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# Branched chain amino acids as source of specific branched chain volatile fatty acids during the fermentation process of fish sauce*

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**Summary.** The source of the formation of branched chain volatile fatty acids (VFA) in fish sauce was investigated. Certain branched VFA were derived from the degradation of specific amino acids as *iso*-butyric acid from valine and *iso*-valeric acid from leucine. Short and long straight chain VFA were significantly higher in the linoleic acid added sample than in the control but did not significantly bring changes to the branched chain VFA. It is suggested that straight chain VFA developed from fish fats. Alanine and isoleucine did not have a clear influence on the production of volatile fatty acids.

**Keywords:** Fish sauce – Volatile fatty acids – Straight chain volatile fatty acids – Branched chain volatile fatty acids – Aerobic fermentation – Anaerobic fementation – Microbial activity

## 1. Introduction

Fish sauce is a clear brown liquid, obtained as hydrolysis product of salted fish after a year of salting. It is commonly used as a condiment in Southeast Asia and an amino acid source of certain social classes in the region. Fish sauces contain 20 g/L of nitrogen, of which 16 g/L are in the form of amino acids (Lafont, 1955 and Sanceda et al., 1990).

Fish sauce has a characteristic aroma which often serves as an indicator to measure the quality of fish sauce, since the very salty taste tends to overpower the other flavor constituents. A number of reports revealed that volatile acids were the most abundant group of volatile compounds in fish sauce (Truong Van–Chom, 1952 and Saisithi et al., 1966). Patis, nuocmam, nampla and shottsuru contained $C_2$ to $C_{10}$ straight and branched-chain volatile acids (Sanceda et al., 1983, 1984, 1986; Dougan and Howard, 1975 and Beddows et al., 1979).

Several conflicting reports on the formation and development of these compounds have been made. For instance, Nguyen -An-Cu and Vialard-Goudou (1953) identified acetic and *n*-butyric acids and suggested that lactic acid bacteria could be involved. The findings of Dougan and Howard (1975) on the determination of individual volatile fatty acids showed that appreciable amounts of straight chain acids were more likely to have been formed by atmospheric oxidation of fish lipids. However, Beddows et al. (1980) reported that it seems unlikely that acetic and *n*-butyric acids could be derived from oxidation of lipid in the manufacture of fish sauce since the quantity of lipid present in the fish was insufficient to account for the amount of volatile fatty acids (VFA). It was found that when fresh fish was mixed with salt and fermented, (no spoilage prior to salting), very little VFA were formed. Saisithi et al. (1966) and Beddows et al. (1980) isolated bacterial species that were able to produce VFA when inoculated on hydrolyzed rockfish (*Seabastodes species or Stolephorus species*). Propionic, *n*-butanoic, and *n*-pentanoic acids appeared to be derived from amino acids via bacterial actions using U-$^{14}$C labeled protein hydrolysates. An obvious difference in the volatile fatty acids profile in fish sauces fermented in the presence (aerobic) and absence of oxygen (anaerobic fermentation) was reported (Sanceda et al., 1992).

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Zarling and Ruchim (1987) reported that *iso*-butyrate was produced from albumin and valine, and *iso*-valerate was produced from albumin and leucine. The dissimilation of leucine, isoleucine and valine to volatile fatty acids was determined in *Fasciola hepatica* and the degradation of branched amino acids to the volatile fatty acids end products was demonstrated. *Fasciola hepatica* was found to metabolize leucine, isoleucine and valine to *iso*-valeric, 2-methylbutyric and *iso*-butyric acids, respectively (Lahoud et al., 1971). Moreover, Britz and Wilkinson (1982) reported that leucine was dissimilated to *iso*-valerate and *iso*-caproic acids by cell suspensions of amino acid fermenting anaerobes, by the "Stickland reaction". It was reported that conversion of amino acid into aroma compounds occur by cell-free extracts Lactobacillus helviticus. A mix branched chain amino acids such as leucine, isoleucine and valine and other aromatic amino acids were transaminated into the corresponding keto acids when amino group acceptor was provided (Klein et al., 2001).

The objective of this study was to investigate the source of branched chain volatile fatty acids during the fermentation process in the manufacture of fish sauce.

