCM2417^T, *S. saprophyticus* JCM2427^T, *S. xylosus* JCM2418^T, *S. epidermidis* JCM2414^T and *S. haemolyticus* JCM2416^T , were obtained from the Japan Collection of Microorganisms. The type strain of *S. nepalensis* CW-1^T was kindly provided by Dr. Busse (Spergser *et al.*, 2003). Unless otherwise indicated all strains were cultured at 30 °C on the NA plates (Becton, Dickinson and Company).

III-1-4 Sequencing of 16S rRNA and *rpoB*

Accession numbers for the sequences of 16S rRNA and *rpoB* from *Staphylococcus* species used in this study are listed in Table III-2. To extract DNAs for PCR from tempelate bacteria, cells were suspended in TTE buffer (TE buffer of 10 mM Tris, 1 mM EDTA, pH 8.0, supplemented with 1% Triton X-100). Cell suspension was boiled for 5 min, immediately cooled on ice, and subjected to DNA extraction by the chloroform extraction method. 16S rRNA and *rpoB* were amplified by PCR using universal primer sets described by Weisburg *et al.* (1991) and Drancourt and Raoult (2002), respectively. PCR products were visualized by ethidium bromide staining following agarose gel electrophoresis and the bands of interest were cut out and purified using polyethylene glycol precipitation. Purified PCR products were sequenced directly as described previously (Satomi *et al.*, 1997) using Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instruction.

III-1-5 Phylogenetic analysis

The 1.0 kbp nucleotide sequences of 16S rRNA covering the base positions 30-1,038 (*Escherichia coli* numbering) and 0.5 kbp nucleotide sequences of *rpoB* covering base positions 2,643-3,241 (*S. aureus* numbering) cited from the GenBank/EMBL/DDBJ

databases (Table III-2) were used for phylogenetic analysis. The sequences of the new isolates were compared with the above sequence data using the BLAST algorithm (Altschul *et al.*, 1990). Multiple alignment, calculation of nucleotide substitution rates (*K*nuc values) described by Kimura (1980), and construction of phylogenetic trees by the neighbor-joining method (Saitou and Nei, 1987) were performed by using the CLUSTAL W computer program (Thompson *et al.*, 1994). Alignment gaps, primer regions used for PCR amplification, and unidentified base positions were not taken into consideration for calculation. The robustness of topology on the phylogenetic trees was evaluated by a bootstrap analysis through 1,000 replication.

III-1-6 DNA-DNA hybridization

DNA-DNA hybridization was performed by the microplate hybridization method (Ezaki *et al.*, 1989). To extract large amounts of DNA to be used for DNA-DNA hybridization experiments, the bacterial strains were cultivated at 30°C for 16 h with shaking in trypticase soy broth (Becton, Dickinson and Company) containing 1.5% glycine, harvested by centrifugation, and suspended in Tris-EDTA (TE) buffer. Suspension thus obtained was treated at 37 °C for lysis with 50 μg/mL labiase (Seikagaku Corporation, Tokyo, Japan) and 1 mg/mL achromopeptidase (Wako Pure Chemicals) until the reaction mixture became viscous. Further purification of chromosomal DNA was carried out according to the standard methods (Sambrook *et al.*, 1989).

III-1-7 PCR assay for detection of *Staphylococcus nepalensis*

By comparing the *rpoB* sequences of 30 *Staphylococcus* species (Table III-2), a suitable PCR primer set (196F, 5'-GTTTAGGAGATACATCCATA-3' covering the base positions 2,888-2,913 and 395R, 5'-AGATATTGAAACAAACAGCATTACT-3' covering the base

positions 3,068-3,087 in *S. aureus* numbering) was designed to specifically identify *S. nepalensis*. The 191 bp PCR amplicon obtained by the aid of these primers was inferred to indicate the presence of *S. nepalensis* template DNA in a sample. PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Corp., MA, USA) in a 100 μL solution containing 200 μM dNTP mixture, 100 μM primers, 0.5 μg DNA as a template, 2.5 U *Taq* DNA polymerase, and PCR buffer (*TaKaRa Ex Taq*® R-PCR Version 1.0, Takara Bio Inc., Otsu, Japan). The reaction consisted of 30 cycles each of 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C, with the final extension step at 72 °C for 7 min. PCR products were visualized by staining with ethidium bromide.

III-1-8 Isolation of bacterial colonies from fish sauce and malt for soy sauce

Aliquots each containing 100 μL of fish sauce were inoculated on the NA plates containing 5% NaCl and incubated at 30 °C for 2 - 3 days. The colonies grown on the NA plates were transferred to another NA plates containing 18% NaCl. The colonies grown on the latter plates were incubated at 30 °C for 2 - 3 days and subjected to PCR assay for detection of *S. nepalensis*.

Four grams of malt were dissolved in 10 mL of sterilized 22% NaCl solution. To 2 mL of this malt solution were add 50 mL of nutrient broth (Becton, Dickinson and Company) containing 18% NaCl in a 500 mL flask and the mixture was incubated at 30 °C for 3 days with reciprocal shaking. Aliquots each containing 100 μL of this fermented broth were diluted with sterilized water, inoculated on the NA plates containing 18% NaCl and incubated at 30 °C for 4 days. The colonies grown on these plates were randomly selected, transferred to another NA plates containing 18 % NaCl, and subjected to PCR assay for detection of *S. nepalensis* as described above.

III-2 Results and discussion

III-2-1 Morphological and biochemical properties

Strains R5G and R4Nu showed milky white and light yellow colony colors, respectively. However, both strains were identified as *Staphylococcus* sp. according to biochemical properties following the Bergey's Mannual: gram-positive coccui, nonmotile, approximately 1μm in diameter, nonsporeforming, positive catalase and negative oxidase acvtivity, acid production from glucose, hydrolysis of urea, arginine and ornithine, nitrate reduction, acetoin production, ß-glucosidase activity, arginine arylamidase activity, alkaline phosphatase activity, pyrrolidonyl arylamidase activity, resistancy to novobiocin, ß-glucronidase activity, and acid production from various kinds of sugar and their derivatives (Table III-1).

III-2-2 Taxonomic studies

The phylogenetic trees based on 16S rRNA and *rpoB* are shown in Figures III-1 and Figure III-2, respectively. The sequence each of the two genes was identical between strains R4Nu and R5G and their phylogenetic position was in the genus *Staphylococcus.* The closest species to both isolates was determined to be *S. nepalensis* based on 16S rRNA and *rpoB* with 99.5 % and 99.0 % sequence identities, respectively. The sequence identity to separate any given two bacteria at the species level based on 16S rRNA is less than 97 % (Stackebrandt and Goebel, 1994) and the nucleotide substitution rate of *rpoB* is much faster than that of 16S rRNA in terms of the third base in a codon (Yamamoto and Harayama, 1998). Therefore, the two isolates in the present study were regarded as the same species of *S. nepalensis*.

The DNA-DNA hybridization experiments also indicated that the three strains of CW-1^T, R4Nu and R5G belong to the same species, showing more than 80 % relatedness values (Table III-3). The DNA-DNA hybridization analysis is the most reliable method for identification of bacterial species and the similarity between any given two strains within the same species is not less than 70 % (Wayne *et al.*, 1987). In conclusion, the two strains of R4Nu and R5G isolated from fish sauce mush made from frigate mackerel were both identified as *S. nepalensis.*

However, there were several differences in the phenotype between *S. nepalensis* isolated from the respiratory tract of goat in the Himalayan region (Spergser *et al.*, 2003) and the present strain isolated from fish sauce mush made from frigate mackeral. For example, the former produced no acid aerobically from ribose and grew in 15% NaCl, whereas the latters produced acid aerobically from the sugar and grew in 22% NaCl.

III-2-3 Possible distribution of *Staphylococcus nepalensis* to various fish sauces

A total of 37 colonies isolated from the NA plates containing 18% NaCl from 19 fish sauces made in Asian countries and one malt sample for soy sauce made in Toyama Prefecture, Japan, were subjected to PCR assay together with the type strains of *Staphylococcus* sp. The DNA fragment amplified using the primer set specific to *rpoB* of *S. nepalensis* had 191 bp as shown in Figure III-3. No PCR products were found from other *Staphylococcus* species including *S. cohnii* subsp. *cohnii*, *S. saprophyticus*, *S. xylosus*, *S. epidermidis* and *S. haemolyticus*, indicating that the primer set was highly specific to *S. nepalensis*.

When analyzed by PCR using the specific primer set, 22 colonies from fish sauces were negative for *S. nepalensis*. However, all of 15 colonies isolated from the malt were

positive to PCR amplification (Table III-4). Because malt is not commonly used for making fish sauce, the above results indicate the possibility that the bacteria isolated from fish sauce mush made from frigate mackerel were originated from the malt contaminating in the fish sauce mush. Although various *Staphylococcus* sp. were isolated in fish sauce and other foods in Asian countries, these were examined by only phenotypic characterization. A further study about the distribution of *S. nepalensis* in food is needed by using molecular biological techniques such as phylogenetic analysis using 16S rRNA and *rpoB* primers and DNA-DNA hybridization.

The genus *Staphylococcus* comprising 38 species are widespread in nature and commonly found on the skin and mucous membranes of human, other mammals, and birds (Holt *et al.*, 1994; Lambert *et al.*, 1998; Vernory-Rozand *et al.*, 2000). They have been also isolated from a wide variety of environmental sources such as water, soil, plant surfaces, meat, and poultry, dairy products, and air (Kloos and Lambe, 1991). Furthermore, they are often found in fermented foods including fish sauce, soy paste, milk products, and salted and dried meat products (Schleifer and Fisher, 1982; Tanasupawat *et al.*, 1991; Vernozy-Rozand *et al.*, 1996; Probst *et al.*, 1998; Vernozy-Rozand *et al.*, 2000). *Staphylococcus* spices are practically used as starter cultures for enhancing the flavor and color of meat products and for improving product safety (Geisen *et al.*, 1992; Berdague *et al.*, 1993).

