Some Characteristics of Proteolytic Enzymes from *Pseudomonas fluorescens*

A. C. Peterson and M. F. Gunderson

Department of Bacteriological Research, Campbell Soup Company, Camden, New Jersey

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Our interest was stimulated by observing the active proteolytic abilities of a culture of *Pseudomonas fluorescens* isolated from a frozen chicken pie. The culture liquefied gelatin rapidly and attacked casein strongly. In addition, the culture displayed considerable lipolytic and amylolytic activity. The culture was isolated from a tryptone-glucose-meat-extract-agar plate after incubation at 5 C for 7 days. Because many investigators have reported pseudomonads as part of the bacterial flora on foods stored at freezing or refrigeration temperatures, it seemed likely that this culture was a part of the normal psychrophilic bacterial flora. In defrosted foods, the effects of the action of enzymes, presumably from psychrophilic bacteria, are very evident. These enzymatic effects are more extensive than might be expected from the number of bacteria present. The degradative action of proteolytic enzymes is one of the most prominent features of defrost and is marked by the formation of extremely disagreeable odors and flavors which render these products unpalatable.

The object of these experiments was: (1) to investigate the effect of some environmental factors on the growth of this culture, (2) to investigate the effect of the environment on the elaboration of the proteolytic enzymes of this culture, and (3) to determine the effect of environmental conditions on the activity of the proteolytic enzymes.

**Materials and Methods**

Tryptone-glucose-meat extract (TGE) broth of pH 7.0 was used as the culture medium. The cultures were grown in low form culture flasks at various temperatures as indicated in the text for periods up to 5 days. Bacterial counts were determined by plating on TGE agar and incubating at the same temperature as the original flask. Plates were incubated at 0 C and 5 C for 14 days, at 10 C for 7 days, and at 20 C and 30 C for 48 hr.

At the same time that samples were taken for plating to determine the number of bacteria per ml for the growth curve experiment, an aliquot was taken for the purpose of determining the presence of extracellular proteolytic enzymes in the medium. This portion was centrifuged for 10 min at 30,000 × g in a Servall centrifuge to remove the mass of bacterial cells. The clear supernatant fluid was rendered bacteria-free by passage through a ceramic no. 02 Sela filter. The samples were then stored at 0 C until assayed.

The extracellular enzymes were precipitated in two fractions from the bacteria-free growth medium. Fraction I was obtained by precipitation with 0 to 50 per cent saturation with ammonium sulfate and Fraction II precipitated by 50 to 85 per cent saturation with ammonium sulfate. The precipitations were carried out at 5 C overnight. The precipitated enzyme proteins were recovered by centrifugation and dialyzed against a large volume of distilled water for 24 hr. They were then dialyzed to their original volume. Extracellular proteolytic enzymes exhibited great stability when stored at 0 C.

Endocellular enzymes were prepared as follows: the bacterial cells were recovered by centrifugation from the broth growth medium and washed 5 times with 0.02 M Sorensen's phosphate buffer, of pH 7.3. The washed cells were then diluted 1 to 4 by volume in the same buffer and sonic treated in a 10 kc Raytheon Sonic Oscillator for 50 min. The sonic extract was centrifuged twice at 30,000 × g for 20 min to remove cellular debris and then passed through a no. 02 Sela filter. The bacteria-free sonic extract was stored at 0 C until used, but lost most of its activity in 2 weeks. Activity could be completely restored by dialysis of the extract against distilled water or 0.02 M phosphate buffer. This suggested poisoning or inactivation by a dialyzable component, perhaps a metallic compound. This was contrary to the experience of several other investigators working with bacterial peptidases who observed metallic ion activation (Ogle and Logan, 1956; Brandsaeter and Nelson, 1956b), principally by calcium (Lenny, 1956; O'Brien and Campbell, 1957), manganese, and cobalt ions (Erlanger, 1957). Activation of a protease from *Clostridium histolyticum* by cysteine and other sulfhydryl activators was reported by Prado et al. (1956).

When the bacteria-free sonic extract was dialyzed immediately after preparation, it could be stored at...

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1 A summary of this work was presented to the 59th General Meeting of the Society of American Bacteriologists held in St. Louis, Missouri, May 10-14, 1959.