## 2. Materials and methods

### *2.1. Materials*

About 13 cm long "maiwashi", a sardine family (Sardinops melanostictus) purchased in the Japanese fish market were used. Another sample, a one year fermented fish sauce partially filtered given by the Lorenzana manufacturer (fish sauce brand in the Philippines) was also used. All the amino acids used were purchased from Waco Chemical Ind. Ltd., Osaka, Japan.

### *2.2. Methods*

#### 2.2.1. Use of basic and branched chain amino acids

Two kinds of experiments using specific basic and branched chain amino acids were carried out.

In one experiment, two sets of experimental preparations were made; one was mixing fish and salt only (control group) and the other was mixing fish and salt with amino acids; alanine, isoleucine, leucine and valine in their original form individually added to the fish mixture before incubation. Each group was prepared, in the absence of air (anaerobic) and the other in the presence of air (aerobic). In the anaerobic preparation, the mixture was placed in layers in air tight glass flasks with weights (the same weight as the salt-fish mixture) placed on top. The container was first loosely closed and nitrogen gas was purged into the container at the rate of 200 ml/min for about one hour to remove air and the container was sealed with two glass stopcocks inserted to a silicone stopper. In the aerobic fermentation, the mixture was placed in layers using similar type of container, weights placed on top and loosely closed. Both containers were kept in an incubator at about 31°C ± 1°C.

#### 2.2.2. Collection of liquid

After a spcific period of fermentation, the solid particles remaining in the mixture was separated from the liquid by first using cheese cloth after which the liquid was again filtered using filter paper. The liquid (fish sauce) was then used for the experiment.

#### 2.2.3. Model experiment with fish sauce as substrate

In this experiment, a crude fish sauce (unprocessed partially filtered fish sauce), fermented for one obtained from one commercial fish sauce producer in the Philippines, was added with the same kind and concentration of amino acids as in the first experiment and the mixtures were aerobically and anaerobically incubated at 31 ± 1°C for 72 and 96 h.

#### 2.2.4. Use of long chain fatty acid

Two kinds of mixtures were prepared, salt-fish mixture (control) and linoleic acid added salt-fish mixture. 2% linoleic acid was added to salt and fish. The mixture was placed in layers, stone of equal weight placed on the top of the mixture and the container was loosely covered. The container was kept in an incubator at about 31°C ± 1°C for 48 hours, 1 week and 1 month after which the liquids were collected as in 2.2.2.

#### 2.2.5. Collection of volatile acids

The volatile acids from the liquid in all the experiments mentioned above were collected using a steam-distillation under reduced pressure method (SDRP). The distillation process was carried out at 40–45°C for 4 h. The distillation flask was chilled throughout the process. The distillates were acidified with 5% HCl, saturated with NaCl and extracted with ether. Anhydrous sodium sulfate was added to the extracts which were then allowed to stand overnight before concentration to 0.5 ml. Concentration was done in the usual manner at 35–40°C.

#### 2.2.6. Gas chromatographic conditions (GC)

Both samples were subjected to gas chromatography analysis. Gas chromatography was accomplished using a Shimadzu 9A model gas chromatograph equipped with a flame ionization detector. Separation of volatile compounds was done on a 0.25 mm i. × 30 m fused silica column coated with CP WAX 52CB. The column temperature was programmed from 60°C held for 4 min, increased to 180°C at a rate of 2°C/min. The injection port was kept at 230°C. Carrier gas was nitrogen with a flow rate of 1.0 mL/min and a split ratio of 1 : 40. A multifunction data processor (C-R6A Chromatopac, Shimadzu, Kyoto, Japan), connected to the GC, was used for a relative quantitative calculation. For a complete identification of the volatile acids, co-chromatography using authentic compounds was employed.

#### 2.2.7. Statistical analysis

Test of significance for the analytical study was done using a Students' t-Test.

## 3. Results and discussion

### *Use of basic and branched chain amino acids*

To clarify the source of branched chain volatile acids, specific branched amino acids were used.

