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Title: Microorganisms Isolated from Sand Filtered Bay Water and the Proteolytic Activity of a Flavobacterium Isolate.

Abstract APPROVED: Dr. J. S. Lee

Monthly samples of sand filtered estuarine water were collected from September 1977 to September 1978 and the microorganisms isolated were compared with those of Yaquina Bay water.

Nearly 66% of the 476 well isolates examined belonged to genera Flavobacterium-Cytophaga and Acinetobacter-Moraxella. Conspicously absent were the members of Vibrionaceae and Enterobacteriaceae in the sand filtered water. The Yaquina Bay water was predominated by microorganisms belonging to Pseudomonas and Vibrionaceae group during the warmer months of Summer and Fall, but Flavobacterium-Cytophaga and Acinetobacter-Moraxella group gained predominance in Winter and Spring.

Three pigmented bacteria from the well identified as Flavobacterium-Cytophaga and an Alteromonas putrefaciens spp isolated from the bay were further characterized taxonomically and their proteolytic activities were studied in detail.

Phenotypic characteristics together with the Guanine plus Cytosine moles percent of 42.2 revealed that one of the most active proteolytic

well isolates belonged to Flavobacterium, section I, and the enzyme produced was an extracellular serine protease, with a mw of 25,000-30,000. This protease production was repressed by the addition of carbohydrates (glucose, sucrose and glycerol) and amino acids (Casamino Acids, glutamate and aspartate) to the growth medium, indicating a control mechanism similar to catabolite repression. However, the additon of dibutyril-cyclic-AMP (5mM) was unable to reverse the glucose-dependent protease repression. The addition of rifampicin (10^{-4}M) to the growth medium caused the inhibition of the extracellular protease production, indicating that the control was at the transcriptional level.

Microorganisms Isolated from Sand Filtered Bay Water and the Proteolytic Activity of a *Flavobacterium* Isolate

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INTRODUCTION

Sand filter systems have been used for years to purify sewage effluents. Aulenbach *et al.* (1978) reported on a system which has been operating for 37 years and exceeded the degree of treatment by most tertiary effluent plants, at a much lower cost. Bouwer *et al.* (1974) described a natural sand-gravel-bed that was responsible for removal of suspended solids, BOD and fecal coliform bacteria from a renovated secondary effluent.

Natural unpolluted water is becoming increasingly scarce. The demand for adequate supply of clean water, however, is especially greater in mariculture. A 6 m deep well encased in polychlorinated vinyl chloride (PVC) pipe of 10 cm in diameter was dug in the sand bar adjacent to the Oregon State University Marine Science Center, Newport, OR. The well was intended to supply filtered seawater to fish and oyster tanks.

This study was undertaken to assess the microbial quality of the filtered water in comparison to that of the bay. Although the chlorination-declorination system had replaced the sand filter system to accommodate the greatly enlarged aquaculture facility at the Center, the sand filter system is still viewed as an inexpensive means of obtaining pathogen free water for aquaculture or seafood processing operations.

The sand-filtered water, however, consistently yielded high proportions of gram-negative pigmented bacteria belonging to the genera

Flavobacterium, Cytophaga and Flexibacter, which are known to be proteolytic (Christensen, 1977). Some Flavobacterium spp were shown to play a role in protein degradation in natural waters (Little et al., 1979). Cytophaga proteases degraded autoclaved feathers (Martin and So, 1969) or aided in sewage purification (Gude, 1980). Proteases from Flexibacter strains isolated from diseased sea bass are known to break down tissues at the site of infection Hikida et al., 1979). However, little is known about the mechanisms and the nature of the proteases produced by this group of microorganisms.

Since the proteolytic activity of these bacteria may cause complications in the use of such water, a systematic study of this enzymatic activity was undertaken. A dye release assay system, originally developed by Rinderknecht et al. (1968) and employed by Little et al. (1979) to study proteolysis in natural water, was adapted to study the extracellular protease synthesis of a Flavobacterium spp. The protease was characterized by polyacrylamide gel electrophoresis and by the effect of different inhibitors on the protease activity.

LITERATURE REVIEW

Microbiology of Estuarine and Coastal Waters

The types of microorganisms isolated from different coastal and estuarine environments could vary widely. Environmental factors obviously cause such diversity but the various analytical procedures used by different investigators further compound the problem. Austin et al. (1979) observed that the choice of the isolation medium exerted a significant influence over the types of bacteria isolated. Similar observations were made by Gouldner (1976), Vaatanen (1977) and Mallory et. al. (1977). However, the generic diversity observed was more likely to be due to environmental differences than by any other factor. Vaatanen (1980a,b) examined the effect of environmental factors on microbial communities present in brackish water of the southern coast of Finland. He concluded that changes in microbial community composition were brought about basically by organic matter content, water temperature, and salinity. Austin et al. (1979) compared the autochtonous flora of two contrasting geographical sites, Tokyo and Chesapeake bays. They found that Vibrio and Acinetobacter spp predominated the Chesapeake Bay microbial flora but Acinetobacter-Moraxella and Caulobacter spp were most frequently isolated from Tokyo Bay. It has been suggested by Simidu et al. (1977) that the low level of vibrios in Tokyo Bay could have been due to antagonism between phytoplanktons and the vibrios.

Among the coastal environments surveyed for their microbial communities were the Artic and Antartic marine waters. Kaneko et al. (1979) found that orange pigmented bacteria resembling Flavobacterium spp

predominated the Artic surface waters. However, the pigmented bacteria were in the minority of the isolates from Antartic coastal waters and, instead, Pseudomonas spp were the predominant isolates (Pfister and Burkholder, 1965).

For a 12 month period, Murchelano and Brown (1970) isolated 649 cultures from the Long Island Sound. Their isolates consisted mainly of Pseudomonas (40.6%), Achromobacter (28.6%), Flavobacterium (23.1%) and Vibrio (4.9%) spp. The remaining fraction consisted of Bacillus, Cytophaga and micrococci. They noted a seasonal change over from Pseudomonas domination in Summer to that of Flavobacterium in Winter. Vibrio spp remained a small but constant fraction throughout the year. Altschuller and Riley (1967) also found a predominance of flavobacteria in Long Island Sound waters during Spring and Winter. Among 54 cultures isolated from Chesapeake Bay during the period of September and December, Cook and Goldman (1976) were able to identify Vibrio (37%), Alcaligenes (15%), Acinetobacter (7.4%), and Pseudomonas (5.67%). No valid genus name was applicable to the "achromobacter-type" nitrate reducers (13%) and denitrifiers (5.6%); the gram-negative non-motile, oxidase negative, fermentative rods (11%); and 3 strains which were non-grouped.

Water Purification by Sand Filtration

James Simpson, working for Chelsea Water Company in London, first introduced sand filtration in 1829. Although it was originally intended to clarify turbid water, its use suddenly increased with the arrival of Asiatic cholera in Europe in 1830. Around 1850 it became known that cholera was spread by drinking water, and the contagious agent could be

removed from water by slow filtration through the sand. Up till about 1920 this was virtually the only treatment practiced to transform surface waters of rivers and lakes into pathogen-free drinking water. After World War I, chlorine and chlorine compounds were introduced to treat the drinking water. However, some communities still depend on sand filtration as a sole means of water purification. Due to its simplicity, it has been proposed as a convenient treatment system of village scale water supplies in developing countries (Huirsman, 1979).

Chlorination of the water intended for rearing bivalve molusk larvae or fishes is not desirable because of the toxic effect of residual chlorine. Water for oyster seed hatcheries also require a salinity higher than 20 parts per thousand (Breese and Malouf, 1975). Estuarine water can provide the desired salinity, but the bacterial contamination often causes mortalities to bivalve mollusk larvae. According to Murchelano et al. (1975) Vibrio spp and Pseudomonas spp are pathogenic to the oyster seeds. Flow-through or recirculating systems using UV-irradiation or ozonation are commonly used in hatchery operations (Mathieson, 1970). However, those processes are expensive and are not fool-proof. Their sanitation efficiency is dependent on factors such as turbidity, organic matter content and microbial load of the water to be treated. It seems that the combination of sand filtration with UV-irradiation or ozonation would improve the efficiency of the purification process.

Sand filtration has also been used to purify contaminated river water (Baars, 1964) and sewage effluents (Bower et al., 1974 and Aulenbach et al., 1978). Effectiveness of water purification processes

is commonly measured by the recovery of coliforms and fecal coliform bacteria (APHA, 1976). After the filtration of a secondary sewage effluent through a natural sand filter bed, no fecal coliform bacteria were recovered from the filtered water (Aulenbach et al., 1978). While the absence of such an indicator bacteria may suggest safety from pathogens, and possibly from viruses, the filtered water is by no means sterile.

The well dug near the Oregon State University Marine Science Center, Newport, was intended to supply unchlorinated and pathogen-free water for aquaculture studies at the Center. The in depth microbiological study undertaken was to assess the microbiological quality of this water and determine its suitability for aquacultural purposes.

Flavobacterium-Cytophaga Complex

The genus Flavobacterium is an uncertain taxonomic concept (Holmes and Owen, 1979). Many gram-negative and pigmented bacteria were erroneously included in this group (Hayes, 1977). Distinction among flavobacterias, cytophagas and flexibacters is by no means certain (Weeks, 1969; McMeekin et al., 1971; and McMeekin and Shewan, 1978). In the 8th edition of Bergey's Manual, Flavobacterium is divided into two sections (Weeks, 1974). Section I contained non-motile species with the G+C content in the range of 26 to 43 mol % (low G+C strains), and Section II contained six species that were either motile or non-motile but had the G+C contents in the range of 63 to 70 mol % (high G+C strains). The manual also lists 27 species incerta sedis.

The non-flagellated flavobacteria (low G+C strains) can be confused with those members of Cytophaga and Flexibacter which fail to

swarm or glide on agar (Hendrie et al., 1968; Weeks, 1969). Numerical phenetic surveys to date (Floodgate and Hayes, 1963; Byron, 1971 and McMeekin et al., 1972) could not resolve this confusion. In order to determine the key characters for the differentiation of flavobacterias, cytophagas and flexibacters, Hirsch and Reichenbach (1980) studied 185 cytophaga-like bacteria, isolated from soil and fresh water habitats. Of 130 different characters tested, many proved to be useless. A reasonable preparation could be achieved based on the following properties: gliding motility, cell morphology, presence of flexirubin-type pigments, and of certain enzymatic activities (catalase, oxidase, lecithinase, urease, phenylalamine deaminase, DNAse, hydrolysis of starch and gelatin liquefaction).

New taxonomic criteria for differentiation of flavobacterias, cytophagas and flexibacters are being developed. According to Callies and Mannheim (1978), the presence of quinones and quinone mediated respiratory functions are promising tools in regrouping the organisms contained in the Flavobacterium-Cytophaga complex. They suggested that genus Flavobacterium should be reserved for organisms producing ubiquinones as the sole respiratory quinones and that genus Cytophaga should be restricted to organisms containing menaquinones. Three distinct fatty acid patterns also exist in flavobacteria, cytophaga-flexibacter and myxobacteria (Fautz et al., 1980). The presence of sphingolipids in certain Flavobacterium spp was reported by Yabuchi et al. (1980). Sphingolipids have been found only among limited groups of bacteria.

It appears that the taxonomical confusion surrounding flavobacteria and cytophagas can only be resolved by methods of molecular taxonomy. Hayes et al. (1977) studied the deoxyribonucleic acid base composition of flavobacteria and related gram-negative yellow pigmented rods. Owen and Snell (1976) studied the deoxyribonucleic acid reassociation among flavobacteria isolated from clinical and environmental sources. By performing hybridizations between DNA and ^{14}C-or ^{3}H-labeled ribosomal RNA, Bauwens (1980) concluded that the genus Flavobacterium was not a biological unity, and that the strains he examined could be regrouped into 4 different r-RNA superfamilies.

Microbial Extracellular Enzymes

The synthesis of microbial extracellular enzymes can be controlled at four different points, namely during transcription of the structural gene into m-RNA, during the transport and attachment of m-RNA to ribosomes, during the m-RNA translation and during the processing of the protein into its final conformation. Among the transcriptional control mechanisms are induction, end product repression and catabolite repression.

Induction or Repression of Synthesis

Extracellular enzymes may be constitutive, partially inducible or fully inducible. Constitutive enzymes include the proteases of Pseudomonas maltophilia (Boethling, 1975), of an Arthrobacter spp (Hofsten and Tjeder, 1965) and a Vibrio parahaemolyticus mutant (Iuchi and Tanaka, 1979). Induction of extracellular proteases by amino acids

has been observed in a marine bacterium (Daatselaar and Harder, 1974) and for a Micrococcus spp (McDonald and Chambers, 1966). Peptides or other proteinaceous substrates induced extracellular proteases in Aeromonas proteolytica (Litchfield and Prescott, 1970 a,b). The best known example of an inducible enzyme is β-galactosidase, described in detail in the classic paper by Jacob and Monod (1961). An extensive discussion on the control of inducible enzyme synthesis is presented by Watson (1975).

For reasons of cellular economy, extracellular enzymes should not be synthesized when readily utilizable substrate is present. Therefore, it is not surprising to find that the synthesis of nearly all extracellular enzymes is sensitive to catabolite and for end product repression.

Catabolite repression can be defined as a phenomenon that causes a reduction in the rate of enzyme synthesis in the presence of glucose or other carbon sources that are readily metabolized (Paigen and Williams, 1970). The molecular mechanism by which glucose brings about catabolite repression is rather involved. When glucose enters the cell it lowers the intracellular concentrations of a specific mediator molecule, namely cyclic adenosine 3'-5'-monophosphate or cyclic AMP (c-AMP). It has been shown that c-AMP binds to a special protein, the c-AMP receptor protein (CRP). This c-AMP-CRP complex facilitates the interaction of DNA dependent RNA polymerase with the promoter region of the operon, which is required for the initiation of the m-RNA synthesis (Pastan and Adhya, 1976). Thus, any significant decrease in the

intracellular c-AMP concentration will lead to an insufficient level of c-AMP-CRP complex and, in the end, the rate of enzyme synthesis is reduced. This type of catabolite repression may be relieved by increasing the c-AMP level, which in a number of cases has been achieved by adding exogenous c-AMP. The way in which glucose lowers the intracellular c-AMP level is not fully understood, but evidence is accumulating that glucose indirectly inhibits the activity of adenylate cyclase, the enzyme responsible for the synthesis of c-AMP from ATP.