2 Ivan Sorvall, Inc., Norwalk, Connecticut.

3 Sela Corporation of America, Philadelphia, Pennsylvania.

4 Raytheon Corp., Waltham, Massachusetts.
0 C for periods up to 2 months without loss of activity. All of these preparations, both endo- and extracellular, were inactivated by heating to boiling for 10 min.

Dry weights on cell suspensions and enzyme preparations were determined by drying for 24 hr at 100 C. Microk jeldahl techniques used standard acid digestion and distillation of ammonia after neutralization as specified by Hawk et al. (1954). The assay technique for measuring amino acids freed by proteolytic enzyme action was that of Anson (1938). Casein was used as the test substrate and the amount of tyrosine, tryptophane, and smaller peptides, containing these two amino acids in positions where they may react with the Folin-Ciocalteau reagent was determined. After reaction with phenol reagent, the intensity of light transmitted was measured at 645 ma.

To cover the substrate pH range used in these experiments, three different buffers were used as follows: phthalate buffer in the range pH 5.0 to 6.15, phosphate buffer of pH 5.7 to 8.0, and borate buffer of pH 7.7 to 9.9. The ends of the buffer ranges were overlapped so that possible effects of the individual buffers themselves could be observed and compensated. The curve therefore is a synthesis of three different buffer curves. At low pH's coagulation of the casein substrate was observed. Quantitative micro-kjeldahl determinations indicated that not all of the substrate was rendered insoluble at pH 5.0, but substrate concentration could have been a limiting factor in assaying proteolytic activity at pH's up to pH 6.

Results and Discussion

Figure 1 shows the growth curves for this microbe when grown in TGE broth at 0, 5, 10, 20, 30, and 37 C. Samples were plated on TGE agar and incubated at the same temperature as the original flask. At 0 C the lag period was only 24 hr. At 5 C it was only 8 hr and substantially less at higher temperatures. This culture did not grow at 37 C. Although the lag phase was very short at 30 C, the number of bacteria per ml was less in the stationary phase than at 20 C. The death phase also was reached earlier at 30 C than at any other temperature. Colonies on the plates incubated at 30 C were minute indicating the adverse effects of this temperature on the bacterium's growth. Therefore, 20 C represented the approximate optimum growth temperature for this organism, although its ability to grow at low temperatures was marked.

Figure 2 presents the data for the liberation of extracellular proteolytic enzymes in the growth medium at the various temperatures at which the rate of growth was determined. The undulating nature of these curves is similar to that observed for Bacterium linens by Friedman et al. (1953). Van der Zant and Nelson (1953) noted increases in soluble nitrogen, tyrosine, and tryptophane when Streptococcus lactis was grown in milk. These products, suggesting proteolytic activity by this microbe, showed only a single peak of maximum activity. These workers were unable to positively demonstrate the presence of extracellular proteolytic enzymes in the growth menstruum. Van der Zant (1957) also observed a single peak of extracellular proteinase production for Pseudomonas putrefaciens. Weill and Kocholaty (1937) reported that secretion of an extracellular
proteinase by *C. histolyticum* reached a maximum during the first 24 hr of growth and then decreased, whereas an aminopolypeptidase was produced in increasing amounts throughout incubation.

Maximum elaboration of the proteolytic enzymes of this culture of *P. fluorescens* appeared to take place at about 24 to 40 hr. The first peak of extracellular enzyme elaboration occurred while the bacterial cells were in the logarithmic growth phase and cell autolysis was minimal. The second peak of enzyme elaboration could result from cell autolysis, since the cells could be in the stationary or death phase depending on the temperature of incubation. For food preservation considerations, it is important to note that maximum proteolytic enzyme elaboration occurred very soon after growth was initiated, before the lapse of very many hours and while the bacterial population was still comparatively low.

Liberation of enzyme in the growth medium was greatest at 0°C and decreased with increasing temperature until the smallest quantities of enzyme were liberated in the menstruum at 30°C. At temperatures and times which might have been expected to afford some protection against bacterial enzymatic damage, maximum proteolytic enzyme production occurred. Because of the quantity of data presented in this illustration, all of the available data was not plotted. Instead, an average of three values over a 24-hr period was used. The tendency to greatly increased enzyme production at low temperatures is directly related to the much smaller activity of those enzymes at low temperatures. As the temperature is increased, the enzyme's greater activity limits the need for as much enzyme in order to achieve a given level of protein attack.