S. xylosus, S. saprophyticus, S. carnosus, and *S. cohnii* have been isolated from fish sauce made in South Asian countries (Schleifer and Fisher, 1982; Itoh *et al.*, 1985; Mura *et al.*, 2000; Chihara *et al.*, 2002). In the previous study, *Staphylococcus* sp., which was isolated from fish sauce mush made from frigate mackerel in Japan and tentatively

identified as *S. xylosus* by phenotypic characterization, was demonstrated to improve fish sauce odor (Fukami *et al.*, 2004a). Biochemical-based commercial kits were employed for the above identification of Staphylococci. However, this method has limitation due to variable characters and ambiguity in the interpretation of end point reaction (Birnbaum *et al.*, 1991). To overcome such ambiguities to identify *Staphylococcus* species and strains, the sequence analysis of 16S rRNA and *rpoB* are often employed with their advantages of sensitivity and specificity (De Buyser *et al.*, 1992; Takahashi *et al.*, 1999; Drancourt and Raoult, 2002). In addition to such molecular biological approaches, it is generally accepted that the DNA-DNA hybridization analysis is the most reliable method for identification of bacterial species.

This is the first discovery to the author's knowledge that *S. nepalensis* is present in food. It is interesting to investigate the distribution of this bacterium to other foods. As mentioned in Chapter II, the bacterium has an ability to improve fish sauce odor and is likely to be also useful for improving an unpleasant odor of other foods such as fermented seafoods. However, *S. aureus* colonization of food has been associated with a form of gastroenteritis. This condition is called staphylococcal food poisoning and results from the ingestion of one or more pre-formed staphylococcal enterotoxins (SEs) on food that has been contaminated with some species of staphylococci, primarily *S. aureus* (Jay, 1992). It is not possible to use it freely because of the restriction by Food Sanitation Law in Japan. To improve volatile compounds in foods using the bacteria, it is necessary to perform the safety tests and SEs production activity (Fueyo, 2001; Blaiotta, 2004).

III-3 Conclusion

The two *Staphylococcus* strains that were isolated in Chapter II from fish sauce mush (moromi) made from frigate mackerel in Japan and proved to improve fish sauce odor were examined for their taxonomic positions. The sequence analysis based on 16S rRNA and *rpoB* showed that the two strains, R4Nu and R5G, had an identical sequence with sequence identities of 99.5 and 99.0 % to the above two genes from the closest species of *S. nepalensis*, respectively. DNA-DNA hybridization test of the two strains showed more than 80 % DNA similarity with *S. nepalensis*, thus confirming the above-mentioned species identification. PCR primers specific to the strain isolated from fish sauce mush were designed from *rpoB* and examined for the distribution of this species to various fish sauces made in Asian countries as well as to fish sauce starter (malt) made from soy beans and barley in Toyama Prefecture, Japan. The amplified DNA fragment bearing the *S. nepalensis* gene was detected in the enriched culture of the malt, but no positive reaction was shown with fish sauce samples. These results suggest that *S. nepalensis* indebted to improve fish sauce odor was originated from the fish sauce starter malt. It is noted that people may have a long experience to use this bacteria in soy sauce.

Chapter IV

General discussion

The present research project started to improve fish sauce which would be widely used in dairy foods and prepared foods by decreasing unpleasant volatile compounds. The volatile compounds in fish sauce were analyzed by using GC and GC-MS and those responsible for the distinctive odor of fish sauce were identified by AEDA and sensory evaluation of QDA. AEDA had not been adopted to identify the odor-active compounds in fish sauce before. Finally, the distinctive odor of fish sauce was reduced, which was accomplished by using bacteria. Then, the method eliminating distinctive odor of fish sauce was examined by using bacteria.

2-Methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, and 3-methylbutanoic acid were principal contributors to the distinctive odor of fish sauce. 2-Ethylpyridine and dimethyl trisulfide were found to contribute to the fishy and fecal notes. The sweaty and rancid notes were attributed to four volatiles including 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide. 2-Ethylpyridine in addition to 2-pentanone and volatile acids were essential to the cheesy note. 2-Ethylpyridine together with 2-methylpropanal and 2-methylbutanal was responsible for the meaty note. The burnt note was developed in the presence of 2-ethylpyridine and dimethyl trisulfide and also in the presence of 2-methylpropanal and 2-methylbutanal. The complicated distinctive notes of fish sauce may be derived from the volatile compounds in fish sauce with high values of FD cooperatively. Furthermore, it was indicated that reduction of the

distinctive odor of fish sauce could be achieved not only by changing the balance of volatile compounds with a high value of FD (a low threshold), but also by reducing these volatile compounds.

Seven volatile compounds associated with the distinctive odor of fish sauce were identified in Chapter I. It was necessary to reduce the 7 volatile for making fish sauce acceptable for dishes. It is common to change volatile compounds for processing fermented meat and cheese by adding starter cultures such as *Lactococcus* sp., *Lactobacillus* sp., and *Staphylococcus* sp. (Geisen *et al.,* 1992; Berdague *et al.,* 1993; Masson *et al.*, 1999). The author tried to isolate the bacteria that have an ability to reduce 7 volatile compounds in fish sauce made in Thailand. As a result, the two bacterial strain, designated R4Nu and R5G, were isolated from the fish sauce mush (moromi) made from frigate mackerel which was preferable to fish sauce made in Thailand. The changes of volatile compounds in Thai fish sauce added with R4Nu were examined in detail. Strain R4Nu reduced the contents of volatile compounds such as 2-ethylpyridine, dimethyl trisulfide, dimethyl disulfide and butanoic acid, and increased the contents of 3-methylbutanoic acid, 3-methyl-1-butanol and 2,6-dimethylpyrazine, when the fish sauce was incubated at 32°C for 24 days. Concomitantly, unfavorable notes of fish sauce were declined by the treatment with the bacterium.

It became clear that strain R4Nu has an ability to metabolize 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, 3-(methylthio)propanal, and dimethyl disulfide. It was also found that 2-methylpropanal was produced from valine, whereas 2-methylbutanal from isoleucine. Furthermore, 3-methyl-1-butanol and

3-methylbutanoic acid were produced from leucine. A highly efficient production of 3-methyl-1-butanol was also observed in fish sauce treated with strain R4Nu. The mechanisms involved in a decrease of dimethyl trisulfide and an increase of 2,6-dimethylpyridine in fish sauce inoculated strain R4Nu are still unknown.

The literature data indicate that α-ketoisocaproic acid is produced from leucine by bacterial deaminases and aminotransferases (Massey *et al.*, 1976). Then, α-ketoisocapronic acid is degraded into 3-methylbutanoic acid by multienzymes complex (Ward *et al.*, 1997) and alternatively into 3-methylbutanal by decarboxylase (Hickey, 1983). In the later pathway, 3-methylbutanoic acid is produced from oxidization of 3-methylbutanal by dehydrogenase. 3-Methyl-1-bunanol is produced from 3-methylbutanal by reduction with aldehyde dehydrogenases (Masson, 1999). As a very high production of 3-methyl-1-butanol in fish sauce was observed with strain R4Nu, the endogenous enzyme may have a relation with such reduction. This compound seems not to affect the odor of fish sauce due to its high threshold. On the other hand, 3-methylbutanoic acid has a low flavor threshold, producing the distinctive odor of fish sauce.

Sulfur compounds are known to produce an off-flavor of white wine (Ferreira *et al.*, 2003) and of aged beer (Gijs *et al.* 2000). It is fresh-onion-like in synthetic matrix and its threshold is extremely low (Devos *et al.*, 1995). Polysulfides are derived from methanthiol either by direct oxidation of sulfur compounds or through reactions with hydrogen sulfide. 3-(Methylthio)propanal is considered to be a precursor of methanthiol and play an important role as the distinctive odor of fish sauce, is probably produced by the strecker degradation of methionine and decomposes to methanthiol and

acrolein (Ballance, 1961). *S. xylosus*, well known as a starter of fermented sausage (Geisen *et al.*, 1992; Berdague *et al.*, 1993), produced dimethyl disulfide in a model solution containing methionine, when incubated at 32°C for 11days as described in Chapter II (data not shown). It was also speculated that dimethyl disulfide and dimethyl trisulfide were produced via 3-(methylthio)propanal and methanthiol by the strecker degradation in the presence of bacteria inheriting in the factory. Since *S. nepalensis* has an ability to reduce the distinctive odor of fish sauce, it may be useful to reduce off-flavor in any fermented foods attributed to such sulfur compounds. It is worth to investigate such possibility.

The two *Staphylococcus* strains isolated from fish sauce mush (moromi) made from frigate mackerel were examined for their species identification. The sequence analysis based on 16S rRNA and *rpoB* showed that the two strains, R4Nu and R5G, had an identical sequence. Their taxonomic positions along with the results from the DNA-DNA hybridization test revealed that the two strains are *S. nepalensis*. PCR primers specific to the strain isolated from fish sauce mush were designed from *rpoB* and examined for the distribution of this species to various fish sauces made in Asian countries as well as to fish sauce starter (malt) made from soy beans and barley in Toyama Prefecture, Japan. The amplified DNA fragment bearing the *S. nepalensis* gene was detected in the enriched culture of the malt, although no positive reaction was shown with fish sauce samples. These results suggest that *S. nepalensis* indebted to improve fish sauce odor was originated from the fish sauce starter malt.

It is interesting to investigate the distribution of this bacterium to other foods. *Staphylococcus* spp., such as *S. xylosus, S. saprophyticus, S. carnosus* and *S. cohnii*,

have been isolated from fish sauce made in South Asian countries (Schleifer and Fisher, 1982; Itoh *et al.*, 1985; Mura *et al.*, 2000; Chihara *et al.*, 2002). It is useful to detect the bacteria using primer sets designed in the present study for 16S rRNA and *rpoB* in the viewpoint of shortening time and reducing laborious work. The strain isolated in this study is thought to have a long history as has been utilized in food. As mentioned in Chapter II, strain R4Nu has an ability to improve fish sauce odor. However, *S. aureus* colonization in food is associated with a form of gastroenteritis. This condition is called staphylococcal food poisoning, resulting from the ingestion of one or more pre-formed staphylococcal enterotoxins (SEs) contained in food, which is primarily caused by *S. aureus* (Jay, 1992). According to Food Sanitation Law, it is not possible to use this bacterium freely. To improve volatile compounds in foods using the bacteria, it is necessary to perform the safety test against SEs production by PCR and DNA fingerprinting (Fueyo, 2001; Blaiotta, 2004).

