The Decomposition of Fats in Mackerel Homogenates Inoculated with Yeasts or Bacteria (Studies on the Spoilage of Foods. VI.)

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By gas chromatographic determination, the amount of free fatty acids, composition of fatty acids derived from glyceride and decomposition rate of fats in mackerel homogenates, individually inoculated with yeasts or bacteria and incubated at 25°C for 7 days, were investigated. The decomposition rate of fats was compared with total volatile basic nitrogen (TVB-N) production in the inoculated homogenates. The following results were obtained. (1) *Candida lipolytica* and *Pseudomonas fluorescens* had the highest decomposition rates of fats among the 5 yeast and 4 bacterial strains tested, respectively. The decomposition rates of fats by them were about 90% after incubation at 25°C for 7 days. (2) Free fatty acids released from glyceride by the yeast strains or the bacterial strains were of the same kind as the fatty acids present in glycerides, and so it was considered that the decomposition of fats mainly depended on cleavage of glycerol ester. (3) Fifteen to twenty-five percent of the released free fatty acids, especially unsaturated free fatty acids, were further decomposed. (4) The decomposition rate of fats by the test strains did not always correspond to the amount of TVB-N produced in the homogenates; for example, *Bacillus subtilis* formed a large amount of TVB-N but showed a small decomposition rate of fats.

Key words: *Candida lipolytica*; *Bacillus subtilis*; *Pseudomonas fluorescens*; gas chromatography (FID); fat decomposition; free fatty acid; mackerel; total volatile basic nitrogen

Introduction

The authors have performed a series of studies on the spoilage of foods by yeasts or bacteria^{1)~8)}. In the previous studies, the proteolytic or lipolytic activities of yeasts³⁾ or bacteria⁶⁾ isolated from seafoods¹⁾ were qualitatively examined. The strains having positive activities were singly inoculated into sterile fish homogenates and incubated at various temperatures, and the sensory signs, pH and total volatile basic nitrogen (TVB-N) produced in the homogenates were determined^{7), 8)}. The yeast

strains tested acted in the same manner as the bacterial strains tested, putrefying the fish homogenate and producing TVB-N.

In this paper, by determining the amounts of free fatty acids and the composition of fatty acid derived from glyceride, the decomposition rate of fats in mackerel homogenates singly inoculated with yeasts or bacteria was estimated. Gas chromatography (GC) was used in order to determine quantitatively the lipolytic activity of yeasts. Moreover, the decomposition rate of fats by the test strains was compared with the degree of organoleptic deterioration of the

homogenates and the amount of TVB-N produced in the same inoculated homogenates.

Experimental

1. Materials

Commercial common mackerel (*Scomber japonicus*) was purchased at a fish market in Tokyo. The fish samples were very fresh.

2. Microbial strains used

The following strains, which were previously isolated from seafoods and identified by the authors^{2), 6)}, were used.

Yeast strains: *Candida lipolytica* M-20-P-5 NHL 11027, *Rhodotorula rubra* U-1-P-6 NHL 11054, *Cryptococcus laurentii* U-1-P-9 NHL 11040, *Trichosporon cutaneum* A-2-P-1 NHL 11063, *Trichosporon pullulans* UC-11-P-1 NHL 11068.

Bacterial strains: *Pseudomonas fluorescens* S-1 NHL 11074, *Bacillus subtilis* S-8 NHL 11075, *Staphylococcus epidermidis* S-6 NHL 11076, *Alcaligenes* sp. S-4 NHL 11077.

3. Reagents

Myristic acid, palmitic acid, oleic acid, eicosenoic acid, eicosapentaenoic acid, erucic acid, docosahexaenoic acid, arachidic acid and linolenic acid were guaranteed analytical grade products from Tokyo Kasei Kogyo Co., Ltd.

BF₃-methanol complex for GC (14%): Wako Pure Chemical Co., Ltd.

Diazomethane solution was prepared from *N*-nitrosomethylurea by the usual method⁹⁾.

N/2 sodium hydroxide-methanol solution: 20 g of sodium hydroxide was dissolved in 1,000 ml of methanol.

N/10 potassium hydroxide-50% ethanol solution: A mixture of N/10 potassium hydroxide solution with ethanol (1 + 1).

Arachidic or linolenic acid test solution: 200 mg of arachidic or linolenic acid was dissolved in 100 ml of ethyl ether (0.2%).

4. Apparatus

Gas chromatograph: Shimadzu Seisakusho Co., Ltd. GC-7AG (flame ionization detection system) equipped with a computing integrator, Chromatopak C-R1A.

Conway's apparatus: The same as in the previous paper⁵⁾.