Figure 3 presents data on the effect of the initial pH of the culture medium on the production of extracellular proteolytic enzymes by this culture of *P. fluorescens*. This figure includes data for the effect of minimum, maximum, and the two optimum pH's on proteolytic enzyme secretion. Intermediate pH curves were not plotted. Tryptone-glucose-meat extract broth was again used as the culture medium. Flasks of medium having initial pH's between 5.5 and 9.0 in 0.5 pH unit increments were used. Additional buffer was not used and the pH of the culture medium was not readjusted to the original values during growth. Terminal pH's were not determined, but undoubtedly varied widely from that of the original pH of the medium. When the number of bacteria per ml of medium was used as the criterion, about pH 8.0 was optimum for growth. When the liberation of extracellular proteolytic enzymes in the menstruum was the criterion, pH 7.0 and 8.5 represented optimum conditions. Acid conditions, particularly as low as pH 5.5 and 6.0, did not favor proteolytic enzyme elaboration. In general, as the pH increased to the optimum, the curves were displaced to the left, so that maximum proteinase production occurred sooner in the growth cycle. Also, the amount of enzyme produced increased very substantially. Past the optimum pH, the amount of enzyme liberated in the medium dropped off sharply. The presence of two widely separated pH optima suggested the presence of at least two different proteolytic enzymes among the the extracellular enzymes. Friedman *et al.* (1953) observed that for *B. linens*, proteinase production was greater at pH 7 than either pH 6 or 8.

Table 1 shows some quantitative data on the proteolytic activity of intact cells, sonic lysate, dialyzed sonic lysate, broth growth medium, and extracellular proteolytic enzymes of Fraction I (precipitated by 0 to 50 per cent saturation with ammonium sulfate) and Fraction II (precipitated by 50 to 85 per cent saturation with ammonium sulfate).

Sonic treatment increased the proteolytic activity, expressed in terms of milligrams of tyrosine and tryptophane per unit volume freed from casein, by the sonic lysate solution by about 36 per cent as compared with intact cells. Proteolytic activity was increased about 550 per cent on a basis of mg of tyrosine and tryptophane freed per mg of protein (enzyme) nitrogen. Dialysis of the sonic lysate increased the activity of the endocellular enzymes about 48 per cent on a volume basis and 9640 per cent on a protein nitrogen basis as compared with the intact cell suspen-
sion. Of the extracellular proteolytic enzymes, those in Fraction I comprised about 20 per cent of the proteolytic activity on a volume basis, as compared with Fraction II proteolytic enzymes. About 96 per cent of the total extracellular proteolytic activity in the growth medium was recovered by the ammonium sulfate fractionation. Activity per mg of protein nitrogen was twice as great in the first fraction of extracellular enzymes as compared with the enzymes in the second fraction. This difference in activity may be due to the presence of different enzymes in the two fractions. Per mg of enzyme protein, the extracellular proteolytic enzymes were much more active than the intact bacterial cells or the nondialyzed sonic lysate. This high activity per unit weight of enzyme protein is of concern in food preservation.

We also investigated the relative activity of endo-
acellular proteolytic enzymes from bacteria grown at 10 C, 20 C, and 35 C. Data in table 2 show that endo-
cellular proteolytic enzymes from P. fluorescens cells grown at 10 C were about 3 times as active per mg of enzyme protein as enzymes from cells grown at 35 C. The adverse effect of the increased temperatures to 35 C is easily seen in the cell yield from equal volumes of culture medium after the same incubation period.

**TABLE 1**

Some quantitative aspects of proteolytic enzymes from Pseudomonas fluorescens

<table>
<thead>
<tr>
<th></th>
<th>Dry Weight (g/ml)</th>
<th>Proteolytic Activity (mg tyrosine and tryptophane per ml)</th>
<th>Kjeldahl Nitrogen (mg N per g dry wt.)</th>
<th>Proteolytic Activity (mg tyrosine and tryptophane per mg N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>0.0764</td>
<td>0.0044</td>
<td>111.7</td>
<td>0.00652</td>
</tr>
<tr>
<td>Sonic lysate</td>
<td>0.0386</td>
<td>0.0060</td>
<td>54.0</td>
<td>0.00928</td>
</tr>
<tr>
<td>Dialyzed sonic lysate</td>
<td>0.0022</td>
<td>0.0065</td>
<td>58.0</td>
<td>0.00908</td>
</tr>
<tr>
<td>Growth medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I*</td>
<td>0.0015</td>
<td>0.0017</td>
<td>35.6</td>
<td>0.0319</td>
</tr>
<tr>
<td>Fraction II†</td>
<td>0.0027</td>
<td>0.0062</td>
<td>184.0</td>
<td>0.0164</td>
</tr>
</tbody>
</table>

Ratio diluted cells to packed volume = 4:1.