Seven volatile compounds identified in Chapter I except 2-pentanone and 2-ethylpyridine in fish sauce were derived from amino acids such as leucine, isoleucine, valine, and methionine. It was also obvious that the bacteria isolated from fish sauce mush made from frigate mackerel were able to metabolize 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, 3-(metylthio)propanal, and dimethyl disulfide as described previously. Additionally, the bacteria had ability to produce aldehydes, alcohol, and acids from branched-chain amino acids such as leucine, isoleucine, and valine.

For production of favorable fish sauce to be widely accepted for cooking, several methods seem possible. Low temperature fermentation and anaerobic fermentation are

recommended for production of fish sauce to suppress the heat degradation and conversion of methanthiol to volatile sulfur compounds such as dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide. Upon this fermentation of fish sauce, the process to use the bacteria reducing 2-ethylpyridine and leaving volatile sulfur compounds seems additionally effective, because 2-ethylpyridine is responsible for the distinctive odor of fish sauce due to its low threshold value. 3-Methylbutanoic acid is produced from leucine by the bacterial enzymes. To prevent the production of 3-methylbutanoic acid, the metabolic pathway to 3-methyl-1-butanol should be accelerated by the activity of decarboxylase and alcohol dehydrogenase. Alternatively, the utilization of aldehyde dehydrogenase and related multienzymes complex to prevent conversion of 3-methylbutanal to carboxylic acids may be possible with *S. nepalensis* strain artificially produced by gene manipulation methods and traditional isolation method of mutants after treatment with mutagenic agents. As 3-methyl-1-butanol produces an important, favorable odor in miso-paste and soy sauce (Ito, 1993), it would be effective to convert aldehydes such as 3-methylbutanal, 2-methylpropanal, and 2-methylbutanal to alcohols including 3-methyl-1-butanol, 2-methylbutanol, and 2-methylpropanol.

2-Ethylpyridine had not been considered in food chemistry as a distinctive odor. This volatile compound was responsible for the grassy note in fish sauce in combination of other volatiles. 2-Ethylpyridine with other volatile compounds also produced smells like sardine although it has not been demonstrated experimentally. Fishy smell is disliked if it exists in seasonings in Japan. The reduction of 2-ethylpyridine seems a key step to produce desirable fish sauce. It seems the best manufacturing method for

making pleasant fish sauce is to treat with the bacteria.

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Table I-1. Volatile compounds in fish sauce characterized by AEDA and GC-MS

Peak No.	RI	Odor charactor	Compound	FD	After deodorization
1	677	fishy	trimethylamine		
2	817	burnt	2-methylpropanal	128	0.38
3	861	phenol			
4	910	burnt			
5	926	strong burnt	2-methylbutanal	64	0.22
6	950	fruity			
7	957	fruity	2-pentanone	64	4.72
8	968	fruity			
9	1050	fruity		8	
10	1055	fruity			
11	1066	melon		32	
12	1083	sweaty			
13	1146	cheesy	1-butanol		
14	1170	rancid	pyridine	16	
15	1183	furity		4	
16	1194	burnt		16	
17	1204	burnt	3-methyl-1-butanol	32	
18	1234	sweaty	2-methylpyridine	4	
19	1250	grassy			
20	1259	sweaty	4-methyl-1-hexanol		
21	1286	grassy	2-ethylpyridine	64	0.13
22	1290	sweaty	cyclopentanol		
23	1324	fruity	2,6-dimethylpyrazine		
24	1333	sweaty	ethylpyrazine		
25	1368	fishy		4	
26	1378	fishy	dimethyl trisulfide	128	0.16
27	1383	rancid	2-ethyl-6-(or-5-)methyl pyrazine		
28	1396	sweaty	2-methyl-5-(1-methylethyl) -pyrazine	8	
29	1435	sweaty		8	
30	1442	rancid			
31	1451	grassy	3-(methylthio)propanal	128	1.9
32	1468	fishy	4-ethyl-6-hepten-3-one	4	
33	1482	sweaty		8	
34	1508	grassy			
35	1570	sweaty			
36	1591	rancid	benzonitrile		
37	1614	cheesy	butanoic acid		
38	1650	cheesy	acetophenone	4	
39	1665	rancid	3-methylbutanoic acid	64	
40	1705	rancid		4	
41	1711	metalic			
42	1788	sweaty			
43	1847	rancid	capronic acid	4	

Fish sauce was deodorized by the method in which fish sauce was alkalized at pH 9.5 with 20% NaOH and evaporated under reduced pressure at 30°C for 4h. The value is the concentration ratio of deodorized fish sauce to non-treated fish sauce. Retention index and flavor dilution are abbreviated as RI and FD, respectively.

Table II-1. Screening of bacteria from fish sauce mush made from frigate mackerel

Strain	Incubation time (day)			
	2	4	8	12
R2G	±	+	+	++
R3G	±	+	+	++
R4G	±	+	+	++
R5G	±	-	-	--
R3Nu	±	+	+	++
R4Nu	-	--	--	--

Symbols for strength of fish sauce odor: ++, very strong; +, strong; ±, same; -, weak; --, very weak .

Table II-2. Comparison of volatile compounds between fish sauces with and without bacterial treatment by the P&T method

	Peak concentration*		
	Control	12.5 days	24.0 days
2-Methylpropanal	2.75 ± 0.030 [a]	2.62 ± 0.04 [a]	2.85 ± 0.02 [a]
2-Methylbutanal	0.83 ± 0.060 [a]	0.77 ± 0.03 [a]	0.86 ± 0.001 [a]
2-Pentanone	0.26 ± 0.030 [a]	0.19 ± 0.01 [b]	0.24 ± 0.01 [a]
2-Ethylpyridine	0.0026 ± 0.0001 [a]	0.0009 ± 0.00001 [b]	0.0011 ± 0.00005 [b]
Dimethyl trisulfide	0.0136 ± 0.001 [a]	0.0076 ± 0.0006 [b]	0.0056 ± 0.0004 [b]
3-(Methylthio)propanal	0.071 ± 0.001 [a]	0.057 ± 0.009 [a]	0.059 ± 0.004 [a]
3-Methylbutanoic acid	0.41 ± 0.001 [a]	0.48 ± 0.001 [b]	0.47 ± 0.01 [b]
Dimethyl disulfide	0.095 ± 0.001 [a]	0.061 ± 0.004 [b]	0.049 ± 0.001 [c]
3-Methyl-1-butanol	0.37 ± 0.01 [a]	3.21 ± 0.08 [b]	3.59 ± 0.001 [c]
2,6-Dimethylpyrazine	0.0068 ± 0.0003 [a]	0.24 ± 0.007 [b]	0.54 ± 0.002 [c]
Butanoic acid	0.77 ± 0.01 [a]	0.592 ± 0.017 [b]	0.51 ± 0.03 [c]

* The values represent the ratios of the peak concentrations in fish sauce to that of the internal standard (cyclohexanol). The bacteria-treated fish sauce was prepared by incubating Thai fish sauce at 32°C in the presence of strain R4Nu as described in Materials and methods. Control values are those of non-treated Thai fish sauce. Values are means ± standard deviations from three different experiments. Different letters within the same row indicate significant differences ($p<0.05$).

Table II-3. Changes of volatile compounds in the fish sauce and in a model solution containing volatile compounds by the treatment with the bacterial strain R4Nu, for 24 days and for 7 h, respectively

	Bacteria-treated fish sauce	Bacteria-treated volatile compounds solution	Origine of compounds	References
2-Methylpropanal	±	−	Valine	Figure II-6
2-Methylbutanal	±	−	Isoleucine	Figure II-4
2-Pentanone	±	±	?	
2-Ethylpyridine	−	−	Phenylalanine ?	a
Dimethyl trisulfide	−	±	Methionine ?	b,c
3-(Methylthio)propanal	±	−	Methionine ?	d
3-Methylbutanoic acid	+	±	Leucine	Figure II-5
Dimethyl disulfide	−	−	Methionine ?	b,c,e
3-Methyl-1-butanol	++		Leucine	Figure II-5
2,6-Dimethylpyrazine	++		?	
Butanoic acid	−	±	Fatty acids	f

a, Hidalgo and Zamora (2004); b, Yu and Ho (1995); c, Nedjma and Hoffmann (1996); d, Mandin*et al.* (1999); e, Casey *et al.* (1965); f, Beddow *et al.* (1980).

Symbols for increase and decrease of volatile compounds in bacteria-treated fish sauce and in the model solution in comparison with those of non-treated counterparts: ++, very increase; +, increase; ±, no change; −, decrease.

Table III-1. Characteristics of strains R5G and R4Nu isolated from fish sauce mash (moromi) made from frigate mackerel

Characteristics	R5G	R4Nu
Gram stain	+	+
Form	cocci	cocci
Endospore	-	-
Motility	-	-
Colony color	milky white	light yellow
Anaerobic fermentation of glucose	+	+
Catalase activity	+	+
Oxidase activity	-	-
Hydrolysis of		
Urea	+	+
L-Arginine-monohydrochloride	-	-
L-Ornithine-monohydrochloride	-	-
Nitrate reduction	+	+
Acetoin production	-	-
β-Galactosidase activity	+	+
Arginine arylamidase activity	-	-
Alkaline phosphatase activity	+	+
Pyrrolidonyl arylamidase activity	+	+
Resistancy to novobiocin	+	+
β-Glucronidase activity	+	+
Acid produced aerobically from:		
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
D-Maltose	+	+
Lactose	+	+
Trehalose	+	+
D-Mannitol	+	+
Raffinose	-	-
Sucrose	+	+
N-Acetylglucosamine	+	+
D-Turanose	+	+
L-Arabinose	+	+
D-Ribose	+	+
D-Cellobiose	-	-
Growth in NaCl		
18%	+	+
20%	+	+
22%	+	+
Esculin hydrolysis	+	+

Symbols: +, positive; -, negative.