Repression of extracellular protease synthesis by the addition of carbon and/or nitrogen sources to the growth media have been reported for Pseudomonas maltophilia (Boethling, 1975), Vibrio parahaemolyticus (Tanaka and Iuchi, 1971), Aeromonas proteolytica (Litchfield and Prescott, 1970a,b), a Micrococcus spp (McDonald and Chambers, 1966), an unidentified marine bacterium (Daatselaar and Harder, 1974), Arthrobacter B22 (Van Hofsten and Tjeder, 1965) and for several members of the genus Bacillus (Priest, 1977).

The Translation-Extrusion Model

An intriguing question associated with extracellular enzyme production in bacteria is how those relatively large molecules are transported across the cytoplasmic membrane. A translation-extrusion model for extracellular enzyme secretion in Bacillus subtilis was postulated by May and Elliott (1968). Additional evidence for this model came from studies on the secretion of B. licheniformis penicillinase (Sargent and Lampen, 1970) and Staphylococcus aureus protein A (Movitz, 1976).

But, only recently this simultaneous translation-extrusion has been demonstrated by the extracellular labeling of the nascent polypeptide chain (Smith et al., 1977; Tai et al., 1979). The growing list of bacterial enzymes synthesized by this model, which were demonstrated by extracellular labeling, include a periplasmic alkaline phosphatase in E coli (Smith et al., 1977), a B. subtilis extracellular α-amylase (Smith et al., 1979) and Corynebacterium diphterium toxin (Smith et al., 1980). It appears that the simultaneous translation-extrusion is a universal model operating among bacteria, and this tightly coupled synthesis-secretion model is similar to the initial steps involved in protein synthesis and secretion in eucaryotic cells (Palade, 1975).

It has been frequently suggested that synthesis of extracellular enzymes takes place on the surface of cytoplasmic membrane (Glenn, 1976 and Priest, 1977). Escherichia coli polyribosomes containing nascent alkaline phosphatase polypeptides were studied by Cancedda and Schlesinger (1974). They observed that 40% of cellular RNA and 55% of cellular m-RNA were attached to cell membrane and were associated with 70-80% of the nascent alkaline phosphatase. Furthermore, the synthesis of extracellular enzymes in Pseudomonas lemoignei (Stinson and Merrick, 1974), P. maltophilia (Boethling, 1975), Bacillus amylolique-faciens (Both et al., 1972) and B. subtilis strain 168 (Glenn, 1976) was found to be 5 to 20 times more sensitive to inhibitors of protein synthesis than the synthesis of the intracellular protein. The increased sensitivity of extracellular enzyme synthesis to inhibitors can be explained if we assume that the membrane-bound polysomes are more accessible to inhibitors than the ones dispersed in the cytoplasm.

Smith et al. (1978) showed that ribosomes are not directly attached to the bacterial cytoplasmic membrane, but were connected to it by the nascent polypeptide chain. There was, however, no gap between the ribosomes and the cytoplasmic membrane.

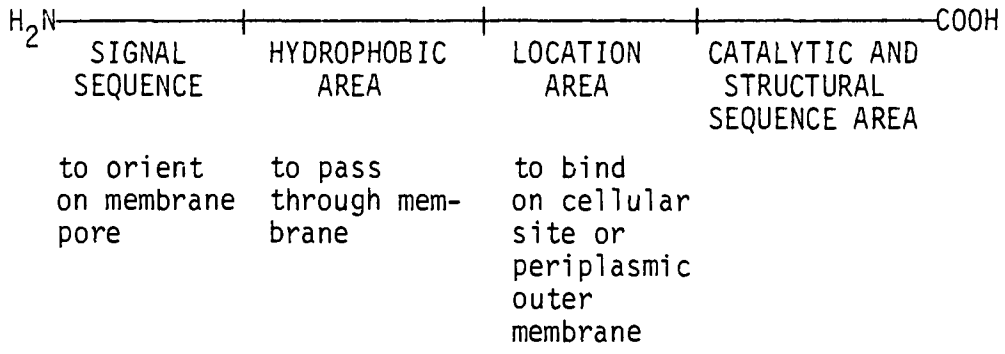
Assuming that procaryote-secreted proteins are synthesized on membrane-bound ribosomes (Tai et al., 1979), a major question is how do m-RNA molecules decide between membrane-bound or cytoplasm dispersed ribosomes, or how and which proteins must be transported across the cell membrane.

The Blobel and Sabatini's "signal hypothesis" (1971) for eucaryotic cells proposes that the m-RNAs for secretory proteins possess a unique sequence of codons located immediately to the right of the initiation codon. Those codons are absent in the m-RNAs coding for cytoplasmic proteins. Recent E. coli gene fusion studies by Silhavy et al. (1977) and Beckwith et al. (1978) indicate that the "signal hypothesis" may also operate in procaryotic cells. They genetically created secretory forms of the E. coli intracellular β-galactosidase. Bassford and Beckwith (1979) showed that a definite amount of amino-terminal amino acid sequence was required for β-galactosidase secretion. Those findings raised the possibility of creating a secretory form of any intracellular enzyme.

Enzymatically removable hydrophobic areas on the amino terminal end of secreted proteins were found in a Bacillus licheniformis penicillinase (Lampen, 1978) and in a structural outer membrane lipoprotein of E. coli (Inouye et al., 1977). The extracellular form of penicillinase with

mw of 29,500 was derived from a precursor "membrane-bound" form (mw 33,000) which had a phosphatidic acid covalently bound to the serine of its amino terminal end (Lampen, 1978). The intracellular form was obtained by specific cleavage (penicillinase release protease) of the 25 amino acid hydrophobic leader which contained the phosphatidic acid.

Combining recent observations of various workers, Ramaley (1979) speculated that the gene for microbial secretory proteins might have the following functional sequence areas:



Once the proteins have crossed the cytoplasmic membrane, processing enzymes (proteases) play a special role in the control of the release of the hydrophobic or anchoring form of the enzyme (Ramaley, 1979). These extracellular proteases are important in the maturation and sculpture of extracellular proteins and they can convert cell-bound enzymes to free extracellular enzymes (Lampen, 1978). The role of processing enzymes in membrane fractions of Corynebacterium diphterium was studied by Smith et al. (1980). They showed that membrane fractions were able to process the translated-extruded precursor of diptheria toxin (mw 68,000) into the active form (mw 62,000).

Classification of Microbial Proteases

Microbial proteases may be classified into four groups, according to the scheme of Hartley (1960), namely 1) serine proteases, 2) thiol proteases, 3) metal (or metal-chelator sensitive) proteases, and 4) acid proteases. Serine proteases show an optimum activity in neutral or alkaline pH range, are sensitive to diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), and usually contain serine and histidine in the catalytic centre. The properties of alkaline proteases of microbial origin were surveyed by Emtseva and Konovalov (1978). The thiol proteases show a neutral pH optimum, are activated by reducing agents, and are sensitive to -SH inhibitors. The metal proteases are inhibited by chelating agents such as EDTA. Acid proteases are predominantly found in molds and yeasts and are most active at pH between 3 and 4.

A detailed review of several microbial protease including their specificity has been given by Morihara (1974). It is of interest to note that most bacterial extracellular proteases have a low molecular weight in the range of 20,000 to 50,000 and a low level of cysteine residues. While pancreatic chymotrypsin has five disulfide bridges, microbial serine proteases have none. This property is fully compatible with the linear translation-extrusion model and with the idea that bacterial exoproteases obtain their active conformation only after the passage through cell membrane (Tai *et al.*, 1979).

Dye Release Method for Enzyme Assay

A simple colorimetric method to detect proteolytic enzymes in biological fluids was developed by Rinderknecht et al. (1968). It is based on the release of the Remazolbrilliant Blue dye covalently bound to an insoluble protein substrate (hide powder), by the action of proteases on peptide bonds. The Hide Powder Azure (HPA) test was sensitive enough to detect the presence of 50-100 ng/ml of chymotrypsin, collagenase or pepsin. Other proteases such as trypsin, fibrinolysin and elastase could be detected at concentrations as low as 1-2 ng/ml.

Different substrates bound to Azure dye have been used to detect α-amylase in serum (Rinderknecht et al., 1967) and solubilizing activity of a cellulase complex (Leisola and Linko, 1976). Recently, this dye release method was used for ecological studies of cellulose decomposition (Moore et al., 1979) and proteolytic activity in lake water (Little et al., 1979).

MATERIALS AND METHODS

Sampling Site and Sample Collection

The intake system which supplies water for the fish and oyster culture tanks located at Oregon State University Marine Science Center, Newport, OR, is shown in Figure 1. The filtering bed for the well consists of sands and gravels. Yaquina Bay and well water samples were collected once every month during the period of September 1977 through September 1978. After collection, the samples stored in sterile plastic jugs (one gallon volume) were analysed within 3 hours. Temperatures were recorded at the time of sampling. Salinity and pH were measured at the laboratory using Quantab titrator strips (Ames Co., Elkhart, IN) and a Beckman Zeromatic II pH meter, respectively.

Microbial Enumeration and Isolation

Heterotrophic Bacteria

For total, viable, aerobic, heterotrophic counts, 0.5 ml of well water or 0.1 ml of bay water diluted with sterile aged bay water, were spread with glass rods on plates of Difco marine agar 2216E (Zobell, 1941). The colony forming units (CFU) values were calculated from the average counts of triplicate plates after 7 days of incubation at 25C. Colonies were picked at randon from plates containing a countable number of colonies and transferred to Difco marine broth 2216E. After 4 days of incubation the broth cultures were transferred onto a marked spot on marine agar 2216E master plates, with sterile 1 ml pipettes. One plate could accommodate 30 such spots.

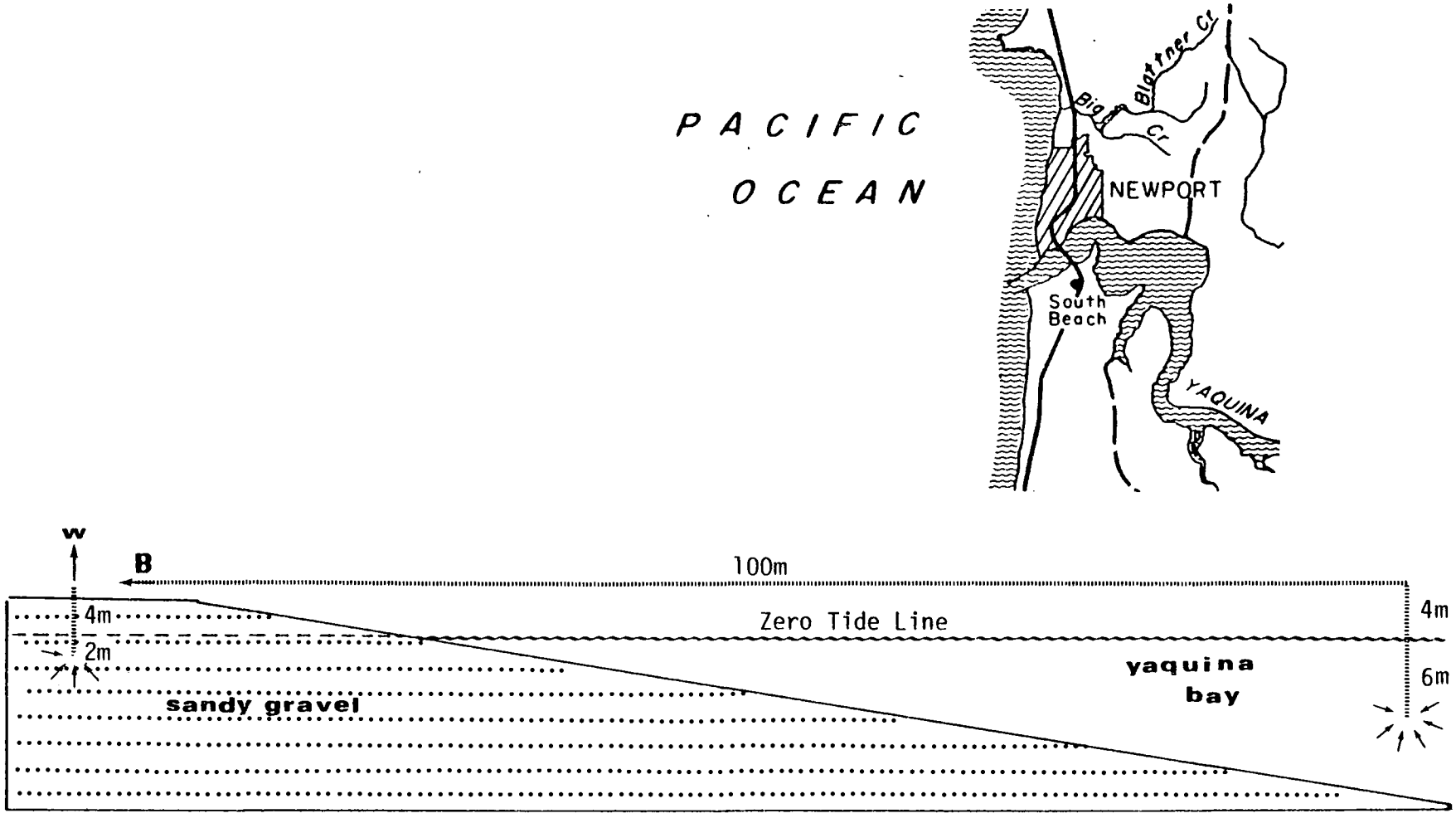


Figure 1. Water supply sources for fish and oyster culture tanks. (B) bay water, (W) well water.

Fecal Coliforms

The fecal coliforms were enumerated with Difco m-FC medium according to the American Public Health Association Standard Methods (1976) with HAWG 04750 Millipore membrane filters. The m-FC plates in duplicate were sealed with waterproof tape, placed into waterproof plastic bags and incubated in a water bath for 24 hrs at 44.5C.