5. Preparation of sterile mackerel homogenate samples

An equal amount of 0.85% saline was added to muscle tissue slices of the mackerel. The tissue was homogenized in a Waring blender for 2 min. Sixty grams of the homogenate was put into a 100 ml Erlenmeyer flask with a foil-covered cotton plug, autoclaved at 121°C for 20 min, and aseptically stirred with a spatula.

6. Preparation of inocula

The 5 yeast strains and 4 bacterial strains were individually streaked onto YM agar (Difco) plates and plate count agar, respectively, and the plates were incubated at 25°C for 4 days, then a fresh colony of each strain was inoculated into YM broth (Difco) and nutrient broth (Difco), respectively, and they were incubated at 25°C for 4 days.

7. Inoculation of strains and sampling of the homogenates

7.1. Each sterile homogenate was individually inoculated with 1 ml of the diluted broth culture to yield an initial microbial load of 10⁵ cells/g. The homogenate was stirred with a spatula under sterile conditions, and incubated at 25°C for 7 days. Then 10 g and 3 g of the homogenates were collected for determination of fatty acid and TVB-N, respectively.

7.2. Fifty ml of arachidic or linolenic acid test solution was taken into a flask and dried up at room temperature. Fifty grams of unsterile homogenate was put into the flask, mixed well and sterilized at 121°C for 20 min. The sterile homogenate containing arachidic or linolenic acid (2 mg/g) was separately inoculated with *C. lipolytica* or *P. fluorescens* to give an initial microbial load of 10⁴ cells/g. The four kinds of flasks were incubated at 25°C for up to 7 days. Ten gram samples for the determination of residual arachidic or linolenic acid were aseptically removed on days 0, 2, 4 and 7 of incubation.

7.3. Each sterile homogenate was individually inoculated with 1 ml of diluted broth cultures of *C. lipolytica*, *P. fluorescens* and *B. subtilis* to obtain an initial microbial load of 10⁴ cells/g, and incubated at 25°C for up to 7 days. From the three kinds of flasks, 10 g samples for the decomposition test of fats and 3 g samples for TVB-N determination were aseptically removed on days 0, 2, 4 and 7 of incubation.

8. Sensory spoilage examination

The degree of softening and odor of the samples were observed by 4 laboratory panelists.

9. Determination of Total Volatile Basic Nitrogen (TVB-N)

The TVB-N was determined by the micro-diffusion method of Conway^{5), 7)}.

10. Decomposition test of fat

10.1. Extraction of fat in sterile mackerel homogenate¹⁰⁾: Five grams of each sample was taken into a blender cup and blended with 50 ml of $\text{CHCl}_3 \cdot \text{CH}_3\text{OH}$ (2+1) mixture for 3 min. After filtration of the homogenate, the residue was blended again with the same solvent. The extracts were combined and made up to 100 ml with CHCl_3 .

10.2. Separation of free fatty acid and glyceride fraction¹¹⁾: Twenty ml of the extracts was dried up under reduced pressure in a water bath at 40°C. The residue was dissolved in 50 ml of petroleum ether, dehydrated with anhydrous sodium sulfate and shaken with 50 ml of *N*/10 potassium hydroxide–50% ethanol solution. The lower layer was used as free fatty acid fraction and the upper layer was used as glyceride fraction in the following tests.

10.3. Preparation of test solution of fatty acids in free fatty acid fraction: Free fatty acid fraction was washed with 20 ml of petroleum ether and shaken with 20 ml of *N* hydrochloric acid and 50 ml of ether. After separation of the ether layer, the aqueous layer was mixed again with 50 ml of ether. The extracts were combined, dehydrated with anhydrous sodium sulfate and dried up under reduced pressure. Five ml of diazomethane solution was added to the residue and the mixture was left to stand for a few minutes at room temperature. Then the solution was made up to 10 ml with ether as test solution A for GC.

10.4. Preparation of test solution for composition of fatty acids in glyceride fraction¹²⁾: Glyceride fraction was washed with 10 ml of water, dehydrated with anhydrous sodium sulfate and dried up under reduced pressure. Ten ml of *N*/2 sodium hydroxide–methanol solution was added to the residue and refluxed for 30 min in a water bath at 65°C. Then 1.0 ml of BF_3 –methanol complex was added to the solution and heating was continued for 5 min. Ten ml each of

hexane and water were added to the content, and the mixture was shaken for 3 min. After separation of hexane layer, the aqueous layer was mixed with 10 ml of hexane and shaken again. The combined hexane layer was made up to 20 ml with hexane as test solution B for GC.

10.5. Determination of fatty acids by gas chromatography: Five μl of test solution A or B was injected into the gas chromatograph and fatty acids were determined by GC under the following conditions.

