STUDIES ON CHITINOCLASTIC BACTERIA IN COASTAL ZONE

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By

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DECLARATION

I hereby declare that this Thesis entitled "Studies on chitinoclastic bacteria in the coastal zones" has not previously formed the basis of the award of any degree, diploma, associateship - fellowship or other similar title or recognition.

Ernakulam,
21April 1982.

(IVY THOMAS)

This is to certify that this thesis is an authentic record of the work carried out by Mrs. Ivy Thomas, M.Sc. under my supervision at the Department of Marine Sciences, University of Cochin and that no part thereof has been presented before for any other degree in any University.

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1. INTRODUCTION

Chitin is a natural carbohydrate polymer which is known to occur as a major structural constituent in the shells of crustaceans, insects and to a lesser extent in plants. It is a close chemical relative of cellulose and it can be modified both chemically and physically to produce materials with a wide variety of potentially useful properties. It occurs in Protozoa, Coelentrata, Mollusca, Chaetognatha, Arthropoda in animal kingdom and fungi and diatom in plant kingdom. It is generally associated with protein and in Crustacea the cuticle may also be highly calcified. Chitin exceptionally constitutes more than half of the organic matter in chitinous structures. Higher concentrations even upto 85% are found in Arthropoda.

It is generally agreed that chitin $(C_6H_9O_4\ NH\ CO\ CH_3)_n$ is a straight chain polymer of N-acetyl-glucosamine (N - acetyl - 2 - amino - 2 - deoxy - D - glucose) units joined to one another by beta - 1, 4 - bonds (Fig. 1).

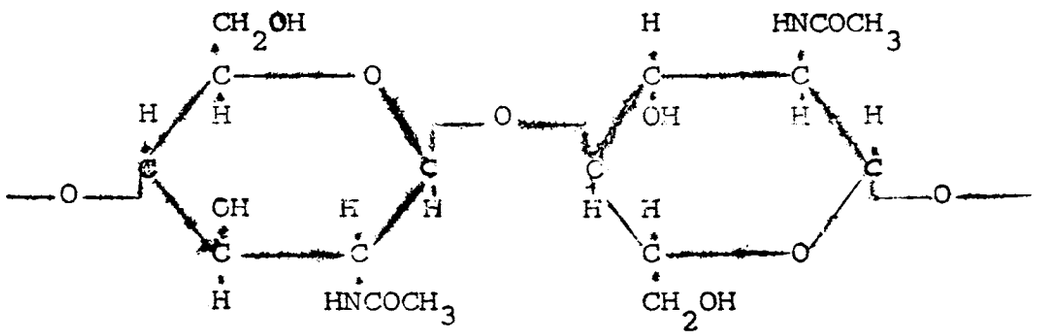

Fig. 1. Units of Chitin.

It is insoluble in water, dilute acids and dilute and concentrated alkaline solutions. But it is soluble in concentrated mineral acid.

On the basis of X-ray diffraction pattern, chitin is divided into three crystallographic types (alfa, beta and gamma forms). The alfa form is known to be very common in arthropods where as beta and gamma forms have reported to occur only in few cases. The distribution of three crystallographic forms does not appear to have any relation to taxonomy. The various chitinous supporting structures exhibit marked differences in morphology chemical composition and physical characteristics.

Chitin and its derivative chitosan have many industrial applications. Chitin in its deacetylated form is used in industry because of its properties like high charged density and potential binding capacity, film forming, coagulation, strong binding to negatively charged polymeric products etc. Because of these properties it is extensively used in ion exchange or chelating solids for chromatography, metal recovery from waste streams, industrial process water purification for recycling, ion exchange membranes for electrodialysis, dye binder for textiles, adhesives etc.

It has many medical applications too. Chitosan (a derivative of chitin) membranes have been used as artificial kidney membranes possessing high mechanical strength in addition to permeability to urea and creatine. The chitin preparations are also used against parasites and as enzymatically decomposible pharmaceutical carriers. Chitosan is a suitable starting material for the production of heparin like blood anticoagulants. The growth and invasive movements of cancer cells could be specifically inhibited with positively charged polysaccharides.

Sirica and Woodman (1971) showed that chitosan can selectively aggregate L - 1210 leukemia cells *in vitro*.

Chitin and chitosan have many applications in the field of agriculture also. The fungicidal effect of chitosan on fungi of varying cell wall composition was reported by Allan and Hadwiger (1979). Chitosan is also known to control *Fusarium* infection in plants (Hatanaka, 1978). Saka (1978) reported that waste mycelium sewage sludge and crab chitin on soil amendments to control the plant parasitic nematodes such as *Meloidogyne incognita* and *Pratylenchus penetrans*.

In the oceans, copepods have a wide distribution and are abundant both in volume and variety. From the data obtained from chemical analysis about five percent of the dry weight of copepod is chitin, the chief constituent of the exoskeleton of the copepods. It has been estimated by Johnstone (1908) that just this one order of planktonic crustaceans produces several million tons of chitin annually. As chitin occurs in the exoskeleton of crustaceans, in some molluscs, coelentrates, protozoans, fungi and yeast the production of chitin in the ocean is believed to be much more abundant.

Jerde and Lasker (1966) have estimated that the population of a single planktonic crustacean Euphausia pacifica produces approximately 1.9×10^{13}g dry weight molt skeleton per year. As per Jeuniaux's (1971) calculation chitin constitutes 58 to 85% of the dry organic weight in the crustacean cuticle and can be estimated that the amount of chitin produced annually as molts of Euphausia pacifica to contain from 4.8×10^{6} to 1.2×10^{7} metric tons of chitin.

ZoBell and Rittenberg (1933) reported that most of the chitin is utilised by biological agents because little of it accumulates in marine sediments. More over, if it were not decomposed there will be a serious drain of Carbon and Nitrogen in their respective cycles. Since there are few animals that can attack chitin directly, it is generally agreed that its decomposition is largely due to microbial action.

The primary means of chitin degradation in aquatic environment is bacterial activity. A number of review articles have been published on microbial chitin degradation (Benton, 1935; Campbell and Williams, 1951; Hood and Meyers, 1973a). Bacteria responsible for this activity have been

isolated and identified by ZoBell and Rittenberg (1938), Campbell and Williams (1951) and Veldkamp (1955) to name a few. Enumeration of such organisms has been however limited. ZoBell and Rittenberg (1938) attempted enumeration of chitinoclasts in marine sediments with little success. Skinner and Davis (1937) and Veldkamp (1955) have enumerated chitinoclasts in soils. Few attempts were also made with fresh water sediments. Okutani (1975) enumerated chitinoclasts in the sediment and water column from Lake Biwa, a fresh water lake in Japan.

The enzymatic lysis of chitin is known to be actually performed by two different enzymes acting consecutively - a chitinase and a chitobiase. This 'Chitinolytic System' can be detected in a given source by incubating it with chitin suspension under optimum pH and temperature and estimating the N-acetyl-glucosamine liberated. Chitinase (EC.3.2.1.14), also classified as an endo - β - N - acetyl-glucosaminidase (Wadstrom, 1971), splits chitin into N-acetyl - D-glucosamine (NAG) dimers and trimers. These may be further broken down by exo-N-acetyl - β - D - glucosaminidase (NAG ase, chitobiase EC. 3.2.1.30).

Enzyme systems which cause the hydrolytic degradation of chitin are also widely distributed in plants, micro-organisms, arthropods and some other invertebrates. Recently the presence of chitinolytic enzymes have been reported in birds, gold fish and some mammals by Jeuniaux (1961, 1962b, and 1965), Dandrifosse et al. (1965) and Jeuniaux and Cornelius (1978).

More recently the presence of nonbacterial chitinolytic enzymes in the digestive tract of Japanese Sea bass (Lateolabrax japonicus), yellow tail (Seriola quinqueradiata), Rainbow trout (Salmo irideus) and some other fishes were reported by Okutani and Kimata (1964a,b), Okutani (1966) and Okutani et al., (1964, 1967 a,b). These observations suggest that simultaneous breakdown of chitin by both bacterial and nonbacterial enzymatic action in the digestive tracts of some animals takes place.

Seki and Taga (1963 a,d; 1965 c) in a series of papers reported on the distribution of chitinoclasts in marine waters and digestive tract of several fishes. Chitinoclasts have been found to be associated with chitin containing organisms (Jones, 1958; Lear, 1963; Seki and Taga, 1963 a,d, 1965 c; Kaneko and Colwell, 1975) and have been implicated in crustacean diseases on the shell and appendages (Sinderman, 1974).

In general, chitinoclastic bacteria may be categorized by their ability to produce a diversity of disease conditions. Numerous shell diseases have been reported to be caused by chitinoclastic bacteria, such as Beneckea, Vibrio, Pseudomonas and Aeromonas species in shrimp, lobster, crab and crayfish (Rosen, 1970; Cook and Lofton, 1973).

With increasing attention on the crustacean culture, quality of water and realistic food conversion rates are essential considerations in order to achieve economic success. The role of chitinoclastic bacteria in such operations is highly critical because they may serve as pathogens causing mass mortalities or serve as incitants of diseases. Chitin digesting Vibrio species (frequently called Beneckea) have been isolated from all marine crustacean exoskeleton lesions properly examined (Hess, 1937; Rosen, 1967, 1970; Cook and Loften, 1973; Young and Pearce, 1975).

Marine chitinoclastic bacteria are exposed to low temperature and high hydrostatic pressures as they subside to the bottom while floating in the open ocean. The thermal and pressure changes must be important ecological

factors for chitinoclastic bacteria when these bacteria are transported to the estuary or bay where considerable concentrations of organic matter are present. Rapid multiplication of the bacteria may occur because they can use a variety of organic materials. In such places the bacteria must be exposed to the changing ecological factors, such as salinity, pH, temperature, etc.

Information on the ecology and biology of chitinoclasts in marine environment is very much limited in India (Srikantaiah and Mohankumar, 1980; Lakshmanaperumalsamy, 1981). Based on these information it is needless to emphasize the necessity to understand the distribution and activity of chitinoclasts in the coastal zone of the marine environment for meaningful and proper management in aquaculture practices. For the present study the coastal zone of Cochin was selected since it has easy access to a variety of biotopes viz. estuarine, neritic and riverine. This study is the first attempt to understand the nature and activity of chitinoclastic bacteria in the coastal zone.

It is proposed to study the occurrence and distribution of chitinoclasts in water, sediment and fauna as related to site characteristics such as temperature, salinity, depth of water, pH, etc. Since no information is available on the chitinolytic properties of coastal strains, it is also proposed to study the chitinolytic activity of the bacterial isolates in relation to various environmental conditions.

It is also planned to work out the taxonomy of some of the representative isolates and certain kinetic properties of their chitinases. It is expected that the results of the study would yield a comprehensive information on the chitinoclastic bacteria in the southern coastal zone of west coast of India.

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2. REVIEW OF LITERATURE

2.1. Distribution of chitinoclastic bacteria:

2.1.1. Distribution of chitinoclasts in water and sediments:

There are a number of reports on the isolation of chitinoclastic microorganisms from different environments like fertilized garden soil, lake waters, sediments, plankton, exoskeletons of insects and crustaceans, intestines of both vertebrates and invertebrates, muds and sands (as reviewed by Benton, 1935; Veldkamp, 1955). It is well known that marine environment is very rich in chitinous material and is an excellent source of chitinoclastic bacteria. Recently Hood and Meyers (1973a) have reviewed the work on the biology of aquatic chitinoclastic bacteria and their chitinolytic activity.

ZoBell and Rittenberg (1938) conducted the initial and notable study in the pelagic zone or deep marine waters and reported the uneven distribution of chitinoclasts in the sediments of California coast. This unevenness in the distribution of chitinoclasts was attributed to the

random distribution of substrates and substrate affinity and colonization of bacteria. The top most sediment layers exhibited a higher bacterial biomass (10^3 cells/g) and the population decreased with increase in core depth. They could not observe any relationship between bacterial biomass and depth of overlying waters or distance from the main land. However, they could find that coarse sediments such as sand supported the largest number of chitinoclastic microorganisms. The possible explanation given was that the chitin particles were concentrated by the sand particles. The percentage of chitinoclast in waters and muds varied from 0.1 to 1% of total bacterial population.