Isolates Identification

Identification of the isolates to family and genus levels, was done by the replica-plating method (Lee and Pfeifer, 1975). The scheme used for their classification is outlined in Figure 2.

Pigmented Isolates: Taxonomy

The yellow and orange pigmented bacteria (strains 15, 20 and 30) were isolated from the well. The salmon-pink pigmented isolate (strain 40), was from the Yaquina Bay itself.

Phenotypic Characterization

The phenotypic characterization of the isolates was made according to the protocol described by Hayes (1977). Flexirubin pigment was tested by dropping 20% KOH onto the colonies (Reichenbach et al., 1974) and the estimation of proteolytic and polysaccharide decomposing potential was made on double layer agar plates as described by Reichardt (1975). Lipolysis of tweens 20, 60 and 80, was measured by the method of Sierra (1957).

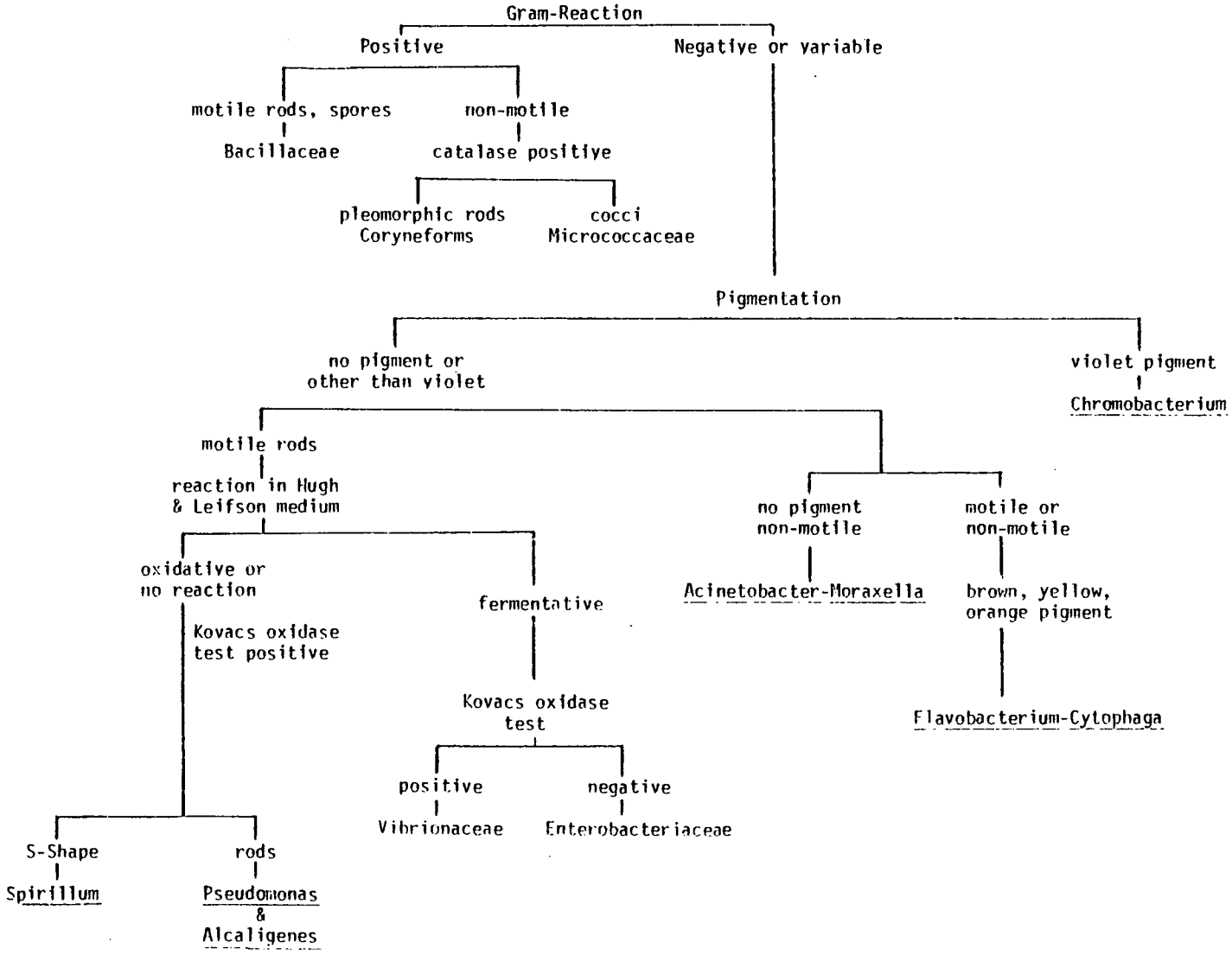


Figure 2. Classification scheme for the identification of the bacterial isolates.

DNA Base Composition

For the determination of guanine plus cytosine (G+C) moles %, the DNA of the cells harvested at the logarithmic growth phase, was prepared according to the Seidler *et al.* modification (1969) of the Marmur and Doty (1961) procedure. The melting temperatures (Tms) were determined in an automatic recording spectrophotometer (Gilford Instruments, model 2000) at 260 nm. Two DNA samples were run concurrently with the *E. coli* WP-2 DNA as a reference. Typical thermal denaturation curves are shown in Figure 3. Tms were calculated from the curve and the difference in Tm of the standard and the sample, i.e., ΔTm, was converted to (G+C) moles % by the relationship of Mandel *et al.* (1970). The (G+C) moles % were determined twice for each DNA sample and averaged.

Pigmented Isolates: Proteolytic Potentials

Media

The Basal Salts Medium (BSM), used to study the proteolytic activity of the four isolates contained, 0.50 g proteose peptone No. 3 (Difco), 0.50 g yeast extract (Difco), and 0.25 g sodium glycerophosphate (Sigma) dissolved in 1l of Basal Salts Solution (BSS). The BSS contained 11.7 g NaCl, 0.74 g $CaCl_2$, 0.38 g KCl and 3.45 g $MgSO_4$ per 1 of distilled water. The pH of BSM was adjusted to 7.0 with a 0.1N NaOH.

Protease Assay

The propylene oxide (Matheson Coleman & Bell, Norwood, OH) sterilized Miracloth bags (Chicopee Mills, Inc., Milltown, NJ) containing the hide powder

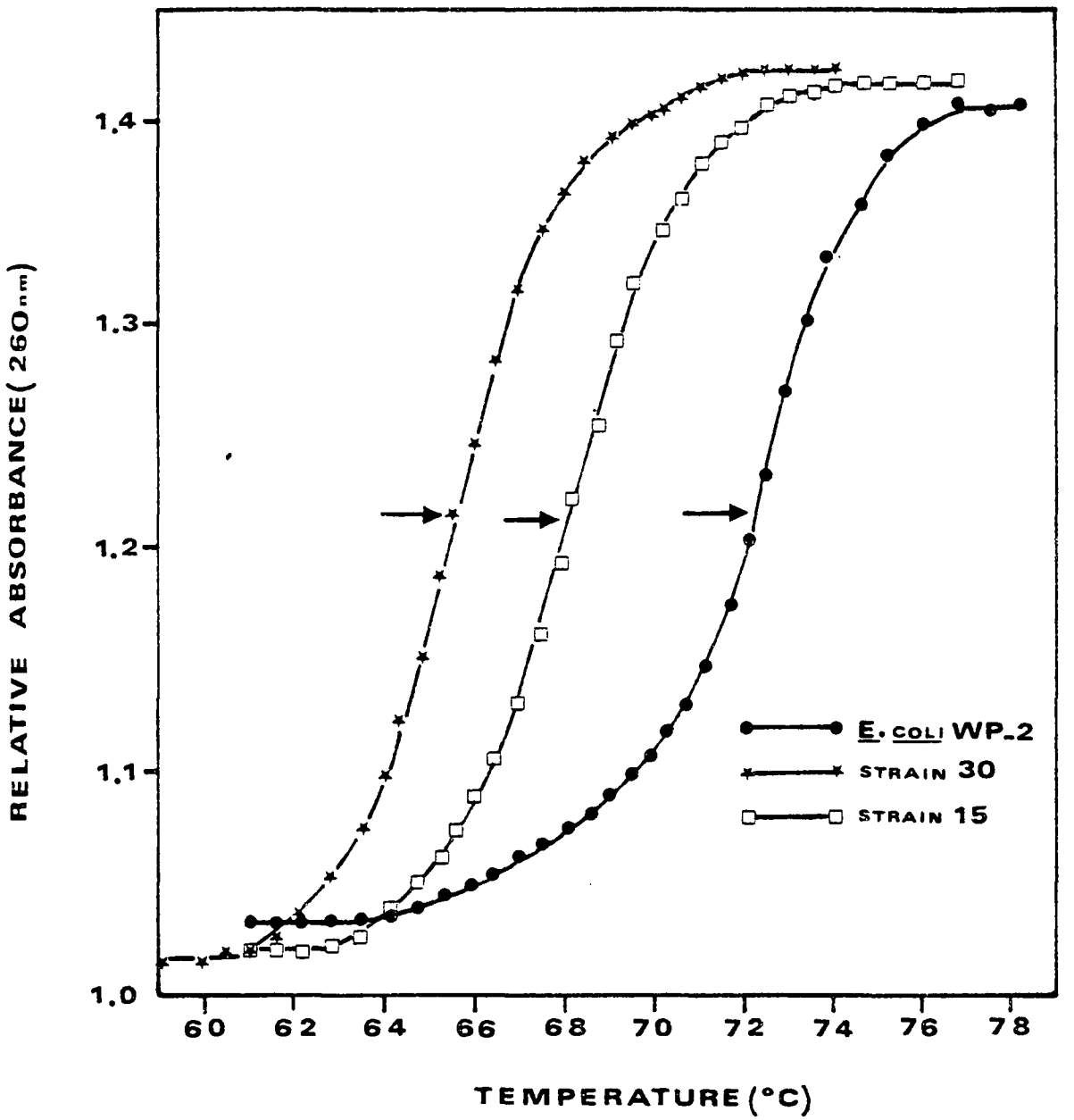


Figure 3. Thermal denaturation curves of DNA from strain 15, strain 30, and Escherichia coli WP-2. Arrows indicate the thermal denaturation temperatures (Tm).

azure (HPA, lot 810273, Calbiochem, San Diego, CA) were prepared according to the procedure described by Little et al. (1979). The HPA bag was aseptically placed in a 300 ml side arm flask (Bellco, Vineland, NJ), which contained the test sample. The flask was incubated at 25C with shaking at 200 rpm in a psychrotherm incubator shaker (New Brunswick Scientific Co., Inc.). The azure dye released from HPA by the proteases was measured spectrophotometrically at 595 nm (Bausch & Lomb, Spectronic 20).

Localization of Protease Activity

The four isolates were grown each in 1l of BSM at 25C with aeration by shaking. After 36 h of incubation 2 g of sterile hide power (Calbiochem) was added to the growth suspension, and the flask was incubated for an additional 48 hours. A 100 ml aliquot of cell suspension was centrifuged (10,000 x g/15 min/4C) and the supernatant was assayed for protease activity (extracellular proteases). The cell pellet was washed once in 20 ml of 0.1 M Tris-HCl (pH 7.0) buffer containing 2 mM Ca^{+2} and centrifuged (25,000 x g/30 min/4C), and resuspended in 10 ml of Tris-HCl buffer. The cell-buffer suspension was chilled in an ice bath and sonicated with a Biosonic III (Bronwill). The sonicated cell suspension was again centrifuged (25,000 x g/30 min/4C) and the supernatant fluid was assayed for protease activity (intracellular and periplasmic proteases). The remaining cell debris was also assayed for protease activity (cell-debris associated proteases). The spectrophotometric readings were made after removing interfering particulate matter by centrifugation.

Strain 15 Protease: Synthesis

Inoculum

BSB medium containing 1% gelatin (W/V) was inoculated with strain 15 and incubated at 25C with shaking at 200 rpm. The cells at logaritmic growth phase were harvested by centrifugation (10,000 x g/15 min/4C) and the resulting cell pellet was washed 3 times with BSS (pH 7.0). The cells were resuspended in minimal volume of BSS (pH 7.0). Inoculum added to medium was to yield approximately 10^9 cells/ml.

Protease Assay

Dye released from Hide Powder Azure (HPA, Calbiochem) by the whole cells, was measured spectrophotometrically at A_{595} after removing the cells by centrifugation (10,000 x g/10 min).

Cell Growth

The cell density was measured spectrophotometrically at 540 nm (Bausch and Lomb, Spectronic 20). The A_{540} value contributed by the solubilized azure dye was subtracted from the total A_{540} value.

BSB Medium

The Basal Salts Buffer (BSB) contained Hepes buffer (N-[2-hydroxyethyl] piperazine -N^1- [2-ethane] sulfonic acid, Sigma Chemical Co.) dissolved in BSS. Sterile side arm flasks containing BSB medium were inoculated with the strain 15 cell suspension and incubated at 25C with

shaking at 200 rpm. Absorbance at 540 nm and 595 nm were measured periodically during incubation.

Studies were conducted in order to select the proper Hepes buffer concentration and the pH which favored maximum protease synthesis. The pH adjustments were made with 0.5 M HCl or 0.5 M NaOH.

Metabolites Addition

The filter sterilized solutions of metabolites listed in Table 5 were aseptically added to side arm flasks containing 100 ml of inoculated BSB (0.10 M Hepes, pH 6.8). Rifampicin (3[4-methyl-1-(piperazinyl)-iminomethyl] rifamycin SV) and dibutiryl cyclic AMP ($N^6O^{2'}$-dibutiryl-adenosine 3':5' cyclic monophosphoric acid) used in this experiment were from Sigma Chemical Company.

Strain 15 Protease: Purification and Characterization

The crude protease from strain 15 was the cultural supernatant obtained by centrifugation (10,000 x g/15 min/4C) of log phase cells in BSB-1% gelatin (W/V).

Protease Inhibitors (Crude Extract)

Part of the supernatant was used to study the effect of inhibitors on protease activity, as described in Table 6. The remainder was used for the purification of the protease, as shown in Figure 4.