Packing material of column: 3% SP-2300/Chromosorb W

Column size: glass tube (2.1 mm ϕ × 1.6 m)

Column temperature: 100~250°C (4°C/min)

Injection port temperature: 270°C

Nitrogen flow rate: 50 ml/min.

Results and Discussion

1. Composition of fatty acids in the uninoculated mackerel homogenate

Compositions of fatty acids in glyceride fraction and free fatty acids fraction, and the composition of main fatty acids in glyceride of the sterile uninoculated mackerel homogenate are shown in Fig. 1 and Table 1, respectively.

Only small amounts of free fatty acids were found in the sterile homogenate, and almost all fatty acids were found in the glyceride fraction. Main fatty acids derived from glyceride in the homogenate were myristic acid, palmitic acid, octadecenoic acid, eicosenoic acid, eicosapentaenoic acid, docosenoic acid and docosahexaenoic acid.

As shown in Table 1, the content of fatty acids in the glyceride fraction was estimated as 98.3% by assuming total fatty acids in the control as 100%. The contents of such polyunsaturated fatty acids as eicosapentaenoic acid and docosahexaenoic acid, which have anti-aneurism and anti-arteriosclerotic activities¹³⁾, were reasonable in comparison with the Standard Tables of Food Composition in Japan¹⁴⁾.

2. Comparison of decomposition rate of fatty acids by yeasts and bacteria

The amounts of 7 main fatty acids in the free fatty acid fraction and glyceride fraction of the mackerel homogenates described above were determined (see Figs. 3 and 4). Gas chromatograms of fatty acids in homogenate inoculated

Fig. 1. Gas chromatograms for fatty acids in sterile uninoculated mackerel homogenate

Table 1. Composition of Main Fatty Acids in Glyceride of Sterile Uninoculated Mackerel Homogenate

Fatty acid	Carbon number	Content (%)	Amount (mg/g)
Myristic acid	14:0	4.7	2.0
Palmitic acid	16:0	19.1	8.2
Octadecenoic acid	18:1	24.0	10.3
Eicosenoic acid	20:1	6.3	2.7
Eicosapentaenoic acid	20:5	7.7	3.3
Docosenoic acid	22:1	4.4	1.9
Docosahexaenoic acid	22:6	20.0	8.6
Others		12.1	5.9
Total		98.3	42.9

GC conditions: column, 3% SP-2300/Chromosorb W (3 mmϕ×1.6 m); temperature, C. T. 100~250°C (4°C/min), I. T. 270°C

with *C. lipolytica* are shown in Fig. 2 as an example.

The decomposition rates of fats by the species tested were compared. Among the five yeast species tested, *C. lipolytica* showed the largest decomposition rate, followed by *Tr. cutaneum* and *Cr. laurentii*. The rates by *Rh. rubra* or *Tr. pullulans* were small. *P. fluorescens* showed the largest rate among the four bacterial species tested, followed by *B. subtilis*, *S. epidermidis*, and *Alcaligenes* sp. As shown in Figs. 3 and 4, the amount of fatty acids in the glyceride fraction decreased and those in free fatty acid fraction increased following the inoculation with *C. lipolytica* and *P. fluorescens*.

Moreover, free fatty acid released from glycerides by the test strains were of the same kind as those in glycerides; therefore, it was considered that decomposition of fat by the test strains

Fig. 2. Gas chromatograms for fatty acids in mackerel homogenate inoculated with *C. lipolytica*

mainly depended on cleavage of glycerol ester.

The total amount of the 7 main kinds of fatty acids in glyceride of the uninoculated mackerel muscle homogenate was defined as 100%, and the ratios (percent) of the free fatty acids to the fatty acids in glyceride of the inoculated homogenates were taken as the decomposition rates of fats. The decomposition rates of fats by the test strains are shown in Table 2.

In the cases of *C. lipolytica* and *P. fluorescens* especially the amount of fatty acids in free fatty acid fraction was large, and that in the glyceride fraction was small. This means that the decomposition rates of fats of the strains are both high. From these results it was clearly evident that *C. lipolytica* which we called a spoilage yeast in our previous papers^{7), 8)}, decomposed fat as effectively as did spoilage bacteria.

3. Decomposition of fatty acid by spoilage yeasts or bacteria

As shown in Table 2, the total amounts of fatty acids were not 100% but 73.3–84.9%. Thus, it was presumed that fatty acids released from glyceride were further decomposed by the test strains to small-molecular compounds which could not be detected under the GC conditions used. In order to confirm that free fatty acids are decomposed by the test strains, arachidic acid ($C_{20:0}$) and linolenic acid ($C_{18:3}$), which were not originally contained in mackerel muscle, were added to sterile mackerel homogenates and the amounts of them remaining are shown in Fig. 5 as a function of time.