* Precipitated by 0 to 50 per cent saturation with ammonium sulfate.

† Precipitated by 50 to 85 per cent saturation with ammonium sulfate.

**TABLE 2**

Relative yield of cells and endocellular proteolytic enzymes activity of Pseudomonas fluorescens grown at various temperatures

<table>
<thead>
<tr>
<th>Cells Grown at:</th>
<th>Yield of Wet Cells Packed Volume</th>
<th>Proteolytic Activity (mg tyrosine and tryptophane/mg N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 C (50 F)</td>
<td>41.0</td>
<td>0.0488</td>
</tr>
<tr>
<td>20 C (68 F)</td>
<td>23.0</td>
<td>0.0212</td>
</tr>
<tr>
<td>35 C (95 F)</td>
<td>0.4</td>
<td>0.0127</td>
</tr>
</tbody>
</table>

Tryptone-glucose-meat extract broth, pH 7.0.

This culture grew well at the low temperatures encountered in defrost, temperatures which might be expected to afford food products some protection but which actually increased enzyme activity.

Figure 4 shows data on the determination of the substrate-in-excess points for these three different enzyme preparations. Substrate-in-excess levels were reached at very low levels of casein for the extracellular enzymes, whereas the endocellular enzymes required about 6 times as much substrate to reach the excess point. This indicates that the extracellular proteolytic enzymes are probably different from the endocellular enzymes. Similar results were reported by van Heyningen (1940) for an endocellular proteinase from C. histolyticum.

When the amount of enzyme was varied and the amount of substrate held constant (figure 5), the amount of tyrosine and tryptophane freed as a result of enzyme action was a linear function of the amount

![Figure 4](http://aem.asm.org/)

*ENDOCELLULAR* & FRACTION I-EXOCYLLULAR *FRACTION II-EXOCYLLULAR*

![Figure 6](http://aem.asm.org/)

*ENDOCYLLULAR* & FRACTION I-EXOCYLLULAR *FRACTION II-EXOCYLLULAR*
of enzyme from Fraction I. Tyrosine and tryptophane freed as a result of enzyme action was a linear function of a limited amount of enzyme from Fraction II, but increased amounts of enzyme did not result in a linear increase in proteolytic degradation products as determined. The amount of tyrosine and tryptophane freed as a result of the action of endocellular enzymes was not a linear function of the enzymes concentrations and suggested that a series of reactions might be involved. Similar results have been reported by Erlanger (1957) for Bacillus brevis and Ogle and Logan (1956) for C. histolyticum.

Because these enzymes were elaborated by a psychrophilic bacterium, we were interested in determining the influence of temperature on the activity of these enzymes after elaboration. For those extracellular enzymes in Fraction I, increasing the temperature increased the rate of protein attack linearly up to 25 C and then the rate decreased slightly as shown in figure 6. For enzymes in Fraction II, increasing the temperature up to 25 C resulted in a nonlinear increase in the rate of protein attack. Further increases in the temperature at which the activity was determined resulted in greatly decreased enzyme activity. Thus in their response to increased temperatures, enzymes in Fraction I differed markedly from those in Fraction II. The influence of temperature on the endocellular enzymes' activity was similar to that observed with Fraction I extracellular enzymes. Thus it can be seen that once the proteolytic enzymes of this culture were elaborated, increases in the temperature greatly accelerated their activity. Friedman et al. (1953) reported that the optimum temperature for extracellular proteolytic enzymes from B. linens was about 38 C, but slight proteolysis was observed at 0 C and 60 C. Optimum temperature for an endocellular proteinase and peptidases from Lactobacillus casei (Brandsaeter and Nelson, 1956a and 1956b) was reported to be 50 C. Mandel et al. (1957), reporting on the exopeptidases of C. histolyticum, observed that these enzymes were very stable even after 1 hr at 80 C. An extracellular proteinase from Bacillus stearothermophilus (O'Brien, 1957) was reported to have its optimum activity at 55 C.