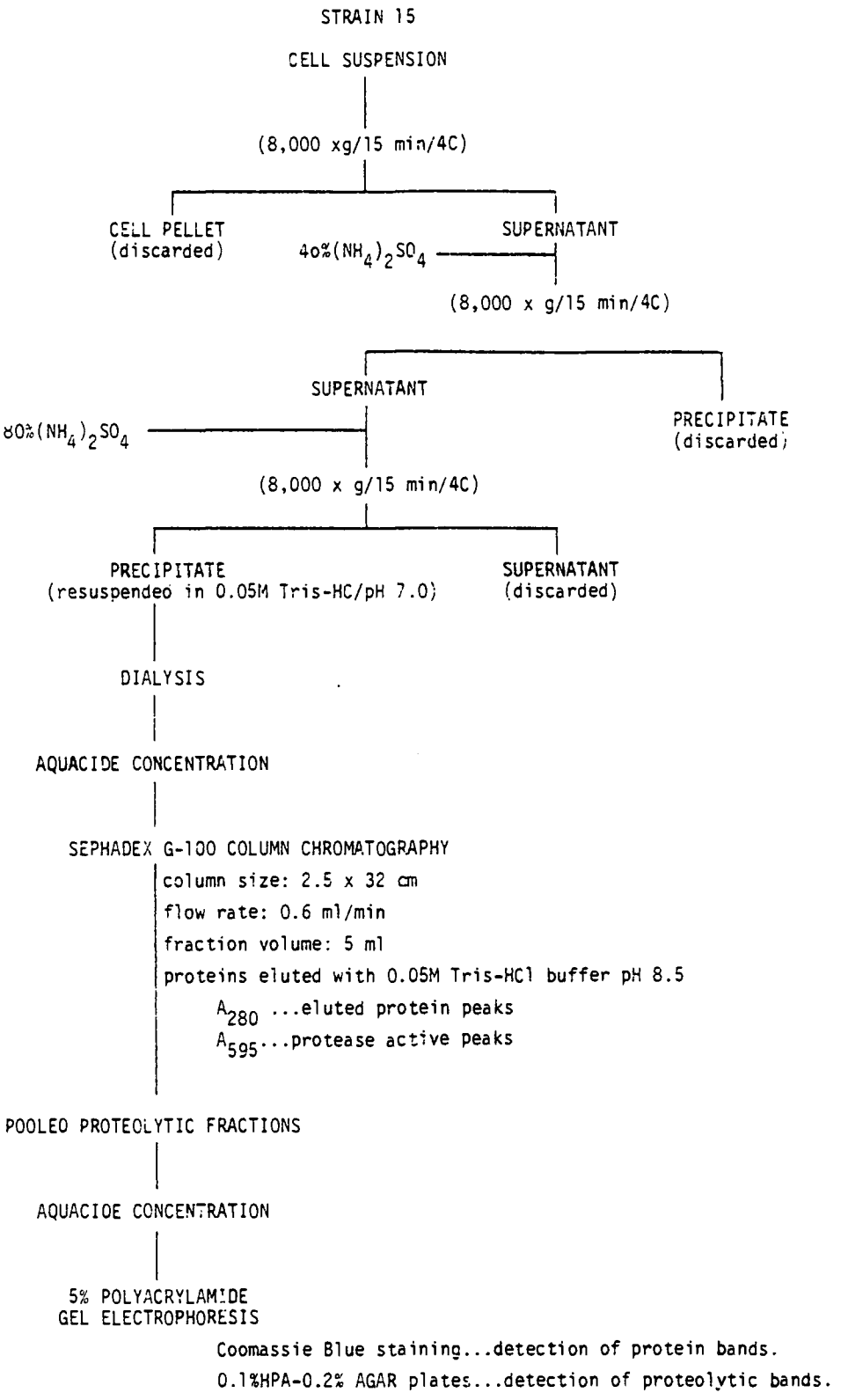


Figure 4. Purification scheme for strain 15 protease.

Ammonium Sulfate Precipitation

In an ice bath, solid ammonium sulfate crystals were slowly added to the supernatant, with stirring until 40% saturation was reached. Any precipitate formed was removed by centrifugation (10,000 x g/15 min/4C) and discarded. To the crude enzyme solution, solid ammonium sulfate was added to reach an 80% saturation. After centrifugation, the supernatant was discarded. The precipitate adhering to the centrifuge bottles was then dissolved in 50 ml of 0.05 M Tris-HCl buffer (pH 7.0).

Dialysis and Concentration

The enzyme preparation was dialyzed overnight against 0.05 M Tris-HCl buffer (pH 8.5) at 4C. The dialyzed extract was concentrated at 4C by placing the dialysis bag in Aquacide powder (Calbiochem, Los Angeles, CA).

Gel Filtration

The enzyme preparation (5 ml) was applied to a Sephadex G-100 column (2.5 x 32 cm) (Pharmacia Fine Chemicals, Inc.) at 4C, which had been equilibrated with 0.05 M Tris-HCl buffer (pH 8.5). The column was eluted with the same buffer at a flow rate of 0.6 ml/min. The fractions (5 ml) were assayed for protein content (A_{280}) and protease activity (A_{595}).

Protease Activity (A_{595})

To determine the protease activity of the different fractions, 1.0 ml aliquots were transferred to 25 ml erlenmeyer flasks, diluted

with 10 ml of 0.05 M Tris-HCl buffer (pH 8.5) and incubated (1 hr/200 rpm/25C) with 0.2% HPA (W/V). After incubation, the reaction was stopped by filtering through Whatman 2^{V} filter paper and the dye released in the filtrate was measured spectrophotometrically at 595 nm.

Pooling and Concentration

The fractions which contained 50% or more of the maximum protease activity were pooled for further study. The pooled fractions were placed in a dialysis bag and concentrated at 4C in Aquacide powder.

Optimum pH for Protease Activity

Samples (0.5 ml) of the concentrated pooled fractions were diluted in 10 ml of 0.1 M Tris-buffer at different pHs. The procedure used for the determination of the protease activity (A_{595}) was described above.

Serine Protease Inhibitors (Purified Extract)

Part of the concentrate was used to study the effect of the serine protease inhibitors, as described in Table 7. The remaining purified concentrate was utilized for further purification by gel electrophoresis.

Discontinuous Polyacrylamide Gel Electrophoresis (PAGE)

The general principles of the experimental procedure followed had been described by Davis (1964), Gabriel (1971) and Wesche-Ebeling (1980). The following stock solutions were prepared in advance and stored at 4C until used.

A. Electrode buffer: 0.025 m tris (tris-hydroxymethylaminomethane), 0.162 M glycine, pH 8.3, 9.0 g tris was mixed with 43.2 g of glycine, in 2.5 l of distilled water. The pH was adjusted to 8.3 with 1.0 N HCl and the final volume was brought to with distilled water.

B. Spacer buffer: 0.062 M tris-HCl, pH 6.7; 22.5 g of tris, 1.5 ml of TEMED (N,N,N^1,N^1-tetramethyleneethylenediamine) and 12.0 ml HCl were added to 2.0 l of distilled water 1.0 N HCl and the final volume was brought to 3.0 l with distilled water.

C. Running gel buffer, 5% (W/V): 3.0 M tris-HCl, pH 3.0; 181.5 g tris, 48.0 ml of 1 N HCl and 1.5 ml of TEMED were combined in 750 ml of distilled water. The pH was adjusted with 1.0 N HCl and the final volume was brought to 1.0 l. Acrylamide/bis solution: 200 g of acrylamide were combined with 10 g bisacrylamide in 750 ml of distilled water, dissolved thoroughly and brought to 1.0 l with more distilled water. Riboflavin solution; 20 mg of riboflavin were dissolved in 500 ml of glass distilled water.

The glass tubes used in the PAGE (0.5 cm x 12 cm) were cleaned in 10% hydrochloric acid, washed thoroughly with distilled water, and soaked in a 0.01% Photoflo (Eastman Kodak, Co.) solution. After drying, the tubes were marked at 9 cm and 10 cm and sealed with Parafilm, and placed vertically in a tube rack. The 5% (W/V) running gel solution was prepared as follows: one volume of the 5% running gel buffer was combined with two volumes of the acrylamide/bis solution, one volume of the riboflavin solution and four volumes of distilled water.

The 5% running gel solution was immediately placed into the tubes up to the 9.0 cm mark with a syringe (No. 20 gauge needle). The gels were carefully layered with distilled water, avoiding the mixing of layering water with gel solution. The riboflavin-catalyzed photopolymerization was accomplished by placing a fluorescent lamp within 10 cm of the tubes containing the solution. Gelatin occurred in ca. 20 min as indicated by a faint opalescence. The top of the gels were dried with paper tissue. The 4% (W/V) spacer gel solution was then prepared as follows: an amount of 0.8 g of cyanogum 41 was dissolved in 20.0 ml of spacer buffer and filtered through Whatman No 1 filter paper. Polymerization was initiated by adding 0.01 g of ammonium persulfate to the solution.

The 4% spacer gel solution overlay was immediately placed into the tubes up to the 10 cm mark with a syringe (No. 20 gauge needle). The gel solution was again carefully overlayered with distilled water. Gelation occurred within 20 min and the top of the gels were dried with paper tissue.

The enzyme samples for electrophoresis consisted of 0.25 ml of 50% sucrose containing a very small amount of bromophenol blue as tracking dye. To this, 1.0 ml of the enzyme extract was added and mixed. The protein content of the enzyme extract was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as standard protein. Aliquots of the enzyme-sucrose-dye solution were applied on the top of each gel.

Electrophoresis Cell (Bio-Rad Laboratories) Model 155 was used for the PAGE. The central cooling core of the apparatus was connected to a

Laude K2/2 constant temperature circulator adjusted to 4C. After the assembly, the apparatus was connected to a constant voltage power supply (EC-Model 454, EC Apparatus Corp.). The cathode lead was attached to the upper chamber and the anode lead to the lower chamber. The initial voltage of 125 v was applied until all the sample had entered the stacking gel (ca. 30 min). The voltage was then increased to 225 v for the remainder of the run (ca. 2 hr).

The tubes were removed from the apparatus when the tracking dye had almost reached the bottom of the gel. The gels were carefully removed from the tubes by gently forcing distilled water between the gels and the glass tubes with a syringe (No. 20 gauge needle).

After electrophoretic fractionation, the proteins in polyacrylamide gel were stained in solution freshly prepared by diluting 1 volume of a 1% stock solution of Coomassie Blue in 20 volumes of 12.5% trichloroacetic acid (Chrambach et al., 1967). After 1-2 hrs of staining, the gel was transferred into 10% trichloroacetic acid and the Rf's were measured. The color intensity of the bands increased during the succeeding 48 hrs of storage.

In order to locate the protease activity, non-stained gels were placed on 1% HPA-2% agar plates, incubated at 25C and checked periodically for the appearance of clear zones of digested HPA.

RESULTS AND DISCUSSION

Bay and Well Water Characteristics

The physico-chemical and microbiological characteristics of Yaquina Bay water and the Marine Science Center well water are compared in Table 1. Salinity and pH were slightly higher for the bay water, indicating perhaps that fresh water had infiltrated into the well. As expected, the temperature fluctuation was smaller for the well water. Although the pH, salinity and temperature values obtained were similar for both environments, it is expected that the estuarine conditions are subject to a greater fluctuation than those of the well. Factors we did not measure such as radiations, Eh or redox-potential, suspended solids and nutrients are also considered to influence the seasonal fluctuation of the estuarine microflora (ZoBell, 1973).

The microbial number of the well water, as expressed in colony-forming units (CFU) was 20 per ml and was 0.4% of that in bay water (4,500 per ml). No fecal coliform (FC) was isolated at any time from the well, while an average of 3.8 FC per 100 ml were found in Yaquina Bay water. The low counts of heterotrophic bacteria and the absence of FC in well water are obviously due to the filtering effect of the sandy-gravel soil (Figure 1). Apparently more than mechanical sieving is involved in the filtration process, but its exact nature is still being debated.

According to Fiori and Babineau (1979) most of the naturally occurring particles such as bacteria, viruses and organic matter have a negative net charge. Sand grains, also, are known to have a negative

Table 1. Summary of physico-chemical and microbiological data of bay and well water.

	Bay Water			Well Water		
Characteristic	Average[a]	Minimum	Maximum	Average[a]	Minimum	Maximum
pH	7.8	7.6	8.0	7.6	7.5	7.8
Salinity ($^{o}/_{oo}$)	25.4	19.0	31.4	23.3	17.0	30.0
Temperature (C)	11.6	8.9	15.0	12.3	11.6	14.4
CFU[b]/ml	4,500	500	11,100	20	13	31
FC[c]/100 ml	3.8	1.3	5.7	none in 5 l of sample		

[a] Monthly samples from September 1977 to September 1978.

[b] CFU, colony forming unit.

[c] FC, fecal coliform.

surface charge (Shackleton, 1977). The surface charge properties of bacteria may be altered by the presence of polyvalent cations (Santoro and Stotzky, 1967) as well as by adsorption of organic materials on the cell surface (Neihof and Loeb, 1974). Working with experimental sand columns, Jenkins et al. (1980) showed that the removal of T_1 coliphages during filtration through sand was enhanced by the addition of calcium ions. Wormald and Stirling (1978) showed that columns packed with beach sediment had a high capacity for removing inorganic phosphate from water samples and converted nitrate to ammonia or to molecular nitrogen even when samples were enriched by up to 20% sewage. Reversible absorption appeared to play a major role in this cleaning process. The number of bacteria was reported to be reduced, but it was not clear if they were removed by the same reversible absorption process or not.

A bacterium reversibly absorbed near a surface withstands repulsion by producing extracellular polymers, which bridge the organism to the surface (Costerton et al., 1978). The exact mechanisms whereby these bacterial polymers interact with the solid surface - or, more specifically, with the macromolecular film on the surface - are not known (Marshall, 1980).