Veldkamp (1955) observed that acid sandy soils always harboured higher concentrations of chitinoclasts and actinomycetes formed the major group of such chitinoclastic population. Hock (1940) reported that mud core samples collected from a depth of 878 metre in the Woods Hole area showed a population of 1.3×10^2 cells/g at the water sediment interface. The population of chitinoclast decreased drastically to 5 cells/g when the depth was increased to 5 cm below the surface of the sediment. On another occasion he found that the population of chitinoclast was 1.5×10^2 cells/ml in 5 fathom waters about one

mile offshore. These results further indicate the inconsistent pattern of distribution of chitinoclastic microorganisms in pelagic waters. Lear (1963) noticed the sparse distribution of chitinoclastic bacteria in regions deeper than 1000 metres off the coast of California and he noticed a correlation between the abundance of bacteria and depth; as the depth increased the population decreased. Bianchi (1971) also observed similar situation in the deep sediments of Mediterranean Sea. Of the 90 samples examined only 10 showed the presence of chitin utilizers: one of the samples however contained 2.4×10^4 cells/g.

One of the most comprehensive studies on the chitin utilizers is that of Seki and his associates. While working in Sagami Bay, Seki and Taga (1965 c) found that each water mass had a characteristic qualitative and quantitative distribution of chitinoclastic bacteria. Maximum number of chitinoclasts were noticed in surface waters and they decreased with depth. They could also observe that the abundance of chitin utilizers varied with temperature. A ten-fold increase in population was noticed from February to May when water temperatures were higher than the winter months. The qualitative variation of chitinoclastic

bacteria in different water masses was attributed to different species of plankton in each layer suggesting a relationship between bacterial types and specific plankton.

The population of chitinoclastic bacteria is significantly higher in shallow waters of coastal zone than in open ocean. Chan (1970) reported higher concentrations of chitin digesters (slightly less than 10% of total bacteria) in sea water and sediment from Puget Sound estuary. Sediments from deep subtidal, intertidal and fresh water areas harboured 2.0×10^4 cells/g, 6.8×10^4 cells/g and 3.7×10^3 cells/g respectively. As in pelagic region highest concentrations of chitin utilizers were at the surface of the sediments and the concentration decreased with depth. Surface waters contained an average of 2.5×10^2 cells/ml while bottom waters contained fewer cells, 2×10^2/ml. Seasonal variations were not noticed at deep sampling stations but in shallow areas the microbial biomass correlated with temperature. The relationship between temperature and abundance of chitinoclast was investigated in Burley Lagoon (Chan, 1970) and the results indicate that the temperature is an important environmental factor influencing the abundance of chitinoclast in shallow

lagoon. Poole and Warnes (1981) reported similar results in East-Central Indiana Borrow Pit lake.

In the neritic waters of Aburatsubo Inlet, Seki and Taga (1963 a) noticed that only 0.4% of the total heterotrophic bacteria were chitinoclastic. No explanation was given to this exceptionally low concentration of chitinoclast in these waters. However, they observed that a considerable number of chitinoclastic bacteria were attached to living copepods suggesting a relation between the chitinoclasts and planktonic crustaceans. An inverse relationship was also noticed between the percentage of chitinoclasts present and chemical oxygen demand (COD). The chitinoclastic bacteria were classified into five species of Beneckea, B. lipophaga predominated in summer and B. hyperoptica appeared in winter and early spring. B. indolthetica and B. chitinovora were always found in association with plankton or suspended matter throughout the year. B. labra was the least common of all the species.

In order to understand the effect of ecological factors on the distribution and growth of chitinoclastic bacteria, Seki and Taga (1963 b) performed a number of experiments. Except B. chitinovora all strains showed 30°C

as their optimum temperature and an inhibition of growth at 40°C. However, the isolates were found to have a higher heat tolerance when compared to other marine bacteria. A temperature of 50°C for 30 minutes exposure was necessary to achieve bactericidal effects. All species exhibited a wide range of pH tolerance with an optimum between 7 and 9. In general, growth was retarded below pH 7 while complete cessation of growth was observed at a pH of 4 (Seki and Taga, 1963 b). These chitinoclasts were also noted to be highly resistant to ultra violet rays. All the above Beneckea species grew well in a media containing 0.5 to 5% NaCl. While 12% concentration of NaCl stopped the cell division. 25% concentration completely killed the cells after 24 hours. These chitinoclastic bacteria were also found to be sufficiently barotolerant. A pressure of 200 atmospheres inhibited the growth but pressures of even 600 atmospheres did not kill the cells (Seki and Taga, 1965 b).

The distribution of chitinoclastic bacteria in the Barataria Bay salt marsh environment was found to be related to factors such as organic matter, chitin deposition and to a lesser extent temperature (Hood, 1973). Highest concentrations of chitinoclast (10^6 cells/g sediment) were

observed in areas of high organic content. The least number of chitinoclast (10^2 - 10^3 cells/ml) was observed in the water column. A concomitant rise in the chitinoclastic bacterial population was noticed with the increase in numbers of chitin producing animals in water column. Beneckea species was found to be the most common bacteria among the chitinoclast. Large concentrations of chitinoclast were found to be associated with intact exoskeleton and the digestive tract of penaeid shrimp.

2.1.2. Association of chitinoclastic bacteria with aquatic animals:

A number of reports show that chitinoclastic bacteria are closely associated with certain marine vertebrates and invertebrates. These reports also suggest a commensal or symbiotic relationship between these microorganisms and plankters. It appears that both external and internal regions of marine animals offer an excellent microenvironment for the growth and survival of chitinoclasts.

Jones (1958) demonstrated that the surface of the radiolarian Castanidin longispinum contained atleast one thousand fold increase in chitin digesters compared to the

biomass of chitinoclasts in the surrounding sea water. Similarly higher concentrations of chitinoclasts were recorded in large copepod species when compared to the surrounding water. They also identified the chitinoclasts as Beneckea species and showed that they were indigenous to copepod and were able to reproduce within the crustacean under certain conditions. Seki and Taga (1963 a) found significant numbers of chitinoclast attached to living copepods within the water column. The observations of Lear (1961) also confirmed this, suggesting that the external surface of planktonic forms serve as the major area of microbial attachment. The adsorption of Vibrio parahaemolyticus, a chitinoclastic bacteria onto chitin and copepods was observed by Kaneko and Colwell (1975). Earlier they also observed a correlation between the population of zooplankton and concentration of V. parahaemolyticus in Chesapeake Bay.

Much work has been done on the microbial flora of the digestive tract of aquatic animals. However, the concept of truly indigenous bacterial population within the digestive tract of fish and other aquatic animals is rather controversial. While Liston (1957) could observe a direct

relationship between bacterial species and species of fish, Potter and Baker (1961) failed to notice such relationship. However, it is generally agreed that the composition of bacterial population in the digestive tract of fish at a given time is mostly dependent on the ingested food (Margolis, 1953). Later workers could show that in some species of aquatic animals at least there is restriction in bacterial types. Aiso et al., (1968) showed that the digestive tract of marine fish as well as plankton had a distinct generic composition ie. Vibrio and Aeromonas and most of them had the capacity to hydrolyse chitin. A large percentage of chitinoclastic bacteria, in few cases exclusively chitinoclasts has been reported in the digestive tracts of both marine invertebrates and vertebrates (Table-1).

Among the marine fishes examined almost 90% of all teleost stomachs and intestines contained chitinoclastic bacteria while the elasmobranchs had a much lower incidence of these bacteria (Chan, 1970).

In addition to this the existence of chitinoclasts in the digestive tract of whales has also been reported (Seki and Taga, 1965 a).

Table 1. Chitinoclastic bacterial population in the digestive tract of animals.

Name of animal	Region	Chitinoclastic bacterial population (Cells/g)	Reference
Octopus	Intestine	1.5×10^5	Seki & Taga (1963d)
Squid	"	4.3×10^5	" " "
Swell fish	"	4.4×10^3	" " "
Japanese Sea bass	Stomach	1.7×10^4	Okutani (1966)
	Intestine	5.4 to 1.6×10^7	" "
	Pyloric caeca	2.3×10^7	" "
Yellow tail	Stomach	2.0×10^4	Okutani et al., (1967a)
	Intestine	1.4×10^5	" "
Variety of estuarine fish	Intestine	1.0×10^7	Chan (1970)

In many cases the chitinoclastic bacteria in the digestive tract of aquatic animals have been identified and species of Vibrio and its closely related species are found to be the major component of the chitinoclast. Sera (1968) reported that chitin decomposing bacteria present in the digestive tract of black sea bream, Acanthopagrus schlegeli, were found to be Vibrio species. Sera and Ishida (1972) later reported the dominance of Vibrio group as chitinoclast in the digestive tracts of many marine fish. In the case of penaeid prawns the chitinoclasts of digestive tract were found to be members of Pseudomonas, Vibrio and Beneckea (Hood and Meyers, 1973 b; 1977 a).

Since now it is established that marine animals harbour restricted bacterial species in their digestive tract and relatively a high microbial biomass, it is necessary to ascertain the role of these bacteria in the nutrition of the animals. The available evidences suggest that the bacteria can contribute to the nutrition of the animals at least in two ways: (1) the bacteria serve as direct food source and (2) the organisms provide growth factors, either by synthesising and releasing it or by

breaking down the substrates by enzymes (Alexander, 1971).

It was established as early as 1931 that bacteria can serve as food for protozoans. Later ZoBell and Feltham (1938) concluded after a series of observations that the abundance of bacteria in marine sediments form an important secondary food source or even an exclusive food source for bottom feeders. Later, evidences accumulated to support this observation (Mac Ginite, 1932; Burke, 1933; Conrey et al., 1972).

In an attempt to define the role of chitinoclastic bacteria within the marine invertebrates (Octopus, squid, etc.) Seki and Taga (1963 d) concluded that only a negligible quantity of 0.003 to 0.0008 percentage of chitin present in the digestive tract was hydrolysed by the bacteria. This shows that the bacteria do not produce enough enzyme to significantly degrade chitin in the diet. However, Hood and Meyers (1973 b, 1977 a) claim that chitinase produced by bacteria degrade chitin and aid the growth of the animal.

Another important role of chitinoclastic bacteria in the aquatic environment, is their ability to cause diseases in fishes and shellfish. These disease producing chiti-

noclasts are detected more frequently with zooplankton and crustaceans. Hess (1937) reported a disease of exoskeleton of lobsters caused by chitinoclasts. These bacteria have been identified as the causative agent of 'brown spot' disease in shrimp (Sinderman, 1971). Numerous shell diseases have been reported in which chitinoclastic bacteria such as Beneckea, Vibrio, Pseudomonas and Aeromonas species were consistently identified with lesions of shrimp, crab, lobster and crayfish (Rosen, 1970; Cook and Lofton, 1973). Sometimes chitinoclasts may be directly toxic or possess the intrinsic capacity to induce a disease condition by certain toxic substance production. For example, V. parahaemolyticus (Krantz et al., 1969; Vanderzant et al., 1970), V. anguillarum (Evelyn, 1971) and V. alginolyticus (Tubiash et al., 1970) have been suggested as causative agents of death in shrimp, crab, molluscs and salmon. A voluminous body of literature accumulated especially in the last decade on the halophilic pathogen, V. parahaemolyticus which is also chitinolytic. This pathogen was responsible for more than 70% of the food poisoning in Japan through fish and shellfish (Sakazaki, 1969). This pathogen was identified in blue crabs in Chesapeake Bay (Krantz et al., 1969), commercial species of shrimp in Gulf of Mexico

(Vanderzant et al., 1970) and oysters (Bartley and Slanetz, 1971). V. parahaemolyticus contaminated crabs and shrimps were attributed to the food poisoning in United States of America. A systematic investigation on the ecology and biology of this pathogen was carried out at Porto Novo in the eastern coast of India and the results confirm the earlier findings on the association of various animals like fish, prawn, crabs with V. parahaemolyticus (Balakrishnan Nair, 1981). He attributed the higher incidence/level of V. parahaemolyticus in freshly caught crustaceans as compared to fishes to the chitinous exoskeleton of prawns and crabs which provide a better substrate for proliferation of the chitinoclastic V. parahaemolyticus and also to the food and feeding habits of crustaceans.

2.2. Taxonomy of chitinoclastic bacteria:

Benecke (1905) was one of the first to describe a bacterium, Bacillus chitinovorous, which exhibited chitinoclastic properties. Numerous reports are available on the non-marine chitinoclastic microorganisms representing a variety of genera such as Flavobacterium, Chromobacterium

(Veldkamp, 1955), and Bacillus (Baxby and Gray, 1968). Members of actinomycetes, Micromonospora, Streptomyces, Nocardia (Veldkamp, 1955) and several fungal species (Gray and Bell, 1963; Otakara, 1964; Leopold and Seichevtova, 1967) have been reported to be chitinoclasts. The following part mainly deals with marine chitinoclastic forms.