**Table 1.** Volatile acids in fish sauces added with amino acids aerobically fermented for one and a half months

| Volatile acids | Amino acids added | | | | |
|---|---|---|---|---|---|
| | Control | Alanine | Valine | *iso*-Leucine | Leucine |
| | Mean ± S.E. (%) | | | | |
| Acetic | 4.02 ± 0.03 | 7.13 ± 0.02 | 5.21 ± 0.02[c] | 5.17 ± 0.03 | 6.11 ± 0.03[c] |
| Propionic | 9.58 ± 0.05 | 9.42 ± 0.03 | 10.02 ± 0.03 | 10.12 ± 0.05 | 10.42 ± 0.03 |
| Isobutyric | 0.20 ± 0.01 | 0.21 ± 0.06 | 1.39 ± 0.03[b] | 0.22 ± 0.07 | 0.32 ± 0.05 |
| *n*-Butyric | 18.06 ± 0.12 | 18.18 ± 0.02 | 19.06 ± 0.10 | 18.16 ± 0.02 | 18.26 ± 0.02 |
| Isovaleric | 0.15 ± 0.02 | 0.13 ± 0.06 | 0.16 ± 0.05 | 0.14 ± 0.02 | 2.74 ± 0.02[a] |
| *n*-Valeric | 0.27 ± 0.03 | 0.27 ± 0.01 | 0.31 ± 0.04 | 0.26 ± 0.01 | 0.25 ± 0.01 |
| Isohexanoic | 0.07 ± 0.03 | 0.04 ± 0.01 | 19.81 ± 0.01[a] | 0.07 ± 0.01 | 0.07 ± 0.01 |

Salt: 30%
Amino acids: 2% of the salt, they were mixed with salt before mixing with fish
[a] Values in the same line are significantly different at $p < 0.001$ from the control
[b] Values in the same line are significantly different at 0.01 from the control
[c] Values in the same line are significantly different at $p < 0.05$ from the control

**Table 2.** Volatile acids in fish sauces added with amino acids and anaerobically fermented for one and a half months

| Volatile acids | Amino acids | | | | | |
|---|---|---|---|---|---|---|
| | Aerobic control | Anaerobic control | Alanine | Valine | *iso*-Leucine | Leucine |
| | Mean ± S.E. (%)[a] | | | | | |
| Acetic | 4.02 ± 0.03 | 1.32 ± 0.02 | 1.13 ± 0.02 | 1.30 ± 0.03 | 1.30 ± 0.01 | 1.31 ± 0.02 |
| Propionic | 9.58 ± 0.05 | 1.58 ± 0.02 | 1.46 ± 0.02 | 1.55 ± 0.04 | 1.56 ± 0.02 | 1.57 ± 0.02 |
| Isobutyric | 0.20 ± 0.01 | 0.40 ± 0.03 | 0.39 ± 0.03 | 0.82 ± 0.02[c] | 0.41 ± 0.01 | 0.36 ± 0.02 |
| *n*-Butyric | 18.06 ± 0.12 | 5.06 ± 0.07 | 5.10 ± 0.03 | 5.16 ± 0.05 | 5.14 ± 0.02 | 5.09 ± 0.04 |
| Isovaleric | 0.15 ± 0.05 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.04 ± 0.02 | 0.05 ± 0.02 | 1.01 ± 0.01[b] |
| *n*-Valeric | 0.27 ± 0.03 | 0.07 ± 0.01 | 0.09 ± 0.02 | 0.07 ± 0.02 | 0.06 ± 0.01 | 0.07 ± 0.01 |
| Isohexanoic | 0.07 ± 0.03 | 0.07 ± 0.02 | 0.07 ± 0.01 | 0.57 ± 0.06 | 0.06 ± 0.02 | 0.07 ± 0.02 |

Salt: 30%
Amino acids: 2% of the salt, they were mixed with salt before mixing with fish
[a] Values are expressed in %
[b] Values in the same line are significantly different at 0.01 from the control
[c] Values in the same line are significantly different at $p < 0.05$ from the control

Fish and salt mixtures added with amino acids were fermented aerobically and anaerobically for one and a half months, and also, a model experiment using a one year old fermented fish sauce was added with amino acids and incubated aerobically and anaerobically for 72 and 96 h. Results showed that addition of valine before fermentation significantly increased the production of *iso*-butyric and *iso*-hexanoic acids and leucine increased that of *iso*-valeric in the aerobically fermented fish mixtures (Table 1). A similar tendency was observed in the anaerobically fermented fish mixtures (Table 2) except that there was no significant increase in the *iso*-hexanoic acid as observed in the aerobically fermented mixture.

