Bacterial Flora and Effect of Lytic Enzyme on Bacterial Growth in Mackerel Muscle during Storage^{*1}

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Bacterial flora in the mackerel muscle homogenates during storage at different temperatures was investigated, and an attempt to apply lytic enzyme as a food preservative was made.

Pseudomonas (32.3%), *Vibrio* (26.3%) and *Corynebacterium* (12.1%) predominated in the mackerel muscle homogenate. The number of bacteria in the mackerel homogenate stored at 20°C increased rapidly and reached a maximum value of about 10⁹ cfu/g (wet weight) of muscle after 12 h. Strains belonging to the genus *Vibrio* were the only bacteria isolated from the mackerel homogenate stored at 20°C for 12 h.

The bacterial population in the mackerel homogenate did not increase during storage at 0°C for 3 days or at -20°C for 60 days. *Pseudomonas* (41.5%) was the most dominant genus at 0°C after 24 h storage, while *Moraxella* (38.8%) and *Pseudomonas* (30.0%) predominated at -20°C after 60 days storage.

The bacterial number in the mackerel homogenate gradually increased at 20°C after a lag phase of about 6 h upon addition of lytic enzyme. Lytic enzyme produced by *Bacillus* sp. V 37 seems to suppress bacterial growth at 20°C for at least 12 h. However, the preservative effect of lytic enzyme retarded after 24 h storage at 20°C.

Many studies have been carried out on the microflora of commercially important species of fish. Simidu *et al.*^{1,2)} reported that *Flavobacterium* (22.5%), *Corynebacterium* (20.0%), *Pseudomonas* (17.8%), *Achromobacter-Acinetobacter* (17.8%) and *Vibrio* (16.4%) were abundant on the skin of flat fish, *Kareius bicoloratus*. After 7 days storage at 2°C, *Pseudomonas* (53.9%), *Vibrio* (25.5%) and *Achromobacter-Acinetobacter* (15.4%) were the predominant genera.²⁾ Horie *et al.*³⁾ demonstrated that about 90% of the bacteria isolated from the skin of several kinds of raw fish consist of the *Pseudomonas* III/IV-H, III/IV-NH, *Vibrio* and *Moraxella*. After spoilage at 0.5°C, the *Pseudomonas* III/IV-H, III/IV-NH and *Vibrio* also dominated in the floras both on the skin and in the muscle.

It was earlier recognized that the major spoilage bacteria of fish are the bacteria normally found in fresh fish. *Pseudomonas* and *Achromobacter* are common to the spoilage in fish.⁴⁻⁸⁾

On the other hand, a number of studies have been made on the application of egg white lysozyme

as a preservative for food stuffs. As for the application of bacteriolytic enzymes other than egg white lysozyme, however, little information is available. Lytic enzyme produced by *Bacillus* sp. V 37 exhibited a remarkable lytic activity on the bacterial cells capable of putrefying kamaboko (boiled fish paste).^{9,10)}

The present study was designated to obtain preliminary information concerning bacterial flora in the mackerel muscle homogenates during storage at different temperatures and some application of lytic enzyme to food preservation.

Methods

Preparation of Fish Muscle Homogenates

Raw mackerel *Scomber japonicus* samples used in this study were purchased from local markets. Fish muscle homogenate was made from 100 g fresh mackerel muscle tissues with skin in 100 ml sterile 0.01 M Tris-HCl buffer (pH 7.0) containing 2% NaCl. For the experimental part, 10 ml of sterile crude lytic enzyme of *Bacillus* sp. V37

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(9,300 units/ml) was added to 100 ml of mackerel homogenate, while 10 ml of sterile 0.01 M Tris-HCl buffer (pH 7.0) was added to 100 ml of mackerel homogenate (control).