Sayler et al. (1975) found that significant proportions of both the total viable bacteria (53%) and the fecal indicator organisms (>80%) isolated from Chesapeake Bay water were directly associated with suspended particles. The material found in estuarine sediments, such as sand, clay, and detritus provide surface for the potential absorption of the suspended particles and microorganisms (Marshall, 1976). This attachment increases the efficiency of bacterial filtration. Once attached to the sediments, the microorganisms allochtonous to the

estuarine water, such as the members of the Enterobacteriaceae family will die-off after a prolonged exposure to adverse environmental conditions such as low temperature, the presence of toxic chemicals and chelating agents (Mitchell, 1968). However, the sorption to sediment materials protect the bacteria from phage attack. Roper and Marshall (1974) demonstrated that this protection was purely physical and not associated with any chemical properties of the colloidal materials. Phages were prevented from direct contact with host bacteria because both were absorbed to sediment. Interruption of predator-prey interactions by such phenomena might account for increased recovery rates for enteric bacteria in certain sediments (Van Donsel and Geldrich, 1971).

Bay Water Versus Well Water Isolates

A total of 871 isolates, 395 from the Yaquina Bay water and 476 from the well water, were selected at random from the initial isolation plates. They were examined and classified according to the scheme shown in Figure 2 and the characteristics of these isolates are listed in Table 2. The majority of the isolates from both sources were gram-negative bacteria and a large percentage of them were pigmented. None of the well isolates were able to ferment glucose as measured by the modified Hugh-Leifson test (Lee and Pfeifer, 1975). Motility by flagella was a positive character for 42.6% of the bay isolates, compared to 22.3% for the well isolates. Bolter (1977) studied the saprophytic bacteria isolated from the Kiel Fjord and Kiel Bight and found that motility was positively correlated with the ability to degrade carbohydrates and proteins. Our finding seems to confirm

Table 2. Characteristics of the bacteria isolated from bay and well water.

Characteristic	Frequency of Occurence[a] (%)	
	Bay	Well
Gram Reaction		
- positive	11.6	5.3
- negative or variable	88.4	94.7
Motility	42.6	22.3
Pigmentation	28.7	34.2
Carbohydrate fermentation (glucose)	19.5	0

[a] A total of 395 bacteria from the Yaquina Bay water and 476 bacteria from the Marine Science Center will water were isolated during the period from September 1977 to September 1978.

such correlation between the motility and the ability to ferment glucose. A combination of all or some of these factors had undoubtedly contributed to the difference in the microbial counts and the types of bacteria found in bay and well water.

Microbial Population of Bay and Well Water

Bacteria consistently isolated from the well throughout the year belonged to genera Flavobacterium-Cytophaga and Acinetobacter-Moraxella and together they comprised 66% of the total (Figure 5). Of the remainder, Pseudomonas spp were the next most numerous.

The histogrames of frequencies of population occurrence (Figure 5) show that the gram-positive rods (Bacillaceae), pleomorphic rods (coryneforms) and cocci (Micrococcaceae) accounted for smaller proportions of the microbial population in both environments. For the bay water samples, isolates belonging to the Micrococcaceae family showed their highest level during the Winter, while the coryneforms count reached their peak in Spring.

Of the gram-negative bacteria, Pseudomonas spp comprised 15% of the bay water isolates and 16% of those isolated from the well. Higher proportions of Pseudomonas spp were isolated during Fall from the Yaquina Bay and during the Winter from the well. They were the majority (73%) of the motile microorganisms present in the well water. The remainder of the isolates belonged to Spirillum spp, Alcaligenes spp and unidentified pigmented rods.

As expected, the bay yielded a more diverse group of microorganisms and they underwent greater seasonal fluctuaions than those of the well.

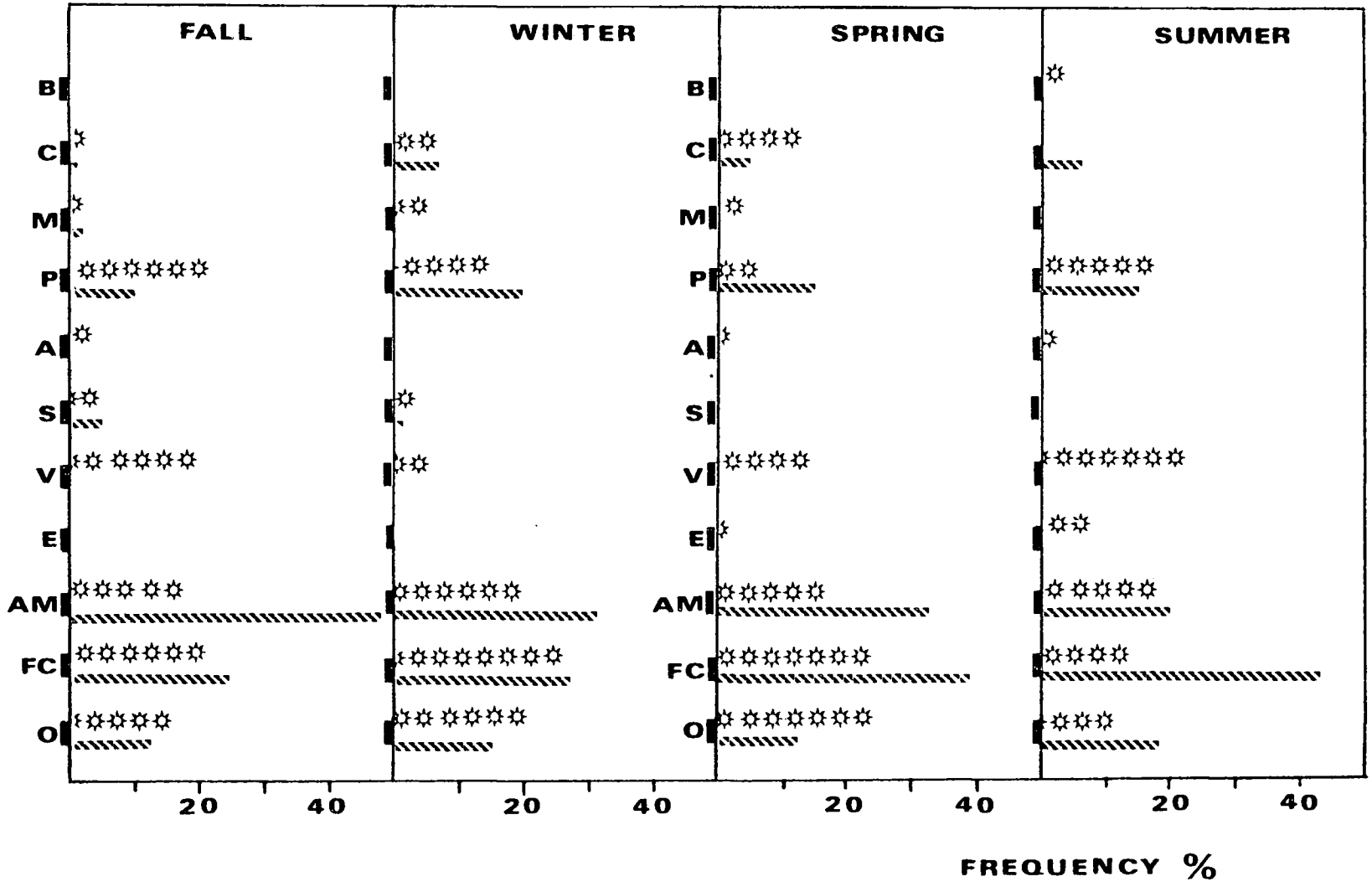


Figure 5. Seasonal microbial flora variation of bay (✲) and well water (≈). Symbols: (B) Bacillaceae; (C) coryneforms; (M) Micrococcaceae; (P) Pseudomonas; (A) Alcaligenes; (S) Spirillum; (V) Vibrionaceae; (E) Enterobacteriaceae; (AM) Acinetobacter-Moraxella; (FC) Flavobacterium-Cytophaga and (O) others.

In a study to determine the effects of the environmental factors on the seasonal variations of microbial populations off the southern coast of Finland, Vaatanen (1980b) observed that the phytoplankton bloom, the fresh water outflow, and the water temperature were the major factors that caused population shift. Species belonging to Flavobacterium-Cytophaga, Acinetobacter-Moraxella, Pseudomonas and Vibrionaceae were the predominant isolates from Yaquina Bay water samples. During the colder months, in winter and early spring, Flavobacterium-Cytophaga and Acinetobacter-Moraxella groups had predominated. The Vibrionaceae started to build up with the spring warm up and reached its maximum during late summer. Seasonal nature of Vibrionaceae bloom in the estuary was well documented by Austin et al. (1979), Cook and Goldman (1976), and Kaneko and Colwell (1974). The predominance of vibrios in Tung Hai (East China Sea), reported by Simidu et. al. (1977) had reached as high as 54% of the total in summer. A high proportion of Vibrio spp was reported for Summer and Autumn samples from Marumsco Bar (Lovelace et al., 1968).

Fermentative Bacteria

Fermentative type bacteria were isolated only from the bay water samples (Table 2). They were assigned to the families Vibrionaceae and Enterobacteriaceae and, according to Reinheimer (1977), this group of bacteria are mainly of the saprophitic types. Their occurrence during the different seasons of the year is shown in Figure 5.

In a taxonomic study of randomly selected fermentative isolates from Chesapeake Bay water, Cook and Goldman (1976) found that the majority of them belonged to Vibrio spp. Those isolates had a close relationship with marine animals which inhabited the coastal waters. They were shown to be present in the digestive tract of fishes (Yoshimizu and Kimura, 1976) and were associated with phyto- and zooplanktons (Kaneko and Colwell, 1973). When nutrients become available from the dead or dying plants and animals, the Vibrio spp appeared to grow faster than the other marine bacteria and became the dominant spp. However, its number also declined rapidly with the depletion of nutrients.

Well Water Isolates

Although 33% of the isolates were tentatively assigned to the Acinetobacter-Moraxella group, they were oxidase positive and sensitive to penicillin and thus appeared to be more closely related to Moraxella than to Acinetobacter. With the exception of M. phenylpyruvica, however, Moraxella species including M. lacunata, M. bovis, M. non-liquefaciens and M. osloiensis were usually isolated from clinical sources and have not been reported to occur in the aquatic environment (Austin et al., 1979). More importantly, the strains isolated in this study could not be matched with any of the Moraxella or Acinetobacter spp described in the 8th edition of Bergey's Manual of Determinative Bacteriology (1974). They seemed to be similar to the Acinetobacter-Moraxella isolates from Chesapeake Bay, described by Austin et al. (1979) and to the atypical and highly pleomorphic Acinetobacter strains from Tokyo Bay described by Simidu et al. (1977).

Pagel and Seyfried (1976) compared aquatic and clinical Acinetobacter spp and showed that the aquatic isolates were biochemically less active than the clinical ones. Our isolates grew poorly on marine agar 2216E and on other media used for characterization of the isolates. They were inactive toward carbohydrates and died-off rapidly after successive transfers in culture media.

Pigmented Isolates

The orange to yellow pigmented bacteria were recovered consistently from the well water (33%) throughout the year. Pigmented bacteria actively degraded macromolecules in aquatic environments (Little et al., 1979; Reichardt, 1980) and pigmented isolates such as Cytophaga psycrophila and Flexibacter columnaris were actively proteolytic and caused lesions on exterior and interior tissues of fishes (Christensen, 1977). In order to determine the potential effect of our pigmented bacteria on fish, 3 isolates from well water (strains 15, 20 and 30) along with a pigmented H_2S producer isolated from bay water (strain 40) were examined in detail.

Taxonomy

The phenotypic and genotypic characteristics of the four pigmented isolates are listed in Table 3. The separation of the non-flagellated flavobacteria from cytophagas remain an open question (Callies and Manheim, 1980). Nevertheless, strains 15, 20, 30 and 40 were tentatively assigned to the genera Flavobacterium, Flexibacter, Cytophaga and Alteromonas, respectively. This tentative classification was based on the scheme provided by McMeekin and Shewan (1978).

Table 3. Characteristics of four pigmented isolates.

Character	strain 15	strain 20	strain 30	strain 40
gram reaction	-	-	-	-
spores formation	-	-	-	-
microcysts formation	-	-	-	-
motility by flagella	-	-	-	+
swarming	-	+	-	-
gliding	-	+	-	-
mol % G+C	42.2	38.3	37.6	(40-50)[a]
pigmentation	orange	yellow	orange	salmon pink
flexirubin	-	+	-	-
cell size: length (µm)	1.5-3.0	4.0-8.0	4.0-8.0	1.5-3.0
diameter (µm)	1.5-0.7	0.5-0.7	0.3-0.7	0.5-1.0
Hugh-Leifson:				
oxidative	+	-	+	+
fermentative	-	-	+	-
production of:				
urease	-	-	-	-
oxidase	+	-	+	+
catalase	-	-	+	+
phosphatase	+	+	+	+
arginine dehydrolase	-	-	-	-
ornithine decarboxylase	-	-	-	+
lysine decarboxylase	-	-	-	-
phenylalanine deaminase	-	-	-	-
H_2S	-	-	-	+
indole production	-	-	-	-
methyl red test	-	-	-	-
Voges-ProsKauer test	-	-	-	-
citrate utlization	-	-	-	-
NO_3 reduction	+	-	-	+
activity on:				
cellulose	-	-	-	-
agar	-	-	-	-
chitin	-	-	+	-
starch	+	+	+	+
casein	+	+	+	+
gelatin	+	-	+	+
tweens 20	+	+	+	+
60	+	+	+	+
80	+	+	+	+
esculin	+	+	-	-
tyrosine	-	-	-	-
utilization of sole carbon sources:				
pentoses:				
ribose	+	-	-	+
xylose	+	-	-	+
arabinose	+	-	+	+
hexoses:				
glucose	+	-	+	+
galactose	+	+	-	+
dissaccharides:				
cellobiose	+	+	-	+
lactose	+	+	+	+
trissaccharide:				
raffinose	+	+	+	+

In the 8th edition of Bergey's Manual of Determinative Bacteriology (Weeks, 1974) the genus Flavobacterium has been divided into two sections. The first contains the non motile-species with 26-43% moles of guanine plus cytosine (G+C) or the so called "atypical cytophagas", and the second, the non-motile and motile peritrichous species with 63-70 moles % G+C. Swarming and motility by gliding or flagella could not be demonstrated for the orange pigmented strain 15 and the G+C content was 42.2 moles %. Therefore, strain 15 was assigned to the genus Flavobacterium, section I. Gliding motility and swarming were inequivocally demonstrated for the yellow pigmented strain 20, which also showed a low G+C of 38.3% and a positive flexirubin reaction. Since flexirubin pigments seem to be restricted to the Cytophagaceae family (Reichenbach et al., 1974), strain 20 is likely to be a Flexibacter or a Cytophaga spp. Due to inability to degrade chitin or agar, strain 20 seems to fit better in the definition of the genus Flexibacter, given by Soriano (1973).

