AN ABSTRACT OF THE THESIS OF

KIE HO RHEE (Name) for the MASTER OF SCIENCE (Degree)

in FOOD SCIENCE (Major) presented on 4/12/74 (Date)

Title: DIMETHYL DISULFIDE PRODUCED IN STERILE FISH MUSCLE BY PSEUDOMONAS PUTREFACIENS

Abstract approved: Jong S. Lee

Sterile fish muscle homogenate was prepared from the individually line caught black rockfish (Sebastes melanops) and inoculated with the pure culture of Pseudomonas putrefaciens strain 17. The inoculated homogenate was incubated at 5°C and the growth and production of volatile sulfur compounds determined by a combined gas-liquid chromatography and mass spectrometry.

A column containing 90-100 mesh diatomaceous earth coated with 10% Carbowax 20M was developed and tested for the quantitative determination of dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS).

The DMDS levels in fish homogenate closely paralleled that of P. putrefaciens growth. The maximum DMDS level of 2.00 μg per 100 g fish homogenate was obtained after 10 days at 5°C in samples treated with 1.5% NaCl.

NaCl was not required by P. putrefaciens but both the growth and DMDS production were stimulated by 1-2% of NaCl.

The DMDS production and the growth of P. putrefaciens were reduced when the homogenate was treated with sodium benzoate (SB) or ethylenediaminetetraacetic acid (EDTA). The maximum levels of DMDS in 0.05% SB treated fish were 0.75 μg per 100 g and 0.85 μg for 0.05% EDTA treatment, respectively.

The SB inhibited the growth rate as well as the maximum growth of P. putrefaciens, while EDTA had no effect on the maximum growth but extended the lag period and reduced the rate of growth. Potassium sorbate (PS) had little effect on DMDS production or the growth of P. putrefaciens. The maximum level of DMDS in fish homogenate, treated with 0.1% PS, was 1.80 μg per 100 g and the growth curve was similar to that of the control.

Dimethyl Disulfide Produced in Sterile Fish Muscle by *Pseudomonas putrefaciens*

by

Kie Ho Rhee

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 1974

Commencement June 1974

APPROVED:

Associate Professor of Food Science
in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented 4/12/54

Typed by Mary Jo Stratton for Kie Ho Rhee

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. J. S. Lee for his guidance during the course of this investigation and the preparation of this thesis; to Dr. L. M. Libbey for his mass spectral analyses; and to the members of the Department of Food Science and Technology for their numerous contributions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DIMETHYL DISULFIDE PRODUCED IN STERILE FISH MUSCLE BY PSEUDOMONAS PUTREFACIENS

INTRODUCTION

Microorganisms are known to play an exclusive role in seafood spoilage (3). The microorganisms responsible for the spoilage, the types of spoilage products, and the chemical indices of spoilage are either unidentified or still being disputed (6, 20, 23).

Recently the volatile sulfur compounds and the Pseudomonas putrefaciens strains, which produce them, have been claimed to be the most significant in seafood spoilage (13, 31). Herbert et al. (20) identified the sulfur compounds produced by P. putrefaciens strains in cod as hydrogen sulfide, methyl mercaptan and dimethyl sulfide (DMS). Miller et al. (31) further increased the list of sulfur compounds to include dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS).

Factors that influence production of sulfur compounds by P. putrefaciens are not clearly known. Castell et al. (11) indicated that some P. putrefaciens strains would not grow without NaCl. Silverrio and Levin (41), however, noted that NaCl was not required for growth but was needed for the formation of the characteristic reddish-brown pigment.

Preservatives used in seafoods, such as sodium benzoate (42, 44) and ethylenediaminetetraacetic acid (EDTA) (24, 34), have long been claimed to retard spoilage with no apparent effect on the microbial growth.

This investigation was carried out to determine the relationship between the growth and sulfur metabolism of P. putrefaciens and some of the conditions that influence this relationship.

LITERATURE REVIEW

Fish Spoilage

The flesh of a healthy fish is sterile but the gills, gut and skin carry a heavy load of bacteria (5). After a fish dies, the regulatory mechanisms preventing invasion of the tissue by bacteria cease to function. In a whole fish the bacteria initially penetrate the gill tissue, multiply in the vascular system, particularly in the caudal vein and the kidney, and eventually reach into the flesh. Another route is the invasion of the intestinal flora through the body cavity. The microorganisms on skin or slime can also enter the flesh through cuts (39).

Microorganisms, once established in the flesh, would begin to utilize a wide variety of low molecular weight compounds, such as carbohydrates, amino acids, and trimethylamine oxide (TMAO). Beatty and Collins in 1939 (2) observed that the spoilage of fish muscle occurred in two stages. Initially, TMAO was reduced to trimethylamine (TMA) and lactic acid exhausted. This was then followed by the hydrolysis and subsequent decomposition of the proteins. Shewan (38) reported that the proteins were attacked with the subsequent production of ammonia and other malodorous compounds, such as hydrogen sulfide, mercaptans and indole. Herbert et al. (20) stated

that seafoods would become organoleptically unacceptable, as the results of the microbial actions on free amino acids and other components of the muscle extractives, long before the proteins have undergone any noticeable change. Castell and Greenough (9) demonstrated that the spoilage odor could be reproduced by the pure cultures of spoilage microorganisms inoculated into partially hydrolyzed proteins that contained monoamino-monocarboxylic acids.

The spoilage of fish and fishery products is claimed to be solely due to the microbial action, with little contribution from the autolytic enzymes (3). Differentiating the tissue enzyme activity from the microbial action could not be made until fish substrate free of bacteria could be obtained. Lerke et al. (22) and Castell and Greenough (7) used sterile press juice and cooked minced fish muscle, respectively. Although these substrates were useful, they could not fully duplicate the natural conditions.

Herbert and Haight (21) attempted to sterilize fish flesh with ethylene oxide. Lobben and Lee (27), however, perfected a method for an aseptical removal of fish flesh from newly caught fish. The substrate thus obtained maintained the integrity of the tissue, minimized protein denaturation, and maintained pH and buffering capacities of the fish muscle intact (20).

Fish Spoilage Microorganisms

Shewan (39) reported that in fresh seafoods, gram-negative heterotrophs, such as Pseudomonas, Achromobacter and Flavobacterium species, had predominated. The Flavobacterium species, however, had disappeared during low-temperature storage and Pseudomonas and Achromobacter species became predominant.

Not all microorganisms found in fish flesh contribute equally to the fish spoilage. Lerke et al. (23) showed that individual members of the genera Pseudomonas and Achromobacter differed markedly in their spoilage capabilities and only a small portion within this group were the active spoilers. Shewan and his co-workers (40) found that Pseudomonas species were more responsible than Achromobacter for the breakdown of fish muscle and the production of the typical spoilage odor.

The precise events of the seafood spoilage are not clearly known. To be classified a "spoiler," the microorganisms must be able to grow on fish at the refrigeration temperatures, at which fillets are normally held, and must be associated with some objectionable changes in color, flavor, odor or texture (6). Castell et al. in 1949 (11) isolated P. putrefaciens from fresh cod and cod fillets stored at 3°C or frozen. Chai et al. (13) reported that in the fresh seafoods P. putrefaciens strains accounted for no greater than 1% of the total bacteria flora, but after 10 days at 1°C they increased to 30% of the total

viable count. P. putrefaciens strains have been known to have the ability to penetrate fish skin rapidly (11), to produce TMA from TMAO, and to produce volatile sulfur compounds (31).

Chemical Tests for Fish Spoilage

Fish spoilage may be measured by the organoleptic test or by the accumulated end products of bacterial metabolism (19). For any microbial metabolic end products to be used as an index of spoilage, they must be closely correlated with the microbial growth, easy to determine, specific for spoilage, and universally applicable to all fish (3). No method proposed to date, however, meets all of these requirements.

Farber and Lerke (17) proposed a rapid test for predicting the shelf life of fish. The test consisted of the determination of volatile reducing substances (VRS) and trimethylamine nitrogen (TMA-N) in fish press juice after having been inoculated with the test culture and incubated at 31°C for 5 hr. The fish would spoil faster if it contained high proportion of the "spoilers." Castell and Anderson (6), however, indicated that the mesophilic species which rapidly decomposed fish at 25°C had produced no apparent spoilage at 3°C even when the initial inoculum contained a very large number of cells.

Collins (14) indicated that the level of acetic acid in flesh might serve as a rapid estimation of the state of preservation. The test,

however, had no advantage over the TMA test and would be subject to wide variation. Liston et al. (26) observed that both the total volatile acid (TVA) and the total volatile base (TVB) would provide useful measures of spoilage but TVB was the more reliable of the two.

TMA is responsible for the characteristic "fishy" odor and has been used as the traditional index of fish spoilage. Fresh fish muscle contains approximately 0.2 mg TMA-N per 100 g but the TMA level increases with spoilage and fish flesh containing TMA-N of 10-20 mg per 100 g tissue is considered unacceptable (16). During frozen storage dimethylamine (DMA) rather than TMA was produced in the muscle of gadoid species (12). Castell and his co-workers (10) suggested that, at least for the gadoid fish, DMA might be used as a measure of frozen-storage deterioration.