Preparation of Crude Lytic Enzyme

Lytic enzyme producing *Bacillus* sp. V 37 was grown in the medium containing polypepton (1.0%), yeast extract (0.5%) and NaCl (0.5 M) at 30°C for 24 h on reciprocating shaker with a shaking rate of 100 strokes per min. The bacterial cells in the culture fluid were removed by centrifugation. Solid ammonium sulfate was added to the supernatant of the culture fluid to 80% saturation, and the solution was allowed to stand overnight at 5°C. The precipitate was dissolved in and dialysed against 0.01 M Tris-HCl buffer (pH 7.0) at 5°C for 5 h. Sterile dialysate (crude lytic enzyme) was obtained by using Millex-GS (0.22 μ m) filter (Millipore Co.). Lytic activity (1 unit) was defined as a decrease in absorbance of 1.00 at 570 nm in 60 min.

Enumeration of Bacterial Number

Enumeration and isolation of heterotrophic bacteria were conducted with a smear plate method using 2% NaCl agar medium. The medium used in this study was composed of polypepton (Daigo Eiyō Kagaku), 5.0 g; yeast extract (Nakarai Chemicals), 3.0 g; NaCl, 20.0 g; agar, 15.0 g in 1,000 ml tap water, pH 7.0. Cultivation was done at 25°C for 2 weeks.

Identification of Bacterial Isolates

The heterotrophic isolates were purified by streaking on agar plates. The generic composition of the isolates was determined according to the scheme of Simidu¹¹⁾ and Bergey's Manuals.¹²⁻¹⁴⁾

Determination of Trimethylamine (TMA)

The determination of trimethylamine (TMA) was carried out according to the microdiffusion analysis method of Yamagata.¹⁵⁾

Results and Discussion

Changes in Viable Bacterial Number

Shewan⁷⁾ reported that during storage of fish in ice (0°C), the number of bacteria in fish muscle increased after a lag phase of 1 to 2 days, reaching maximum values of about 10^7 to 10^8 cfu/g after 9 to 10 days. However, viable count in the muscle stored at 25°C rapidly increased and reached a maximum level of about 10^8 cfu/g within 1 to 2

Fig. 1. Changes in bacterial number and trimethylamine content in the mackerel muscle homogenate stored at 20, 0 and -20°C.

days.⁷⁾

A similar tendency was also observed in this study. As shown in Fig. 1, the numbers of bacteria in the mackerel muscle homogenate stored at 20, 0 and -20°C were counted at fixed time intervals. The initial count in the homogenate was 2.7×10^4 cfu/g (wet weight) of mackerel muscle. The number of bacteria in the homogenates increased and reached the maximum level of about 10^9 cfu/g after 12 h storage at 20°C, but did not increase during 3 days storage at 0°C. Viable count in the homogenate stored at -20°C gradually decreased to about 10^8 cfu/g after 60 days.

The concentration of TMA (trimethylamine)-N after 12 h storage at 20°C was higher than the threshold value of spoilage (about 3 mg/100 g). However, TMA-N level did not exceed the threshold value of spoilage in the homogenates stored at 0°C for 3 days or at -20°C for 60 days.

Changes in Microflora in Homogenates Stored at Different Temperatures

Of the 373 strains of bacteria and yeasts, 99 strains were isolated from fresh mackerel muscle homogenate (zero time), 100 strains from the homogenate stored at 20°C for 12 h, 94 strains from the homogenate stored at 0°C for 24 h and 80 strains from the homogenate stored at -20°C

Table 1. Morphological and physiological characteristics of heterotrophic microbial isolates from the mackerel muscle homogenates stored at different temperatures

Storage time	0 h	12 h at 20°C	24 h at 0°C	60 days at -20°C
Number of strains isolated	99	100	94	80
Form				
Rod	91	100	93	76
Sphere	7	0	1	1
Yeast	1	0	0	3
Gram				
negative	76	100	77	73
positive	23	0	17	7
Flagellation				
Polar	56	100	60	24
Peritrichous	8	0	4	3
Spore formation	3	0	4	1
Catalase positive	99	100	94	80
Oxidase positive	75	100	76	69
Hugh & Leifson (Glucose)				
Oxidative	32	0	39	36
Fermentative	27	100	18	1
Gas production from glucose	0	0	0	0
O/129 sensitivity	26	100	18	0