Gliding motility could not be detected in the orange pigmented strain 30. Difficulties in demonstrating spreading and gliding by Cytophaga spp have been reported (Christensen, 1977). Strain 30 showed a low G+C content (37.6%), chitin hydrolysis and a cell morphology typical of Cytophaga spp; it also showed the ability to ferment glucose, a property shared by Cytophaga fermentans (Weeks, 1974) and C. aquatilis spp. nov. isolated from the gills of freshwater fish (Strohl and Tait, 1978).

Strain 40 was assigned to the genus Alteromonas (Bauman et al., 1972) on the basis of its oxidative metabolism and motility via polar flagella. The other characteristics of this strain, such as strong H_2S production, salmon pink endopigment and the ability to decarboxylate ornithine, are characteristics associated with A. putrefaciens (Van Spreekens, 1977).

Proteolytic Activity

Figure 6 compares the extracellular protease activities of the 4 isolates, by the amount of azure dye released from Hide Powder Azure (HPA) when incubated with the culture supernatants at 25C and shaken at 200 rpm. The proteolytic activity of strain 15 was the highest among the 4 isolates, while strain 20 showed almost no activity. It was surprising to note that the Alteromonas isolate, strain 40, showed very little proteolytic activity, despite the fact that A. putrefaciens is known to be one of the most active fish spoilage bacteria (Van SpreeKens, 1977).

Since "extracellular" enzymes of gram-negative bacteria can be contained in the periplasmic space (Costerton et al., 1974), the intracellular-periplasmic contents and the cell debris obtained from the 4 isolates were assayed for their proteolytic activities against HPA. From Table 4, it can be seen that, with the exception of strain 15, the amount of dye released by the intracellular-periplasmic proteases, or by the cell debris associated proteases, was very small or undetectable. The rate of dye release (regression line slope) by strain 15 supernatant proteases was 15 times greater than that shown by the intra-

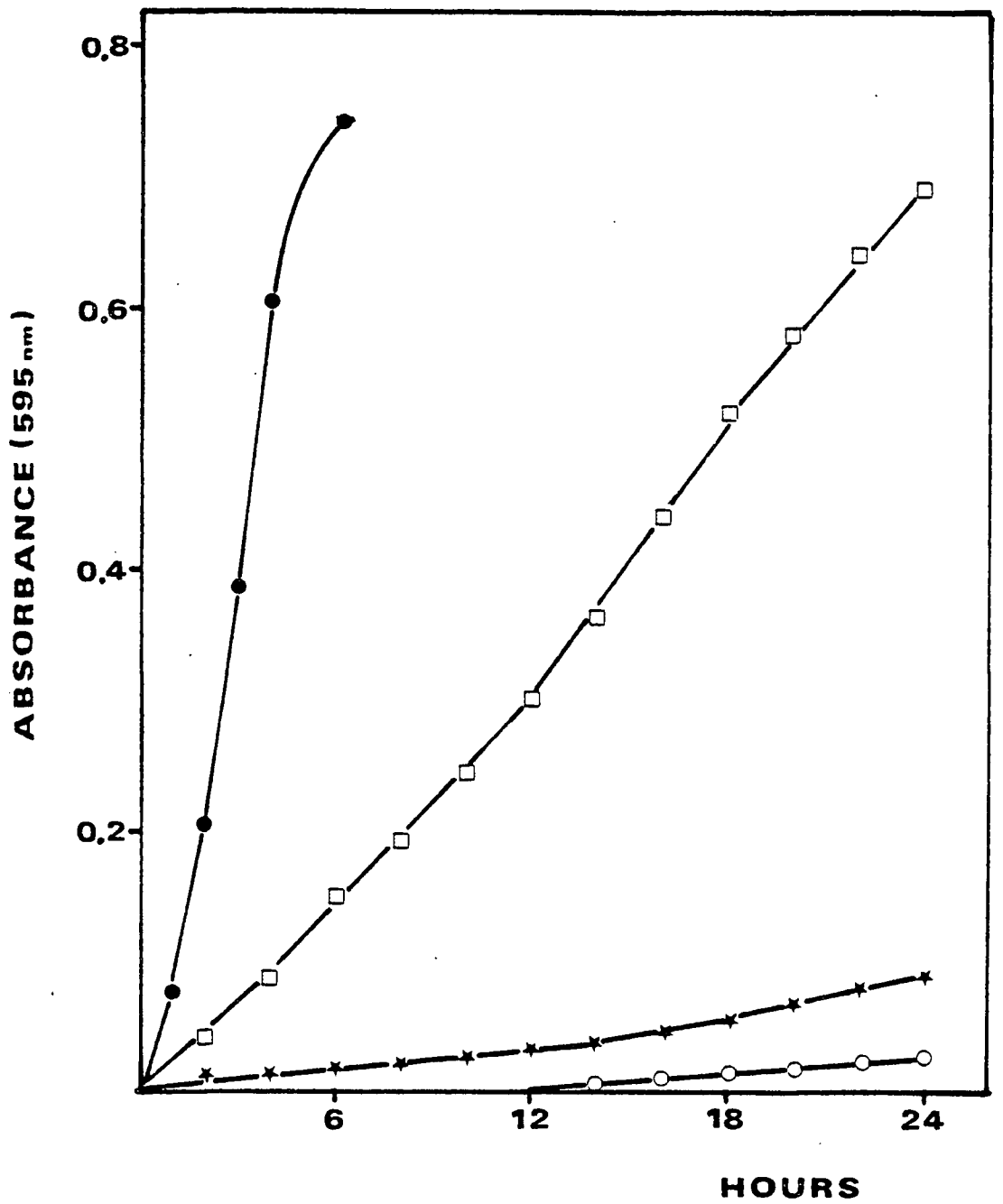


Figure 6. Dye release by the extracellular proteases (culture supernatant) of four pigmented isolates. Symbols: (●) strain 15; (□) strain 30; (○) strain 20 and (✱) strain 40.

Table 4. Localization of protease activity of four pigmented isolates.

Strain	Culture Supernatant		Sonicated cells			
			Supernatant		Cell debris	
	A_{595}[a]	ΔA_{595}/hr[b]	A_{595}	ΔA_{595}/hr	A_{595}	ΔA_{595}/hr
15	0.750	0.150	0.750	0.010	0.450	0.005
20	0.005	0	0.005	0	0.005	0
30	0.300	0.030	0.010	0	0.005	0
40	0.030	0.003	0.005	0	0.005	0

[a] A_{595} = azure dye release measured by absorbance at 595 nm after 12 hr incubation.

[b] ΔA_{595}/hr = slope of regression line.

cellular-periplasmic proteases or 30 times greater than the cell debris associated proteases. According to Ramaley (1979) secretory proteins are not present in cell cytoplasm, because they are excreted as soon as they are synthesized. Intracellular proteases are often in an inactive form (Holzer et al., 1975). Therefore, the dye release obtained by the action of our intracellular-periplasmic proteases could well have been due to the extracellular proteases located within the periplasmic space.

On the basis of the strong proteolytic activity, and its extracellular nature, strain 15 was selected for further study. The mechanism of protease(s) production and subsequent purification and characterization of the protein(s) were attempted with this strain.

Protease Synthesis by Strain 15

BSB Medium Formulation

Hepes buffer (N-[2-hydroxyethyl]piperazine-N'-[2-ethane]sulfonic acid) was selected on the basis of its reported stimulatory effect on protein synthesis in cell free suspension without precipitating cations, as well as for its inability to act as a metabolite when added to cell suspensions (Good et al., 1966). The effect of Hepes buffer concentration on protease synthesis was examined with 0.10 M and 0.15 M Hepes added to the Basal Salts Solution (BSS) at pH 7.0. As shown in Figure 7, during the first 8 hrs of incubation at 25C and 200 rpm shaking, the rate of dye release was greater with 0.10 M than with 0.15 M of Hepes buffer. Since a maximum protease synthesis in a short time period was desired, 0.10 M Hepes was selected for the BSB formulation. Similarly,

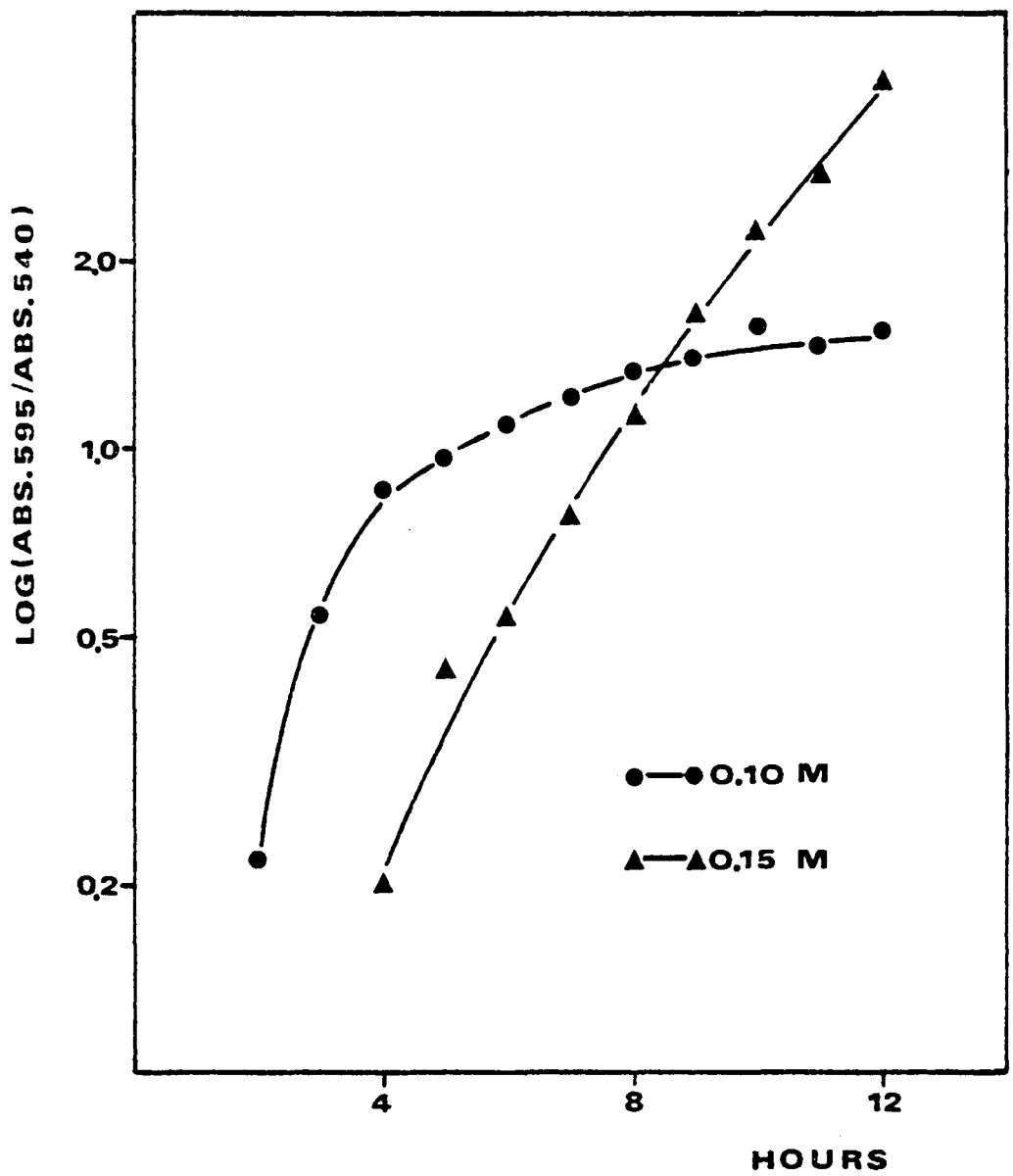


Figure 7. Effect of Hepes buffer addition to the Basal Salts Solution (pH 7.0) on the rate of dye release (A_{595}) during cell growth (A_{540}) of strain 15.

the pH of 6.8 (Figure 8) was selected as the optimum pH within the working range of Hepes buffer (6.8-8.2) for protease synthesis by strain 15.

Metabolites Addition

Carbohydrates and Amino Acids - The effects of additional carbon and nitrogen sources to the BSB-HPA medium, on the azure dye release (A_{595}) and the cell growth (A_{540}) of strain 15 are presented in Table 5. The degree of repression of protease production and for its secretion was shown by the lower % activity. In general, addition of amino acids promoted cell growth but not the proteolytic activity. The carbohydrates, except sucrose, did not promote additional cell growth but, except for pyruvate, they drastically inhibited protease production. Therefore, the production of protease in strain 15 was apparently controlled by the HPA substrate and the inducers derived from it, and repressed by the added metabolizable carbon and/or nitrogen sources. Enzyme synthesis was most strongly repressed by glucose. Repression by sucrose was almost as strong as that caused by glucose. Glycerol was relatively less effective and pyruvate was almost totally ineffective as a catabolite repressor.