Table III-2. List of accession numbers in the GenBank/EMBL/DDBJ databases of 16S rRNA and *rpoB* from *Staphylococcus* species

No.	Species or subspecies	Strain	Accesion no.	
			16S rRNA	rpoB
1	*S. arlettae*	ATCC 43957 ^{T}	AB009933	AF325874
2	*S. aureus* subsp. *anaerobius*	ATCC 35844 ^{T}	D83355	AF325894
3	*S. aureus* subsp. *aureus*	ATCC 12600 ^{T}	D83357	X64172
4	*S. auricularis*	MAFF 911484 ^{T}	D83358	AF325889
5	*S. capitis* subsp. *capitis*	ATCC 27840 ^{T}	L37599	AF325885
6	*S. capitis* subsp. *urealyticus*	ATCC 49326 ^{T}	AB009937	
7	*S. caprae*	ATCC 35538 ^{T}	AB009935	AF325868
8	*S. carnosus*	ATCC 51365 ^{T}	AB009934	
9	*S. caseolyticus*	MAFF 911387 ^{T}	D83359	
10	*S. chromogenes*	MAFF 911474 ^{T}	D83360	AF325892
11	*S. cohnii* subsp. *cohnii*	MAFF 911487 ^{T}	D83361	AF325893
12	*S. cohnii* subsp. *urealyticus*	ATCC 49330 ^{T}	AB009936	
13	*S. condimenti*	DSM 11674 ^{T}	Y15750	
14	*S. delphini*	ATCC 49171 ^{T}	AB009938	
15	*S. epidermidis*	ATCC 14990 ^{T}	D83363	AF325872
16	*S. equorum*	ATCC 43958 ^{T}	AB009939	AF325882
17	*S. felis*	ATCC 49168 ^{T}	D83364	AF325878
18	*S. gallinarum*	ATCC 35539 ^{T}	D83366	AF325890
19	*S. haemolyticus*	MAFF 911476 ^{T}	D83367	AF325888
20	*S. hominis*	DSM 20328 ^{T}	X66101	AF325875
21	*S. hyicus*	ATCC 11249 ^{T}	D83368	AF325876
22	*S. intermedius*	MAFF 911388 ^{T}	D83369	AF325869
23	*S. kloosii*	ATCC 43959 ^{T}	AB009940	AF325891
24	*S. lentus*	MAFF 911385 ^{T}	D83370	AY036973
25	*S. linens*	DSM 15097 ^{T}	AF527483	
26	*S. lugdunensis*	ATCC 43809 ^{T}	AB009941	AF325870
27	*S. lutrae*	DSM 10244 ^{T}	X84731	
28	*S. muscae*	CCM 4175 ^{T}	S83566	AF325884
29	*S. nepalensis*	CW-1 ^{T}	AJ517414	
30	*S. pasteuri*	ATCC 51129 ^{T}	AB009944	
31	*S. piscifermentans*	ATCC 51136 ^{T}	AB009943	
32	*S. pulvereri*	ATCC 51698 ^{T}	AB009942	AF325879
33	*S. saccharolyticus*	ATCC 14953 ^{T}	L37602	AF325871
34	*S. saprophyticus*	MAFF 911473 ^{T}	D83371	AF325873
35	*S. schleiferi* subsp. *coagulans*	ATCC 49545 ^{T}	AB009945	
36	*S. schleiferi* subsp. *schleiferi*	DSM 4807 ^{T}	S83568	AF325886
37	*S. sciuri*	ATCC 29062 ^{T}	S83569	AF325881
38	*S. sciuri* subsp. *sciuri*	DSM 20345 ^{T}	AJ421446	
39	*S. simulans*	MAFF 910161 ^{T}	D83373	AF325877
40	*S. succinus* subsp. *casei*	DSM 15096	AJ320272	
41	*S. succinus* subsp. *succinus*	DSM 14617 ^{T}	AF004219	
42	*S. vitulinus*	ATCC 51145 ^{T}	AB009946	
43	*S. warneri*	ATCC 27836 ^{T}	L37603	AF325887
44	*S. xylosus*	MAFF 911482 ^{T}	D83374	AF325883

Table III-3. DNA-DNA hybridization values of *Staphylococcus* strains R4Nu and R5G in comparison with their related species

Species	Strain	Percentage similarity	
		R4Nu	CW-1^T
Staphylococcus sp.	R4Nu	100	95
Staphylococcus sp.	R5G	91	97
Staphylococcus nepalensis	CW-1^T	89	100
Staphylococcus xylosus	JCM2418^T	31	31

Table III-4. PCR assay for *Staphylococcus nepalensis* in fish sauce made in Asian countries and malt for soy sauce of Japan

No.	Fish sauce and malt	Producing country	Colony number	PCR amplification
1	Fish sauce	V	3	-
2	Fish sauce	V	1	-
3	Fish sauce	V	1	-
4	Fish sauce	V	0	
5	Fish sauce	V	0	
6	Fish sauce	V	1	-
7	Fish sauce	T	0	
8	Fish sauce	T	1	-
9	Fish sauce	T	2	-
10	Fish sauce	T	0	
11	Fish sauce	T	0	
12	Fish sauce	T	0	
13	Fish sauce	T	1	-
14	Fish sauce	T	0	
15	Fish sauce	T	0	
16	Fish sauce	T	1	-
17	Fish sauce	P	0	
18	Fish sauce	J	8	-
19	Fish sauce	J	3	-
20	Malt	J	15	+

Symbols: V, Vietnam; T, Thailand; P, Philippine; J, Japan; +, positive; -, negative. The malt sample was made from fermented soy beans and barley with *Aspergillus oryze* in Toyama Prefecture, Japan. Colonies were isolated from the NA plates containing 18% NaCl at 30 °C incubated for 2 - 3 days as described in Materials and methods.

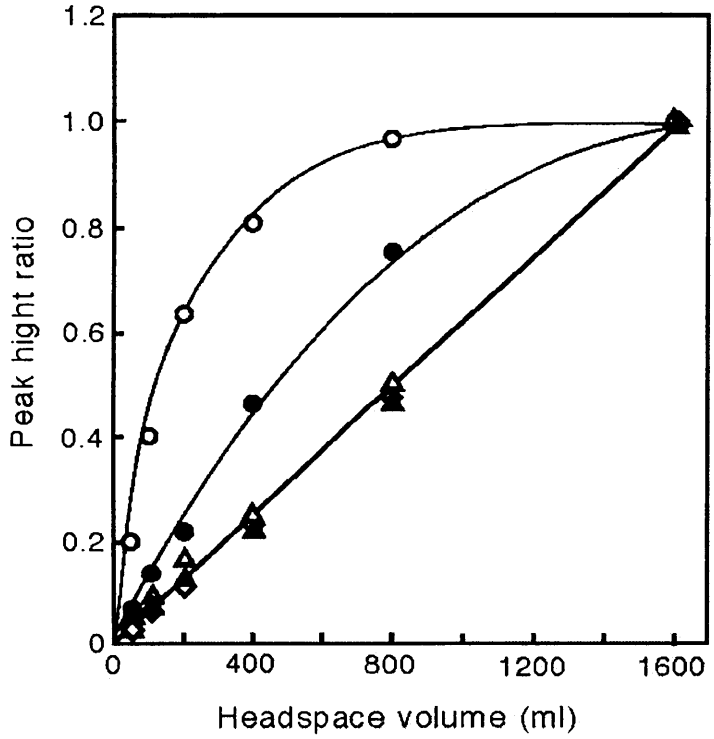


Figure I-1. Relationships between the peak area and purged gas volumes (internal standard =1.0)

○, 2-Methylpropanal (2); ●, 2-methylbutanal (5); ▲, 2-pentanone (7); △, dimethyltrisulfide (26); ◇, 3-(methylthio)propanal (31). The peak number corresponds to that in Table I-1.