Table 2. Generic percentage distribution of heterotrophic microbial isolates from the mackerel muscle homogenates stored at different temperatures

Storage time	0 h	12 h at 20°C	24 h at 0°C	60 days at -20°C
Number of strains isolated	99	100	94	80
<i>Pseudomonas</i>	32.3	0	41.5	30.0
<i>Vibrio</i>	26.3	100.0	19.1	0
<i>Moraxella</i>	5.1	0	9.6	38.8
<i>Acinetobacter</i>	1.0	0	1.1	7.5
<i>Flavobacterium</i>	8.1	0	10.6	15.0
<i>Alcaligenes</i>	4.0	0	0	0
<i>Corynebacterium</i>	12.1	0	12.8	2.5
<i>Bacillus</i>	3.0	0	4.3	1.2
<i>Micrococcus</i>	6.1	0	1.1	1.2
<i>Staphylococcus</i>	1.0	0	0	0
Yeast	1.0	0	0	3.8

for 60 days.

As shown in Table 1, all strains isolated from the homogenate stored at 20°C for 12 h were gram-negative rods and motile with polar flagella. They were also able to ferment glucose. In all cases, most of the isolates were oxidase positive, asporogenous rods. Strains capable of producing gas in the medium containing glucose were not isolated.

Table 2 lists the generic composition of aerobic heterotrophic isolates from the mackerel homogenates stored at 20, 0 and -20°C. *Pseudomonas* (32.3%), *Vibrio* (26.3%) and *Corynebacterium* (12.1%) predominated in the fresh mackerel

muscle homogenate (zero time), and gram-positive organisms constituted 23.2% of the isolated microorganisms. In the mackerel homogenate stored at 0°C for 24 h, *Pseudomonas* (41.5%) was the most dominant bacterial genus. *Vibrio* (19.1%), *Corynebacterium* (12.8%), *Flavobacterium* (10.6%) and *Moraxella* (9.6%) were also abundant. Only *Vibrio* was isolated from the mackerel homogenate stored at 20°C for 12 h.

On the other hand, *Moraxella* (38.8%), *Pseudomonas* (30.0%) and *Flavobacterium* (15.0%) were found to be the dominant genera in the mackerel homogenates stored at -20°C for 60 days.

Table 3. Effect of lytic enzyme on bacterial count in mackerel muscle homogenate during storage at 20°C

Storage time (h)	Number of bacteria (cfu/g)	
	Control	Lytic enzyme addition
0	2.55×10^3	2.55×10^3
6	5.20×10^4	3.63×10^3 (6.98%)
12	9.38×10^8	$<1.00 \times 10^5$ (<1.07%)
24	5.84×10^8	2.36×10^8 (40.4%)

Percentage value (%) shows ratio of bacterial number of the homogenate, to which lytic enzyme has been added, to that of control at each storage time.

Okuzumi *et al.*¹⁶⁾ reported that *Flavobacterium-Cytophaga*, *Moraxella*, *Micrococcus* and *Staphylococcus* dominated after frozen storage of fish. *Pseudomonas* and *Vibrio* rapidly disappeared during frozen storage, compared with *Flavobacterium-Cytophaga*, *Moraxella* and coccus groups.¹⁸⁾ The members of *Vibrio* have been reported to produce trimethylamine.^{17,18)} A high amount of TMA in the mackerel homogenate stored at 20°C for 12 h may be mainly due to the *Vibrio* group.

These results are almost similar to the observations reported recently.

Effect of Lytic Enzyme on Bacterial Count in Mackerel Homogenate

As shown in Table 3, the number of bacteria in the mackerel homogenate stored at 20°C increased with storage time, and reached the value of 5.84×10^8 cfu/g (wet weight) of mackerel muscle. In the mackerel homogenate, where *Bacillus* sp. V 37 lytic enzyme has been added, the bacterial population gradually increased after a lag phase of about 6 h. After 12 h of storage at 20°C, the bacterial number was only less than 1.00×10^5 cfu/g, corresponding to less than 1.07% of the control value. However, preservative effect of the lytic enzyme retarded after 24 h of storage at 20°C.