The decomposition rate of arachidic acid by the two strains was several percent, but that of linolenic acid was 15–25%. Thus, decomposition of fatty acids by the strains did occur, and

Fig. 3. Main fatty acids in the free fatty acid fraction and the glyceride fraction of mackerel homogenates inoculated with yeast strains

Fig. 4. Main fatty acids in the free fatty acid fraction and the glyceride fraction of mackerel homogenates inoculated with bacterial strains

the rate was larger for unsaturated fatty acid (linolenic acid) than for saturated fatty acid (arachidic acid).

4. Relationship between decomposition rate of fats and TVB-N production by the yeasts and bacteria

The decomposition rates of fats, the amounts

of TVB-N and the external sensory signs of the inoculated homogenates are summarized in Table 3.

It was clear that the decomposition rates of fats by *C. lipolytica* and *P. fluorescens* were very large, as were the TVB-N values; moreover, the degree of softening of the homogenates in-

oculated with the above strains was also remarkable. On the other hand, the decomposition rate by *B. subtilis* was small, but TVB-N value was large and the sensory signs were quite marked.

Therefore, it was recognized that relation between the decomposition of fats and the TVB-N values was not the same for all the strains tested. Further experiments were carried out

with *C. lipolytica*, *P. fluorescens*, and *B. subtilis*. The results are shown in Fig. 6.

The TVB-N values of the homogenates increased markedly during storage. The decomposition rate by *B. subtilis* was lower than those by *P. fluorescens* and *C. lipolytica*. These phenomena are consistent with the results in Table 3. It was suggested that the yeast *C. lipolytica* has a spoilage action similar to that of *P.*

Table 2. Free Fatty Acids and Fatty Acids in Glyceride of Mackerel Homogenates Inoculated with Yeasts or Bacteria

Test strain*	Free fatty acid (%)	Fatty acid in glyceride (%)	Total** (%)
<i>Tr. pullulans</i>	0.8	84.1	84.9
<i>Rh. rubra</i>	2.4	80.2	82.6
<i>Cr. laurentii</i>	7.3	73.2	80.5
<i>Tr. cutaneum</i>	27.5	48.3	75.8
<i>C. lipolytica</i>	63.6	9.8	73.4
<i>Alcaligenes</i> sp.	4.1	76.7	80.8
<i>S. epidermidis</i>	5.6	73.0	78.6
<i>B. subtilis</i>	8.8	64.5	73.3
<i>P. fluorescens</i>	65.1	10.5	75.6

* Each mackerel homogenate was inoculated with a test strain, and incubated at 25°C for 7 days.

** These values are based on total fatty acids in glyceride of sterile mackerel homogenates (control) as 100%.

Table 3. Relationship among Decomposition of Fats, Production of Total Volatile Basic Nitrogen (TVB-N) and Sensory Spoilage Examination of Mackerel Homogenates Inoculated with Yeasts or Bacteria

Strain*	Decomposition of fats** (%)	TVB-N (mg/100 g)	Sensory sign	
			Softening	Odor
<i>Tr. pullulans</i>	6.6	15.1	—	—
<i>Rh. rubra</i>	10.9	38.9	—	—
<i>Cr. laurentii</i>	18.7	21.0	—	—
<i>Tr. cutaneum</i>	46.3	54.0	—	+
<i>C. lipolytica</i>	89.1	277.2	++	++
<i>Alcaligenes</i> sp.	14.8	11.2	—	—
<i>S. epidermidis</i>	18.9	20.9	—	—
<i>B. subtilis</i>	28.3	210.0	++	++
<i>P. fluorescens</i>	90.6	280.0	++	++

* Each mackerel homogenate was inoculated with a test strain, and incubated at 25°C for 7 days.

** Decomposition rates were estimated from the decrease of fatty acids in glyceride. The total content of the seven main fatty acids (shown in Table 1) in glyceride of the sterile uninoculated mackerel homogenate was taken as 100%.

Fig. 5. Time courses for degradation of free fatty acids added to mackerel homogenate —: arachidic acid; ----: linolenic acid
○: *P. fluorescens*; ●: *C. lipolytica*
2 mg/g of arachidic acid or linolenic acid, and 10^4 cells/g of *P. fluorescens* or *C. lipolytica* were added to mackerel homogenates, and incubated at 25°C for 7 days.

Fig. 6. Time courses for decomposition of fats and formation of total volatile basic nitrogen in mackerel homogenate

The samples were inoculated with yeast or bacteria and incubated at 25°C for up to 7 days.

○: *C. lipolytica*; ◐: *P. fluorescens*; ●: *B. subtilis*

fluorescens.

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