As early as 1938, ZoBell and Rittenberg, isolated about thirty-one chitinoclastic bacteria but they were not classified. However two of their isolates were identified as Vibrio species. Hock (1941) described two species of bacteria, Bacterium chitinophilum and B. chitinochroma. Campbell and Williams (1951) isolated Gram-negative chitin decomposers, fermenting glucose (gas, -; acid, +) and identified as Pseudomonas and Achromobacter based on their flagella type. The identification of strains as Pseudomonas was rather unsatisfactory since this genus is restricted to bacteria having oxidative metabolism. The new species assigned to Achromobacter were subsequently placed into a newly created genus Beneckea (Breed et al., 1957).

Six isolates from Japanese waters were classified as Agarbacterium, Beneckea and Pseudomonas (Kihara and Morooka, 1962). Seki and Taga (1963 a) also described thirty nine strains of chitinoclastic bacteria. They

isolated species resembling V. alopsis which was first described by ZoBell and Upham (1944) and Pseudomonas cryothasia (Campbell and Williams, 1951). Okutani (1966) described and proposed the name to six chitinoclastic bacteria from the digestive tract of marine fish as follows: Vibrio gerris, V. orphus, V. labrakos, Aeromonas skiaina, A. chitinophthora and Alginomonas channe. Other chitin utilizers isolated were similar to those previously named: Bacterium lepidorthosae (Campbell and Williams, 1951), Aeromonas liquefaciens, A. punctata, A. hydrophila (Breed et al., 1957); V. piscium (Breed et al., 1957), V. anguillarum (Sakazaki et al., 1970), V. parahaemolyticus (Sakazaki et al., 1963).

Chan (1970) separated the chitinoclasts from Puget Sound estuary into the following genera: Vibrio, Pseudomonas, Cytophaga, Aeromonas, Photobacterium and Streptomyces. He also reported the abundance of Vibrio species, similar to V. marinus (redefined by Colwell and Morita, 1964), V. gerris, V. alginolyticus and isolated a strain which was named Oceanomonas alginolyticus by Miyamoto et al., (1961) but Sakazaki (1968) designated it as a second biotype of V. parahaemolyticus which is its present name.

Eventhough the genus Aeromonas is very closely related to genus Vibrio, both can be differentiated based on their GC ratios (DNA base composition). Aeromonas contains 50 to 60 moles ratio GC (Hill, 1966), while Vibrio has 40 to 50 moles ratio GC (Colwell, 1970). After a detailed study, 145 strains of marine bacteria representing Beneckea, Vibrio, Aeromonas, Pseudomonas and Photobacterium were reassigned to Beneckea (Bauman et al., 1971). Chitinoclastic activity is also widespread among the luminous marine bacteria (Spencer, 1961). The luminous bacteria which exhibited chitinoclastic activity include strains of Photobacterium splendidum, Ph. sepiae, Ph. harveyi, Ph. pierantonii, Ph. fischeri, Ph. phosphoreum and Vibrio albensis. The role of luminous bacteria in the degradation of chitin in nature is obscure.

It is known that part of chitin synthesized in the sea may decompose while still in suspension but the majority sediments, and is incorporated into anaerobic muds where it is eventually degraded. It has been proved now that anaerobic bacteria also take part in chitin degradation (Clarke and Tracey, 1956; Billy, 1969; Timmis et al., 1974). The chitinoclastic anaerobic bacteria identified so far are: Clostridium septicum, C. perfringens, C. novyi type A

(producing active lethal toxin), C. sporogenes, C. tertium, C. chitinophilum.

Poole and Warnes (1981) reported that majority of the chitinoclastic bacteria isolated from a freshwater habitat were Gram-negative - Pseudomonas, Chromobacterium, Flavobacterium, Moraxella and Serratia spp. Gram-positive organisms showing chitin hydrolysis were predominantly actinomycetes.

2.3. Chitin degradation:

2.3.1. The structure of chitin:

The chitin is differentiated into 3 different states based on their purity i.e. "native chitin", "de calcified chitin" and "chitin". Pure chitin is very rarely found in natural environment. It normally occurs in combination with proteins and certain inorganic salts especially calcium salts. This type of chitin is referred as native chitin. The composition of native chitin in the cuticle of animals varies with species. For example, hard exoskeleton of crabs contains more salts and protein than the soft exoskeleton of shrimp. The pure chitin in the exoskeleton is highly organised into linear or fibrous micelles and based on their

X-ray diffraction pattern. The chitin is classified into α, β and γ types. Each chitin is composed of chains of the N-acetyl-glucosamine unit linked in the 1-4 β glucosidic manner but arranged in different physical structure (Richards, 1951). The chitin from the insects and crustaceans is of α type and contains two helical polysaccharide chains oriented in opposite directions with screw axe's along the chain direction. The bonding phenomena has been explained by Dweltz (1960), Carlstrom (1962) and Ramakrishnan and Prasad (1972). The β type is produced by members of Coelentrata, Annelida, Mollusca and Brachiopoda and is associated with collagen cuticle (Hackman, 1964). The γ type is prevalent in cuttle fish shell and its X-ray diffraction pattern is more disoriented when compared to other chitins (Hackman, 1960).

The exact nature of association between protein and chitin is not clearly understood. However, Hackman (1960) suggested that the protein is linked by stable covalent bond resulting in a glycoprotein complex. Subsequently he also showed that the protein appeared to be linked to the chitin chains through the aspartyl and hystidyl residues (Hackman, 1964). This protein can be removed either by hot water treatment or by treatment with 5% KOH, depending

on the type of cuticle (Richards, 1951). Some of the microbial chitinases attached chitin only when its associated protein was removed (Richards, 1951; Bull, 1970).

Calcium carbonate exists in chitinous exoskeleton of crustaceans as calcite and its percent composition varies from 16 - 60% of the dry weight of the cuticle depending on the species. Other inorganic salts such as $MgCO_3$, $Ca_3(PO_4)_2$, SiO_2, $(Al,Fe)_2O_3$, MgO, CaO, P_2O_5 and $CaSO_4$ may also be present in trace quantities (Richards, 1951).

2.3.2. Enzymatic mechanisms involved in the degradation of chitin:

A number of enzymes are known to be responsible for the breakdown of chitin by an exocellular enzyme system and the end products assimilated and is utilized for intracellular metabolic processes by the cell. Based on a number of reports, Hood and Meyers (1977 a) suggested the following pathway for the enzymatic breakdown of chitin:

Prehydrolytic factor
Chitin ----------> chitin (susceptible)

chitinase
Chitin ----------> chitodextrins

* chitinase
Chitodextrins ----------> chitobiose
(short chain
soluble chitin residues)

* chitobiase
Chitobiose ----------> N-acetyl glucosamine

* Deacetylase
N-acetylglucosamine ----------> Glucosamine

* Deaminase
Glucosamine ----------> Glucose*

* Cellular uptake occurs.

Like many other polysaccharides one or more enzymes degrade the substrate to a biose stage and further to a monomer stage by another enzyme. Reynolds (1954) observed that an exocellular chitinase produced by a Streptomyces species degraded the chitin with the formation of monomer (N-acetylglucosamine) and dimer (N,N-diacetylglucosamine). Chitodextrin, glucosamine and glucose were not detected as end products of enzymic hydrolysis of chitin. Berger and Reynolds (1958) found a chitinase system in Streptomyces griseus with two chitinases with similar activities and a chitobiase. While working with chitinase extracted from a snail Kimura et al., (1967 a) showed the presence of three products (N-acetylglucosamine and two oligosaccharides) from

chitin hydrolysis. Okutani (1966) showed the end products of a chitinase system from an animal (a marine fish - Lateolabrax japonicus) and a bacterial source (Vibrio gerris and Aeromonas chitinophthora) were N-acetylglucosamine, its oligosaccharide (possibly N, N-diacetylchitobiose) and glucosamine. However, he suggested that the glucosamine detected was due to deacetylation of chitin during preparation. Similar results were reported by Hackman (1964) and Veldkamp (1955). However Veldkamp (1955) detected the accumulation of acetic acid in the culture fluids of Pseudomonas chitinovorans in the presence of chitin under conditions in which bacterial metabolism was inhibited. It was concluded that the accumulation of acetic acid in the culture fluid was a result of deacetylation of N-acetylglucosamine to glucosamine and acetic acid by deacetylase.

Monreal and Reese (1969) suggested the necessity of a prehydrolytic factor similar to that reported for cellulolytic systems. Chitinase from Serratia marcescens showed increased activity with swollen chitin when compared to crystalline chitin. The data indicated that a special enzyme (CH_1) was necessary to convert the crystalline chitin to a susceptible form for glycanase. No synergistic effect was noticed on

combining the chitinolytic factors; however Jeuniaux (1955, 1959 a) observed such effects when several chitinase fractions were combined.

The chitinase of Serratia marcescens was found to be highly specific acting only on 1,4 polymer of N-acetyl-glucosamine (Monreal and Reese, 1969). These results suggest that the chitinase system consists of (1) chitinase which includes a random endoglycanase releasing soluble intermediates from chitin and (2) a glycosidase which hydrolyses this intermediate to a monomer stage.

The final steps in the break down of chitin have not yet been clearly understood. The presence of exocellular deactylase or deaminase in an organism which produces a chitinase has not yet been recognized. ZoBell and Rittenberg (1938) detected ammonia and acetic acid in the culture media of marine chitinoclastic bacteria when grown in the presence of chitin. Large quantities of acetic acid and lactic acid along with other organic acids were found accumulated in the media when marine chitinoclastic bacteria were grown on chitin as the sole carbon source (Okutani and Kitada, 1968 a,b). It was also observed that acetic acid accumulated to a lesser extent even in media without chitin. These results suggest

that the organic acid detected in the culture media are mostly metabolic intermediates produced by intracellular reactions. However an exocellular specific deacetylase capable of attacking acetyl group on the N-acetylglucosamine unit in E. coli was recognized (Faulkner and Quastel, 1956; Dobrogoxz, 1968). Wu and Wu (1971) showed that deacetylated glucosamine was transported into the cell by a specific enzyme system.

The information on the deamination of glycosamine is very much limited. Eventhough the assimilation of amine compounds by the marine bacteria has been shown, the exact mechanism has not yet been clearly demonstrated (Meyers and Nicholson, 1970). Budd and Spencer (1968) observed that marine bacteria utilized methyl amine by a demethylation process and not by deamination or amine oxidase mechanism. Eventhough Reisert (1972) detected glucose and N-acetylglucosamine in the culture fluid of a fungus Chytrimyces species grown with chitin, the presence of glucose was attributed to its release during germination of the spore rather than breakdown products.

2.3.3. The rate of chitin degradation:

The process of degradation within an aquatic ecosystem plays an important role in the cycling of nutrients within the ecosystem. Chitin decomposition is significant when we consider the large quantity of chitin generated in the aquatic ecosystem and its relatively high nitrogen content. The release of organically bound nitrogen and carbon is a substantial factor to be considered in carbon-nitrogen cycling in a given ecosystem. The rate of chitin degradation is known to be governed by a number of biological and chemical factors.

For substrates where the primary mechanism of breakdown is mainly by an enzyme it is rather difficult to discuss the degradation in 'nonenzymatic' terms. Not only enzymes are limited by their indigenous properties but also by those limiting factors in growth and development of the organism. The example given by Hood and Meyers (1973 a) is illustrative of this fact. "If an organism produces an extra cellular enzyme whose cell free characteristics include inactivation at pH 6 - 7, but the organism is neither found in nature nor can survive at pH's other than 6 and 7, the enzyme, for all practical purposes is non-functional within that biological system". With these considerations only the process of chitin degradation is discussed below.

A number of publications especially those of Seki (1965 a,b) and Seki and Taga (1963 c) have attempted to determine the rates of chitin degradation in the oceanic environment. When pure cultures of Beneckea species were grown in a chitin medium under simulated conditions the rate of decomposition was 30 mg chitin/24 hrs/10^{10} bacterial cells. It is interesting to note that only little variation in the rate of degradation was noticed with the bacterial type, the initial concentration of inoculum and initial concentration of chitin. However it was reported that the surface area of the particle inversely affected the rate of decomposition, i.e. smaller the particle faster the degradation.

Chitin degradation is also known to occur even in deep seas. In a radiolarian ooze obtained from the abyssal region (36°59'N, 152°36'E) of the Pacific Ocean and which was stored for about two months before the bacteriological analysis a few chitinoclastic bacteria were found per gram dry weight of the ooze (Taga and Seki, 1965). However, hydrostatic pressure is known to affect the rate of chitin degradation; a pressure of about 200 atmospheres decrease the rate nearly by 40%. Seki and Taga (1963 c) calculated the disappearance of chitin at optimal temperature. Since 0,033 cm long chitin strips occur predominantly in the ocean its rate of decomposition was

calculated as 27 mg/24 hrs/g of chitin at 25°C. As per their calculation the complete mineralization of chitin may take 40 to 70 days.