As shown in Table 2, only *Vibrio* was isolated from the mackerel homogenate stored at 20°C for 12 h. *Bacillus* sp. V 37 exhibited a remarkable lytic action on the living cells of *Vibrio parahaemolyticus*.¹⁰⁾ The low bacterial number in the mackerel homogenate to which lytic enzyme has been added may be due to the lysis of *Vibrio* cells. Although bacterial flora in the mackerel homogenate stored at 20°C for 24 h was not determined,

mackerel homogenate stored at 20°C for 24 h may be occupied by bacteria other than *Vibrio* or by lytic enzyme-resistant bacterial groups.

Further study is required on the changes in the microflora of the mackerel homogenates to which lytic enzyme has been added, during storage at different temperatures.

References

- 1) U. Simidu, E. Kaneko, and K. Aiso: *Nippon Suisan Gakkaishi*, **35**, 77–82 (1969).
- 2) U. Simidu: In “*Kaiyōbiseibutsu*” (ed. by N. Taga), Univ. Tokyo Press, Tokyo, 1974, pp. 45–65.
- 3) S. Horie, M. Okuzumi, M. Kimura, M. Akahori, and M. Kawamae: *J. Food Hyg. Soc. Japan*, **13**, 410–417 (1972).
- 4) C. H. Castell and G. W. Anderson: *J. Fish. Res. Bd. Can.*, **7**, 370–377 (1948).
- 5) C. H. Castell and M. F. Greenough: *J. Fish. Res. Bd. Can.*, **16**, 21–31 (1959).
- 6) P. Lerke, R. Adams, and L. Farber: *Appl. Microbiol.*, **13**, 625–630 (1965).
- 7) J. M. Shewan: In “*Fish as Food*” Vol. 1 (ed. by G. Borgstrom), Academic Press, New York, 1961, pp. 487–560.
- 8) J. M. Shewan, G. Hobbs, and W. Hodgkiss: *J. Appl. Bacteriol.*, **23**, 463–468 (1960).
- 9) I. Sugahara, K. Hayashi, and T. Kimura: *Bull. Fac. Fish., Mie Univ.*, No. 2, 59–66 (1975).
- 10) I. Sugahara, K. Hayashi, T. Kimura, and N. Takayama: *Bull. Fac. Fish., Mie Univ.*, No. 9, 23–29 (1982).
- 11) U. Simidu: *Mar. Sci.*, **5**, 588–594 (1973).
- 12) R. E. Buchanan and N. E. Gibbons: *Bergey's Manual of Determinative Bacteriology*, 8th ed., Williams and Wilkins, Baltimore, 1974, p. 1246.
- 13) N. R. Krieg and J. G. Holt: *Bergey's Manual of Systematic Bacteriology*, Vol. 1, Williams and Wilkins, Baltimore, 1984, p. 964.
- 14) P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt: *Bergey's Manual of Systematic Bacteriology*, Vol. 2, Williams and Wilkins, Baltimore, 1986, pp. 965–1599.
- 15) M. Yamagata: In “*Suisan Seibutsukagaku-Shokuhingaku Jikkensho*” (ed. by T. Saito, H. Uchiyama, S. Umemoto, and T. Kawabata), Koseisha Koseikaku, Tokyo, 1974, pp. 281–286.
- 16) M. Okuzumi, S. Horie, K. Imai, and K. Matsubara: *J. Food Hyg. Soc. Japan*, **15**, 22–29 (1974).
- 17) T. Fujii, Y. Ishida, and H. Kadota: *Nippon Suisan Gakkaishi*, **43**, 1241–1247 (1977).
- 18) T. Fujii and Y. Ishida: In “*Biseibutsu no Seitai*” Vol. 4, Univ. Tokyo Press, Tokyo, 1977, pp. 45–60.