Tarr in 1939 (43) questioned the value of TMA as a quality index. Shaw and Shewan (37) and Lobben and Lee (27), among others, have noted that lower amines such as DMA, TMA and ammonia are not always produced by the organisms causing the strong odors characteristic of spoiling fish.

Role of Sulfur Compounds in Fish Spoilage

As our understanding of the chemical components of the fish muscle and the activities of fish spoiling bacteria increased, the explanation of fish spoilage became no longer simple. Many

microorganisms do not reduce TMAO to TMA but still produce organoleptic spoilage. Shaw and Shewan (37) concluded that a TMA value did not indicate the extent of spoilage but the extent to which the TMA producing organisms had been active.

Castell and Greenough (9) showed that Pseudomonas fragi which neither reduced TMAO nor hydrolyzed protein, nevertheless produced fruity and oniony odors in media containing partially hydrolyzed protein. The organism was also able to produce odors suggesting rotten vegetables from methionine, and hydrogen sulfide-like odors from cysteine, cystine and glutathione. Miller et al. (30) have recently identified the compounds responsible for these various odors in P. fragi-inoculated sterile fish muscle. They were dimethyl sulfide (DMS), acetaldehyde, ethyl acetate, ethyl alcohol, and dimethyl disulfide (DMDS). The strong sulfide odor that persisted during storage was due to the accumulation of methyl mercaptan, DMS and DMDS.

Castell et al. (8) reported that the characteristic potato-like odor in spoiling fish could be reproduced by growing P. perolens in a wide variety of culture media. Protein hydrolysates and many of the amino acids were particularly good substrates both for the growth and odor production. Miller et al. (32) showed that the compound responsible for the musty, potato-like odor was 2-methoxy-3-isopropylpyrazine. P. perolens, in addition, produced other sulfur

compounds such as methyl mercaptan, DMDS and dimethyl trisulfide (DMTS).

Levin (25) reported that the ability to produce hydrogen sulfide was a singularly useful criterion for identifying P. putrefaciens. P. putrefaciens was found to be the only organism capable of producing hydrogen sulfide among the microorganisms isolated from haddock. Herbert et al. (20) quantitatively determined the production of volatile sulfur compounds such as hydrogen sulfide, methyl mercaptan and DMS in spoiling cod muscle. The quantities of the sulfur compounds detected in spoiled fish were well above the odor threshold levels and the hydrogen sulfide-producing Pseudomonas species were responsible for their productions. Miller et al. (31), however, failed to detect DMS in fish flesh inoculated with a pure culture of P. putrefaciens. Instead, they showed the production of hitherto unreported sulfur compound, DMTS, by P. putrefaciens.

Segal and Starkey (36) reported the methyl mercaptan formation as a result of microbial decomposition of methionine. They also found that methyl mercaptan was readily oxidized to DMDS. Miller et al. (32) proposed that further direct oxidation of DMDS without cleavage would produce thiosulfinate ester and it would yield two molecules of unstable sulfenic acid intermediates upon hydrolysis. The oxidation of sulfenic acid, in the presence of hydrogen sulfide, would yield DMTS.

Effect of Preservatives on Fish Spoilage

Fish is a highly perishable commodity. To maintain the freshness of the fish, various antimicrobial agents have been tried, in combination with the low temperature storage, with varying degree of success (47).

Tarr and Bailey (44) found that fish iced with benzoic acid had slightly lower bacterial counts but a markedly lower TMA content than the fish iced without benzoic acid. Tarr and Sunderland (45) further demonstrated that benzoic acid, benzoates and p-hydroxyethylene benzoate suppressed the formation of TMA in fish muscle undergoing spoilage, without significantly affecting the number of bacteria. Fellers and Harvey (18) pointed out that a 2-minute dip in a 0.15% benzoate solution would prolong the keeping quality of fish fillets. Similarly, Debevere and Voets (15) showed that the addition of 0.135 to 0.4% of potassium sorbate in cod fillets also inhibited spoilage.

Recently, ethylenediaminetetraacetic acid (EDTA) has received attention as a fish preservative. Levin (24) demonstrated that EDTA in nutrient broth inhibited the growth of the facultative psychrophiles, *P. putrefaciens*, *P. fragi* and *P. fluorescens*. He found, however, that there was no change in the microbial population. One percent EDTA solution as a dip extended the shelf life of haddock (35), petrale sole, and ocean perch fillets (34), again without any notable effect on the microbial population.

The indirect evidences presented above seem to indicate that the preservatives may selectively inhibit the microbial metabolic activities that lead to fish spoilage, with little effect on their growth potential. The sterile fish system, employed in this laboratory, afforded an opportunity to examine this hypothesis.

MATERIALS AND METHODS

Microorganism

Pseudomonas putrefaciens 17 was selected as the test microorganism because of its ability to produce H_2S (13) and other volatile sulfur compounds (31) in fish. The organism grew readily and was actively proteolytic at refrigeration temperatures and was the most active H_2S producer among several strains of P. putrefaciens obtained from R. E. Levin, Department of Food Science and Technology, University of Massachusetts, Amherst. The cultural and differential characteristics of this organism are summarized in Table 1.

Table 1. Characteristics of P. putrefaciens 17.

1.	Gram-negative and polarly flagellated
2.	Produce H_2S
3.	Liquefy gelatin
4.	Reduce litmus milk and nitrate
5.	Possess deoxyribonuclease, ribonuclease and cytochrome oxidase
6.	Acid not produced from mannitol

Sterile Fish Homogenate

Black rockfish (Sebastes melanops), line-caught off the coast of Newport, Oregon, were immediately killed on board by a blow at the

head, individually wrapped in clean towels to prevent puncture damage to the skin, and packed in ice and transported to the laboratory. After overnight storage at 0°C, the whole fish was washed thoroughly with water, swabbed with 70% ethyl alcohol, and air dried under a sterile laminar flow of air in a sterility test cabinet (BioQuest). After excess moisture had evaporated, a portion of the skin was aseptically removed from a side of the fish along the rectangular incision. Additional incisions were then made within the exposed flesh, and the strips of muscle tissue from this area cut out. The fish muscles were placed in sterile jars and immediately frozen at -30°C until used.

Sterile fish homogenate was prepared by blending one part of fish muscle with two parts of water. NaCl and test chemicals were dissolved in water prior to blending to give the desired final concentrations.

Inoculum

Cells of *P. putrefaciens* 17, grown on trypticase soy (TS) agar (BBL) for 48 hr at 25°C, were collected and suspended in sterile distilled water to give an optical density (O. D.) of approximately 1.0 at 420 nm. The above cell suspension was inoculated and mixed thoroughly into fish homogenate to give approximately 10^4 viable cells per g. Ten-g quantities of this homogenate were dispensed into

sterile, screw-capped vials (Kimble No. 60957, size No. 1) and incubated at 5°C. The replicate vials were removed periodically and the microbial count and the volatile sulfur compounds determined.

Microbial Analyses

Viable Count

The entire contents of the vial were mixed with 10 ml of sterile distilled water to give a 1:1 dilution. A series of 1:10 dilutions were prepared from this mixture in 9 ml of distilled water, and 0.1 ml of the proper dilutions was spread plated in duplicate on solidified TS agar.

The plates were incubated at 25°C and the colonies were counted after 48 hr.

Growth Experiment

Cells of *P. putrefaciens* 17, grown on TS agar for 48 hr at 25°C, were inoculated into tryptone-peptone-yeast extract-glucose-NaCl (TPN) broth and incubated at 25°C for 24 hr. Side arm flasks (Bellco) containing 50 ml TPN broth and various combinations of preservatives were inoculated with a drop of 24 hr cell suspension and incubated at 5°C with shaking in a Psycrotherm Incubator Shaker (New Brunswick Scientific Co., Inc.). Growth was recorded as O.D. at 420 nm. The

preservatives used in this study were sodium benzoate (SB) (Mallinckrodt Chemicals Works), potassium sorbate (PS) (Union Carbide Chemical Company), ethylenediaminetetraacetic acid (EDTA) (Matheson Coleman and Bell), and fumaric acid (FA) (Matheson Coleman and Bell).

In addition, four Pseudomonas species, two Achromobacter species and two Vibrio species were inoculated into media acidified with FA to give the final pH values of 4.0, 4.5, 5.0, 5.5, and 6.0. Growth response was then recorded after a week at 5°C.

Gas-Liquid Chromatography

Varian Aerograph 1200 gas chromatograph with flame ionization detector was used throughout this investigation. A stainless steel column (10 ft x 1/8 in. O.D.), containing 90-100 mesh diatomaceous earth (Anakrom ABS) coated with 10% Carbowax 20 M, was prepared. The column is a slight modification of that used by Maruyama (28) for the identification of volatile sulfur compounds of cooked Brassicaceous vegetables.