The amino acids glutamate and aspartate, when added singly caused only a slight decrease in dye released. However, the combination of glutamate and aspartate was very effective in repressing the protease formation. These 2 amino acids can lead to the synthesis of intermediates of the Krebs cycle, and together they could serve as the

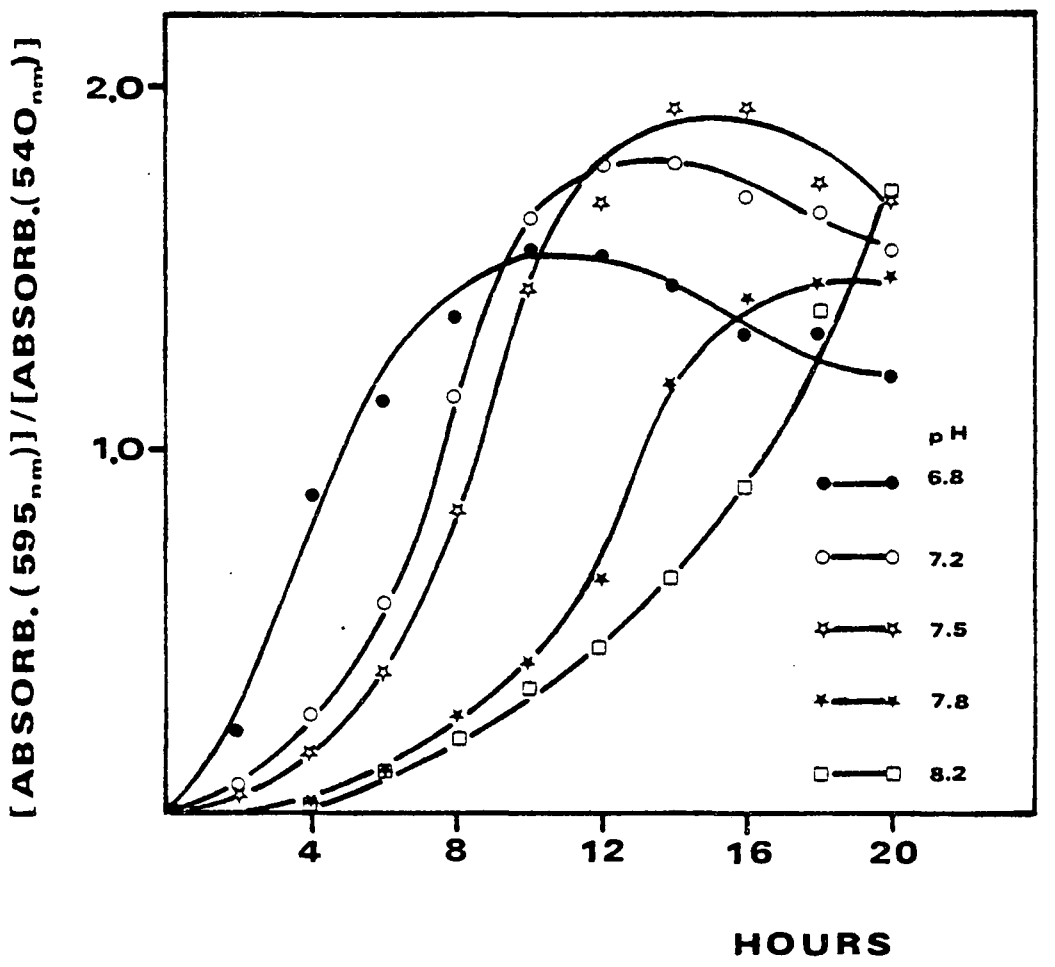


Figure 8. Effect of the pH of the Basal Salts Buffer (BSB) medium on the rate of dye release (A_{595}) during cell growth (A_{540}) of strain 15.

Table 5. Effect of added amino acids and carbohydrates on the dye release (A_{595}) by proteases and cell growth (A_{540}) of strain 15 on BSB-HPA medium[a]

Additions	A_{595}[b]	A_{540}[b]	A_{595}/A_{540}	Relative activity (%)
control	0.047	0.370	1.27	100
Aminoacids				
10 mM aspartate	0.610	0.510	1.20	94
10 mM glutamate	0.570	0.480	1.19	94
5 mM Asp. + 5 mM glut.	0.235	0.460	0.51	40
0.2% Casamino Acids	0.435	0.610	0.71	56
Carbohydrates				
10 mM pyruvate	0.440	0.360	1.22	96
10 mM glycerol	0.250	0.390	0.64	50
10 mM sucrose	0.160	0.615	0.26	20
10 mM glucose	0.075	0.370	0.20	16

[a]the BSB-HPA medium contained 11.7g NaCl, 0.74 $CaCl_2$, 0.38g KCl, 3.45g $MgSO_3$ and 23.83 Hepes buffer. The pH was adjusted to 6.8 with 0.5N HCl. A sterile HPA bag containing 0.2g HPA was aseptically added to the cell suspension.

[b]the A_{595} and A_{540} readings were taken at 12 hrs incubation.

starting point for the synthesis of 12 other amino acids. Therefore, if the carbon and nitrogen needed for strain 15 growth could be synthesized from glutamate and aspartate, there would be no need for this bacterium to break down HPA and the protease production would be repressed. Casamino Acids were less effective as a repressor, than the asparate and glutamate mixture. Similar effects of Casamino Acids were observed with Aeromonas proteolytica (Litchfield and Prescott, 1970a, b) and with an unidentified marine isolate (Daatselaar and Harder, 1974). A possible reason for this could be that the Casamino Acids contain, together with the amino acids, a number of small peptides, which could act as inducers for the proteases production.

Dibutyryl Cyclic-AMP - The decrease in dye release activity caused by the addition of carbohydrates and amino acids (Table 5) suggests that a mechanism similar to catabolite repression was operating in the control of extracellular proteases production in strain 15. Cyclic-AMP has been shown to play an important role in the synthesis of a number of catabolic enzymes in Escherichia coli and other microorganisms by reversing the catabolite repression of glucose and other substrates (Pastan and Perlman, 1970). Attempts to reverse catabolite repression of extracellular protease synthesis, by the addition exogenous cyclic-AMP, in Pseudomonas maltophilia (Boethling, 1975) and Vibrio parahaemolyticus (Tanaka and Iuchi, 1971) had failed. Bromke and Hammel (1979) suggested that the possible reason for the failure was the inability of the cells

to take up the added exogenous cyclic-AMP. However, even by using a more permeable derivative, the dibutyryl cylic-AMP (5 mM), we were not able to reverse the glucose-dependent catabolite repression of the strain 15 protease production. This suggests that the control operating in strain 15 protease production is not regulated by the classical catabolite repression system described by Rickenberg (1974).

Rifampicin - If HPA is an inducer of protease in strain 15, then the new protease-specific m-RNA would be synthesized in the presence of HPA. This new m-RNA synthesis could be prevented by the addition of rifampicin, an inhibitor of transcription (Riva and Silvestri, 1972). Figure 9 shows that when the cells were grown in the presence of rifampicin, with HPA as the sole source of carbon, both growth and protease synthesis were minimal. Since the proteases were not synthesized, HPA could not be used, and the cells did not grow. When 10^{-4}M glucose, plus $5x10^{-4}$M glutamate plus $5x10^{-4}$M aspartate was added to the BSB-HPA medium, as extra sources of carbon and nitrogen, growth occurred even in the presence of 10^{-4}M rifampicin. However, the amount of dye released was less than that with the 10^{-4}M rifampicin alone. Rifampicin thus appeared to have selectively inhibited the extracellular protease-specific m-RNA formation.

The synthesis of extracellular enzymes in Pseudomonas lemoignei (Stinson and Merrick, 1974), P. maltophilia (Boethling, 1975), Bacillus amyloliquefaciens (Both et al., 1972), B. subtilis strain 168 (Glenn, 1976) and Serratia marcescens (Bromke and Hammel, 1979) was found to be

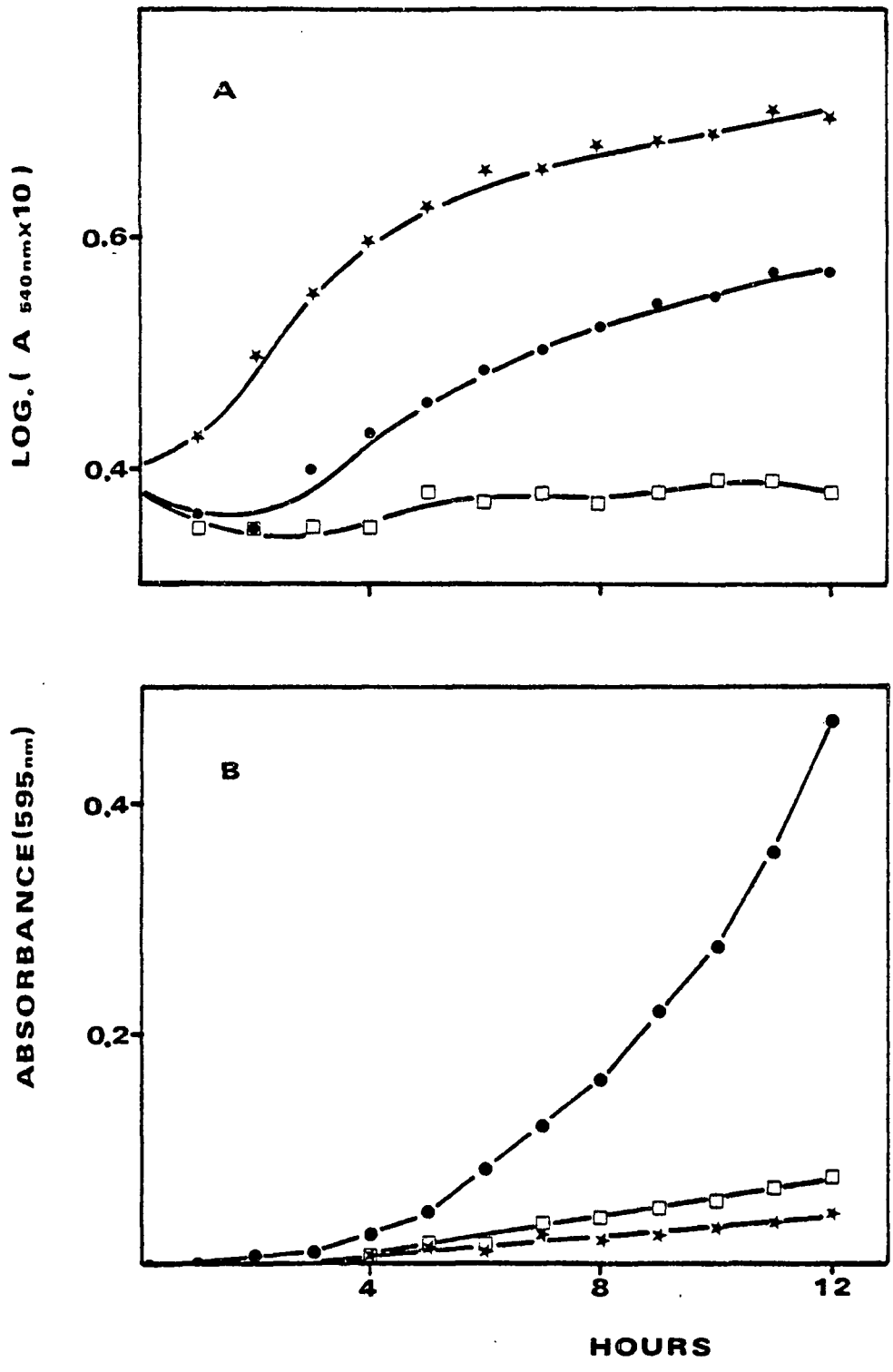


Figure 9. Effect of rifampicin on (A) cell growth (A_{540}) and (B) azure dye release (A_{595}). Symbols: (●) no additions; (□) plus 10^{-4}M rifampicin; (✱) plus 10^{-3}M glucose, 5 x 10^{-4}M aspartate, 5 x 10^{-4}M glutamate and 10^{-4}M rifampicin.

considerably more sensitive to inhibitors of protein synthesis than the synthesis of intracellular enzymes. It has been suggested that the greater sensitivity of the extracellular enzyme synthesis may be due to the fact that the membrane bound ribosomes are more accessible to the inhibitors than are the cytoplasmic ribosomes. This explanation appears to be in agreement with the work of Tai et al. (1979), who showed that the bacterial extracellular enzymes were synthesized in the membrane-bound polysomes.

Characterization of Strain 15 Protease

Protease Inhibitors

The effect of four common enzyme inhibitors on the activity of strain 15 crude protease extract (supernatant) is presented in Table 6. The residual activities with reference to the control (no inhibitor added) were calculated for the different inhibitors. The phenylmethylsulfonyl fluoride (PMSF), a less toxic substitute for the diisopropyl phosphofluoridate (DFP) serine protease inhibitor (Fahrney and Gold, 1963), completely destroyed the protease activity against HPA. Since the PMSF sulfonilates the serine residue at the active site, this would indicate that the strain 15 protease is a serine protease.

Of the microbial extracellular proteases, the best studied as a group are the serine proteases, which can be divided into at least four groups: trypsin-like proteases, alkaline proteases, myxobacter α-lytic protease and staphylococcal protease (Morihara, 1974). Among the best known are the subtilisins, which are alkaline proteases of broad

Table 6. Effect of inhibitors on crude extract protease activity of strain 15

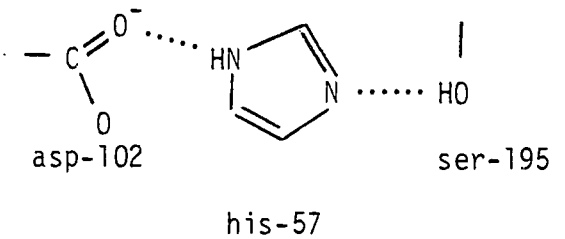
Protease type	Inhibitor[a]	Inhibitor concentration (M)[c]	Regression line slope	Residual activity
	control	-	0.35	100
Metal chelator proteases	EDTA[b]	10^{-4}	0.36	105
		10^{-2}	0.28	80
	o-phenan-throline	10^{-4}	0.20	60
Thiol proteases	p-mercuri-benzoate	10^{-3}	0.28	80
Serine proteases	PMSF[b]	10^{-3}	0	0
		10^{-2}	0	0

[a] Samples (100 ml) of crude enzyme extract (supernatant) were pre-incubated with the inhibitors (25C/200 rpm/1 hr) in 300 ml side arm flasks. HPA bags (0.2% w/v) were added to the preincubated mixture and further incubated for another hour. Residual protease activity was calculated from the regression line slopes of dye release measured at 595 nm.

[b] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; PMSF, phenyl-methylsulfonyl fluoride.