Figure 7 presents data on the activity of endocellular enzymes determined as a function of time and temperature. Increases in the temperature up to 37 C resulted in increases in the activity. At this temperature, a rate limiting reaction was noted and the activity decreased with time. Thus for the endocellular enzymes, a temperature between 30 C and 37 C was optimum for maximum activity. We did not determine if substrate exhaustion was responsible for this decreasing activity at 37 C, although Van der Zant and Nelson (1953) have considered the freeing of compounds acting with Folin-Ciocalteau reagent as an early phase of proteolytic activity. We have not yet made similar studies of the effect of time and temperature on the activity of the extracellular enzymes. It is interesting to note that, except for Fraction II extracellular proteolytic enzymes, the activity of both endo- and extracellular enzymes was increased even beyond the temperature at which this microbe would grow. It can also be observed that the extent of protolysis was low at freezing even though not completely stopped. These results are very similar to those reported by Van Heyningen (1940) for C. histolyticum, Brandsaeter and Nelson (1956b) for L. casei, and Viswanatha and Liener (1956) for a ciliated protozoan, Tetrahymena pyriformis.

Figure 8 presents data for the effect of substrate

\[ \text{mg. TYROSINE AND TRYPTOPHANE X10}^{-3} \text{ PER ml.} \]

on pH for Fraction I and Fraction II extracellular enzymes from Pseudomonas fluorescens.
pH on the activity of extra- and endocellular enzymes from *P. fluorescens*. Peaks of proteolytic activity at about pH 7.8, 8.8 and possibly 6.2 were noted for extracellular enzymes in Fraction II. Possible optima at pH 6.2, 7.8, and 8.8 were observed for enzymes in Fraction I. Endocellular enzymes gave a pH optimum picture very much like that observed with Fraction I, suggesting that part of the extracellular enzymes in the growth menstrum may be endocellular enzymes secreted by cell autolysis. Dialysis of the enzyme preparations removed several of the activity peaks observed previously at other pH's with the nondialyzed enzymes.

Thus so far our studies on the effect of amount of substrate, amount of enzyme, activity per unit weight of enzyme and effect of temperature on activity have indicated that the endo- and extracellular enzymes preparations may have contained as many as three different enzymes. However, the activity response of Fraction I extracellular enzymes to different substrate pH's was very similar to that of the endocellular enzymes.

For *B. linens*, optimal pH for extracellular proteinase activity was reported to be 7.2 to 7.3 (Friedman et al., 1953). Brandsaeter and Nelson (1956a, 1956b) reported a pH optimum range of 7.0 to 7.5 for endocellular proteinase from *S. lactis*, pH 5.5 to 6.5 for endocellular proteinase from *L. casei*, and pH optimum of 7.0 to 8.0 for endocellular peptidase activity from *L. casei*. Lenney (1956) reported pH optima of 3.8, 5.0, and 6.5 for endocellular proteinases from yeast. An endocellular ornithine peptidase from *B. brevis* (Erlanger, 1957) had its pH optimum in the range 8.5 to 9.0. Felix et al. (1956) reported a carboxypeptidase in brewers yeast with a pH optimum of 6.0. A pH range of 6.9 to 7.2 was reported as optimum for activity of an extracellular proteinase from *T. pyriformis* (Viswantha and Liener, 1956), from *B. stearotherophilus* (O'Brien and Campbell, 1957) and of exopeptidases from *C. histolyticum* (Mandel et al., 1957). Berger et al. (1938a, 1938b) have demonstrated that a number of bacteria secreted extracellular proteolytic enzymes into the growth media. They observed that the optimum pH value of the endocellular enzymes of most of the organisms examined for peptide hydrolysis was between 8 and 9, except for *Lactobacillus pentosus* and *Propionibacterium pentosaceum* which had acidopeptidases having pH optima of 5.5 and 6.0. Brandsaeter and Nelson (1956a) made the interesting observation that for endocellular proteinase from *L. casei*, pH effects on enzyme activity were temperature dependent. This may well have been a reflection of greatly lowered general activity at low temperatures, but requires some other explanation at 30, 40, and 50 C.