In the model experiment carried out for 72 h, addition of valine increased the values of *iso*-butyric acid and leucine increased that of the *iso*-valeric acid although the values for both were lower in the anaerobic compared to the aerobic sample (Table 3). The same kind of phenomenon was observed in the 96 h aerobically incubated sample (data not shown). Zarling and Ruchim (1987) reported that *iso*-butyrate and *iso*-valerate are produced by the degradation of amino acids in human stools, the former from valine and the latter from leucine. Saizithi et al. (1966) reported that some bacteria isolated from fish sauce could produce volatile acids from amino acids but they failed to identify the acids produced. They further showed that the

**Table 3.** Volatile acids in a model experiment with fish sauce as substrate (%)[a]

| Samples | 72 h incubation | | |
|---|---|---|---|
| | *iso*-Butyric | *iso*-Valeric | *iso*-Hexanoicc |
| NaCl + water | * | * | * |
| NaCl + water + Valine | * | * | * |
| NaCl + water + Leucine | * | * | * |
| NaCl + water + *iso*-Leucine | * | * | * |
| Fish sauce | 1.20 ± 0.02 | 2.50 ± 0.04 | 0.67 ± 0.02 |
| Fish sauce + Valine | 2.92 ± 0.03 | 2.51 ± 0.02 | 0.65 ± 0.02 |
| Fish sauce + Leucine | 1.22 ± 0.03 | 4.12 ± 0.03[c] | 0.67 ± 0.02 |
| Fish sauce + *iso*-Leucine | 1.22 ± 0.01 | 1.70 ± 0.01 | 0.58 ± 0.010 |
| | 96 h incubation | | |
| | *iso*-Butyric | *iso*-Valeric | *iso*-Hexanoic |
| NaCl + Water | * | * | * |
| NaCl + Water + Valine | * | * | * |
| NaCl + Water + Leucine | * | * | * |
| NaCl + Water + *iso*-Leucine | * | * | * |
| Fish sauce | 1.20 ± 0.02 | 2.50 ± 0.04 | 0.67 ± 0.02 |
| Fish sauce + Valine | 2.91 ± 0.03 | 2.52 ± 0.02 | 0.67 ± 0.02 |
| Fish sauce + Leucine | 1.22 ± 0.03 | 4.12 ± 0.03 | 0.66 ± 0.02 |
| Fish sauce + *iso*-Leucine | 1.21 ± 0.01 | 2.51 ± 0.01 | 0.66 ± 0.01 |

[a] A one year fermented fish sauce was used as substrate
[b] Means of three replicates
[c] Significantly different at $p < 0.01$ from the control
[d] 30% NaCl water solution
* No volatile fatty acids were detected in the samples
Respective amino acids at 2% were added to the substrate right before aerobic incubation

total viable counts decreased as fermentation time advanced.

Theoretically, the reactions between $\alpha$-dicarbonyl compounds, like the deoxyones obtained in the Maillard reaction, and the amino acids are classed under the term Strecker reaction. This reaction involves transamination and gives amino ketones, aldehydes and $CO_2$. It occurs in foods at higher concentrations of amino acids and under more drastic reaction conditions such as higher temperatures or under pressure. The aldehydes formed, often called Strecker aldehydes, can act as food odorants. This reaction is presented in Fig. 1. The increase in the values of the branched chain volatile acids in the amino acid added fish mixtures in this study might follow this formation route but has yet to be confirmed. The presence of aldehydes in fish sauce had been reported (Sanceda et al., 1984). Although the mechanism of the formation of these branched chain volatile acids were not carried out in this paper, they could probably be caused by deamination of amino acids to carboxyl group as intermediate products and further decarboxylyzed to volatile acids.