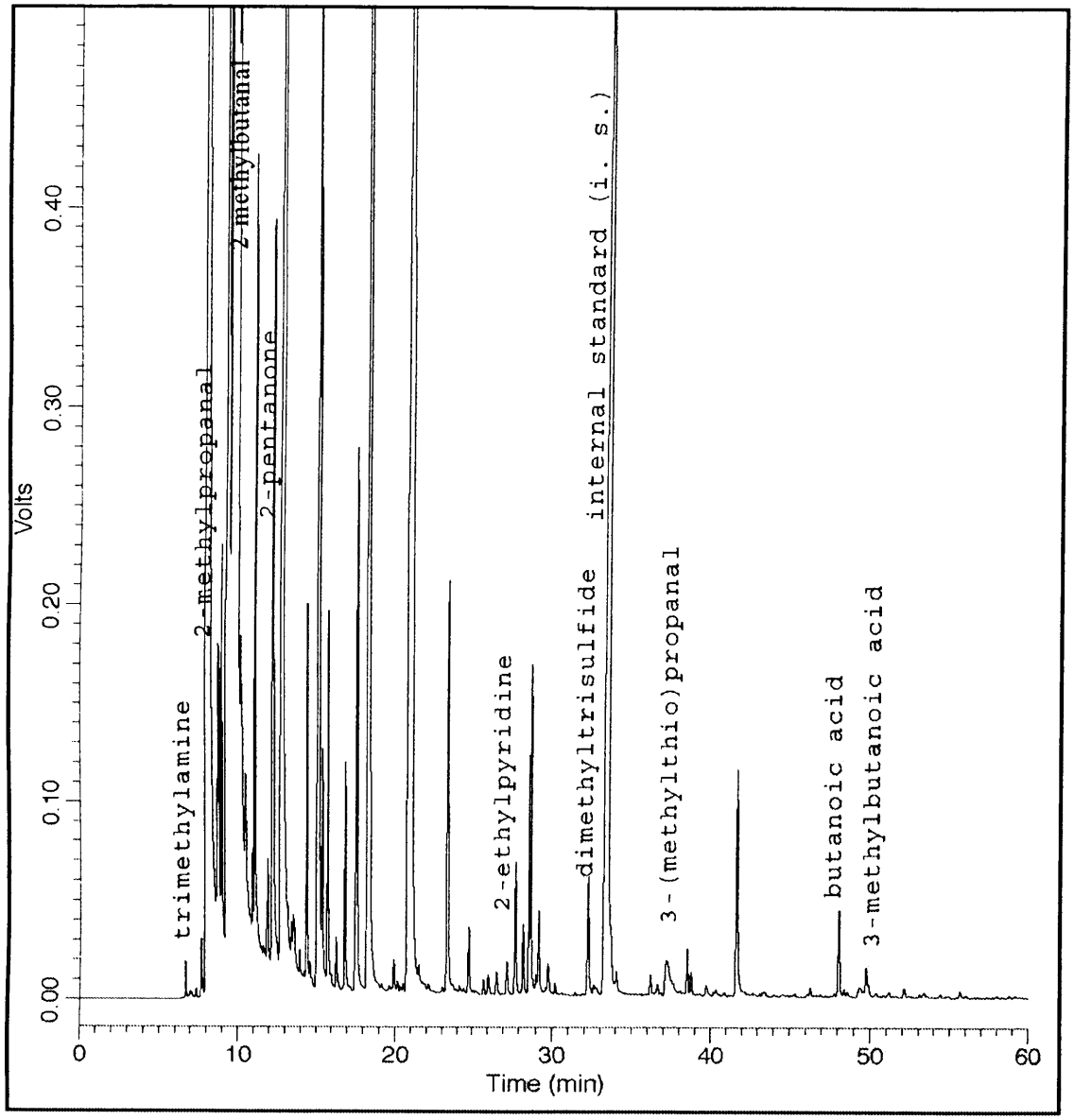


Figure I-2. Gas chromatogram of headspace volatile compounds of fish sauce.

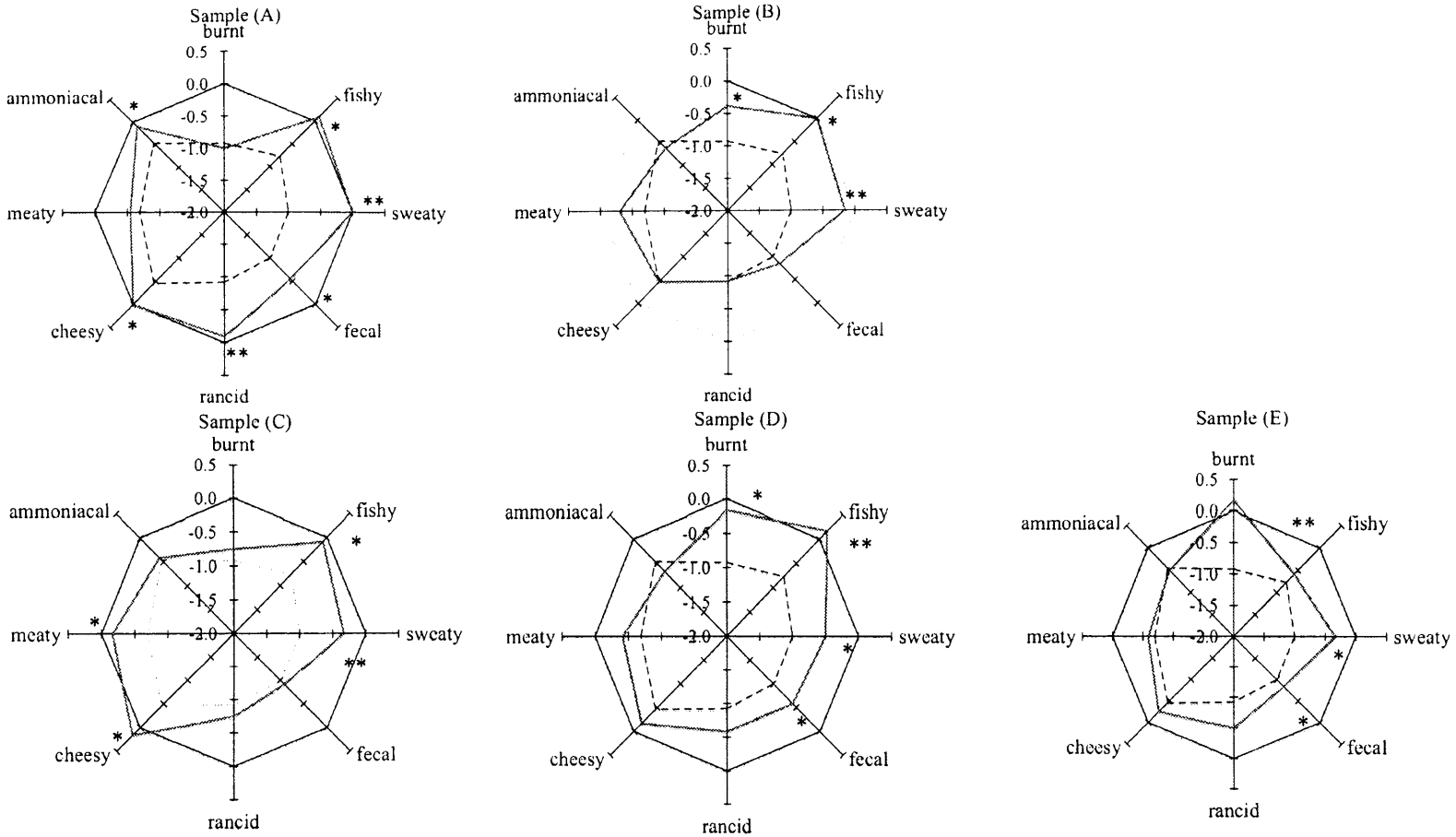


Figure I-3. QDA of fish sauce odor: (—) non-treated; (---) deodorized; (▬) addition samples. Addition samples were prepared with deodorized fish sauce to which four odorants, 2-methylpropanal (370.7 ng/mL), 2-methylbutanal (38.5 ng/mL), 2-ethylpyridine (1.4 ng/mL), and dimethyl trisulfide (7.5 ng/mL), were added: sample A, all four volatiles added (2-methylpropanal, 2-methylbutanal, dimethyl trisulfide and 2-ethylpyridine); sample B, three volatiles added (2-methylpropanal, 2-methylbutanal, and dimethyl trisulfide); sample C, three volatiles added (2-methylpropanal, 2-methylbutanal, and 2-ethylpyridine); sample D, two volatiles added (2-ethylpyridine and dimethyl trisulfide); sample E, two volatiles added (2-methylpropanal and 2-methylbutanal). Significance between deodorized and addition samples was assessed by Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$.

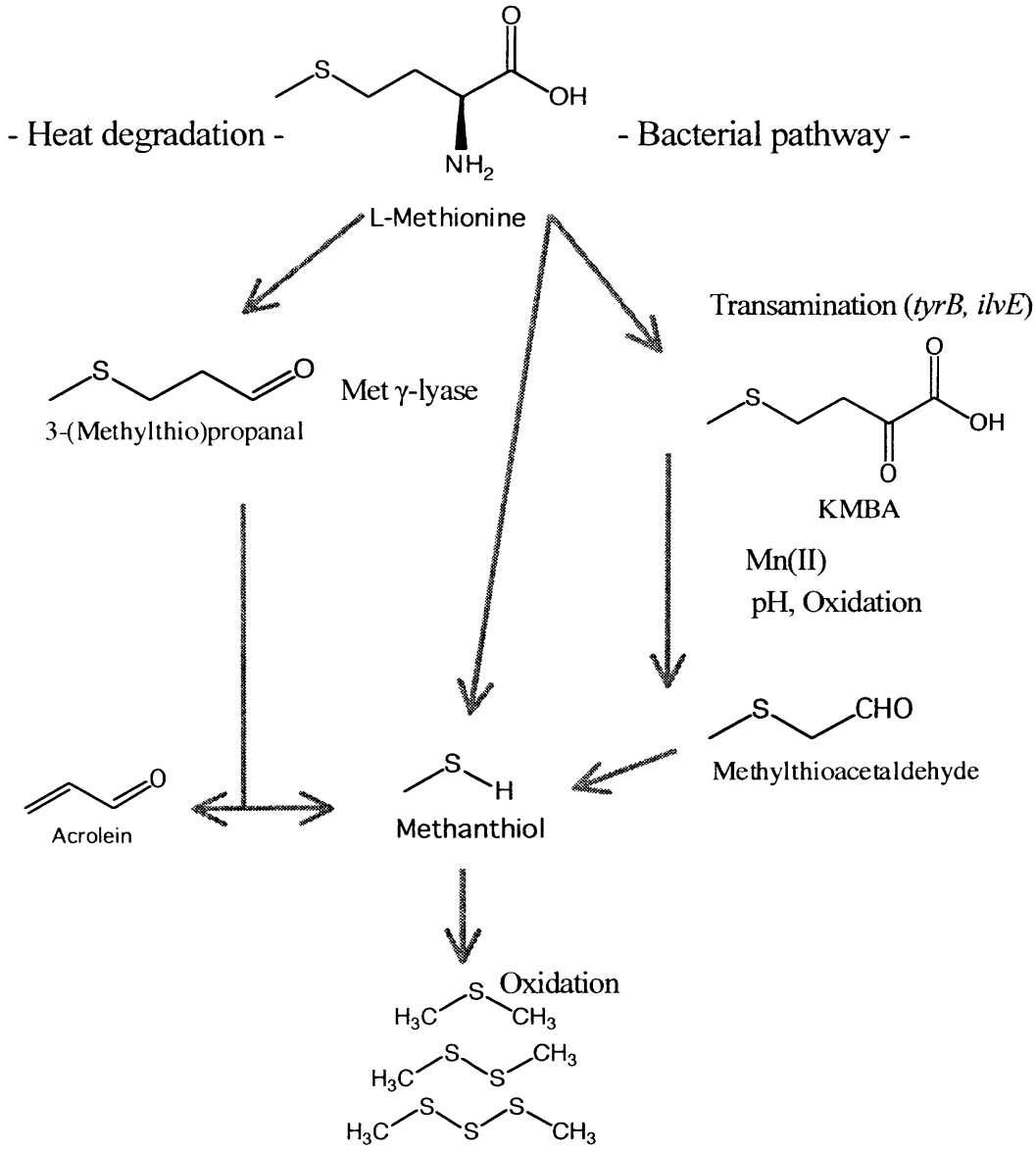
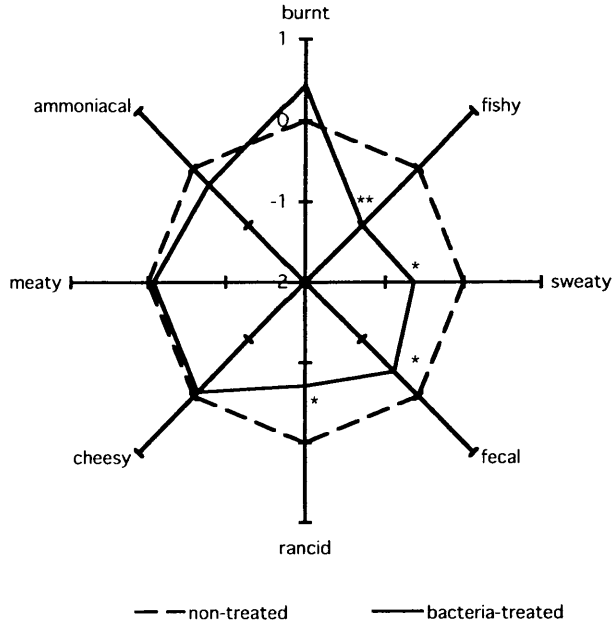