[c] Inhibitor concentration is expressed as final concentration in reaction mixture.

specificity produced by different strains of B. subtilis (Smith and Markland, 1971). The most striking feature of those enzymes is that the structure of their active sites is similar to that found in the pancreatic serine proteases (trypsin, chymotrypsin and elastase), a group of enzymes of independent evolutionary origin (Smith et al., 1970). Microbial and pancreatic serine proteases hydrolyse peptides in a similar fashion because they have the same catalytic triad in their active sites (Stroud, 1974). In this triad, called the "charge relay system", the hydrogen bonding of his-57 to asp-102 increase the reactivity of ser-195 (Wright et al., 1969).



This "charge relay system" has subsequently been found in all the microbial serine proteases investigated for their structure (Fersht, 1977).

Gel Filtration and Molecular Weight Determination

The G-100 Sephadex pattern of the fractionated, dialysed concentrated protein extract is shown in Figure 10. The molecular weight of the protease was estimated to be in the range of 25,000-30,000 by the Andrews (1965) gel filtration method, using bovine serum albumin and ribonuclease reference proteins.

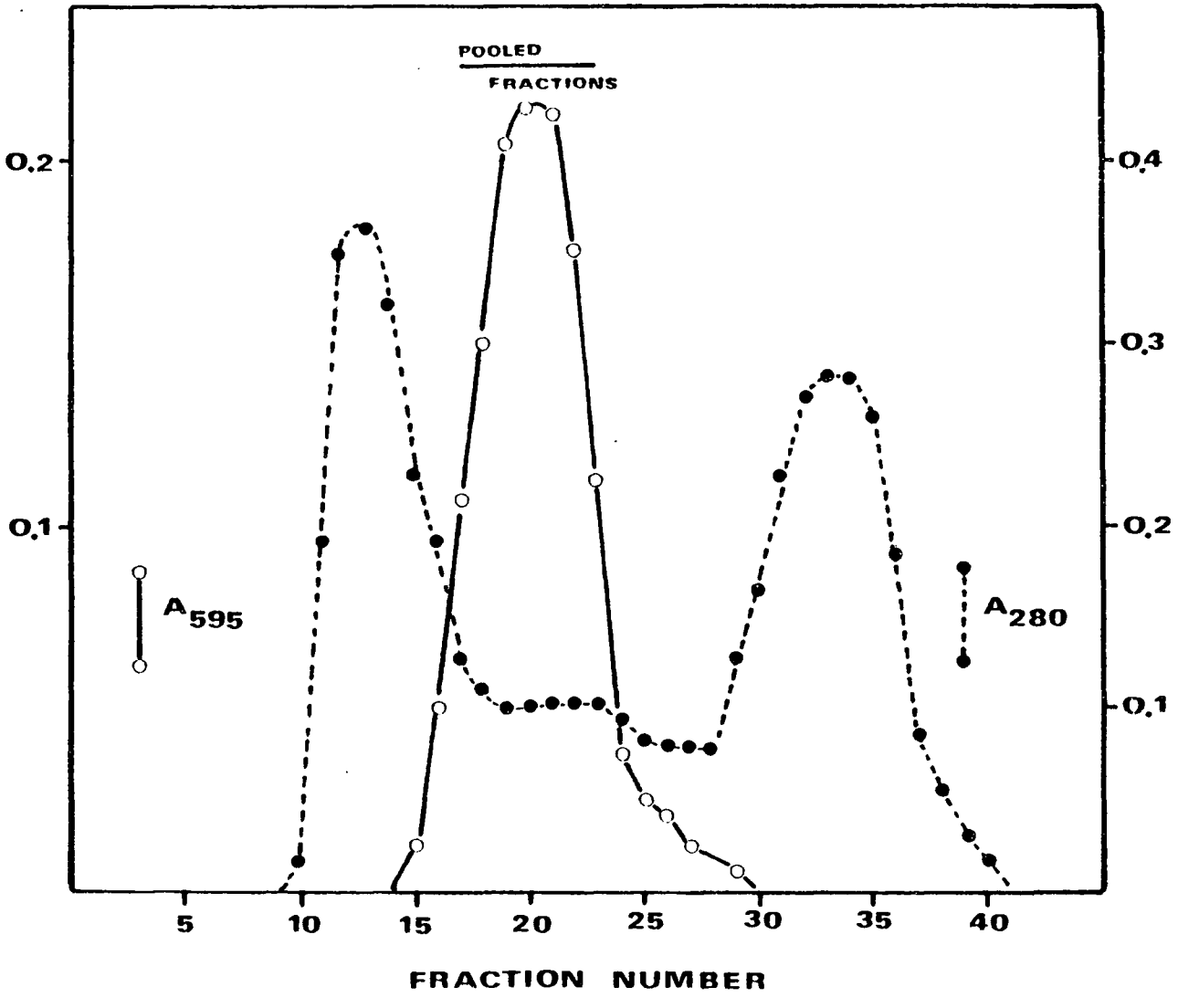


Figure 10. Elution pattern of the fractionated enzyme extract, from a Sephadex G-100 column.

Optimum pH for Enzyme Activity

As shown in Figure 11, strain 15 protease activity was maximum at near pH 8.5. Therefore, it could be classified as an alkaline serine protease. These proteases are produced by bacteria, yeasts and molds. Their molecular weight range between 15,000-30,000 and they are most active at high or pH (∿10). Little is known about the alkaline serine proteases of gram-negative bacteria (Morihara, 1974). A subtilisin-like alkaline serine protease, produced by P. maltophilia, was the first such enzyme to be studied in some detail (Boethling, 1975). This extracellular subtilisin-like serine protease had the molecular weight of 35,000 and the pH optimum at 10.0, and, as was with subtilisins, showed a broad substrate specificity.

Serine Protease Inhibitors

The effect of serine protease inhibitors on the protease activity of the partially purified enzyme is shown in Table 9. The failure to alkylate the strain 15 protease with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) or 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), the reagents specific for the active center histidine in chymotrypsin and trypsin, indicated that it was not a trypsin or chymotrypsin-like serine protease. A partial loss of hydrolytic activity against HPA was obtained by pre-incubation of the enzyme extract with L-benzyloxycarbonyl-phenylalanine chloromethylketone (ZPCK). When this substance was tested on subtilisin BPN', a slow progressive inactivation was observed (Shaw and Ruscica, 1968). Evidences, therefore, suggest that the strain 15 protease be a subtilisin-like serine protease.

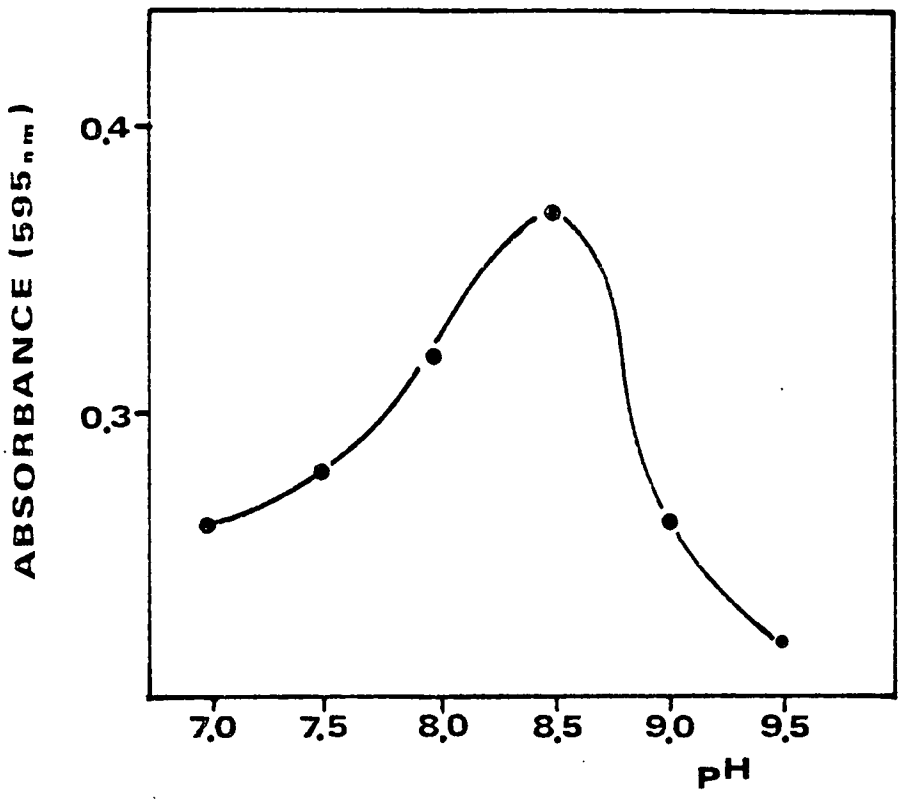


Figure 11. Effect of pH on the activity of the partially purified strain 15 protease.

Table 7. Effect of inhibitors on the protease activity of strain 15.

Protease type	Inhibitor[a]	Inhibitor concentration (M)[c]	A_{595}	% residual activity
	control	-	0.220	100
serine proteases	PMSF[b]	10^{-2}	0	0
trypsin-like	TLCK[b]	10^{-2}	0.220	100
chymotrypsin-like	TPCK[b]	10^{-2}	0.215	97
subtilisin-like	ZPCK[b]	10^{-2}	0.180	81

[a] Samples (0.5 ml) of concentrated pooled fractions (Fig. 10) were diluted in 10 ml of 0.1M Tris-HCl buffer (pH 8.5) and pre-incubated with the inhibitors (25C/200 rpm/1 hr) in 25 ml erlenmayer flasks. HPA (0.2% w/v) was added to the pre-incubated mixture. After 1 hr incubation the reaction was stopped by filtrating the mixture through filter paper Whatman 2^{v}. The dye released in the filtrate was measured by absorption at 595 nm.

[b] Abbreviations used: PMSF, phenylmethysulfonyl fluoride; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; ZPCK, L-benzyoxycarbonyl-phenylalanine chloromethyl ketone.

[c] Inhibitor concentration is expressed as final concentration in reaction mixture.

Gel Electrophoresis

Figure 12 shows the eletrophoretic patterns of the enzyme extract and the partially purified enzyme extract. In each case only one proteolytically active band was detected, and it was located in the same region in the gel.

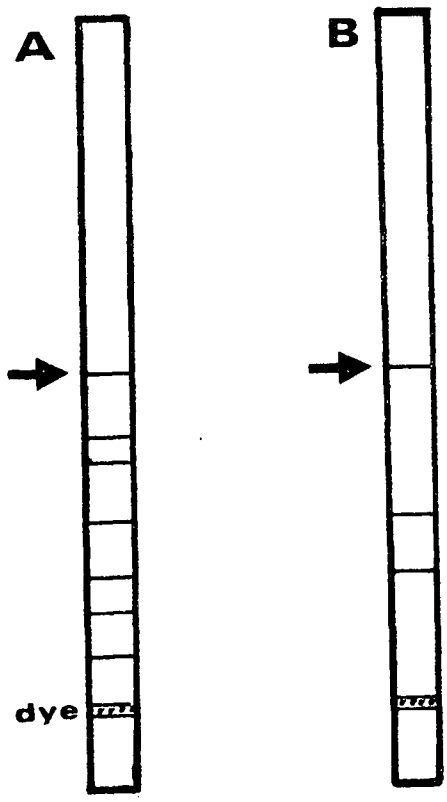


Figure 12. Electrophoretic patterns on 5% polyacrylamide gels:

(A) 100 µg of fractionated and concentrated enzyme extract.

(B) 50 µg of purified and concentrated enzyme (Sephadex G-100 proteolytic fractions). The arrows indicate proteolytically active bands.

SUMMARY

A total of 476 microorganisms isolated from sand filtered estuarine water were studied and compared with 395 Yaquina Bay isolates. Pigmented bacteria were persistently recovered (34.2%) from the filtered water throughout the year. These proteolytic microorganisms were further characterized and their proteolytic activities examined in detail. The results and conclusions are summarized below:

1. Filtration through sand caused quantitative and qualitative changes in microbial population. The number of microorganisms present in sand filtered water (average 20 CFU/ml) was 0.4% of that obtained from the bay water (average 4,500 CFU/ml). Fecal coliforms (FC) found in bay water (average 3.8 FC/100 ml) were never recovered from the filtered water.
2. Flavobacterium-Cytophaga and Acinetobacter-Moraxella spp predominated the bay water microbial flora during the colder months of Winter and Spring. Pseudomonas spp and Vibrionaceae were the predominant isolates during the Summer and Autumn. Flavobacterium-Cytophaga and Acinetobacter-Moraxella spp were consistently isolated from the filtered water. No Vibrionaceae or Enterobacteriaceae spp were isolated from sand filtered water.
3. Three pigmented isolates from the well water that comprised 33% of the total and an estuarine pigmented H_2S producer were taxonomically analyzed. On the basis of their phenotypic

characteristics and the DNA base ratios, those strains were assigned to the genera Flavobacterium, Flexibacter, Cytophaga and Alteromonas.

4. Proteolytic activities of the four pigmented isolates were examined by a colorimetric method based on azure dye release from Hide Powder Azure (HPA). The Flavobacterium spp was the most active and it produced an extracellular protease.

5. Maximum protease synthesis occurred at pH 6.8 with the addition 0.10 M Hepes buffer to the basal medium. Addition of carbohy drates and aminoacids promoted the Flavobacterium cell growth, but reduced the amount of protease produced. The protease synthesis was strongly repressed by glucose. Casaminoacids or the combination of glutamate and aspartate also effectively inhibited the protease synthesis. Catabolite repression ap peared to have been involved in the control of protease synthesis, but the repression of protease synthesis could not be reversed by dibutyril-c-AMP. Rifampicin (transcription in hibitor) prevented growth and extracellular protease synthesis. With the addition of carbon and nitrogen sources, however, growth occurred but no protease was synthesized.

6. The Flavobacterium extracellular protease had a mw of 25,000-30,000 and was completely inhibited by PMSF, a serine protease inhibitor. Maximum protease activity was obtained at near pH 8.5, and it was not affected by TLCK or TPCK, inhibitors of the trypsin and chymotrypsin serine proteases, respectively. The subtilisin inhibitor, ZPCK, was able to partially inhibit

the protease activity. Gel electrophoresis revealed only one proteolytically active band. The Flavobacterium protease thus appeared to be an extracellular, subtilisin-like, alkaline serine protease.

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