In this study, the production of *iso*-hexanoic acid was significantly higher in the valine added sample incubated aerobically ($p < 0.001$) and anaerobically ($p < 0.05$) than in their controls. It was difficult to explain this result since valine lacks the carbon skeleton to produce *iso*-hexanoic acid. Britz and Wilkinson (1982) reported that freshly compared cell suspensions of clostridia and *Peptostreptococcus anaerobius* converted leucine to *iso*-valeric and *iso*-caproic acids in the absence of other amino acids. It was further reported that the presence of alanine and valine in incubations effectively increased the concentration of *iso*-caproic acid at the expense of *iso*-valeric acid, implying that leucine acted there primarily as a proton acceptor.

Some species of clostridia proved to produce acetic, propionic, and *n*-butyric acids when grown in threonine media (Elsden, Hilton, 1978). Furthermore, they reported that clostridia species which oxidize valine to

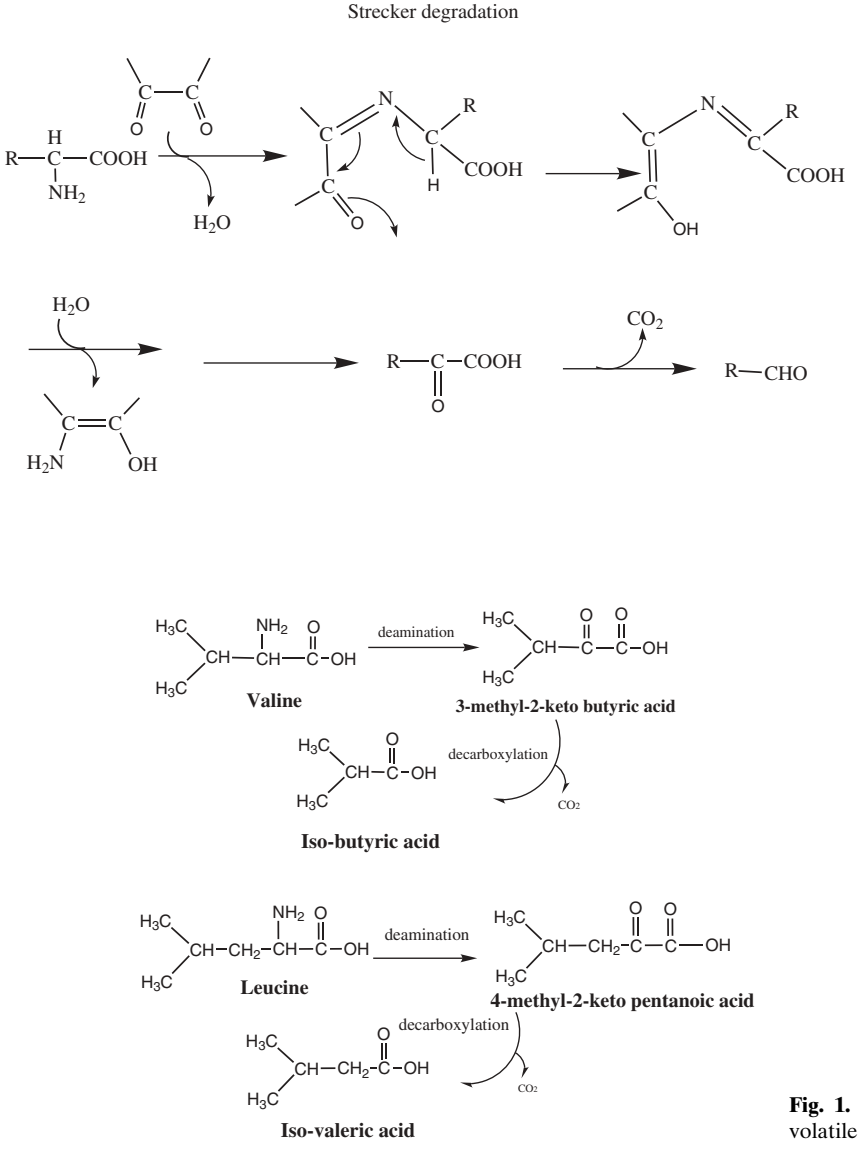


**Fig. 1.** Possible formation route of branched chain volatile fatty acids

*iso*-butyric acid also oxidize leucine to 3-methyl butyric acid and isoleucine to 2-methyl butyric acid. They also reported that *iso*-caproic acid fraction produced by some species is shown to be derived from leucine. In this study, our finding showed that addition of valine to fish mixture fermented aerobically resulted in a significant increase in the production of *iso*-hexanoic acid. However, *iso*-hexanoic acid was not significantly increased by the addition of leucine. Addition of alanine and iso-leucine did not show a clear effect in the production of branched chain acids.