Figure I-4. Proposed pathway for the formation of volatile sulfur compounds (VSCs) in fish sauce. KMBA: α-keto-γ-methylthiobutyrate; VSCs, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS); *tyrB*, aromatic aminotransferase; *ilvE*, transaminase B; Met γ-lyase, methionine γ-lyase.

In mouth



By sniffing

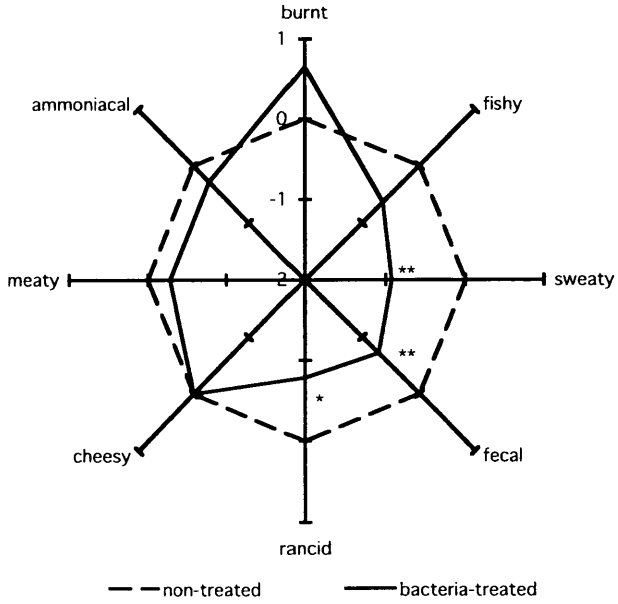


Figure II-1. QDA of the odor in the bacteria-treated (—) and non-treated (---) fish sauces.

The bacteria-treated fish sauce was prepared as follows: 100 mL culture of strain R4Nu was added to 550 mL of fish sauce and incubated at 32°C for 24 days with gentle stirring. The bacteria-treated fish sauce was passed through 0.45 μm filter before sensory evaluation. Two types of sensory evaluation were carried out, one in mouth and the other by sniffing. The significance between the bacteria-treated and non-treated fish sauces was analyzed by Student's *t* -test: * $p< 0.05$, ** $p< 0.01$.

(A) 2-Methylpropanal
(B) 2-Methylbutanal
(C) 2-Ethylpyridine
(D) 2-Pentanone
(E) Dimethyl trisulfide
(F) 3(Methylthio)propanal
(G) 3-Methylbutanoic acid
(H) Dimethyl disulfide
(I) Butanoic acid

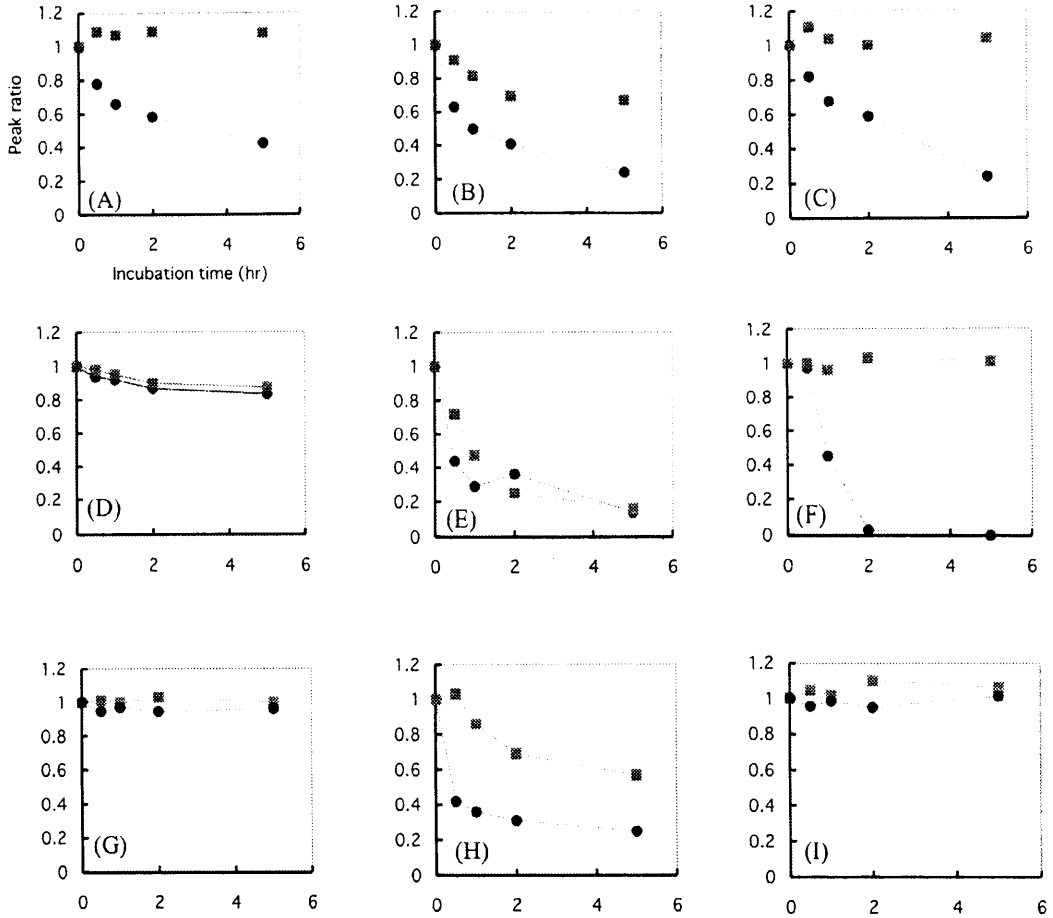


Figure II-2. Catabolism in a model solution for volatile compounds responsible for the distinctive odor of fish sauce.

▨, Addition of inactivated strain R4Nu; ◎, addition of strain R4Nu. The reaction mixture containing 35 mL of 22% (w/v) NaCl plus 0.05M phosphate buffer (pH 5.4), 350 μL diluted volatile compounds mixture and 1 mL of bacterial solution or 1 mL of heat inactivated bacterial solution as a negative control, was incubated at 32 °C with gentle stirring. Five mL of the reaction mixture was filtered through a 0.25 μm membrane. Volatile compounds extracted by SPME was measured by GC (see Materials and methods).

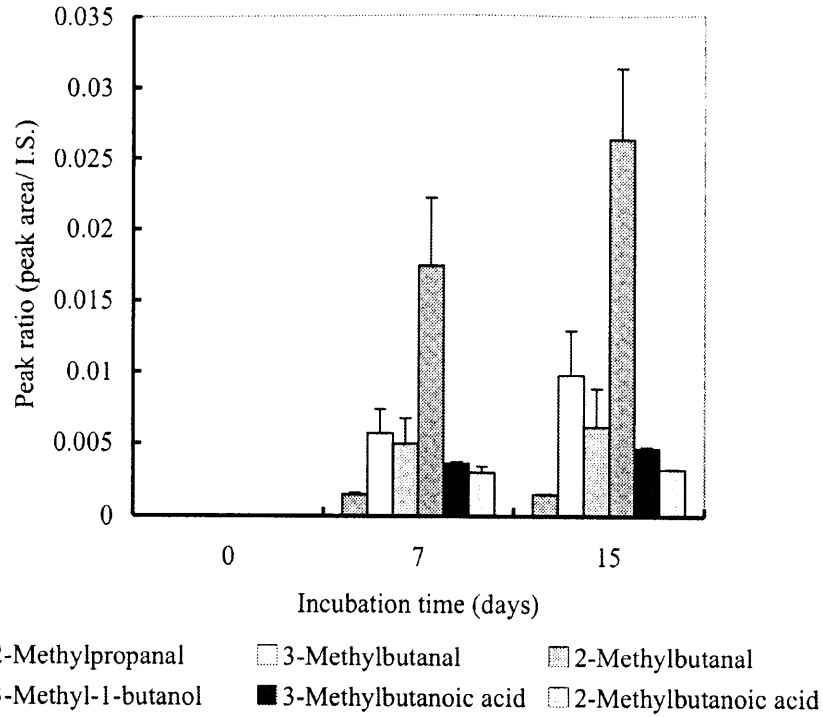


Figure II-3. Production of volatile compounds from a synthetic fish sauce. The synthetic fish sauce containing various amino acids at the same concentrations as found in fish sauce was prepared in 0.05M sodium phosphate (pH 5.4) containing 22% NaCl and passed through a 0.25 μm membrane filter for sterilization. To 100 mL of the synthetic fish sauce was added 2 ml bacterial solution and the mixture was incubated at 32 °C with gentle stirring. Volatile compounds extracted by SPME were measured by GC-MS.