**SUMMARY**

A culture of *Pseudomonas fluorescens* was isolated which had definite psychrophilic attributes and a very active enzyme constitution. This culture was found to produce heat labile endo-and extracellular proteolytic enzymes. The extracellular proteinases were recovered by ammonium sulfate precipitation. Extracellular proteolytic enzyme elaboration was shown to be inversely proportional to the temperature at which the culture was grown, at least from 0 C to 30 C. The extracellular proteolytic enzymes were found to be more active per unit weight than the endocellular enzymes. Temperatures above room temperature were demonstrated to have adverse effects on cell production and endocellular proteolytic activity. Increases in temperature greatly accelerated the action of proteolytic enzymes of this culture once they were elaborated. On the basis of pH optima and other criteria, several different endo- and extracellular enzymes were produced by this culture of *P. fluorescens*.

**REFERENCES**


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Microbiology of Shellfish

Bacteriological Study of the Natural Flora of Pacific Oysters (Crassostrea gigas)\(^1,\)\(^2\)

R. R. COLWELL AND J. LISTON

College of Fisheries, University of Washington, Seattle, Washington

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There is an extensive literature on the public health aspects of shellfish bacteriology (Dodgson, 1928). A great deal of excellent work has been carried out on the incidence and survival of such groups as the enteric pathogens (Salmonella, Shigella) and related coliform indicator organisms in shellfish grown under various conditions (Foote, 1895; Fabre-Demorgue, 1912; Kelly and Arcisz, 1954). As a result of this work, the practical conditions necessary for prevention and control of shellfish-borne infection are now well established. However, there is an almost complete lack of information concerning the bacterial types not derived from sewage associated with shellfish.

A comparison of the number of colonies obtained on count plates prepared from shellfish incubated at room temperature (\textit{circa} 20 to 25 °C) and at 37 °C indicates that nonmesophilic bacteria probably comprise the bulk of the bacterial population of shellfish. One type of noncoliform microorganism said to be peculiar to shellfish belongs to the group of large \textit{Spirochaetae}. This type of microorganism was described by Fantham (1907) from mussels, \textit{Spirochaeta anodonta}, and by Dimitroff (1926) from oysters, \textit{Saprospira} and \textit{Cristispira}. In some published reports concerning the presence of coliform organisms in shellfish, casual reference has been made to the presence of other bacteria. Thus Joseph (1914) described the occurrence of spore-bearing, asporogenous, pigmented, and nonpigmented bacteria in market oysters, Berry (1916), and Geiger \textit{et al.} (1926) noted the presence of \textit{Proteus}, \textit{Alcaligenes}, and \textit{Pseudomonas fluorescens} together with other common “water bacteria,” also in market oysters. Eliot (1926) found that the green fluorescent, yellow pigmented, nonpigmented, and “vibrio” groups of microorganisms rapidly increased in number during the spoilage of market oysters at 20 °C. Tanikawa (1937) found that typical water bacteria of the genera \textit{Achromobacter}, \textit{Pseudomonas}, \textit{Flavobacterium}, and \textit{Micrococcus} were of greatest importance in the spoilage of market oysters held at 0 °C. The results of these spoilage studies are remarkably similar to the bacteriological findings for fin fishes held at similar temperatures (Shewan and Liston, 1956), and, in the latter case, it has been quite well established that the spoilage organisms are derived from the flora of the living fish which is predominantly composed of asporogenous gram-negative rods (Georgala, 1958). By analogy it seems not unreasonable to suspect that the spoilage bacteria in oysters are related to the normal bacterial population present in the living animal.

The purpose of this study was to determine the composition of the natural bacterial flora of oysters held under controlled natural conditions in various areas of Washington. Coliform counts were carried out to obtain some information concerning the degree of pollution of the environment, but the major portion of the investigation was concerned with noncoliform bacteria.

\textbf{Materials and Methods}

Yearling Pacific (that is, Japanese) oysters, \textit{Crassostrea gigas}, were obtained from Purdy, Washington, and were placed in floating trays in three different areas of Washington: Hood Canal, Oyster Bay, and Willapa Bay; a control group was maintained in the salt-water aquarium at the College of Fisheries. Samples of three oysters and 150 ml of seawater were taken from the aquarium weekly and from the floats every

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