*Use of long chain fatty acid*

To further support the findings above, a supplementary experiment on the addition of linoleic acid was done. Addition of linoleic acid to fish mixture before incubation resulted in an increase in straight chain volatile acids and the increase continued as fermentation progressed but not in the branched chain ones (Table 4). The values of the acids were higher in the linoleic acid-added samples than in the control. Also long straight chain volatile acids ($C_7$ to $C_{10}$) detected in the linoleic acid added sample were much higher than in the control but none of their branched chain acids were found (data not shown. These phenomenon im-

**Table 4.** Volatile acids in the linoleic acid-added fish liquid (T)[a]

| Acids | *Incubation period* | | |
|---|---|---|---|
| | 48 hr | 1 week | 1 month |
| | control | | |
| Acetic | tr | 0.13 ± 0.02 | 0.74 ± 0.03 |
| Propionic | tr | 0.10 ± 0.01 | 0.58 ± 0.02[c] |
| Isobutyric | tr | tr | 0.14 ± 0.02 |
| *n*-Butyric | 0.21 ± 0.02 | 1.91 ± 0.02 | 3.81 ± 0.04[c] |
| Isovaleric | 0.31 ± 0.01 | 0.51 ± 0.05 | 0.64 ± 0.06 |
| *n*-valeric | 0.24 ± 0.01 | 0.18 ± 0.03 | 0.24 ± 0.01 |
| Isohexanoic | nd | tr | 0.09 ± 0.01 |
| *n*-Hexanoic | nd | trr | tr |
| | lincleic acid added[b] | | |
| Acetic | 0.10 ± 0.01 | 0.22 ± 0.05 | 0.96 ± 0.04[c,d] |
| Propionic | tr | 0.15 ± 0.04 | 0.64 ± 0.01[d] |
| Isobutyric | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.15 ± 0.02 |
| *n*-Butyric | 0.20 ± 0.01 | 1.99 ± 0.02 | 4.03 ± 0.02[c,d] |
| Isovaleric | 0.53 ± 0.03 | 0.51 ± 0.05 | 0.66 ± 0.03 |
| *n*-valeric | 0.20 ± 0.01 | 0.24 ± 0.06 | 0.24 ± 0.02 |
| Isohexanoic | nd | tr | tr |
| *n*-Hexanoic | nd | 0.11 ± 0.06 | 0.11 ± 0.01 |

[a] Values are means of three replicates
[b] Linoleic acid was added before incubation
[c] Values in the 1 month linoleic acid-added and control are significantly different at $p < 0.05$ from the 48 hr incubation in the control
[d] Values in the 1 month linoleic acid-added liquid are significantly different at $p < 0.05$ from the 1 month control
*tr*, Values are less than 0.01%. *nd*, Not detected

ply that straight chain acids might be derived from fat. Fish fats hardly contain branched chain fatty acids.

A slight increase in the amount of *iso*-valeric was observed, however, this branched acid is unlikely to be formed from straight chain volatile acids. They are theoretically believed to be derived from the degradation of amino acids. It has been reported that certain volatile acids can be produced by the Stickland reaction on specific amino acids with some Clostridia sp. It was demonstrated that *n*-butyric acid could not be produced by this reaction due to lack of an amino acid with an appropriate carbon skeleton (Nisman, 1954).

The rate of oxidation in fermented foods may be affected by many factors. Access to oxygen is one factor. Marcus and Frederickson (1968) reported that termination reactions in the course of lipid oxidation could be different and produce different oxidative products at different oxygen concentrations. It has also been theorized that halide ions may activate the myeloperoxide-$H_20_2$-halide system associated with blood components in fish (Kanner and Kinsella, 1983), thus initiating lipid oxidation.

## 4. Summary

It seemed that normal volatile acids were derived from fish fats. Addition of valine and leucine increased the production of *iso*-butyric and *iso*-hexanoic, and *iso*-valeric acids, respectively, suggesting that these branched chain acids were produced from the degradation of amino acids.

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