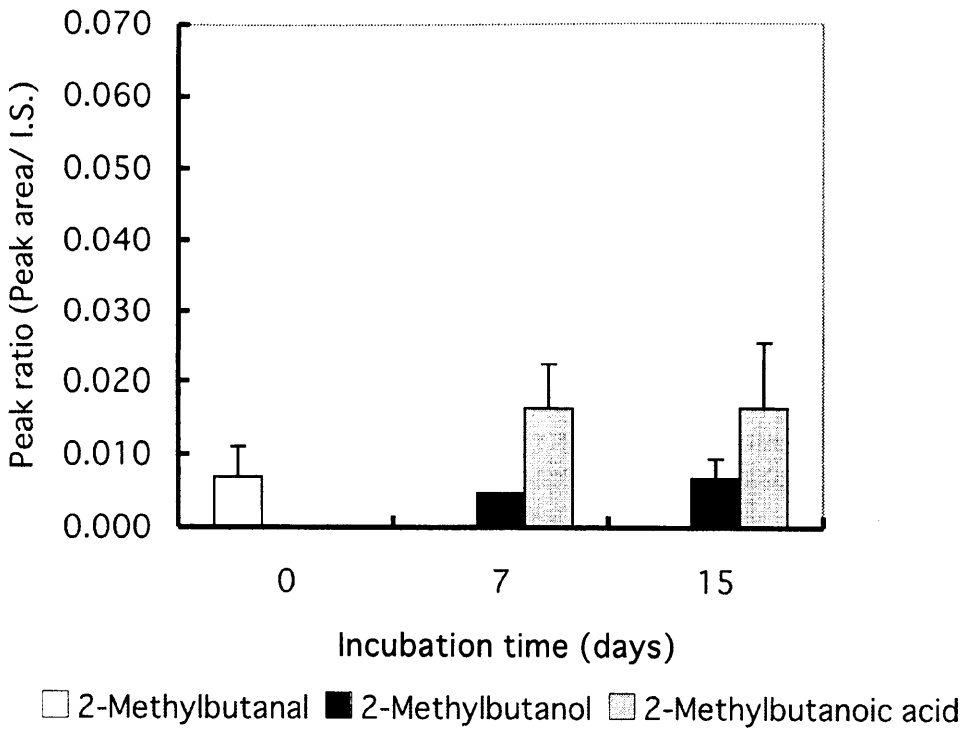


Figure II-4. Production of volatile compounds from isoleucine. Isoleucine was dissolved in 0.05M sodium phosphate (pH 5.4) containing 22% NaCl and passed through a 0.25 μm membrane filter for sterilization. To 100 mL containing 300mg isoleucine was added 2 ml bacterial solution and the mixture was incubated at 32 °C with gentle stirring. Volatile compounds extracted by SPME were measured by GC-MS.

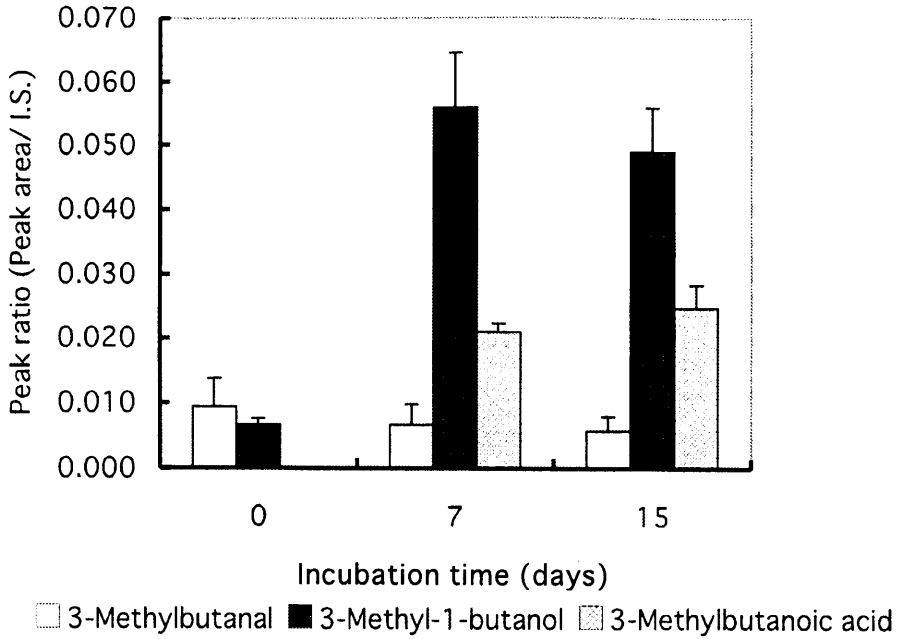


Figure II-5. Production of volatile compounds from leucine. Leucine was dissolved in 0.05M sodium phosphate (pH 5.4) containing 22% NaCl and passed through a 0.25 μm membrane filter for sterilization. To 100 mL containing 300mg leucine was added 2 ml bacterial solution and the mixture was incubated at 32 °C with gentle stirring. Volatile compounds extracted by SPME were measured by GC-MS.

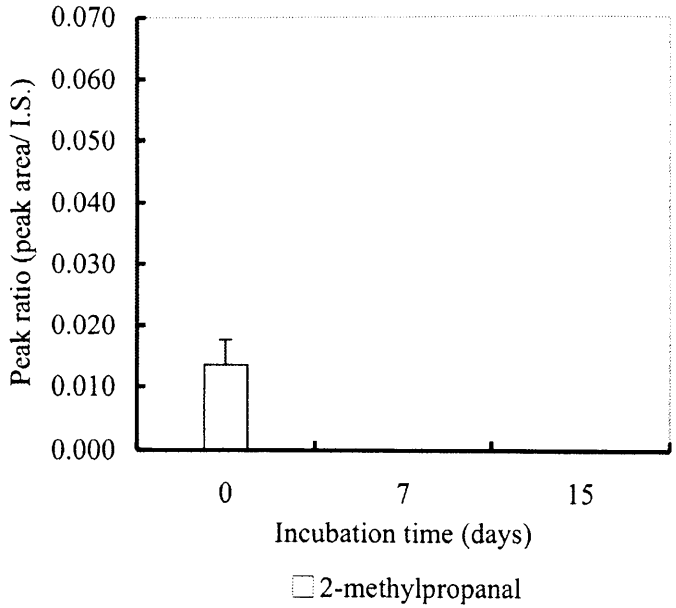


Figure II-6. Production of volatile compounds from valine. Valine was dissolved in 0.05M sodium phosphate (pH 5.4) containing 22% NaCl and passed through a 0.25 μm membrane filter for sterilization. To 100 mL containing 500mg valine was added 2 ml bacterial solution and the mixture was incubated at 32 °C with gentle stirring. Volatile compounds extracted by SPME were measured by GC-MS.

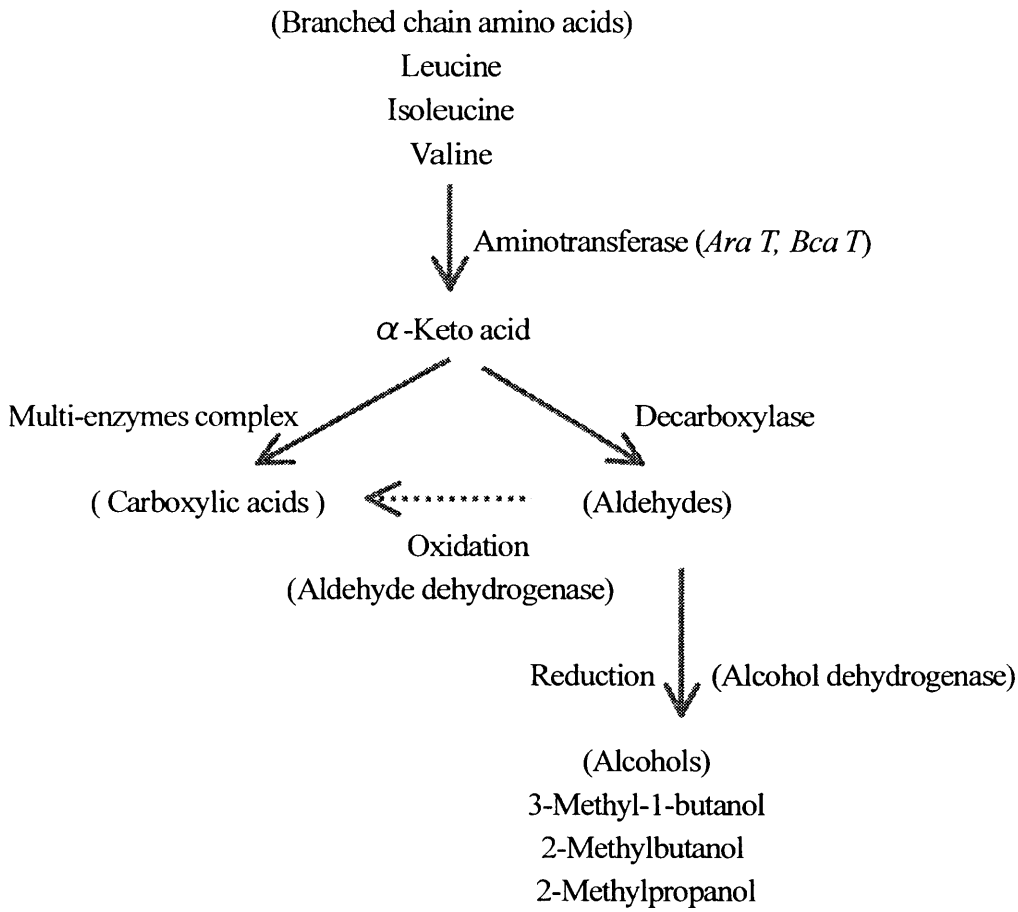


Figure II-7. Proposed pathway for the formation of volatile compounds from branched chain amino acids. *AraT*, aromatic aminotransferase; *BacT*, branched chain aminotransferase.

A

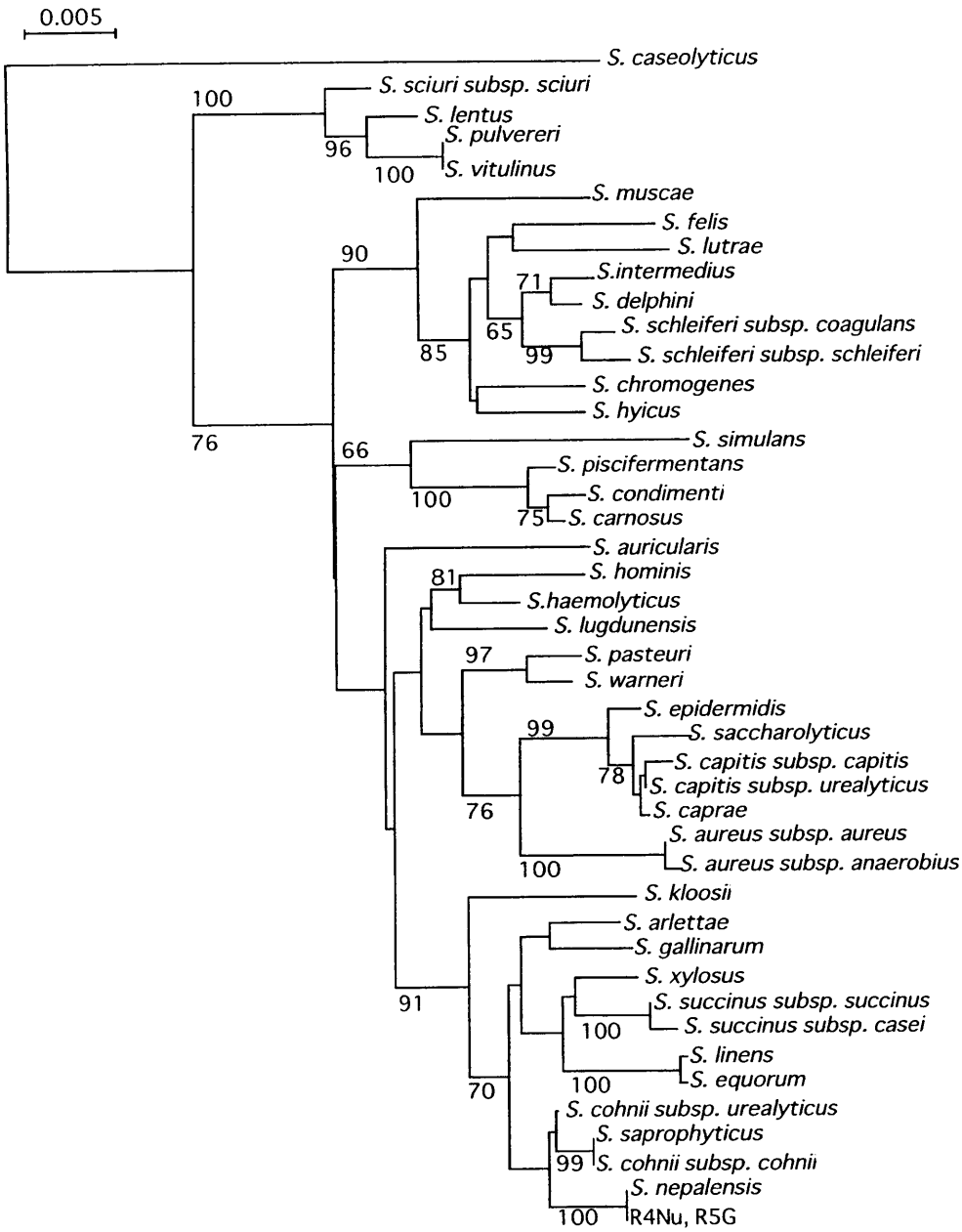


Figure III-1. The phylogenetic trees based on 16S rRNA. The tree was constructed by the neighbor-joining method and the scale bar indicating 0.005 unit of genetic distances for 16r RNA, was computed by the Kimura's two parameter method. The numbers in the tree indicate bootstrap values in 1,000 replication and only values more than 60 % are shown.

B

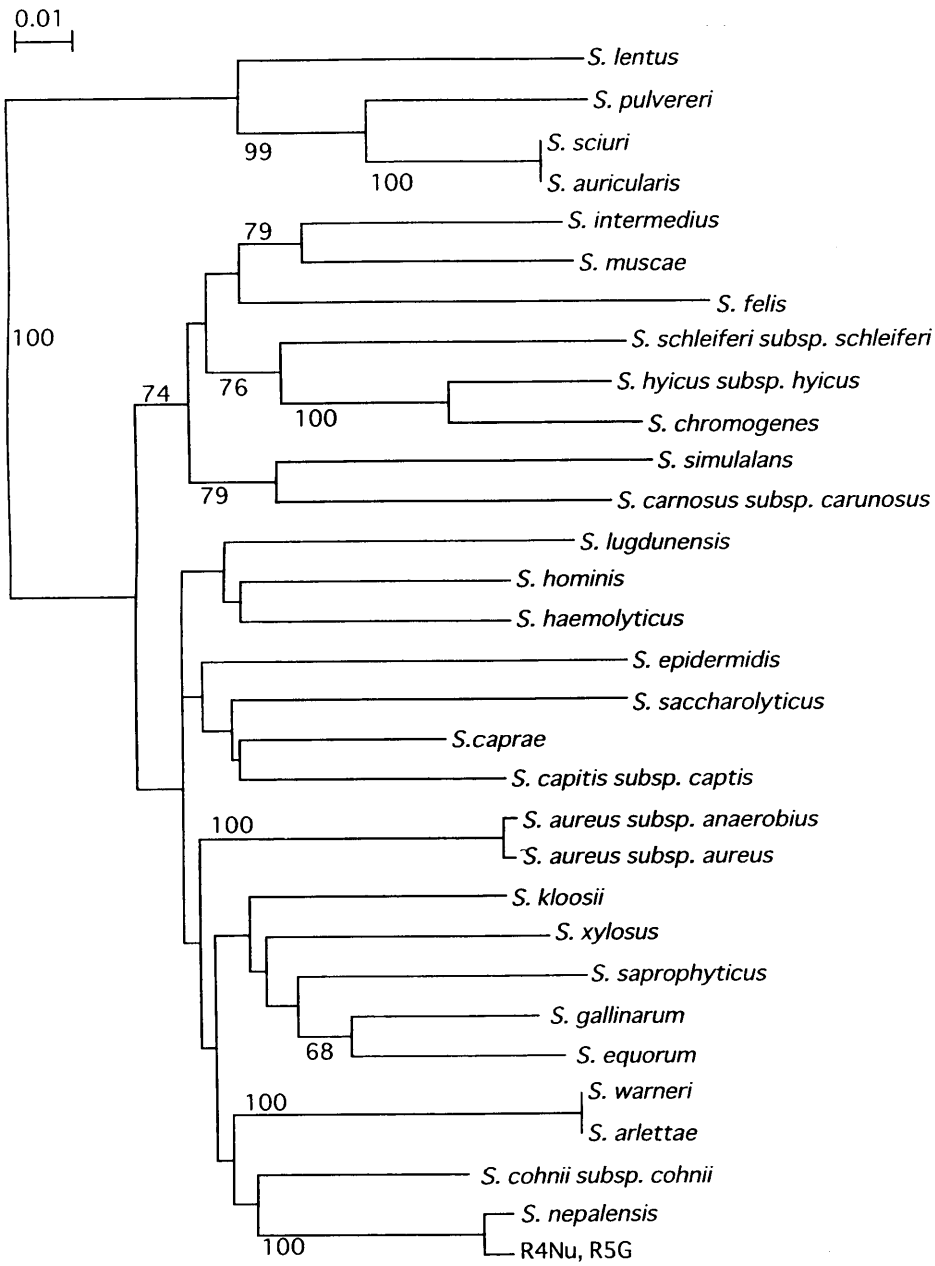


Figure III-2. The phylogenetic tree based on *rpoB*. The tree was constructed by the neighbor-joining method and the scale bars indicating 0.01 unit of genetic distances for *rpoB*, was computed by the Kimura's two parameter method. The numbers in the tree indicate bootstrap values in 1,000 replication and only values more than 60 % are shown.

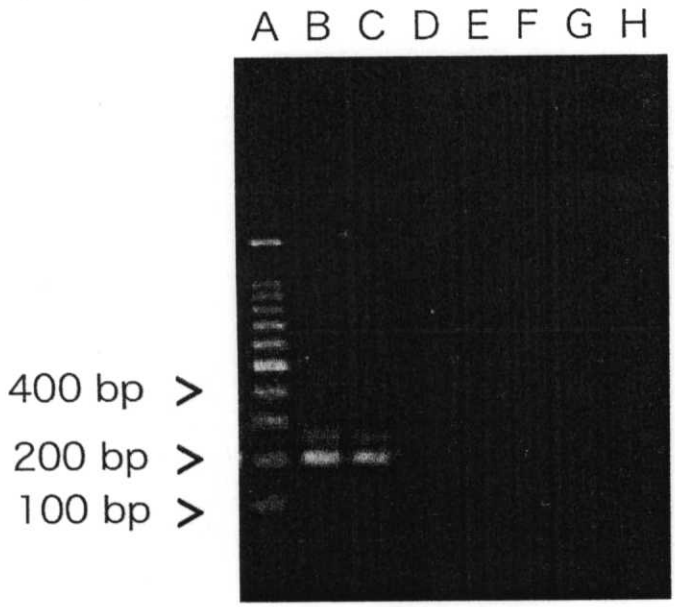


Figure III-3. Specificity of the PCR primer set for detection of *Staphylococcus nepalensis*. A, 100 bp ladder; B, strain R4Nu; C, *S. nepalensis* CW-1^{T}; D, *S. cohnii* subsp. *cohnii* JCM2417^{T}; E, *S. saprophyticus* JCM2427^{T}; F, *S. xylosus* JCM2418^{T}; G, *S. epidermidis* JCM2414^{T}; H, *S. haemolyticus* JCM2416^{T}. Numbers in the left indicate the molecular weights of standard DNA fragments.