Standard curves with peak heights obtained with known concentrations of volatile sulfur compounds added to sterile fish were prepared. The peak heights thus determined were proportional to the concentrations of dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS) (Figure 1).

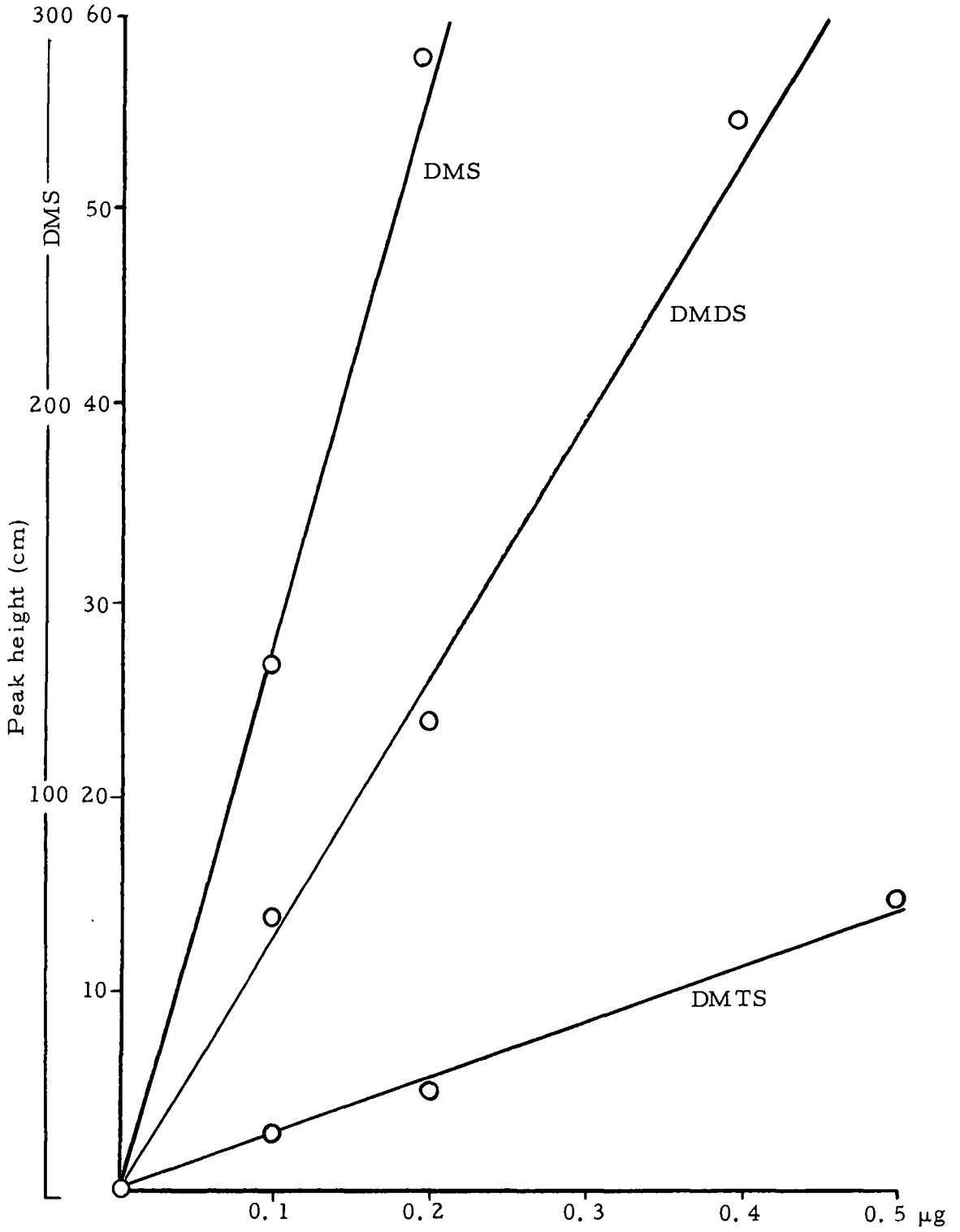

Figure 1. Recorder response for various concentrations of DMS, DMDS and DMTS added to fresh fish.

Sample vials were removed from the incubator at intervals and frozen at -15°C. At the end of an experiment, all vials were subjected to the following gas chromatographic analysis procedure. Seven ml of distilled water was added to 10 g of the sample in the vial and a few milligrams of 1-tetradecanol (Eastman Organic Chemicals) was added to control foaming. The mixture was then acidified with concentrated HCl to pH 3-4 to convert the free amines that might have been present into nonvolatile acid salts and to minimize the interference by the tailing peaks (29).

A gas entrainment on-column trapping procedure, developed by Morgan and Day (33) for the identification of relatively low-boiling compounds, was used in this study. Each sample was tempered at 60°C for 10 min and the volatiles were condensed on the column packing by purging with nitrogen gas at the rate of 12 ml per min for 10 min. The volatiles were trapped in a portion of the column submerged in a Dewar flask containing a slurry of dry ice in 2-methoxyethanol. In quick succession the column was disconnected from the entrainment assembly and connected to the injection port. The oven was then turned on for 5 min at 70°C with the carrier gas off and after the oven temperature had equilibrated the carrier gas was turned on.

The column was operated isothermally at 70°C for 10 min with the nitrogen gas flow rate of 20 ml per min, then the temperature was

programmed to 100°C at 0.5°C per min. The detector and the injector port temperatures were 240° and 190°C, respectively.

Mass Spectroscopy

A Finnigan Model 1015C gas liquid chromatography and mass spectroscopy (GLC-MS) system, which included a Varian Aerograph Model 1400 GLC, was used to identify DMDS. The GLC-MS interface was a Gohlke all glass, jet orifice splitter which allowed the optimal amounts of sample components to pass to the ion source. The operating conditions were: filament current 300 μA, electron voltage 70 eV, and multiplier voltage 1.6 kV. Spectra were scanned from m/e 12 to m/e 275 in 1.0 sec.

RESULTS

Effect of NaCl on *Pseudomonas putrefaciens* Growth

The growth curves of *P. putrefaciens* 17 in TGY broth at 5°C, in the presence of 0 to 5% NaCl, are presented in Figure 2. *P. putrefaciens* 17 grew well without NaCl, but 1 to 2% NaCl seemed to enhance the growth. When 1 to 2% NaCl was present, the length of lag period was shortened and the level of attainable growth increased. With 5% NaCl, the growth was poorer in comparison to the control.

Similar growth experiments in fish homogenates were conducted and the results are presented in Figure 3. In the presence of 5% NaCl, the growth of *P. putrefaciens* 17 was noticeably poor, due to the extended lag period of up to 6 days at 5°C. The growth level after 15 days, however, approached that of 0 and 1.5% NaCl.

Dimethyl Disulfide (DMDS) Production

Table 2 shows the production of DMDS in *P. putrefaciens* 17-inoculated fish homogenate. The DMDS production was noted most in fish homogenate containing 1.5% NaCl after 10 days at 5°C. A typical flame ionization detector (FID) chromatogram of the volatiles produced by *P. putrefaciens* 17 in sterile fish muscle is illustrated in

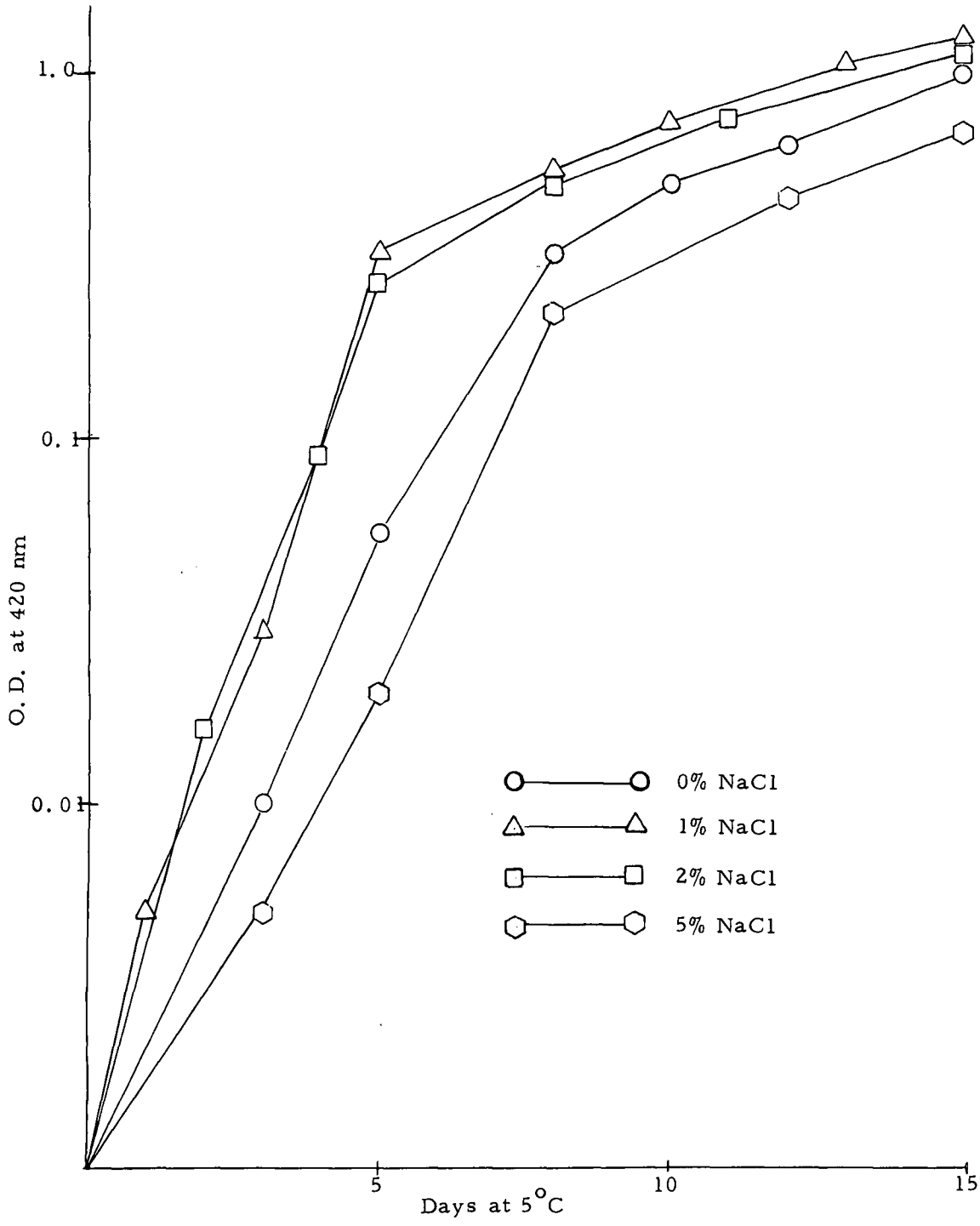

Figure 2. Growth of P. putrefaciens 17 in TPN broth containing various concentrations of NaCl.

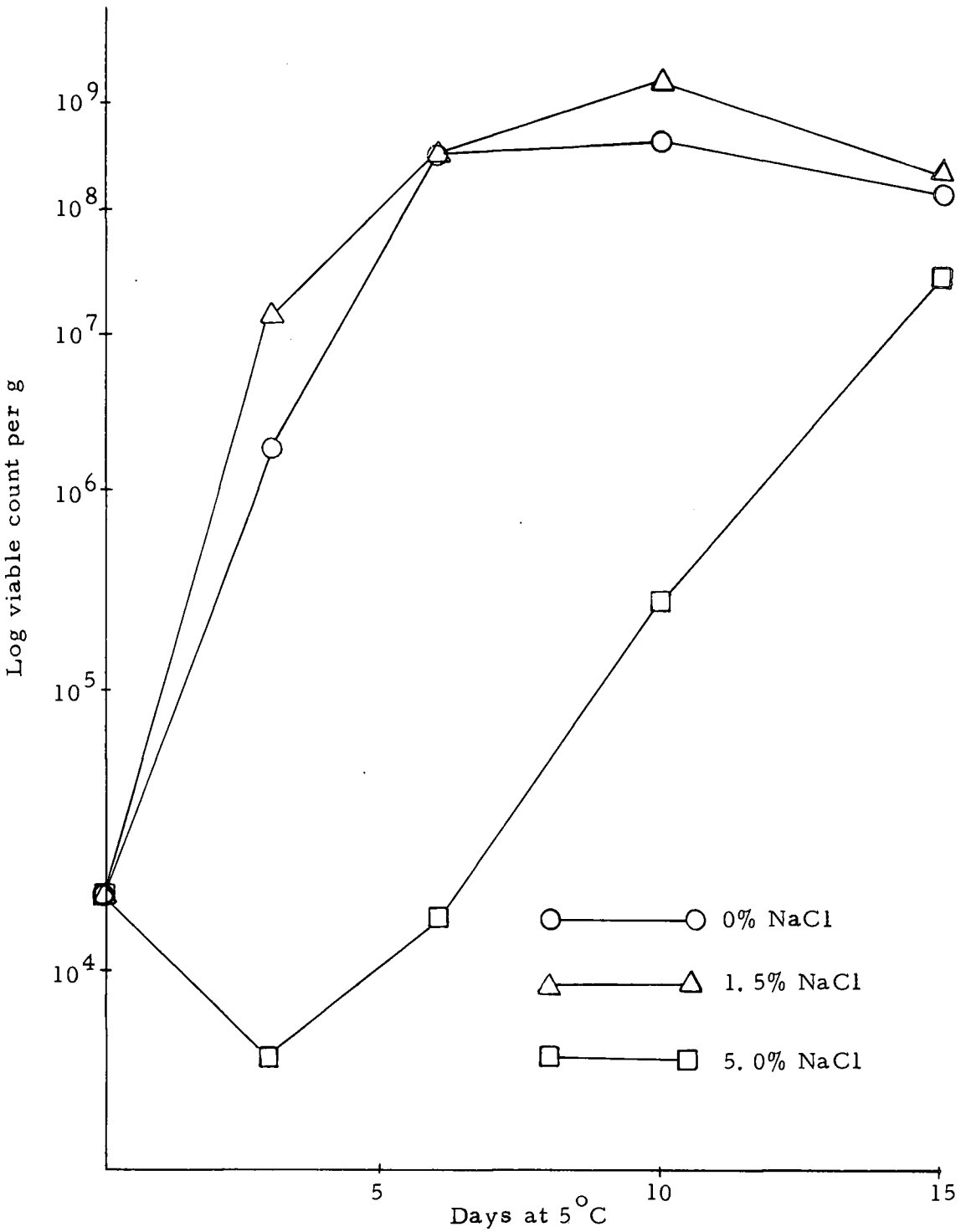

Figure 3. Growth of P. putrefaciens 17 in NaCl-treated fish homogenates.

Table 2. Effect of NaCl on dimethyl disulfide (DMDS) production in P. putrefaciens 17-inoculated sterile fish homogenate.

NaCl (%)	DMDS µg/100 g fish muscle[a]			
	3	6	10	15 days at 5°C
0	0.70	0.85	0.85	0.95
1.5	0.60	0.80	2.00	0.60
5.0	0.80	1.00	1.30	1.05

[a]An average of at least two independent determinations.

Figure 4. The production of DMDS was poor in fish homogenate containing no NaCl and in 5.0% NaCl. The eventual reduction of DMDS level after 15 days, which was observed consistently, might have been due to further transformation of DMDS into other sulfur compounds, as noted by Miller et al. (32).

No DMDS production was noted in sterile uninoculated fish, indicating that P. putrefaciens 17 was solely responsible for its production.

Effect of Sodium Benzoate (SB) and Potassium Sorbate (PS)

The growth curves of P. putrefaciens 17 in TPN broth at 5°C in the presence of SB and PS are presented in Figure 5. The rate of growth was reduced in proportion to the increasing concentrations of SB and PS. Although the growth rates were reduced, cells in the presence of 0.05% SB or 0.1% PS eventually reached the maximum

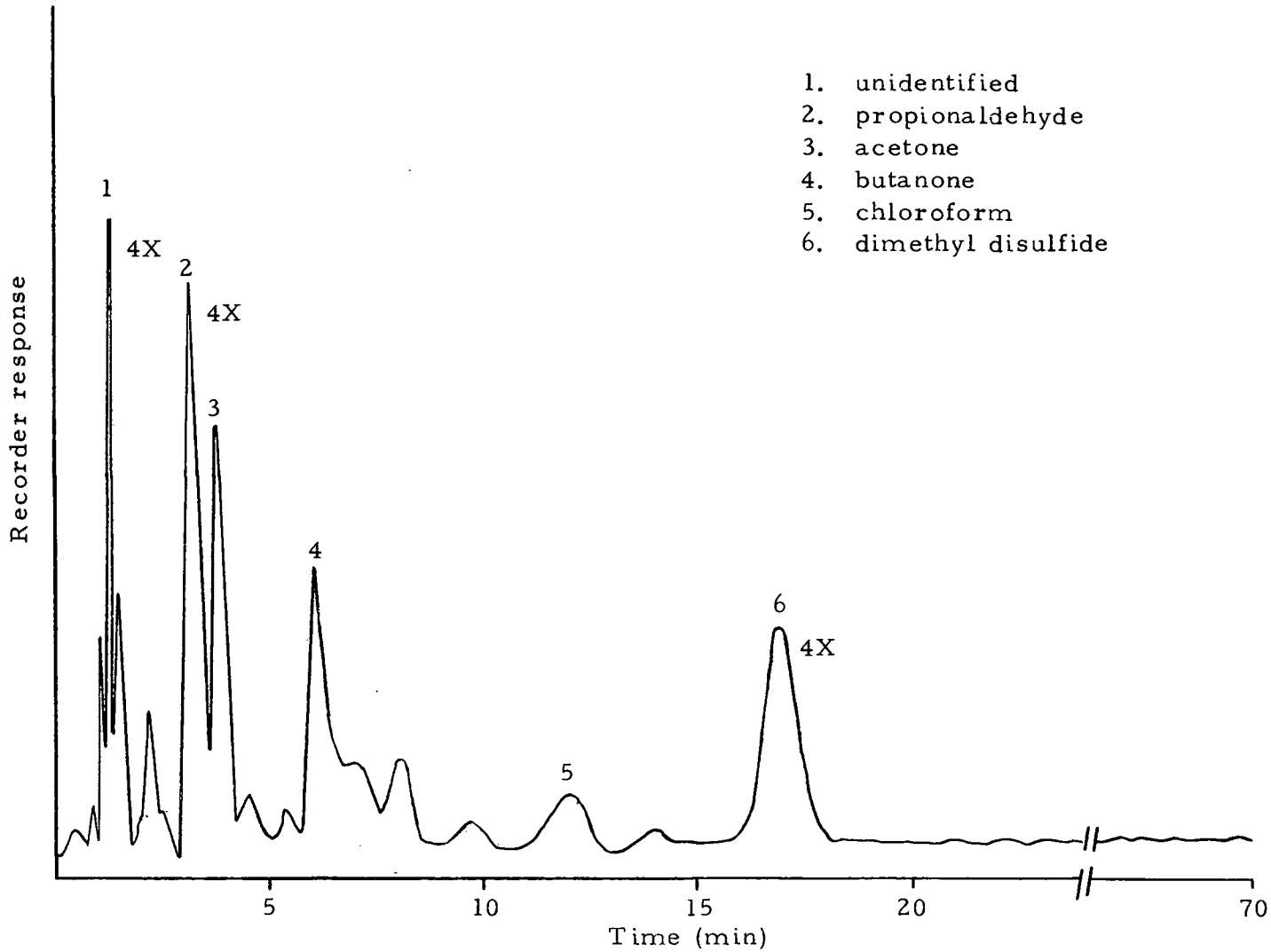

Figure 4. FID chromatogram of volatiles produced by P. putrefaciens 17 in sterile fish muscle incubated at 5°C for 15 days.

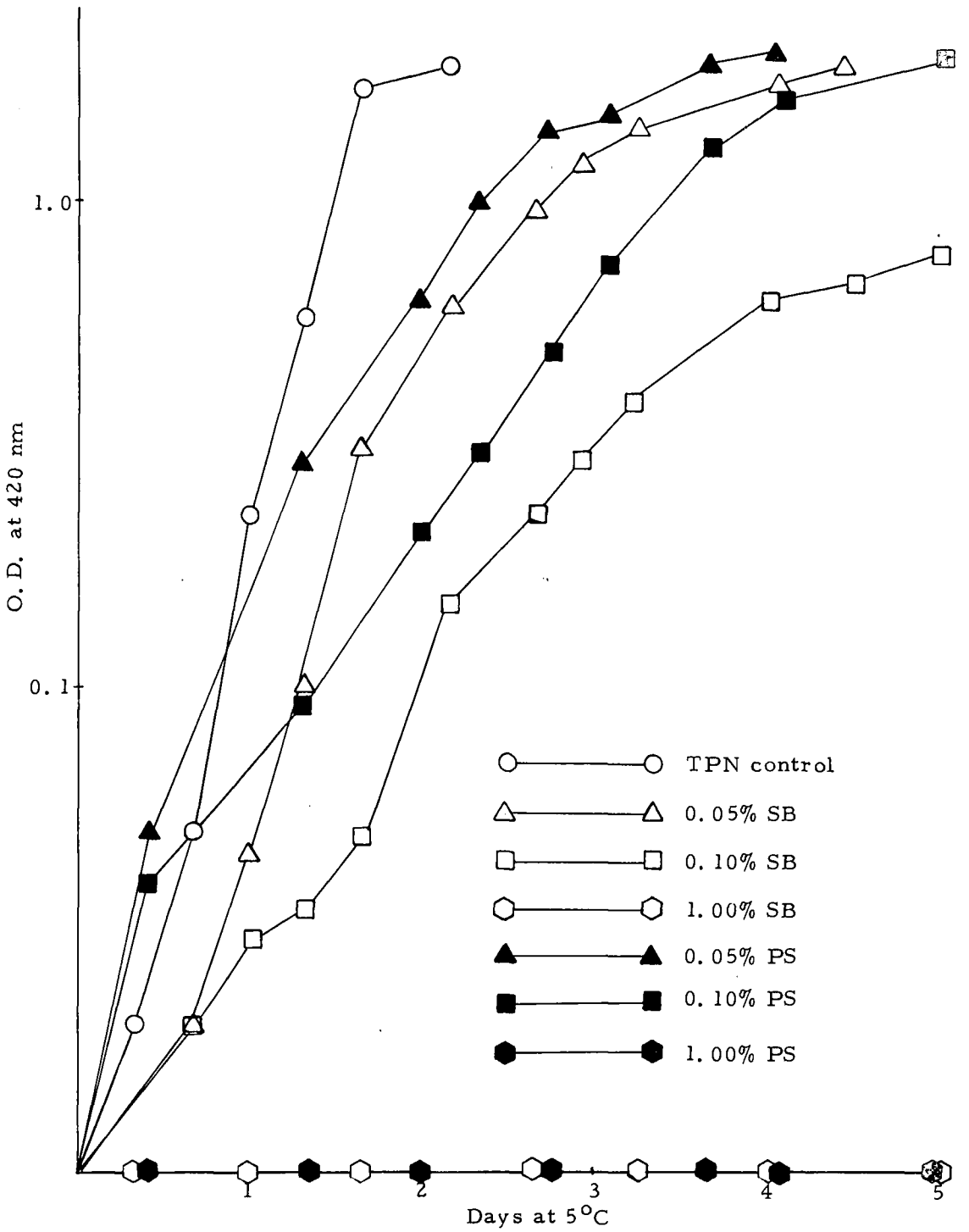

Figure 5. Growth of P. putrefaciens 17 in TPN broth containing sodium benzoate (SB) and potassium sorbate (PS).

growth level of the control. SB was more effective than PS against P. putrefaciens 17; however, at the concentrations of 1.0%, both SB and PS completely inhibited the growth of P. putrefaciens 17 in broth at 5°C.

The growth curves of P. putrefaciens 17 in fish homogenates in the presence of SB and PS are presented in Figure 6.

The growth rate of P. putrefaciens 17 was not affected by 0.05% SB or 0.1% PS but the maximum level of growth was approximately one log scale lower with 0.05% SB. When the concentration of SB was increased to 0.1%, the lag period as well as the maximum growth was noticeably influenced.

In contrast to the limited effects on growth, the SB, and to a lesser extent PS, markedly influenced the production of DMDS in inoculated fish (Table 3). The inhibition of DMDS production was partial with 0.05% SB and 0.1% PS, but 0.1% SB completely inhibited the production of DMDS in inoculated fish homogenate during 15 days of observation at 5°C.

The pigment formation was rapid in fish homogenate without preservatives; however, the pigmentation was delayed in fish samples treated with preservatives. The reddish-brown pigments became apparent after 10 and 15 days in fish homogenates, with PS and SB, respectively, compared to 6 days for the control.

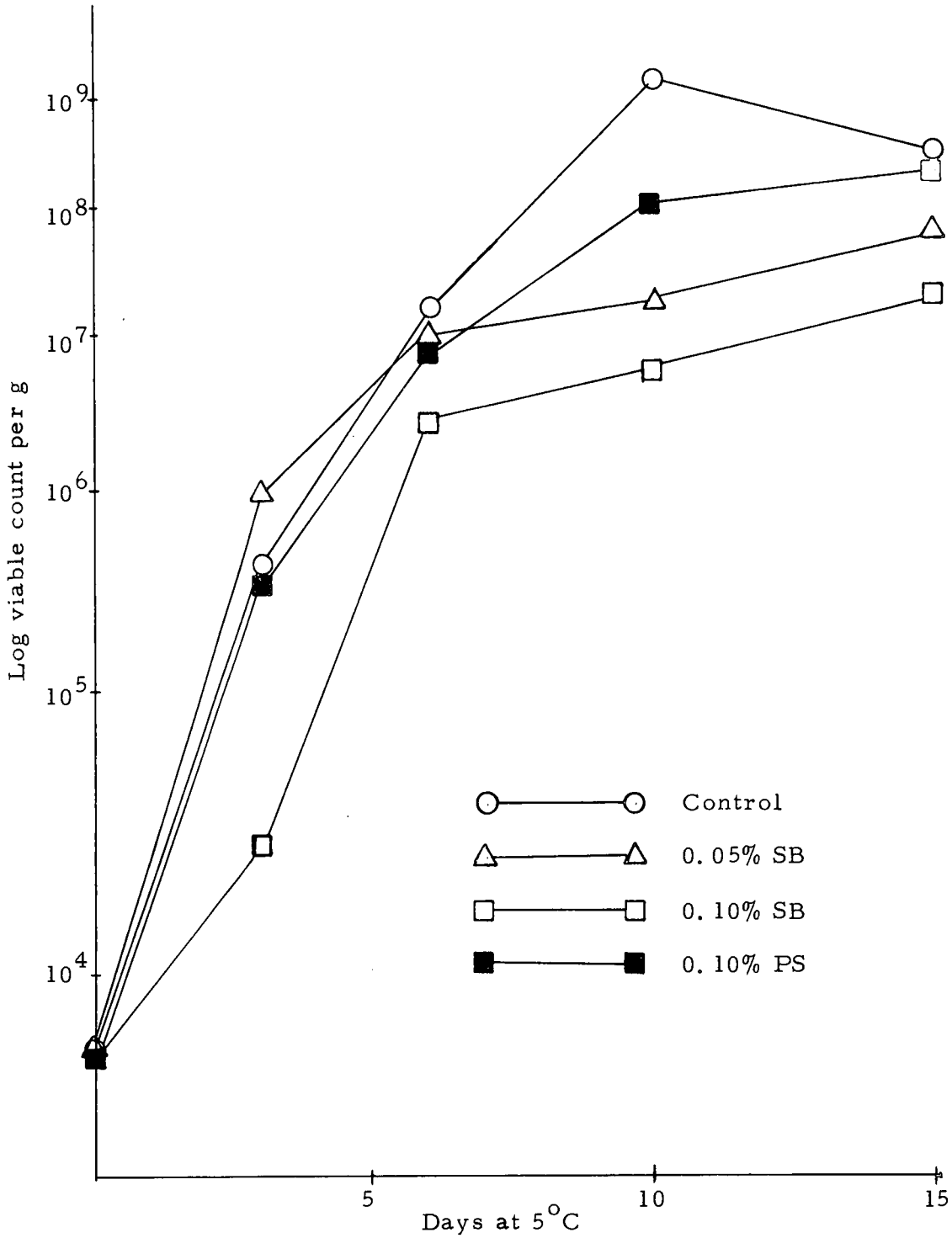

Figure 6. Growth of P. putrefaciens 17 in fish homogenate treated with SB and PS.

Table 3. DMDS production in P. putrefaciens 17-inoculated sterile fish homogenate treated with SB and PS.

Preservative[a] (%)	DMDS µg/100 g fish muscle[b]			
	3	6	10	15 Days at 5°C
none	0.60	0.80	2.00	0.60
0.05 SB	0.20	0.20	0.75	0.25
0.10 SB	0.20	0.30	0.30	0.20
0.10 PS	0.40	0.45	1.00	1.80

[a]SB = Sodium benzoate; PS = Potassium sorbate.

[b]An average of at least two independent determinations.

Effect of Acid and Sodium Benzoate

The antimicrobial effectiveness of sodium benzoate (SB) increases in proportion to the increase in acidity (4). SB, therefore, is usually applied in combination with acidifying agent.

In order to simulate the commercial practice, a 1:1 mixture of SB and fumaric acid (FA) was prepared, and this mixture was tested against P. putrefaciens 17.

When 0.05% and 0.1% of the mixture were incorporated into TPN broth, the pH values were reduced to 5.4 and 4.6, respectively. P. putrefaciens 17 failed to grow in these media at 5°C.

The same concentrations of SB-FA mixture were incorporated into fish homogenate and the growth of P. putrefaciens 17 determined. The pH of fish homogenate was reduced from 7.0 to 6.6 by the 0.05%

mixture and to 6.4 by the 0.1% mixture. In contrast to that observed in TPN broth, P. putrefaciens 17 grew well in the presence of 0.05% SB-FA mixture, but failed to grow in the presence of 0.1% mixture (Figure 7).

The difference in the growth responses of P. putrefaciens 17 in broth and fish homogenate may be due to the greater buffering capacity of the fish protein, which would reduce the effectiveness of FA.

P. putrefaciens 17 and Pseudomonas species were the least affected by the lower pH (Table 4). Compared to Achromobacter, which are commonly associated with seafoods, the growth limiting pH of Pseudomonas species was 0.5 to 1.0 units lower.

Table 4. Effect of pH on the growth of selected microorganisms in TPN at 5°C.

Microorganism[a]	pH[b]					
	4.0	4.5	5.0	5.5	6.0	6.8 (control)
13 (Pseudomonas I)	-	+	+	+	+	+
15 (Pseudomonas II)	-	+	+	+	+	+
18 (Pseudomonas III)	-	+	+	+	+	+
21 (Pseudomonas IV)	-	-	-	+	+	+
43 (Achromobacter)	-	-	-	-	+	+
44 (Achromobacter)	-	-	-	-	+	+
VP-S (Vibrio)[c]	-	-	+	+	+	+
VP8700 (Vibrio)[c]	-	-	+	+	+	+
P. putrefaciens 17	-	-	+	+	+	+

[a] From stock culture
[b] + = growth; - = no growth
[c] Incubated at 25°C

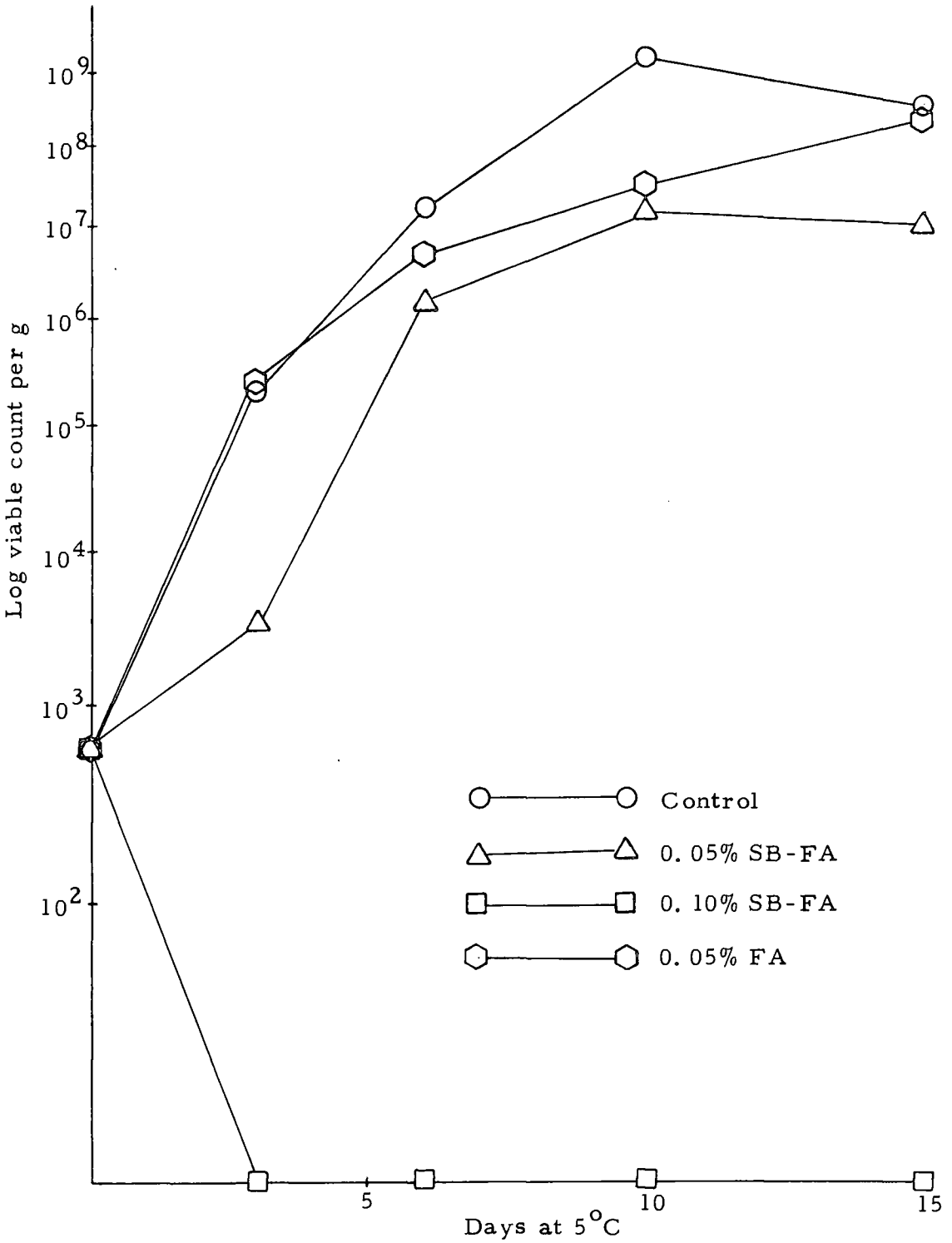

Figure 7. Growth of P. putrefaciens 17 in fish homogenates treated with SB-FA or FA.

The DMDS production in fish homogenate treated with SB-FA mixture was low, reflecting the poor growth of P. putrefaciens 17 in such preparation (Table 5).

Table 5. DMDS production in P. putrefaciens 17-inoculated fish homogenate, treated with sodium benzoate (SB) and fumaric acid (FA) mixture.

SB-FA (%)	DMDS µg/100 g fish muscle[a]			
	3	6	10	15 days at 5°C
control	0.60	0.80	2.00	0.60
0.05	0.50	0.65	0.80	0.80
0.10	0.50	0.45	0.50	0.20

[a]An average of at least two independent determinations.

Effect of EDTA

Ethylenediaminetetraacetic acid (EDTA) was claimed to be effective against P. putrefaciens species (24). Figure 8 shows the growth curves of P. putrefaciens 17 in fish homogenate treated with 0.05% and 0.1% EDTA. In contrast to SB or PS, EDTA only extended the length of lag period. After this delay, the cell count increased rapidly and reached the level of the control at the 15th day.

The DMDS production in the presence of EDTA, shown in Table 6, was also poor.

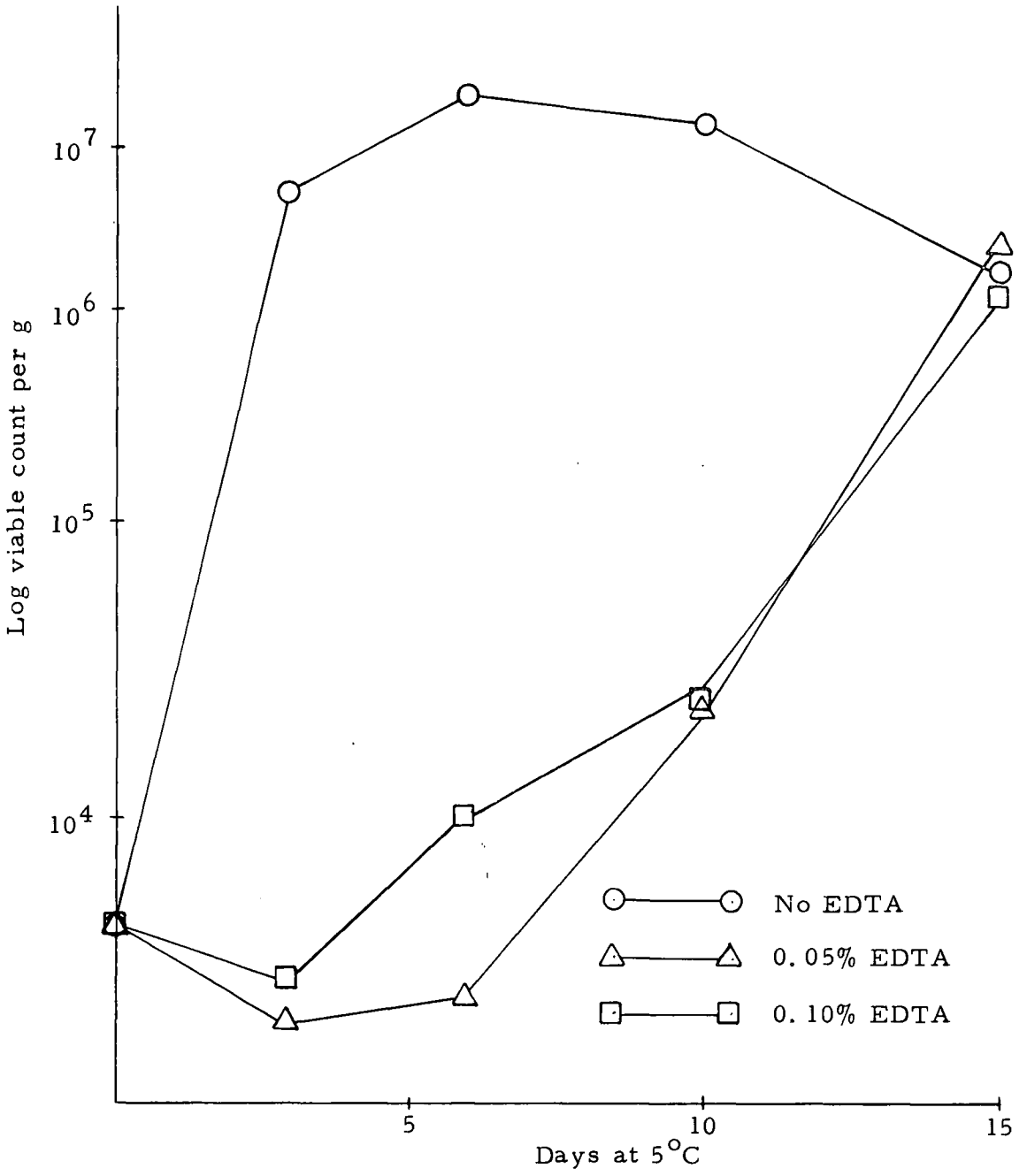

Figure 8. Growth of P. putrefaciens 17 in fish homogenates treated with Na_4EDTA.

Table 6. DMDS production by P. putrefaciens 17 in fish homogenates treated with Na_4EDTA.

EDTA (%)	DMDS µg/100 g fish muscle[a]			
	3	6	10	15 days at 5°C
none	0.60	0.80	2.00	0.60
0.05	0.50	0.60	0.30	0.30
0.10	0.45	0.60	0.45	0.40

[a]An average of at least two independent determinations.

DISCUSSION

Dimethyl disulfide (DMDS) was selected as the spoilage indicator, as it could be detected quantitatively by the column employed, and its production closely paralleled that of microbial growth.

Other sulfur compounds were undoubtedly produced by P. putrefaciens. The low molecular weight sulfur compound, methyl mercaptan, which could not be detected due to its extreme volatility, was shown to be the precursor of DMDS formation (36). The trisulfide compound, dimethyl trisulfide (DMTS), which was reported to have been produced by P. putrefaciens culture (31), was not detected during this study. The storage period of 15 days was perhaps too short to detect DMTS production.

DMDS was also the most malodorous compound among all the chemicals suggested as the indices of spoilage. The odor threshold of DMDS was 7.6 ppb (46) and the respective odor thresholds of spoilage products such as acetic acid and trimethylamine (TMA) were 24.3 ppm and 1.7 ppm respectively (1).

Within the legally permissible concentrations, potassium sorbate (PS) had little or no effect on the growth and DMDS production of P. putrefaciens 17. Sodium benzoate (SB) and EDTA, on the other hand, showed some promise.

SB suppressed the growth of P. putrefaciens 17 when the effective concentration was increased. Either by increasing the concentration or by providing acidic conditions which increased the cell permeability, the growth rate as well as the maximum level of growth could be reduced. More significantly, the production of DMDS was retarded by low concentrations of SB, which was not sufficient to suppress the microbial growth.

EDTA acted differently on P. putrefaciens 17. In the presence of EDTA, the length of lag period was increased but the ultimate level of growth reached was the same as that of the control. The production of DMDS in the presence of EDTA was slowed but was not completely inhibited.

SUMMARY AND CONCLUSION

Sterile fish muscle of the black rockfish (Sebastes melanops) was inoculated with the pure culture of P. putrefaciens 17 and dimethyl disulfide (DMDS) production was determined during storage at 5°C. The results and conclusions may be summarized as follows:

1. DMDS production in inoculated fish closely paralleled that of P. putrefaciens 17 growth. The levels of DMDS produced at 3, 6, 10 and 15 days at 5°C were 0.60, 0.80, 2.00 and 0.60 μg per 100 g, respectively, in fish muscle treated with 1.5% NaCl. The cell counts of P. putrefaciens 17 at the respective incubation periods were 1.5×10^7, 5.2×10^8, 2.0×10^9 and 2.6×10^8 per g fish muscle. No DMDS was produced in sterile uninoculated fish.
2. Contrary to the earlier claim, NaCl was not required for the growth or DMDS production of P. putrefaciens. NaCl concentrations of 1 to 2%, however, stimulated both the growth and DMDS production.
3. Sodium benzoate (SB) and ethylenediaminetetraacetic acid (EDTA) inhibited the growth and DMDS production of P. putrefaciens 17. In the presence of 0.1% SB, the growth rate and the maximum growth level of P. putrefaciens 17 were approximately 65% and 1.7% of the respective controls. The

maximum level of DMDS produced in the presence of 0.1% SB was 0.3 μg compared to that of 2.0 μg for the control. In the presence of 0.1% EDTA, the inoculated P. putrefaciens did not assume the logarithmic growth until after 6 days at 5°C. The ultimate level of growth, however, was the same as the control.

4. Potassium sorbate (PS) at 0.1% concentration had very little effect on the growth or DMDS production of P. putrefaciens 17.

BIBLIOGRAPHY

1. Baker, R. A. Threshold odors of organic chemicals. J. Amer. Water Works Assoc. 55:913-916. 1963.

2. Beatty, S. A. and Collins, V. K. Studies of fish spoilage. VI. The breakdown of carbohydrates, proteins and amino acids during spoilage of cod muscle press juice. J. Fish. Res. Bd. Canada 4:412-423. 1939.

3. Beatty, S. A. and Gibbons, N. E. The measurement of spoilage in fish. J. Biol. Bd. Canada 3:77-91. 1937.

4. Bosund, I. The bacteriostatic action of benzoic and salicylic acids. V. Influence of pH on the total uptake of benzoic acid by Proteus vulgaris and baker's yeast. Physiol. Plant. 13:793-799. 1960.

5. Burgess, G. H. O. and Shewan, J. M. Intrinsic and extrinsic factors affecting the quality of fish. In: Proteins as human foods, ed. by R. A. Lawrie. The AVI Publishing Company Inc., Westport, Conn., 1970. p. 186-199.

6. Castell, C. H. and Anderson, G. W. Bacteria associated with spoilage of cod fillets. J. Fish. Res. Bd. Canada 7:370-377. 1948.

7. Castell, C. H. and Greenough, M. F. The action of Pseudomonas on fish muscle. 1. Organisms responsible for odours produced during incipient spoilage of chilled fish muscle. J. Fish. Res. Bd. Canada 14:617-625. 1957.

8. Castell, C. H., Greenough, M. F., and Jenkin, N. L. The action of Pseudomonas on fish muscle. 2. Musty and potato-like odours. J. Fish. Res. Bd. Canada 14:775-782. 1957.

9. Castell, C. H. and Greenough, M. F. The action of Pseudomonas on fish muscle. 4. Relation between substrate composition and the development of odours by Pseudomonas fragi. J. Fish. Res. Bd. Canada 16:21-31. 1959.

10. Castell, C. H., Neal, W., and Smith, B. Formation of DMA in stored frozen sea fish. J. Fish. Res. Bd. Canada 27:1685-1690. 1970.

11. Castell, C. H., Richards, J. F., and Wilmot, I. Pseudomonas putrefaciens from cod fillets. J. Fish. Res. Bd. Canada 7:430-431. 1949.

12. Castell, C. H., Smith, B., and Neal, W. Production of DMA in muscle of several species of Gadoid fish during frozen storage, especially in relation to presence of dark muscle. J. Fish. Res. Bd. Canada 28:1-5. 1971.

13. Chai, T., Chen, C., Rosen, A., and Levin, R. E. Detection and incidence of specific species of spoilage bacteria on fish. II. Relative incidence of Pseudomonas putrefaciens on haddock fillets. Appl. Microbiol. 16:1738-1741. 1968.

14. Collins, V. K. Studies of fish spoilage. VIII. Volatile acid of cod muscle press juice. J. Fish. Res. Bd. Canada 5:197-202. 1941.

15. Debevere, J. M. and Voets, J. P. Influence of some preservatives on the quality of prepacked cod fillets in relation to the oxygen permeability of the film. J. Appl. Bact. 35:351-356. 1972.

16. Dyer, W. J. and Mounsey, Y. A. Amines in fish muscle. II. Development of TMA and other amines. J. Fish. Res. Bd. Canada 6:359-367. 1945.

17. Farber, L. and Lerke, P. Studies on the evaluation of freshness and on the estimation of the storage life of raw fishery products. Food Tech. 15:191-196. 1961.

18. Fellers, C. R. and Harvey, E. W. Effect of benzoated brine dips on keeping quality of fish fillets. Food Res. 5:1-12. 1940.

19. Good, C. M. and Stern, J. A. Effect of iced and frozen storage upon the trimethylamine content of flounders. Food Tech. 9:327-331. 1955.

20. Herbert, R. A., Hendrie, M. S., Gibson, D. M., and Shewan, J. M. Bacteria active in the spoilage of certain sea foods. J. Appl. Bact. 34:41-50. 1971.

21. Herbert, R. A. and Haight, R. D. Effects of ethylene oxide on the tissue and flora of fresh and freeze dried cod fillets. J. Appl. Bact. 30:224. 1967.

22. Lerke, P., Adams, R., and Farber, L. Bacteriology of spoilage of fish muscle. I. Sterile press juice as a suitable experimental medium. Appl. Microbiol. 11:458-462. 1963.

23. Lerke, P., Adams, R., and Farber, L. Bacteriology of spoilage of fish muscle. III. Characterization of spoilers. Appl. Microbiol. 13:625-630. 1965.

24. Levin, R. E. The effectiveness of EDTA as a fish preservative. J. Milk Food Tech. 30:277-283. 1967.

25. Levin, R. E. Detection and incidence of specific species of spoilage bacteria on fish. I. Methodology. Appl. Microbiol. 16:1734-1737. 1968.

26. Liston, J., Chapel, J. G., and Stern, J. A. The spoilage of Pacific Coast rockfish. 1. Spoilage in ice storage. Food Tech. 15:19-22. 1961.

27. Lobben, J. C. and Lee, J. S. Roles of microorganisms in the deterioration of rockfish. Appl. Microbiol. 16:1320-1325. 1968.

28. Maruyama, F. T. Identification of dimethyl trisulfide as a major aroma component of cooked Brassicaceous vegetables. J. Food Sci. 35:540-543. 1970.

29. Miller, A. III, Scanlan, R. A., Lee, J. S., and Libbey, L. M. Volatile compounds produced in ground muscle tissue of cannery rockfish (Sebastes pinniger) stored on ice. J. Fish. Res. Bd. Canada 29:1125-1129. 1972.

30. Miller, A. III, Scanlan, R. A., Lee, J. S., and Libbey, L. M. Identification of the volatile compounds produced in sterile fish muscle (Sebastes melanops) by Pseudomonas fragi. Appl. Microbiol. 25:952-955. 1973.

31. Miller, A. III, Scanlan, R. A., Lee, J. S., and Libbey, L. M. Volatile compounds in sterile fish muscle (Sebastes melanops) by Pseudomonas putrefaciens, Pseudomonas fluorescens, and an Achromobacter species. Appl. Microbiol. 26:18-21. 1973.

32. Miller, A. III, Scanlan, R. A., Lee, J. S., Libbey, L. M., and Morgan, M. E. Volatile compounds produced in sterile fish

muscle (Sebastes melanops) by Pseudomonas perolens. Appl. Microbiol. 25:257-261. 1973.

33. Morgan, M. E. and Day, E. A. Simple on-column trapping procedure for gas chromatographic analysis of flavor volatiles. J. Dairy Sci. 48:1382-1384. 1965.

34. Pelroy, G. E. and Seman, J. P., Jr. Effect of EDTA treatment on spoilage characteristics of petrale sole and ocean perch fillets. J. Fish. Res. Bd. Canada 26:2651-2657. 1969.

35. Power, H. E., Sinclair, R., and Savagaon, K. Use of EDTA compounds for the preservation of haddock fillets. J. Fish. Res. Bd. Canada 25:2071-2082. 1968.

36. Segal, W. and Starkey, R. L. Microbial decomposition of methionine and identity of the resulting sulfur products. J. Bact. 98:908-913. 1969.

37. Shaw, B. G. and Shewan, J. M. Psychrophilic spoilage bacteria of fish. J. Appl. Bact. 31:89-96. 1968.

38. Shewan, J. M. The bacteriology of fresh and spoiling fish and some related chemical changes. In: Recent advances in food science, ed. by J. Hawthorn and J. M. Leitch. London, Butterworths, 1962. p. 167-193.

39. Shewan, J. M. The microbiology of fish and fishery products - A progress report. J. Appl. Bact. 34:299-315. 1971.

40. Shewan, J. M., Hobbs, G., and Hodgkins, W. The Pseudomonas and Achromobacter groups of bacteria in the spoilage of marine white fish. J. Appl. Bact. 23:463-468. 1960.

41. Silverrio, R. and Levin, R. E. Evaluation of methods for determining the bacterial population of fresh fillets. J. Milk Food Tech. 30:242-246. 1967.

42. Stewart, J. E. and Castell, C. H. Inhibition of bacterially produced trimethylamine. J. Fish. Res. Bd. Canada 17:595-596. 1960.

43. Tarr, H. L. A. The bacterial reduction of TMAO to TMA. J. Fish. Res. Bd. Canada 4:367-377. 1939.

44. Tarr, H. L. A. and Bailey, B. E. Effectiveness of benzoic acid ice for fish preservation. J. Fish. Res. Bd. Canada 4:327-334. 1939.

45. Tarr, H. L. A. and Sunderland, P. A. The role of preservatives in enhancing the keeping quality of fresh fillets. Fish. Res. Bd. Canada Prog. Rept. No. 39, p. 13-16. 1939.

46. Wilby, F. U. Variation in recognition odor threshold of a panel. J. Air Pollution Control Assoc. 19:96-100. 1969.

47. Yukio, T. and Buhei, Z. Spoilage of fish and chemical preservation. Adv. Food Res. 7:41-82. 1957.