Using a simulated model sea bed system Liston et al. (1965) showed a rapid loss of $CaCO_3$ initially from a chitin substrate followed by a slower loss of protein and chitin. The chitin degradation rate of Puget Sound sediment was 18.8 mg chitin/day. But with a mixed coastal sediments it was 4.5 mg chitin/day (Liston et al., 1966). Using the carbon conversion rate they could find that the microbiota involved in the chitin degradation in nearshore sediments was different from offshore sediments. Subsequently Chan (1970) demonstrated that Vibrio species from the Puget Sound estuary degraded chitin at a rate of 80 - 130 µg/hour/10^{10} bacterial cells at 22°C. Seki (1965 b) conducted some closed system experiment and observed areas of higher chitin decomposition i.e. 75 mg chitin degraded/30 days, just below the water sediment interface than in the water column or in the deeper sediment zones. Since this was a closed system experiment the results of which cannot be directly compared to the in situ conditions. However, the data support the presence of microenvironments capable of carrying out greater chitin mineralization.

In situ rates of chitin degradation of native chitin in the salt marsh ecosystem were extremely high i.e. 87 mg/day/g chitin (Hood, 1973). Hood and Meyers (1977 b) also studied *in vitro* chitin degradation rates for predominant chitin degrading bacteria from the estuarine environment. They reported that untreated (native) chitin offered the best substrate for microbial degradation while pure chitin was degraded much more slowly. Another interesting observation which they recorded was with increased quantities of seeded chitin an increase in decomposition rate was noted. The average rate of degradation for the isolates in pure cultures was 38.8 mg/day/10^{10} cells at 22°C. This value is comparable to that reported by Seki and Taga (1963 c) from strains isolated in Aburatsubo Inlet (30 mg/day/10^{10} cells at 25°C) and by Chan (1970) from strains isolated from Puget Sound estuary (19.2 - 31.2 mg/day/10^{10} cells at 22°C).

The relative potentials of chitin degradation rates in sea water and sediments from the Barataria Bay site (Hood and Meyers, 1977 b) is comparable to those found in Aburatsubo Inlet in Japan (Seki, 1965 b). Data suggest that little variation in chitin degradation potential exists within these estuarine types. *In situ* studies revealed several factors which influenced the rate of chitin decomposition.

A strong correlation was observed between decomposition rate and environmental water temperature. The degradation rate was maximum in late summer when the water temperature averaged 30°C while lowest rates were recorded in mid winter when the temperature was 10°C (Hood and Meyers, 1977 b). In the ocean of the temperate zone chitin may be calculated to be completely mineralized within 140 days in the surface waters at 15°C, within 370 days in the intermediate waters at 5°C and within 500 days in the deep waters at a few degrees centigrade if the effect of hydrostatic pressure is not taken into account (Seki, 1965 a)

Goodrich and Morita (1977 a) reported on chitinase activity associated with marine fish and estimated, based on digestive tract bacterial analyses, that a single species of fish (Enophrys bison) would be responsible for the decomposition of as much as 16 metric tons of chitin annually. Poole and Warnes (1981) studied the microbial mineralization rates of chitin in a fresh water habitat. Results indicate that the sediment water interface was an active site of chitin mineralization in the lake environment also. The samples seeded during summer showed the fastest rate of decomposition with greater than 50% loss after two weeks and greater than 95% loss after 7 weeks of incubation in situ. The highest rate

of decomposition during this period was 43.6 mg/day. Samples seeded during the spring and fall showed considerably slower rates with only 30% weight loss after 9 weeks of incubation *in situ*.

From the results of these workers (Seki, 1965 a,b; Seki and Taga, 1963 c; Chan, 1970; Hood, 1973; Hood and Meyers, 1977 b; Poole and Warnes, 1981) it is apparent that chitin degradation is primarily a function of temperature and organic matter. Factors such as salinity and pH which do not fluctuate to any appreciable amount in the marine environment have little effect on the growth of chitinoclastic bacteria or on the rate of chitin decomposition.

2.4. Distribution of chitinase system:

2.4.1. General aspects:

Chitinases are known to be distributed widely in bacteria, fungi, plants, invertebrates and vertebrates. Since microorganisms form a convenient source of chitinase, these systems are more generally studied than the other systems. Among plants a chitinase system has been reported in beans and other seeds (Powning and Irzykiewicz, 1965). Abeles *et al.* (1971) reported glucanase and chitinase activity

in bean leaves. Egg plants and peppers (Koroleva et al., 1979) and wheat germ (Molano et al., 1979) have also been reported to contain chitinase systems. A number of fungi have been identified to elaborate chitinase - Basidiomycetes (Tracey, 1955); Aspergillus niger (Otakara, 1963; Thomas et al., 1979); Chyrtrimyces hyalinus (Reisert, 1972); Verticillium albo-atrum (Vessey and Pegg, 1973); Sclerotinia sclerotiorum (Rai and Dhawan, 1978); Beauveria bassiana (Leopold and Samsinkova, 1973). The cray fish parasite, a fungus, Aphanomyces astaci is known to excrete chitinase (Unestam, 1966, 1968; Soderhall et al., 1978). Members of Actinomycetes, especially Streptomycetes, have also been reported as chitinase producers. Streptomyces griseus (Berger and Reynolds, 1958); S. violaceus (Wigert, 1962); Streptomyces species (Skujins et al., 1970); S. orientalis (Tominaga and Tsujisaka, 1976) are all chitinase producers.

Among the vertebrates, most species whose diet contains organisms containing chitin (e.g. insects and fungi) synthesize chitinolytic enzyme in their digestive system. These enzymes are principally secreted by the gastric mucosa but in some species also by pancreas. These chitinolytic enzymes can be defined as true chitinases devoid of any significant lysozymic activity (Cornelius et al., 1975).

In lower vertebrates the correlation between secretion of chitinases and nature of diet is clearly seen in amphibians and reptiles. This correlation is less obvious in fishes (Micha et al., 1973). In higher vertebrates also the relation between the chitinase production and nature of diet is seen. Among birds and mammals so far studied insectivorous or omnivorous species always secrete chitinases (Jeuniaux, 1961, 1962 b, 1963). Cornelius et al. (1975) reported that out of the six species of mammals belonging to order Carnivora chitinase was found to be associated with the gastric mucosa of only two species (Canidiae: dog and fox) which are not adapted to strictly meat diet. A glycol-chitin-splitting enzyme without lysozyme (muramidase) activity was found in serum from various animals. Goat, cow, hen, sheep and pig possessed high activity and no activity was found in serum from man, monkey, horse, dog, cat, rabbit and guinea-pig (Lundbald et al., 1974, 1979 a).

The occurrence of chitinase in the digestive juice of snail, Helix pomatia has also been reported (Zechmeister and Toth, 1939; Strasdine and Whitaker, 1963; Lundblad et al., 1976). A chitinase from the snail Helix peliomphala was partially purified by Kimura et al. (1967 a, b). Chitinolytic enzyme activity in the larval development of silk worm,

Bombyx mori has also been studied (Kimura, 1973). Recently chitinase was recorded in the hunting spider, Cupiennius salei (Mommsen, 1980).

2.4.2. Chitinase of marine animals:

A number of marine vertebrates and invertebrates have been reported to contain chitinase. Okutani and Kimata (1964 a, b) examined a number of aquatic animals (Lateolabrax japonicus, Seriola quinqueradiata, Hippoglossoides dubius, Stichaeus grigorjewi, Gadus macrocephalus, Mustelus manazo, Polypus dofleni, Ommastrephes sp.) for the presence of chitinase in various organs and concluded that stomachs, the livers and the spleens of all fish tested and the stomach, liver and the buccal mass of cephalopoda tested exhibited chitinase activity. A series of investigations by Okutani (1966), Okutani et al. (1967 a, b) and Sera and Okutani (1968) described the properties and mechanisms of the chitinase systems of the Japanese sea bass (Lateolabrax japonicus), the Yellow tail fish (Seriola quinqueradiata), the Rainbow trout (Salmo irideus) and the sea bream (Acanthopagrus schlegedi). Highest activity was reported in the stomach regions whereas little activity was observed in pyloric caecea and the intestines.

A chitinase system was demonstrated in the gastric juice of the American lobster, Homarus americanus, by Brockerhoff et al. (1970). A highly active chitobiase was detected but very little chitinase activity was noted. Earlier Kooiman (1964) reported chitinase activity for related species, Astacus fluviatilis and Homarus vulgaris. High amounts of chitinases and chitobiases have been found in gastroderm of four species of sea-anemones (Jeuniaux, 1962 a). The chitinolytic activity of the gastroderm was found to be as high as those of the gastric mucosa of some insectivorous vertebrates, in terms of wet weight of tissues. Strong chitinase activity was found in the gastric mucosa of elasmobranchs (Squalus acanthias, Etmopterus spinax and Raja radiata) and the teleost (Coryphaenoides rupestris) (Fange et al., 1976, 1979, 1980). A remarkably high chitinase activity occurred in the pancreas of stomachless holocephalan fish, Chimaera monstrosa. Hormonal control of chitinolytic activity in the integument of Balanus amphitrite has been observed (Freeman, 1980).

These reports suggest that a variety of animals secrete their own chitinases. However as pointed out earlier, the presence of chitinase in an animal can be correlated to their chitin containing diet. Later workers started looking at the role of bacteria in the gastro-intestinal tracts of these

animals. Microorganisms may serve as a direct source of nutrient for the animal as well as the elaboration of extracellular *in situ* chitinase system. In the shrimp (*Penaeus setiferus*), the chitinase produced by the predominant gut bacteria, *Beneckea neptuna* was found to be an inducible chitinase whereas the animal had an indigenous constitutive chitinase and chitobiase systems (Hood and Meyers, 1977 a). However, the occurrence and activity of chitinase in the stomach contents of *Enophrys bison* and *Platichthys stellatus* were attributed to the chitinoclastic bacteria present in the organ (Goodrich and Morita, 1977 a, b).

2.4.3. *Chitinase in sea water and sediments*:

Since chitinoclastic bacteria are reported to be more widely prevalent in the marine environment, Goodrich and Morita (1977 a) made the first attempt to measure the chitinase activity in sea water and sediment samples of Yaquina Bay, Oregon, USA. In all sediment and water samples from Yaquina Bay, no detectable levels of chitinase activity were noted. Subsequently, Chandramohan and Thomas (1980) analysed fifteen sediment samples, collected from various regions of Cochin backwater but failed to detect any chitinase activity. However Goodrich and Morita (1977 a) reported that chitinase activity could be detected in offshore sediments (400 m).

2.5. Production and properties of chitinases:

2.5.1. Cultural conditions and production of Microbial chitinases:

The first step in the study of microbial chitinases is the identification of conditions which promote production of enzyme. Such cultural conditions were reviewed by Monreal and Reese (1969). The factors which are known to affect the production of chitinases in microbial cultures include (1) source of chitin, (2) type of chitin, (3) particle size, (4) initial concentration of chitin, (5) incubation period, (6) temperature, (7) pH and (8) certain organic and inorganic chemicals.

Serratia marcescens elaborated little enzyme when grown on mushroom chitin or on beetle (Tribolium) chitin and it produced higher quantities of enzyme with shrimp chitin. Maximal yields were obtained on highly purified commercial chitin. Baxby and Gray (1968) also observed increased growth of bacteria on shrimp chitin than on lobster chitin. However, it should be noted that in these studies the shrimp chitin which they used was a highly purified one whereas the lobster chitin was only partly purified. The reduction of substrate particle size resulted in increased chitinase activity. Maximal yields of enzyme occurred on 1.5% to 2.0% chitin. However for the

fungus Chytriomyces, only 0.2% substrate was required for highest enzyme production (Reipert and Fuller, 1962). Highest activity was detected on the substrate chitin, whereas with the soluble dimer (N, N-diacetyl chitobiose) nearly one third of the maximum yield was recorded. The monomer (N-acetyl-glucosamine) gave less than 7% of the activity while chitosan, glucosamine, cellulose, cellobiose, glucose and lactose yielded very little activity. Therefore Monreal and Reese (1969) have suggested that the probable inducers of chitinase system are short chain units (three or more N-acetylglucosamine units).

Maximum enzyme yield of Beneckea neptuna was at 25 - 27°C at 4 to 5 days, with 0.5% chitin concentration (Hood and Meyers, 1977 a). The initial pH of the medium did not have any appreciable effect on enzyme production. Although maximum chitinase activity by B. neptuna was at 25°C, considerable yield was obtained even at 20°C. Very little difference was observed in enzyme yield at pH 6.0 and 7.0. Eventhough 5 days were required for maximum enzyme production, the chitinase yields were relatively high even after one day. Since substrates such as chitosan, N-acetylglucosamine, glucosamine, glucose and peptone stimulated little chitinase activity, the data would suggest that the enzyme system

is induced by chitin units, an observation which supports the earlier finding of Monreal and Reese (1969). However the chitinase of the fungus Beauveria bassiana produced chitinase without the addition of chitin to the medium, indicating the constitutive nature of the enzyme (Leopold and Samsinkova, 1970).

Clarke and Tracey (1956) reported that glucose in the culture medium depressed chitinase production by a factor of 3 to 5. Similarly Okutani and Kitada (1968 a,b) observed the acetate and lactate in the culture medium inhibited chitinase production of several chitinoclastic marine bacteria but little inhibition was noticed when the bacteria were grown on succinate. While studying the aerobic decomposition of chitin by isolated chitinoclastic bacteria, Seki and Taga (1963 c) observed that peptone and glucose exerted very little effort on chitin decomposition. They also reported that the maximum activity was noticed on the second day and the rate of chitin decomposition decreased with increased particle size. Further they observed a lowering of the pH in the media after bacterial growth and accumulation of a considerable amount of ammonia. Since hydrostatic pressure is known to affect the rate of decomposition of chitin and the bacterial population the production of chitinase by

bacteria may also be affected by such pressures (ZoBell and Oppenheimer, 1950; Oppenheimer and ZoBell, 1952; Seki and Taga, 1963 c).

Highest chitinase yields were obtained in 4 - 5 days for Serratia marcescens and several other species (Monreal and Reese, 1969) and for the fungus Beauveria bassiana (Leopold and Samsinkova, 1970). Reynolds (1954) reported that Streptomyces species produced maximum quantities of chitinase in 6 days growth.

A Vibrio species isolated from sea shore mud exhibited maximum production of inducible chitinase at 30°C in a two days culture with medium containing 0.2% colloidal chitin (Uchida et al., 1979). The initial pH for maximum enzyme production varied with the species of microorganism. Generally bacteria showed optimum chitinase production at neutral pH 7.0 to 7.5, fungi at pH 4.5 (Monreal and Reese, 1969). The chitinase production by Bacillus thuringiensis increased when the organism was cultivated at pH 7.2 (Chigaleichik, 1976). Reyes et al. (1977) studied autolysis of Neurospora crassa under different cultural conditions and release of chitinase. Chitinase activity was never found in fermentor cultures. In shake culture, this enzyme was found after 22 days of autolysis whereas in stationary cultures it was excreted after 8 days of autolysis.

2.5.2. Properties of Microbial chitinases:

It is a well established fact that extraneous protein content of an enzyme extract causes marked change in the substrate breakdown rate and the effect of pH on the activity of the enzyme as pointed out by Tracey (1955) the enzyme must be purified before characterization however such purification is tedious and time consuming and because of these reasons many of the reports on the properties of chitinase have involved crude extracts. With this limitation the properties of some of the microbial chitinases are given below:

Chitinases from several species of Streptomyces have been isolated and purified to determine many of the physical and chemical properties (Jeuniaux, 1957, 1958, 1959 b; Skujins et al., 1970). The pH optimum for the chitinase of Streptomyces antibioticus was found to be 6.2 (Skujins et al., 1970). This enzyme was found to be relatively stable while drying and heating with inactivation at 65°C within 3 hours. The sequence of ion inhibition was as follows: $Mg^{++} < Co^{++} < Zn^{++}$. It was also observed that Na^{+} inhibited the enzyme more effectively than Ca^{++}. However in small quantities Ca^{++} stabilizes the enzyme (Jeuniaux, 1959 b; Skujins et al., 1970). Wigert (1962) reported that the activity of chitinase from a Streptomyces species was reduced by Cu^{++} and activated by Mg^{++}.

The molecular weight of the Streptomyces chitinase was found to be 29,000 based on the sedimentation test (Skujins et al., 1970). Since chitinase reaction takes place only after adsorption of the enzyme to the surface of the substrate and the Michaelis-Menten equation is not applicable to enzymes which act at surfaces the Km of the chitinase is considered to be unrealistic (Skujins et al., 1970). Two chitinase enzymes (I and II) isolated from Streptomyces orientalis have molecular weights of ~ 33,000 and 25,000 and isoelectric focussing point at pH 8.80 and 8.65 respectively. Both the enzymes have been reported to have an optimum pH of 5.5 - 6.5 and they were stable at pH 6.0 - 8.0 at 40°C for 3 hours (Tominaga and Tsujisaka, 1976). Chitinase of Streptomyces griseus and chitinase and chitobiase of Actinomyces species had an optimum pH of 6.2 and 6.0 respectively (Berger and Reynolds, 1958; Tiunova et al., 1973).

The mode of degradation of glycol-chitin and chitin by two enzyme fractions isolated from Aspergillus niger was investigated by Otakara (1964). One of the enzymes rapidly cleaved the endo- β - glucosaminidic bonds in the polysaccharide chain forming chitodextrin and oligosaccharides, while the other one produces monosaccharide as a main product in the degradation. A study of the chitinase of another

fungus Chyrtriomyces hyalinus revealed greatest activity at a pH similar to those in Streptomyces i.e. pH 5.5 (Reisert, 1972). Activity was found to be negligible below 10°C, optimal at 25°C and completely lost at 45°C. Eventhough N-acetylglucosamine and glucose did not inhibit the activity, Cu^{++} and Cd^{++} caused total inhibition. Co^{++}, Li^{++}, Mg^{++} and Na^{+} resulted in decreased activity in the same order. Similar results were also reported for the chitinase of the parasitic fungus Aphanomyces astaci (Unestam, 1968).

The chitinases of several marine bacteria were isolated and their properties were studied either with purified or partially purified preparations. The partially purified chitinase from Aeromonas chitinophthora and Vibrio gerris exhibited a pH optimum of 5.5 - 6.0 and 7.0 respectively; stability maintained within a range of pH 5.0 - 9.0; and temperature optimum of 40°C (Okutani, 1966). A crude preparation of chitinase from Serratia marcescens showed the maximum activity at pH 6.4 and 50°C with 50% loss of activity at 50°C for one hour at pH above 7.2 and below pH 4.8 (Monreal and Reese, 1969). Chigaleichik and Pirieva (1978) observed differences in the properties of chitinases isolated from pigmented strains of Serratia marcescens and their non-pigmented variants.

Properties of chitinase from a marine bacterium, Vibrio alginolyticus was studied using a chromogenic substrate 3,4 dinitro-phenyl-tetra-N-acetyl chitio-tetraoside (I) (Aribisala and Gooday, 1978). The chitinase exhibited maximum activity at pH 5.5 and 56°C with a substrate concentration of ~ 0.23 mM. Higher concentration of the substrate inhibit the reaction. Double reciprocal Lineweaver-Burke plots indicated higher activity than expected in substrate concentration ≤ 5 mM; the apparent Km value obtained by linear extrapolation was ~ 7.3 mM. Colloidal chitin and α chitin (both 4 mg/ml) completely inhibited the hydrolysis of I, the apparent Km value for I increasing to 600 and 18 mM respectively. Chitinase was produced by both Pseudomonas aeruginosa and Vibrio anguillarum only in the presence of chitin as a carbon source (Nagahata and Shimahara, 1979). The enzymes from these two bacteria showed pH and temperature optima at 6.0 and 60°C respectively eventhough they have somewhat different activity curves. Both enzymes were found to be stable at pH 6 to 9 and ≤ 40°C.

Crude chitinase preparations were obtained from culture filtrates of Enterobacter sp. and Vibrio sp. which were isolated from the intestinal tract of grey mullet (Mugil cephalus) (Mowlah et al., 1979). The Enterobacter

preparation after dialysis for over night lost virtually all the chitinase activity whereas the enzymic activity of Vibrio preparation did not decrease during similar dialysis. The addition of Ca^{++} prevented the loss of chitinase activity during dialysis in Enterobacter preparation. $CaCl_2$ was the best co-factor among the chemicals tested. Using $CaCl_2$ it was possible to isolate two different chitinases from Enterobacter by polyacrylamide gel electrophoresis. The Vibrio preparation showed only one band in the absence of $CaCl_2$. The enzyme preparation from Enterobacter lost its activity at 50°C, and the Vibrio preparation did not loose its activity at similar temperature.

Ohtakara et al. (1979) reported on the purification and some properties of chitinase from Vibrio species isolated from seashore mud of the Ariake Sea. The optimum pH of the enzyme was in the range of pH 6.0 to 8.0 for colloidal chitin and 10.5 for glycol chitin. The chitinase was stable at alkaline pH from 9 to 11, but was unstable at 60°C or more. The isoelectric point was 3.7 and the molecular weight was estimated to be 63,000. The chitinase hydrolyzed chitin, colloidal chitin, glycol chitin, chitotetraose and chitotriose but not chitosan and chitobiose. None of the metal ions tested markedly stimulated chitinase activity. The enzyme

was inhibited by Sn^{++}, Cu^{++} and Ag^{+} and completely inhibited by Hg^{++}. Monoiodoacetic acid and P-chloromercuribenzoate did not inhibit the enzyme but EDTA showed slight inhibition of chitinase activity.

Crude enzyme preparation from Bacillus R-4 digested cell walls of Rhizopus, Mucor, Trichoderma and Piriculania (Tominaga, 1977). Column chromatographic studies showed that the Bacillus enzyme preparation consisted of protease and chitosanase. The chitosanase had a molecular weight of 31,000 isoelectric point at pH 8.30, a pH optimum at 5.6 and a temperature optimum at 40°C (Tominaga and Tsujisaka, 1975; Tominaga, 1977). The chitinase of Bacillus thuringiensis isolated from the culture filtrate exhibited a maximum rate of hydrolysis of colloidal chitin at pH 8.0 and 60°C (Chigaleichik, 1976).

The chitinase of Beneckea neptuna isolated from gut of Penaeus setiferus was found to be a moderately actively inducible one (Hood and Meyers, 1977 a). Examination of the cell free enzyme of the bacterium revealed an optimal pH of 7.0 and a temperature optimum of 40°C. When N-acetyl-glucosamine was assayed (Reissing et al., 1955) a pH optimum of 6.0 was noticed suggesting that a chitobiase, the enzyme responsible for the hydrolysis of the chitin dimer, requires an optimum pH 6.0 for maximum activity.

2.5.3. Properties of chitinases from marine vertebrates and invertebrates:

A series of investigations by Okutani (1966), Okutani et al. (1967 a,b) and Sera and Okutani (1968) described the properties, and mechanisms of chitinase system of the Japanese sea bass (Lateolabrax japonicus), the yellow tail fish (Seriola quinqueradiata) the rainbow trout (Salmo irideus) and the sea bream (Acanthopagrus schlegedi). The chitinase of the sea bass exhibited maximum activity at pH 4 and 50°C. Its stability range was pH 3.0 - 8.0 at 60°C for 30 minutes. The chitinase preparation from yellow tail also exhibited similar results. The trout chitinase was found to be slightly less stable when compared to other enzymes and exhibited an optimum pH of 4.5 and temperature of 30°C. The optimum pH and temperature of sea bream chitinase was 3.4 - 4.0 and 60°C respectively. Lundblad et al. (1979 b) worked on the chitinase in the lymphomyeloid tissue of marine fishes. Chitinase was most active at pH 1.0 in Leydig's organ from Raja radiata (elasmobranch) and at pH 2.7 in the epigonal organ from Squalus acanthias (elasmobranch) when glycol chitin was used as the substrate.

The plasma chitinase of Gadus morhua (cod) and Myxine glutinosa (cyclostome) exhibited a pH optimum of 4.5 whereas pancreas chitinase of Chimaera monstrosa (holocephalan) had an optimal activity at pH 3.0 and Raja radiata lymphomyeloid tissue chitinase at pH 0.7 using glycol chitin as the substrate (Fange et al., 1976).

Some of the fishes were re-examined by Fange et al. (1979) and they reported as follows: Coryphaenoides rupestris (teleost) gastric mucosa (glycol) chitinase had one optimum activity at pH 1.25 whereas S. acanthias (glycol) chitinase had two pH optima, at pH 1.6 and 3.6. Chimera monstrosa pancreatic chitinase had a very strong optimum around pH 8.0 - 10.0 and one less strong at pH 3.0. The isoelectric point was approximately 4.9. The molecular weight of the C. monstrosa pancreatic chitinase was estimated to be approximately 43,000.

The properties of a partially purified chitinase from a snail Helix species was worked out by Kimura et al. (1967 a, b). They reported that deproteinisation and decalcification of chitin increased the activity of chitinase by about 80% and they attributed this increase to the purity of the substrate. Certain ions like Fe^{++}, Mn^{++} and Zn^{++} were demonstrated to increase activity. Later Lundblad et al.

(1976) reported that the chitinase from Helix pomatia showed two active peaks. These chitinases, with molecular weight ~ 26,000 and 13,000 had somewhat different pH activity curves with optima at 4.2 and 4.3. By isoelectric focussing, the first peak with molecular weight ~ 26,000 was divided into two chitinase active regions with pI at 5.7 and 3.8. The second peak with molecular weight ~ 13,000 had a pI at 7.3.

The chitinase in the gastric juices of American lobster Hommarus americanus exhibited an optimum pH of 3.0 - 8.0 and a temperature of 37°C with chitin azure as the substrate. Kooiman (1964) reported chitinase activity at optimum pH of 3.0 - 4.0 for related species Astacus fluviatilis and Hommarus vulgaris.

Enzymatic studies revealed high chitinase activity by the chitinoclasts associated with shrimp as well as a moderately active indigenous chitinase produced by the hepatopancreas of the shrimp in the salt marsh environment (Hood, 1973). Optimum activity of both enzymes was found to be at 40°C and pH 7. The penaeid enzyme was inhibited by Co^{++} and the bacterial enzyme was inhibited by Na^{+}. The bacterial enzyme was shown to be induced while the shrimp

enzyme was constituted. Hood and Meyers (1977 a) worked on the chitinase of the shrimp Penaeus setiferus and reported that it exhibited greater activity at pH 5.0 - 7.0 compared with the bacterial enzymes (pH 7.0 - 9.0). These results indicate that the hepatopancreas chitinase is different from that of the bacteria.

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3. MATERIALS AND METHODS.

The present investigation was planned to study the occurrence, distribution and biology of chitinoclastic bacteria in the coastal zones of Cochin area. This study was divided into three phases. Firstly, the occurrence and distribution of chitinoclastic bacteria in sediments, waters of Vembanadu Lake, prawn culture pond and nearshore area were investigated over a period of one year. Secondly chitinoclastic bacteria and chitinolytic activity associated with a number of estuarine and marine fishes and prawns were estimated. Lastly a number of isolates exhibiting chitinoclastic property were selected from different sources, identified and the chitin degrading properties of selected bacteria were investigated in detail.

For the enumeration of chitinoclastic bacteria and total heterotrophs a number of samples from different area were collected and analysed. Totally eight sampling stations were fixed for routine collection right from Aroor in south to Eloor in north which includes a prawn culture pond (Station 7) at Narakkal. In addition, a number of sediment samples

from 15 stations were collected and analysed for chitinase activity and chitinolytic bacterial population. Enumeration of chitinoclast was carried out by pour plating with chitin agar and incubating them at *in situ* temperatures in the laboratory. Chitinoclastic colonies were easily recognized by the clear lytic zones surrounding each colony and they were readily enumerated.

Colonies showing chitin digestion on agar plates were isolated for further study. Isolates were selected from many samples of water, sediment and fauna from Cochin back-waters and prawn culturing pond. Each isolate was streaked several times to ensure purity, coded and then subjected to various tests. Physiological and biochemical tests along with morphological characteristics were used to assign generic identification by following standard identification schemes.

In addition, the chitinase activity in sediments and intestinal contents of prawns and fishes were estimated to understand the *in situ* activity. Reprecipitated chitin from prawn shells was used as the medium for isolation of chitinoclastic bacteria and also for enzyme assays. Chitino-clastic activity was measured by the release of the monomer N-acetylglucosamine resulting from the hydrolytic depoly-merization.

Various important ecological factors such as temperature, salinity, pH and dissolved oxygen were monitored in various stations during the period of investigation. Further, the effects of such factors on the growth and chitinolytic activity of selected bacteria were also studied.

3.1. Description of the study area:

The backwaters of Kerala consist of shallow, semi-enclosed and extensive body of brackish water running parallel to the coastline located in the tropical zone. The portion between Alleppey and Azhikode (09°30'-10°10'N, 76°15' - 76°25'E) is the major zone and forms a large basin into which several rivers empty themselves. It covers an area of about 512 square kilometres. At Cochin it is connected to the Arabian Sea on the western side, about 450 m wide which is the entrance to the Cochin harbour. At the northern extremity (Azhikode) it has another connection with the sea and at the southern side it terminates into a large body of fresh water - the Vembanad Lake.

The coastline is of an emergent type formed of a number of long narrow sand bars running parallel to the coastline often in several rows. The sand bar and the

harbour channel at Cochin are periodically dredged to facilitate the traffic of the port.

Only eight stations were selected for routine sample collection based on the accessibility and nature of stations. The distance between the extreme stations Arbor and Eloor, along the coastal zone of Cochin, is about 18 Km. (Fig. 2). This region is a centre of tremendous fishing activity and a number of fish processing industrial units are located. Moreover, the stations were selected in such a way that they represent areas influenced by a variety of human activities. Three stations i.e. Fisheries Harbour, Mattancherry Channel and Tanker Jetty are located around the Wellington Island. The Naval Headquarters, civilian air port, harbour, warehouses, fertilizer handling plants, railway yards, cold storage plants and a number of commercial establishments are located in this island. It is connected to the western side (Mattancherry) and to the eastern side (Ernakulam) by bridges. This area is also subjected to heavy boat and ship traffic. Because of the close proximity to the city, this area is considerably polluted by sewage, industrial effluents and wastes, oil etc.

The major flushing out of the estuary takes place during the peak monsoon period and when it is over the ecosystem comes back to its original state. It is obvious that the hydrographical and biological features of an area such as this is influenced to a major extent by these monsoons.

Station No. 1 (Aroor):

A number of prawn peeling sheds are situated in this area. The prawn shells thus accumulated are not dumped away as waste into the sea but are used as manure to the coconut trees fringing the zone. Besides, the area is also inhabited by a large number of fisher folk.

Station No. 2 (Fisheries Harbour):

The whole sale fish market of Cochin is situated at this station as it is the major fish landing site. In peak season, hundreds of mechanised fishing boats unload their catch here. Hence the area seems to get greatly polluted by the loading and unloading operations.

Station No. 3 (Mattancherry Channel):

The Mattancherry Channel lies in between the fisheries harbour and barmouth. It is the main shipping channel for the cargo and passenger ships. Being very close to the barmouth, the station is highly under tidal influence. Moreover, this station lies in the main harbour area.

Station No. 4 (Barmouth):

Barmouth is bound by Vypeen in north, Fort Cochin in south, Arabian Sea in west and Vembanad Lake in east. This is the main entrance to the Cochin harbour and the depth in the centre is about 15 m. However, sampling was done along the shallow edges of the Barmouth. A number of Chinese dip nets are operated daily along the shores of Vypeen and Fort Cochin bordering the station.

Station No. 5 (Tanker Jetty):

This station is in the Ernakulam Channel. Oil tankers unload their crude oil here and it is pumped to oil refinery situated several Kms away. As a result, the surface waters and sediment surface are most often covered with

oil films. The Cochin Shipyard, one of the major ship builders of the country, is also situated very near to the station.

Station No. 6 (Narakkal Channel):

This is a smaller channel, compared to other channels viz. Mattancherry Channel and Ernakulam Channel. It is mainly used for the passage of small boats.

Station No. 7 (Narakkal Pond):

There are a number of closed ponds, each measuring about 80 - 100 sq. m. and 1 m deep owned by private parties and Kerala State Fisheries. As the ponds are situated very close to the sea they are protected by sea walls. Eventhough they are all closed ponds, during monsoon there is a mixing of water from the close-by channels. A continuous culture of penaeid prawns in these ponds is practised throughout the year. One of the centrally located ponds was selected for the present study.

Station No. 8 (Eloor):

Most of the industries like the chemical and fertilizer plants (FACT, TCC, etc) are concentrated in this region. The water in this area is therefore greatly influenced by the effluents from these industries. The water in this region has been reported to contain arsenic, copper and other poisonous metals as the major pollutants.

3.2. Estimation of Physico-chemical parameters:

Physico-chemical parameters monitored during the period of investigation at all stations were temperature (water and sediment), salinity (surface and bottom water), dissolved oxygen, pH (water and sediment) and turbidity. Temperature was measured on board using a thermometer with 0.5°C accuracy. Salinity was determined following the standard argentimetric method (Harvey, 1955) with necessary correction and the dissolved oxygen content was estimated by Winkler's titration (APHA, 1965). pH and turbidity were determined using a pH meter (Elico Model L1 - 10) and a 30 cm diameter Secchi disc respectively. From Secchi disc readings, turbidity was expressed in terms of light

attenuation coefficient (k) which was calculated using the formula of Poole and Atkins (1929):

$$\text{Attenuation Coefficient (k)} = \frac{1.7}{\text{Secchi disc reading in m}}$$

3.3. Sampling methods:

Samples were collected for a period of one year. Eventhough no definite periodical collections were made, the collections were distributed throughout the year (1980). Surface and bottom water samples were collected from each station using sterile Casella type bottle samplers. The samples were immediately transferred to sterile conical flasks. The sediment samples were taken with a Van Veen grab and the central portion of the sample was asceptically transferred to sterile containers. The samples were transported to the laboratory in an ice box within three hours, from the time of collection for various analyses. Fresh prawns were caught from different stations using cast net and transferred to sterile polythene bags. Fishes were obtained by trawling or by Chinese dip net or cast net and transported to the laboratory in sterile polythene bags. They were analysed immediately or held in ice till analysis whenever there was a delay. Gastrointestinal

tracts of both prawns and fishes were analysed. All dissections in the laboratory were carried out using aseptic techniques. In the case of fishes, the specimens were surface washed first with sterile water to remove excess slime and debris. The incision area was then swabed with 95% ethyl alcohol. For every incision and dissection, heat sterilized forceps and scalpels were used. The contents of the tract were transferred to sterile flasks. In the case of prawns, in addition to gastrointestinal tract, the shell surface was also sampled and analysed. In this case the whole animal was transferred to 100 ml sterile sea water blank and shaken for five minutes. Further dilutions for plating were made from this.

3.4. Enumeration of total heterotrophic and chitinoclastic bacteria:

The total heterotrophic and chitinoclastic bacterial population in the samples were estimated following the conventional pour plate technique after serial dilutions with sterile 50% sea water.

The composition of the medium was:

Peptone	-	3.0 g
Beef Extract	-	0.5 g
Agar	-	2.0 g
Colloidal chitin	-	.5 g
50% Sea water	-	100.0 ml

The total number of viable colonies appearing on these plates were considered as total heterotrophic bacterial population and the colonies with clear zones around them were counted as chitinoclastic bacteria. In case of sediment, one gram sample was transferred to 99 ml sterile 50% sea water blanks and further serial dilutions were prepared with 90 ml blanks. After serial dilutions the contents of the first flask were filtered through a pre-weighed Millipore membrane filter (pore size 0.45 μ) and dried at 80°C to a constant weight. The bacterial populations were expressed on dry weight basis. The samples (1.0 ml) from each dilution and medium were mixed thoroughly in sterile petri dishes and allowed to solidify. Triplicate plates for each dilution were prepared and incubated aerobically at room temperature (28 $\pm$ 2°C) for five days. Colony forming units in plates, where the number of colonies were 30 - 300, were counted and the average number of bacteria per gram/100 ml of sample was calculated.

3.5. Preparation of colloidal chitin:

The chitin was prepared from the exoskeleton of prawns. Prawn shells were washed first with warm water and decalcified in 1% (v/v) HCl for four days. Everyday the HCl was removed and fresh HCl was added. After decalcification the shells were washed to remove HCl and placed in 2% (w/v) KOH for 10 days. During this period the chitin-KOH mixture was brought to boiling on the 3rd, 6th and 10th day. After 10 days the chitin was washed free of KOH and boiled for 15 minutes with ethyl alcohol. The chitin strips thus obtained were dried and preserved for further use.

Colloidal chitin was prepared by dissolving chitin strips prepared as above in 50% (v/v) H_2SO_4 and precipitating the chitin by a fifteen fold dilution with water. The precipitated chitin was centrifuged at 1500 rpm and washed thoroughly with distilled water till it was free from acid. Care was taken not to allow the colloidal chitin to dry. This chitin was used for medium preparation as well as for enzymatic assays. The excess chitin was steamed at about 100°C for 15 minutes and kept in glass jars at 4 - 8°C for future use.

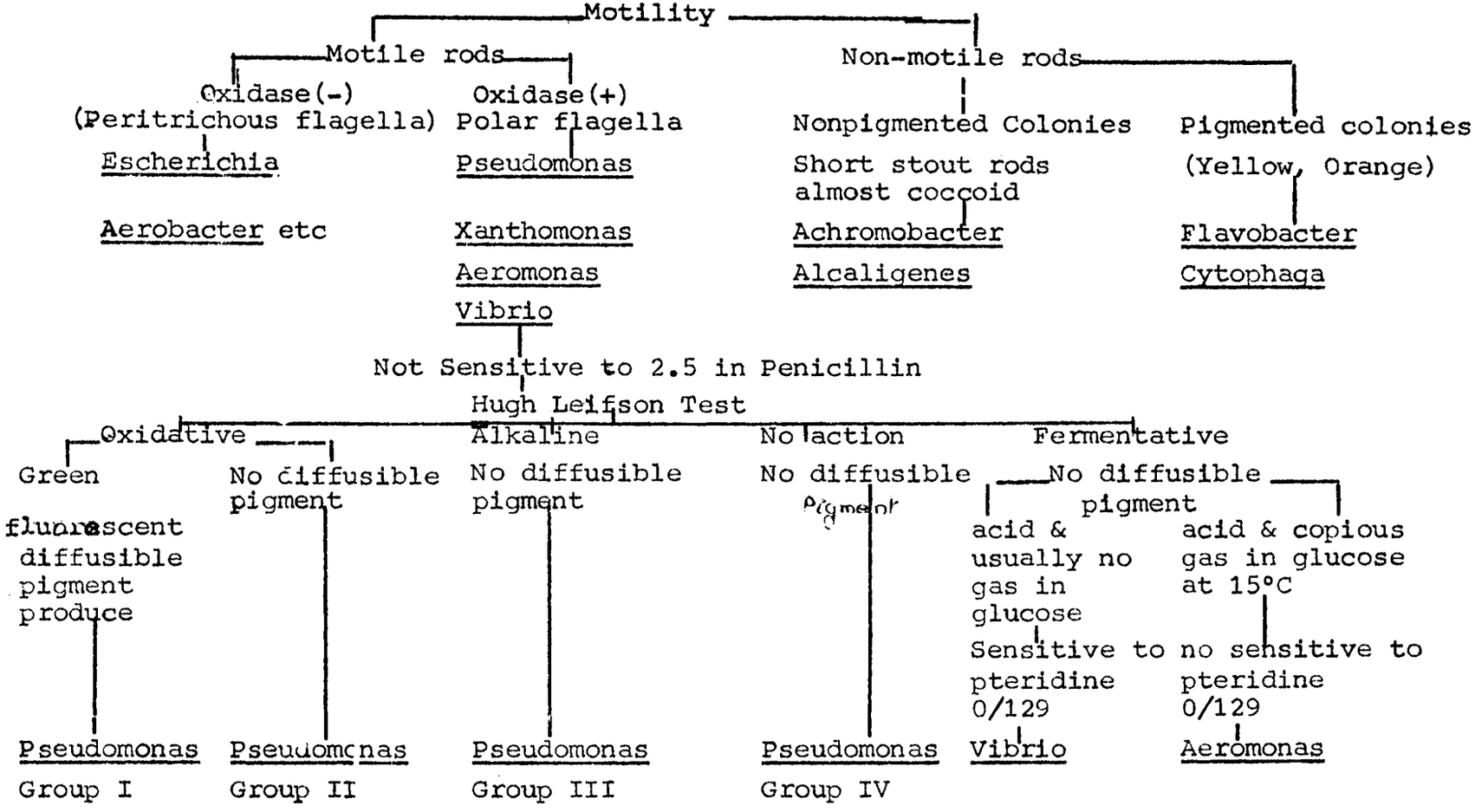
3.6. Purification and maintenance of cultures:

Chitinoclastic colonies isolated from the plates were streaked on the chitin agar medium and checked for their purity. If there was contamination they were restreaked and pure cultures were isolated. The cultures were maintained on chitin agar slants prepared with 50% sea water.

3.7. Identification of chitinoclastic bacterial cultures:

Colonies showing definite chitinoclastic activity were selected from plates of highest dilution. At the highest dilution only a few chitinoclastic colonies representing the most abundant types would appear on the chitin agar. Under this condition all the active colonies were picked, purified and maintained on chitin agar slants. Pure cultures of selected chitinoclastic bacteria were identified by the scheme of Shewan (1963) as outlined in Table 2. Preliminary studies indicated that the marine chitinoclasts were almost exclusively Gram-negative asporogenous rods. Hence this scheme was found suitable to identify most of the isolates

Table: 2. SCHEME FOR DIFFERENTIATING SOME GRAM-NEGATIVE BACTERIA*



* Adopted after Shewan (1963)

3.8. Assay of chitinase activity in sediments and the digestive tract of prawns and fishes:

For chitinase assay, the sediment samples were diluted to 1:100 (w/v) in 50% minimal sea water salts solution (MSWS) or tap water and the gut contents of animals were transferred to test tubes and diluted to 1:10 (w/v) with 50% MSWS. These preparations were used as enzymes in enzyme assay. Modifications of standard procedure (Jeuniaux, 1966) as employed by Goodrich and Morita (1977 a) were used to determine the levels of chitinase activity in the sediments and in the digestive tract of animals. Chitinase assay mixture was prepared in 50 ml Erlen Meyer flask and their composition was as follows:

Chitin suspension (5 mg/ml)	- 1 ml
Citric acid (0.6 M), dibasic Sodium Phosphate (1.2 M) buffer (pH 5.1)	- 1 ml
Sample (Enzyme source)	- 0.1 to 2.0 ml
MSWS (minimal sea water salts)	- Total volume upto 4.0 ml
Toluene	- 0.5 ml

Toluene was added to prevent bacterial action during assay. Duplicate flasks were incubated at 37°C in a water bath for 2 hrs in the case of gut samples and 24 hrs in the

case of sediment samples. For controls the volume of chitin suspension or enzyme was substituted with equal volume of MSWS. After incubation, the flask contents were thoroughly shaken, centrifuged at 23,000 x g for 5 minutes. The product of chitin degradation N-acetyl-D-glucosamine (NAG) in 0.5 ml of supernatant solution was estimated.

3.8.1. Preparation of minimal sea water salts solution (MSWS):

This solution contained most of the major ions of sea water and it was prepared according to Goodrich and Morita, 1977 a).

The composition was as follows:

NaCl	-	24.0 g
$MgSO_4 \cdot 7\ H_2O$	-	7.0 g
$Mg\ Cl_2 \cdot 6\ H_2O$	-	5.3 g
KCl	-	0.7 g
Distilled water	-	1000 ml
pH	-	7.5

3.8.2. Estimation of N-acetyl-D-glucosamine (NAG):

The N-acetyl-D-glucosamine assay developed by Morgan and Elson (1934) and modified by Ressig et al. (1955) was employed. 0.5 ml of sample was mixed with 0.1 ml of potassium tetraborate (0.8 M) and heated in a boiling water bath for 3 minutes. Afterwards it was cooled under tap and 3.0 ml of DMAB reagent was added. The mixture was incubated at 37°C in a water bath for 20 minutes. The intensity of the colour developed was read at 585 µm in a Hitachi Model - 200 Spectrophotometer. Standard curve was prepared with analytical grade NAG in the similar way and unknown quantities in samples were estimated using this curve.

3.8.3. Preparation of DMAB reagent:

10.6 g of analytical grade p-Dimethyl aminobenzaldehyde (DMAB) was dissolved in 100.0 ml of glacial acetic acid containing 12.5% v/v concentrated HCl. This stock solution was diluted with 9 volume of glacial acetic acid before use.

3.9. Assay of bacterial chitinase:

3.9.1. Enzyme preparation:

The method employed was essentially that of Hood and Meyers (1977 a). Bacterial chitinase activity was measured by the release of NAG from re-precipitated chitin. The cultures were grown in liquid medium of the following composition:

Peptone	-	1.5 g
Yeast Extract	-	0.5 g
Sodium Chloride	-	2.5 g
Colloidal chitin	-	0.5 g
Tap water	-	100 ml

Young cultures (12 - 24 hrs old) were used for inoculation of the above medium and incubated for 7 days at room temperature (28 $\pm$ 2°C) with occasional shaking during incubation. Control flask were kept without inoculation. At the end of the incubation period the contents of the flasks were centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 minutes. The cell free supernatant was used for precipitating the enzyme. Saturated ammonium sulphate solution was added to 70% saturation and kept over night in a refrigerator. The precipitated enzyme was

centrifuged at 15,000 rpm for 15 minutes and the supernatant was discarded. The precipitate was dissolved in a known quantity of distilled water and dialysed against 2 litres of distilled water at 4°C for a period of 24 hours. This preparation was used as the enzyme.

3.9.2. Enzyme assay:

The enzyme assay mixture consisted of colloidal chitin (5 mg/ml) - 1.0 ml; citrate-phosphate buffer (pH 6.2) - 1.0 ml; enzyme solution - 1.0 ml; and 1.0 ml of MSWS. 0.2 ml of toluene was also added as a bacteriostatic agent. The tubes containing the enzyme assay mixture were incubated at 37°C in a water bath for 2 hours. Suitable controls were also maintained. After incubation the contents were centrifuged (10,000 rpm) and the amount of NAG released into the solution was estimated. The tests were run in duplicates and the average values were taken. Chitinase activity was expressed as µg of NAG liberated in one ml of the incubation mixture under assay conditions.

3.9.3. Protein estimation:

The protein content of enzyme preparation was estimated as follows: 0.5 ml of 10% TCA was added to 0.5 ml of enzyme solution to precipitate the protein. It was centrifuged

for 20 minutes at 10,000 rpm and the precipitate was dissolved in 3.0 ml of 0.1 N NaOH and the concentration was read at 278 µm against a blank of 0.1 N NaOH in a Hitachi Model 200 Spectrophotometer. Standard curve was prepared with bovine serum albumin in the same way.

3.10. Growth and physiology of chitinoclastic bacteria:

3.10.1. Effect of temperature on growth of chitinoclastic bacteria:

The effect of temperature on growth of the chitinoclastic bacteria was assessed by growing them at different temperatures. Nutrient broth (peptone - 1.5 g; beef extract - 0.5 g, and 50% sea water - 100 ml, pH - 7.5) was prepared and 5 ml aliquots were distributed into test tubes. After autoclaving the tubes were inoculated with loop full of 12 - 24 hours old cultures and incubated at various temperatures i.e. 4°, 10°, 15°, 20°, 25°, 30°, 37° and 40°C for 24 hrs. Duplicate tubes were maintained for each culture and for each temperature. The growth was measured by optical density at 450 µm in a Hitachi Model 200 Spectrophotometer.

3.10.2. Effect of pH on growth of chitinoclastic bacteria:

The effect of pH on growth of chitinoclastic bacteria was determined as follows: 5.0 ml aliquots of nutrient broth were distributed in test tubes. After autoclaving, the pH of the medium in different test tubes was adjusted to various levels i.e. 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11, with either sterile 1 N HCl or 1 N NaOH. A loop full of 12 - 24 hour old culture was inoculated into different tubes. Duplicate tubes were maintained for each pH value. The inoculated tubes were incubated at the room temperature ($28 \pm 2°C$) for 24 hours. Duplicate tubes were maintained for each culture and for each pH. The growth was measured by optical density at 450 µm in a Hitachi Model 200 Spectrophotometer.

3.10.3. Effect of Sodium chloride on growth of chitinoclastic bacteria:

The salinity requirements of chitinoclastic bacteria were examined in Campbell's basic medium (Campbell and Williams, 1951) (g/l; K_2HPO_4 - 1.0; $MgSO_4$ - 0.5; $CaCl_2$ - 0.1; Ferric phosphate - trace; Polypeptone - 0.1; NaCl - various concentrations and distilled water - q.S; pH - 7.8) having different NaCl concentrations (0 - 15%). The medium with different NaCl concentration was dispensed in 5 ml

aliquots into test tubes and autoclaved. Loop full of 12 - 24 hour old culture was inoculated and incubated at room temperature (28 ± 2°C) for 24 hours. Duplicate tubes were maintained for each culture and for each concentration of NaCl. The growth was measured by optical density at 450 µm in a Hitachi Model 200 Spectrophotometer.

3.10.4. Effect of temperature on survival of chitinoclastic bacteria:

The temperature tolerance of chitinoclastic bacteria was determined by the following procedure:

The bacteria used for this experiment were precultured for 12 - 24 hours in peptone sea water medium (peptone 0.1% in 50% sea water) at room temperature (28 ± 2°C). After 24 hours the culture solution was distributed in 5 ml aliquots in different test tubes and they were kept at 40°, 50° and 60°C in water bath. At different time intervals i.e. 10, 20, 30, 40 and 60 minutes, one loop full bacteria from each tube kept at different temperatures was removed and inoculated into fresh peptone sea water medium. They were incubated at room temperature (28 ± 2°C) for a period of 5 days. Duplicate tubes were maintained for each treatment. The growth was measured by optical density at 450 µm in a Hitachi Model 200 Spectrophotometer.

3.10.5. Effect of pH on survival of chitinoclastic bacteria:

The survival of chitinoclastic bacteria at various pH values was tested as follows: Buffers of different pH values ranging from 2.6 to 10.6 using citrate-phosphate buffer and glycine-NaOH buffer were prepared. They were distributed in 5 ml aliquots into various test tubes. 0.1 ml of 12 - 24 hour old culture suspension (grown in peptone sea water medium) was inoculated into each test tube and incubated at room temperature (28 $\pm$ 2°C). At predetermined intervals i.e. 6, 12, 18, 24 and 30 hours, a loop full of the culture from each tube was transferred to fresh peptone sea water medium and incubated for 5 days. Duplicate tubes for each treatment were maintained. The growth was measured by optical density at 450 µm in a Spectrophotometer.

3.10.6. Effect of Sodium chloride on survival of chitinoclastic bacteria:

The survival of chitinoclastic bacteria at various sodium chloride concentration was tested as follows: A known quantity of culture suspension prepared from 12 - 24 hour old culture was placed in each tube. After contacting for 6, 12, 18, 24, and 30 hours each time at various concentrations of sodium chloride prepared in distilled water

(0 - 15%) at room temperature (28 ± 2°C) one loop full of the bacteria was inoculated into fresh peptone sea water medium. Duplicate tubes were maintained for each treatment. The growth was measured by optical density after 5 days at 450 µm in a Spectrophotometer.

3.11. Cultural conditions and chitinase production:

3.11.1. The effect of type, initial concentration and particle size of chitin on chitinase production of chitinoclastic bacteria:

The effect of different chitin types such as powdered chitin, colloidal chitin and powdered chitosan was investigated as follows: Each type of chitin was added at 0.1%, 1% and 3% levels to the nutrient broth. The media were dispensed in 150 ml quantities into 500 ml Erlen Meyer flasks and autoclaved. A loop full of 12 - 24 hour old culture was added to each flask and incubated for 7 days at 37°C with occasional shaking. Suitable control flasks were maintained. At the end of incubation period the growth was measured by optical density at 450 µm and the contents of the flasks were centrifuged at 10,000 rpm for 15 minutes. The amounts of NAG, glucosamine, glucose and ammonia present in the supernatant solution were estimated. To the rest of supernatant solution, ammonium sulphate was added to 70%

saturation and the enzyme was prepared and assayed as detailed earlier. The effect of different size particles of chitin on chitinase production of the bacteria was tested by incorporating chitin particles of different sizes (0.5 and 1.0 mm) in the place of other chitin types. The rest of the procedure was similar to that given above.

3.11.2. Effect of other substrates on chitinase production:

The effect of certain chitin derivatives i.e. chitin and chitosan, N-acetylglucosamine, glucosamine and glucose on the production of chitinase by chitinoclastic bacteria was tested by incorporating different substrates at varying concentrations (0.1% to 3.0%) in the nutrient medium. The medium contained only one substrate at a time. The media were dispensed in 100 ml aliquots into 250 ml Erlen-Meyer flasks and autoclaved. The flasks were inoculated with loop full of 12 - 24 hr old cultures and incubated at room temperature (28 $\pm$ 2°C) under static conditions for 7 days. The contents of flasks were centrifuged at 10,000 rpm for 15 minutes and the enzymes were salted out from the culture filtrate as described earlier and assayed.

3.11.3. Effect of temperature on chitinase production of chitinoclastic bacteria:

The effect of temperature on chitinase production of the chitinoclastic bacteria was assessed by growing them at different temperatures. Nutrient broth with chitin (Peptone - 1.5 g; Yeast extract - 0.5 g; NaCl - 2.5 g; Colloidal chitin - 0.5 g; and tap water - 100 ml; pH 7.5) was prepared and 150 ml quantities were distributed into 500 ml Erlen Meyer flasks. After autoclaving the flasks were inoculated with loop full of 12 - 24 hour old cultures and incubated at various temperatures i.e. 4, 30, 37 and 40°C for 5 days. Duplicate flasks were maintained for each treatment. The growth was measured by optical density at 450 µm in a Hitachi Model 200 Spectrophotometer. The contents of the flasks were centrifuged at 10,000 rpm for 15 minutes and the residual concentration of NAG, glucosamine, glucose and ammonia in the cell free culture solution was estimated. The chitinase elaborated into the medium by the bacteria was salted out and the activity was assayed as described earlier.

3.11.4. Effect of pH on chitinase production of chitinoclastic bacteria:

The effect of pH on chitinase production of chitinoclastic bacteria was determined as follows: Nutrient broth with chitin was prepared and 150 ml quantities were distributed into 500 ml Erlen Meyer flasks. After autoclaving, the pH of the medium in different flasks was adjusted to various levels i.e. 5, 7, and 9 with either sterile 1 N HCl or 1 N NaOH. A loop full of 12 - 24 hour old culture was inoculated into each flask. Duplicate flasks were maintained for each pH value. The inoculated flasks were incubated at the room temperature (28 $\pm$ 2°C) for 5 days and the growth was measured by optical density at 450 µm in a Spectrophotometer. The contents of the flasks were centrifuged at 10,000 rpm for 15 minutes and the residual concentration of NAG, glucosamine, glucose and ammonia in the cell free culture solution was estimated. The chitinase elaborated into the medium by the bacteria was salted out and the activity was assayed as described earlier.

3.11.5. Effect of Sodium chloride on chitinase production of chitinoclastic bacteria:

The effect of Sodium chloride on chitinase production of chitinoclastic bacteria was determined as follows: Nutrient broth with chitin and added NaCl at different concentrations (0, 1%, 3%, 5% and 7%) were prepared and dispensed into 500 ml Erlen Meyer flasks and autoclaved. Loop full of 12 - 24 hour old cultures were inoculated and incubated at room temperature (28 $\pm$ 2°C) for 5 days. Duplicate flasks were maintained for each culture and for each concentration of NaCl. The growth was measured by optical density at 450 µm in a Spectrophotometer. The contents of the flask were centrifuged at 10,000 rpm for 15 minutes and the residual concentration of NAG, glucosamine, glucose and ammonia in the cell free culture solution was estimated. The chitinase elaborated into the medium by the bacteria was salted out and the activity was assayed as described earlier.

3.11.6. Estimation of residual NAG in the cell free culture solution:

0.5 ml of the cell free culture solution was taken and its NAG content was estimated as outlined earlier (refer page 76).

3.11.7. Estimation of residual glucosamine in the cell free culture solution:

Glucosamine was estimated following the Morgan-Elson Method modified by Good and Bessman (1964).

0.5 ml of cell free culture solution was mixed with 0.5 ml of distilled water and 0.2 ml of acetic anhydride solution was added. To this mixture 1.0 ml of borate buffer (1.2 M Boric acid - 0.56 M KOH in 1 litre of water; pH 9.2) was added and kept in a boiling water bath for 3 minutes. Afterwards, it was cooled in ice bath for 5 minutes and 8 ml of DMAB reagent was added and incubated at 37°C for 20 minutes. The colour developed after 20 minutes was read against a blank at 570 µm in a Hitachi Model 200 Spectrophotometer. A standard curve was prepared using glucosamine hydrochloride (BDH). The glucosamine contents of samples were calculated using the standard curve.

3.11.8. Estimation of residual glucose in the cell free culture solution:

Glucose was estimated by following the Nelson-Somogyi method (Somogyi, 1952).

0.5 ml of cell free culture solution was taken in a test tube and the volume was made to 2.0 ml with

distilled water. 2 ml of alkaline copper reagent was added and heated in a boiling water bath for 10 minutes. It was cooled quickly for one minute and 2 ml of arsenomolybdate reagent and 4 ml of distilled water were added. After allowing it to stand for 15 minutes the developed colour was read at 520 µm in a Hitachi Model 200 Spectrophotometer. A standard curve was prepared with analytical grade glucose (BDH).

3.11.9. Estimation of residual ammonia in the cell free culture solution:

The residual ammonia present in the cell free culture solution was estimated by direct Nesslerisation (APHA, 1971). 0.5 ml of cell free culture solution was added to 9.5 ml of ammonia free distilled water in a test tube. 0.5 ml of Nesslers reagent (BDH) was added to the above solution and if necessary further dilutions with distilled water were made. The intensity of colour was read at 410 µm in a Hitachi Model 200 Spectrophotometer. Standard curve was prepared using analytical grade ammonium sulphate (BDH).

3.12. Generation time of chitinoclastic bacteria:

The generation time of chitinoclastic bacteria was estimated using two media (Nutrient medium with chitin and Nutrient broth without chitin). A known concentration of cells was inoculated into 50 ml of medium and incubated at room temperature (28 ± 2°C) in a rotary shaker. At different time intervals (0, 30, 60, 90 min.) aliquots were removed and the growth was measured by optical density at 450 µm in a Hitachi Model 200 Spectrophotometer. Generation time in hours was determined from the exponential phase of the curve of a semilogarithmic plot.

3.13. Determination of chitin degradation in vitro:

The method followed for this study, was essentially that of Hood and Meyers (1977). Each organism was grown in a nutrient broth containing yeast extract (0.05%) peptone (1%) and sea water (pH 7.6) for 24 hours at room temperature (28 ± 2°C), centrifuged, washed in sterile sea water and resuspended in 100 ml sterile sea water. The same medium was incorporated with colloidal chitin at 0.325% level and distributed in 100 ml aliquots

into 250 ml Erlen Meyer flasks. After autoclaving, the flasks were inoculated and incubated at room temperature (28 ± 2°C) under static conditions. Control flasks containing chitin and basal medium were also maintained. At 24 hours intervals the cell biomass was determined using the standard plate count method and nutrient agar in sea water. The flasks were removed at different intervals, heated to boiling for 10 minutes; filtered and washed with 2% HCl. After washing, the chitin was collected by filtration, dried at 105°C for 12 hours and the dry weight was determined gravimetrically. The remaining chitin subtracted from the initial concentration represented the amount of substrate decomposed.

3.14. Enzymatic properties of bacterial chitinase:

3.14.1. Time course of the activity:

Time course of the activity of chitinolytic enzymes obtained from the bacteria was established by analysing the end product concentration in the enzyme assay mixture at predetermined intervals.

3.14.2. Effect of temperature on the activity:

Effect of temperature on the activity of chitinolytic enzyme obtained from the cultures was studied by

incubating the enzyme assay mixture at different temperatures (4 - 40°C).

3.14.3. Effect of pH on the activity:

The effect of pH on the hydrolysis of chitin by crude chitinase obtained from the cultures was studied by varying the pH values of the assay mixture by using different buffers. 0.1 M citric acid - 0.2 M dibasic sodium phosphate, pH 2.6 - 7.0, 0.2 M glycine - 0.2 M NaOH - pH 8.6 - 10.6.

3.14.4. Effect of substrate concentration on the activity:

Effect of substrate concentration on the activity was studied by varying the substrate concentration in the enzyme assay mixture keeping other factors constant. The concentrations of colloidal chitin used were from 0.05 to 10.0 mg/ml.

3.14.5. Effect of Enzyme concentration on the activity:

Effect of enzyme concentration on the activity was studied by varying the quantity of enzyme in enzyme assay mixture while keeping the other factors constant.

3.14.6. Effect of different molar concentration of buffer on the activity:

Effect of different molar concentration of buffer on the activity was studied by varying the molar concentration of the buffer (citrate-phosphate buffer) from 0.05 to 0.5. The other conditions remained unchanged.

3.14.7. Effect of temperature on the stability of chitinase:

Enzymes were preincubated in citrate-phosphate buffer (pH 5.6) at various temperatures (20, 30, 40, 50, 60 and 70°C) for 20 minutes and the residual activity was measured under the standard conditions.

3.14.8. