

Roles of Microorganisms in the Deterioration of Rockfish¹

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Seven species of microorganisms indigenous to fish were inoculated into sterile fish muscle, obtained from *Sebastes alutus* and *Sebastes alascanus*, and the production of total volatile acids (TVA) and total volatile bases (TVB) was determined during storage at 1 C. No production of TVA and TVB was noted in sterile fish, fish inoculated with *Pseudomonas* type III species (PIII-985), or fish inoculated with *Flavobacterium* (F-1070). The inactivity of PIII-985 was due to its low maximal growth level, whereas the inactivity of F-1070 was due to its slow growth rate. *Pseudomonas* type I species (PI-950), *Pseudomonas* type II species (PII-952), and two *Achromobacter* (*Acinetobacter*) species (A-981 and A-70) grew and actively produced TVA and TVB. TVB levels increased steadily even after the microbial number reached a maximum, whereas TVA levels declined toward the end of the storage period. It appeared that a minimal microbial growth level of 10⁸ cells/g was needed before detectable levels of TVA or TVB could be produced. Regardless of genera, microorganisms which grew well in fish were able to produce TVA and TVB.

The spoilage of fish is due to microbial action, with little or no contribution from autolytic enzymes (3, 5, 11, 18, 23, 25, 28).

Microbial species primarily associated with fish spoilage have been found to belong to the genera *Pseudomonas*, *Achromobacter*, and *Flavobacterium* (1, 9, 20, 27). Little is known, however, of the relative roles of each genus in the spoilage of fish.

To differentiate the roles of *Pseudomonas*, *Achromobacter*, and *Flavobacterium* in degradative processes, cultures of representative species were inoculated into sterile fish muscle obtained by aseptic exsection. We measured and compared production of total volatile acids (TVA) and total volatile bases (TVB) in these samples at 1 C.

MATERIALS AND METHODS

Fish substrate. Two species of rockfish of the family *Scorpaenidae*, *Sebastes alutus* and *Sebastes alascanus*, were used in this study. *Sebastes* samples were obtained from the Seafoods Laboratory of the Department of Food Science and Technology, Oregon

State University, Astoria, Ore. *Sebastes alutus* samples were obtained by otter trawl on board the R. V. Yaquina, an oceanography research vessel of Oregon State University. A sudden depletion of *S. alutus* species during the course of this study necessitated the substitution of a related species, *S. alascanus*. Although no systematic comparison between the two fish species could be made under this circumstance, the TVA and TVB values of sterile fish muscle did not change in either species during storage at 1 C. A comparative study by Liston et al. (22) with Pacific rockfish species has shown that the differences between the species are minor.

Sterile fish flesh was obtained in the following manner. The whole fish was washed in running water and thoroughly swabbed with 95% ethyl alcohol. A portion of skin was aseptically cut loose from one side of the fish and pulled off. A section of exposed flesh was aseptically excised under an ultraviolet lamp. Flesh samples from several fish were pooled and ground in a sterile meat grinder.

The sterility of the sample was tested in duplicate by inoculating 10 g of ground fish from each lot of 500 g into 90 ml of TPN broth containing 5% peptone (Difco), 2.5% yeast extract (Difco), 1% glucose, and 5% NaCl. The broth was then incubated at 27 C for 48 hr or longer. The contaminated lot produced turbidity within 48 hr, and samples that did not produce turbidity within 48 hr remained unchanged during subsequent incubation.

To initiate the experiment with samples that retained freshness, designated microbial cultures were

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inoculated before the outcome of the sterility test was known. When the fish homogenate proved to be unsterile, the experiment was discarded and new samples were obtained. When freshly caught fish were used, however, the technique yielded sterile samples in 9 of 12 trials.

Microbial inoculum. The microorganisms used in our study, *Pseudomonas* (PI-950, PII-952, and PIII-985), *Achromobacter* (A-981 and A-70), and *Flavobacterium* (F-1070), were isolated from ocean perch by Lee et al. (20). They were classified according to Shewan et al. (26), except for *Pseudomonas* PIII-985. We did not attempt to differentiate further between *Pseudomonas* types III and IV. The criterion separating the two types is based on alkaline or neutral reaction on Hugh-Leifson medium. Such differentiation is difficult due to various levels between neutral and frank alkaline reactions.

To minimize contamination by media, microbial inocula were prepared from surface growth. The cultures were grown at 27 C in TPN broth. After two successive transfers in broth, 1 ml of the active growth was spread on TPN agar and was incubated for 24 hr at 27 C. Cells were scraped off the agar and were suspended in sterile distilled water; the concentration was adjusted to approximately 10^8 cells/ml by optical density. The ground fish was inoculated with this suspension to give an initial microbial load of 10^6 cells/g in order to simulate the level of microbial contamination in fresh fillet. After thorough mixing, 10-g samples of fish were weighed into vials. Inoculum prepared by this method contained the minimal amount of impurities from the growth media, and the water added to the fish was less than 0.001 ml/g. Vials were stored at 1 C for periods up to 32 days. During this period, duplicate vials were removed at designated intervals for TVB, TVA, and viable count determinations.

Viable count. Viable counts were made by the drop plate technique with 0.2% peptone (Difco) water as the diluent (20). Portions (0.1 ml) of a series of dilutions were plated on TPN agar, and viable counts were made after 48 hr at 27 C. With PI-950 and PII-952, however, 24 hr of incubation was sufficient.

Chemical tests. TVB and TVA were chosen as the chemical indices of spoilage because of their general acceptance (3, 5, 7, 8, 14, 16, 17). Procedures were modified in order to accommodate the reduced sample size. Modified test procedures were as follows.

TVB. The procedure was essentially that of Stansby et al. (29). A 10-g amount of fish was placed in 25 ml of 60% ethyl alcohol, and four glass beads were added. The mixture was then shaken vigorously, allowed to stand for 1 hr, and centrifuged; the supernatant fluid was decanted into a 50-ml volumetric flask. The precipitate was shaken with 10 ml of 60% ethyl alcohol and was centrifuged; the supernatant fluid was added to the volumetric flask. This procedure was repeated, and the total volume was made to 50 ml with 60% ethyl alcohol. The ethyl alcohol extract was placed in a 100-ml semimicro-Kjeldahl flask containing 1.25 g of sodium tetraborate (99.5%), four glass beads, and four drops of Antifoam 20 (General Electric). The flask was connected to a condenser and

was heated with a gas microburner. The first 20 ml of the condensate was collected in 5 ml of 4% boric acid and was titrated with 0.05 N HCl, with methyl red-bromocresol green as the indicator.

TVA. The method used was a modification of the procedure reported by the Association of Official Agricultural Chemists (2). A 10-g sample of fish was placed in a 125-ml Erlenmeyer flask containing 30 ml of CO₂-free distilled water and was shaken; then 5 ml of 0.1 N H₂SO₄ was added. An 8-ml amount of 20% phosphotungstic acid was added to this mixture, and the total weight was made to 65 g by the addition of water. After thorough mixing, the content was filtered through Whatman no. 2 filter paper, and 30 ml of the filtrate was placed in a 125-ml distillation flask. The filtrate was then acidified with an equal volume of 0.1 N H₂SO₄ and was steam-distilled. The first 40 ml of the distillate was titrated with 0.01 N NaOH, with 1% phenolphthalein solution as the indicator.

RESULTS AND DISCUSSION

Sterile fish. After 62 days, the TVB level of sterile fish stored at 1 C was 7.6 mg of nitrogen/100 ml, as compared to the original level of 4.1. The TVA level, on the other hand, remained at 2 to 3 throughout the 62-day storage period.

The role of autolytic enzymes in the degradation of fish muscle has been regarded as minor (4, 5, 11, 18, 23, 24). Our findings from sterile fish tend to substantiate these claims.

Our findings also indicate that, under the conditions tested, TVA and TVB values are in fact a result of microbial action. The values may serve, therefore, to indicate degradative activities depending on the particular microorganisms involved.

Naturally contaminated fillet. The study with the commercial fillet was conducted to provide a basis of comparison for inoculated samples.

The natural flora did not initiate logarithmic growth until after 5 days of storage at 1 C. The microbial count at the stationary phase was 10^9 (Fig. 1). The TVB level of the sample increased with continued storage, but increase was maximal when microbial growth reached the stationary phase (Fig. 3).

Inoculated fish. As expected, *Pseudomonas* type I (PI-950) grew well in fish substrate. Compared to the natural flora, PI-950 did not exhibit a lag period, and the stationary phase was reached at a higher cell concentration. Logarithmic growth by PI-950 was initiated within a day and reached the count of 10^{10} at the stationary phase (Fig. 1).

Growth rates by PI-950 and natural flora were similar during the log periods. Both cultures doubled in number in approximately 12 hr.

The shorter lag period for PI-950 may be explained by the fact that the cells inoculated into fish substrate were actively growing at the time of

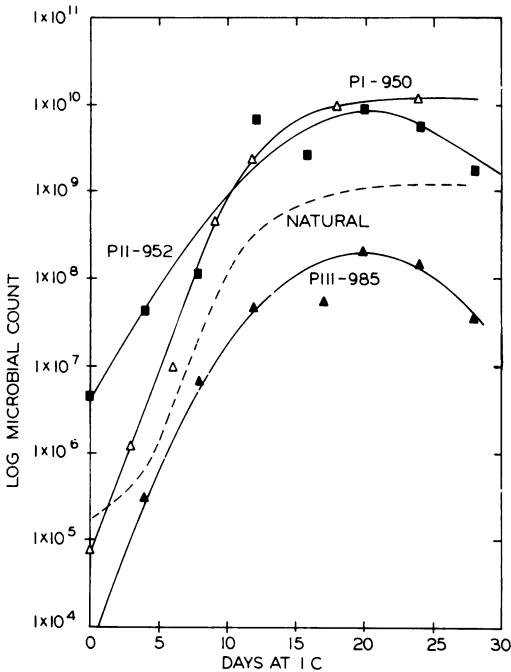


FIG. 1. Microbial growth in sterile rockfish inoculated with *Pseudomonas* species PI-950, PII-952, or PIII-985. Dotted line indicates microbial growth curve in naturally contaminated fillet.

inoculation, and that PI-950 was one of the predominant species isolated from perch (20).

The maximal growth level reached by PI-950 was one above the level of the natural flora (Fig. 1). Since an earlier study by Lee et al. (20) has shown that microorganisms found in commercial fillet after storage are made up almost exclusively by *Pseudomonas* species similar to PI-950, the reduced total microbial growth in the natural population might have been influenced by some factor which could not be reproduced in a pure culture.

TVB production during storage did not increase in the same proportion as microbial growth (Fig. 3). As was observed with the mixed culture, however, the buildup of microbial numbers, to a given level, preceded the increase in TVB.

When the count of PI-950 exceeded 10^{10} , a TVB of 107 mg of nitrogen/100 ml was reached after 24 days of storage at 1°C. The natural flora produced the equivalent level of TVB in 17 days with a microbial count of 5×10^8 . Therefore, it appeared that microbial species other than *Pseudomonas* contributed to the TVB level produced in naturally contaminated fish.

TVA levels did not increase while PI-950 was in the logarithmic growth stage; nevertheless,

TVA production was initiated before that of TVB. As the storage period progressed, however, the TVA level dropped from 16.7 to 1.2. In contrast, the TVB production remained at a high level. The drop in the TVA value at an advanced stage of spoilage was also noted by Hillig et al. (18), in flounder stored at 2°C for over 13 days.

The growth pattern of *Pseudomonas* type II (PII-952) was similar to that of PI-950. It did not show a visible lag period, and compared to the flora of the naturally contaminated fillet the stationary phase was reached at higher cell concentration. Logarithmic growth by PII-952 was initiated within a day, and reached a count of 10^{10} as did PI-950 (Fig. 1). Similarities between PI-950 and PII-952 are not surprising, since they were differentiated solely by the production of an ultraviolet fluorescent pigment in *Pseudomonas* type I.

The maximal level of TVB produced in PII-952 inoculated fish was somewhat lower than that of PI-950 inoculated fish (Fig. 3). Production of TVB was again preceded by a buildup of microbial numbers.

The onset of TVA increase in PII-952 inoculated fish was slower than that in fish inoculated with PI-950, but ultimate levels reached were nearly double that produced by PI-950 (Fig. 4). TVA and TVB levels in PII-952 inoculated fish increased simultaneously. TVA values in fish inoculated with PII-952 also declined as the storage period progressed.

The microbial count of *Pseudomonas* type III (PIII-985) in fish did not reach the high levels of *Pseudomonas* species types I and II. The maximum reached was 3.2×10^7 cells/g, which was one log below the level of the natural flora (Fig. 1). Since PIII-985 also did not show an initial lag phase, the low growth maximum may indicate an inherent limitation for growth of this organism. Spoilage microorganisms have been reported to grow preferentially on soluble substrates rather than on products of structural protein hydrolysis (19, 21). If such stepwise degradation of fish muscle takes place, microorganisms with substrate limitation would be expected to contribute only part of the total degradation processes.

TVB and TVA produced in PIII-985 inoculated fish were negligible (Fig. 3 and 4).

The taxonomic status of *Achromobacter* species described in this paper is uncertain. If the proposals by Brisou and Prévot (6) and Thornley (30) are adapted, the *Achromobacter* species described herein (A-70 and A-981) would have to be reclassified under phenons 3 and 4 of the genus *Acinetobacter*. A-70 was oxidase-negative and was unable to utilize glucose, xylose, and lac-

tose either fermentatively or oxidatively. A-981, on the other hand, was oxidase-positive and utilized glucose, xylose, and lactose oxidatively. Lactose oxidation, however, was very slow. Both species grew at 0 C but were unable to grow at 37 C. For both species, the optimal temperature for growth was between 26 and 28 C.

A taxonomic study of this group of microorganisms will be reported later. Therefore, the discussion here will be confined to their actions on fish flesh.

The growth rate of A-981 in fish was comparable to that of PI-950 and PII-952 (Fig. 1 and 2). The level of TVB produced in A-981 inoculated fish, however, was the lowest among the organisms that reached a count of 10^8 cells/g or above (Fig. 3). The TVB level in A-981 inoculated fish averaged 77 mg of nitrogen/100 ml after 27 days. This level of TVB, although not accompanied by a putrid odor, would be sufficient to classify the fish as unacceptable (13).

A-981 produced approximately the same amount of TVA as did PI-950, but only about half that produced by PII-952 (Fig. 4). As with PI-950 and PII-952, the TVA level declined toward the end of the storage period. A-981, therefore, could not be differentiated from PI-950

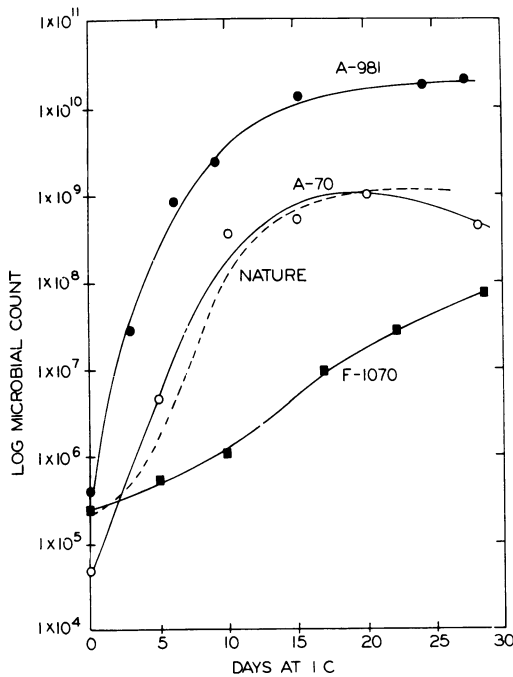


FIG. 2. Microbial growth in sterile rockfish inoculated with achromobacter species A-981 and A-70 and Flavobacterium species F-1070. Dotted line indicates microbial growth curve in naturally contaminated fillet.

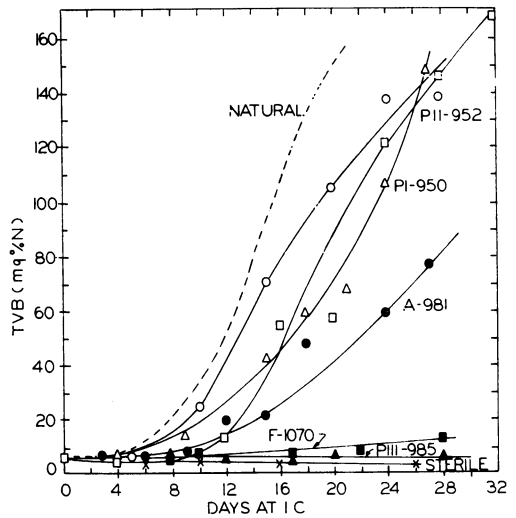


FIG. 3. TVB production in sterile rockfish inoculated with bacteria. Dotted line indicates TVB produced in naturally contaminated fillet.

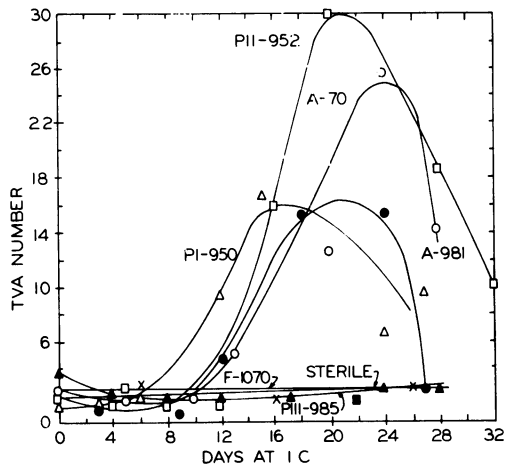


FIG. 4. TVA number change in sterile rockfish inoculated with bacteria.

and PII-952 on the basis of TVA production alone, when determinations were made at the terminal stage of storage.

The maximal level of growth reached by A-70 was 10^9 . This was one log below levels observed with A-981, PI-950, and PII-952, but was comparable to that in the natural flora (Fig. 2).

Despite its poorer growth, A-70 inoculated fish produced TVB more rapidly than did naturally contaminated fillet (Fig. 3). A-70 inoculated fish again lacked the putrid odor despite the high TVB levels.

The maximal level of TVA reached by A-70

was 25.6 (Fig. 4). This was second only to that of PII-952. The TVA again decreased toward the end of the storage period.

The yellow-pigmented, gram-negative rod, *Flavobacterium* F-1070, did not grow well in fish tissue. The maximal number reached after 28 days at 1 C was 7.7×10^7 (Fig. 2). The limited growth was probably due to a slow growth rate, rather than to a low maximal level, as was observed with PIII-985. Since the level of growth did not reach the minimum that seemed to be required for active production of TVA and TVB, it was not surprising to find that limited amounts of TVA and TVB were produced in F-1070 inoculated fish (Fig. 3 and 4).

Microbial roles in fish spoilage. Several interesting points have emerged from this study. Microorganisms that grew well in fish produced high levels of TVA and TVB. The levels of TVB and, to a limited extent, TVA produced by three genera of microorganisms, *Pseudomonas*, *Achromobacter*, and *Flavobacterium*, can be attributed almost entirely to the growth capabilities of these organisms. The growth capacity can be measured either by the maximal level of growth attainable or by the rate of growth. In the latter case, it is possible that TVA and TVB production can reach high levels if the inoculated fish are stored for a longer period of time (10). A difference in capacity to promote TVB production, however, was apparent among microorganisms that grew actively in fish (Fig. 5).

Microbial numbers increased prior to the production of TVA and TVB. It appears that a critical number of 10^8 cells/g is needed before detectable levels of TVA and TVB can be produced. The lag period preceding the active production of TVA and TVB limits the usefulness of these tests as a means of predicting storage potentials of fish. Since TVA undergoes further degradation at advanced stages of storage, TVA production would not be a reliable measure for determining the state of spoilage in fish. The volatile acids found in fish are primarily formic and acetic acids, with smaller amounts of propionic and butyric acids (15). Since microorganisms are able to utilize these low molecular weight acids in the presence of air, packaging and conditions of storage would also influence the TVA levels.

The slow growth of F-1070 provides an explanation for the difference in the ratio of pigmented and nonpigmented bacteria in fresh and stored fish (12). Microorganisms found in fish, however, cannot be classified as either spoilage or nonspoilage species unless comparisons are made with the same number of bacteria and conditions approximate those of natural fish. Other

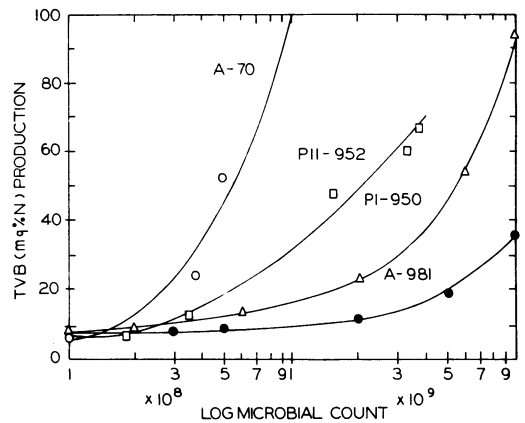


FIG. 5. Relationship between microbial numbers and TVB.

factors, such as differences in growth rate and differences in growth temperatures, would influence the microbial numbers in an arbitrary system and would render comparison useless.

As shown with *Pseudomonas*- and *Achromobacter*-inoculated fish, TVA and TVB values cannot be directly related to odor. Thus, more reliable chemical tests are needed for the detection of spoilage.

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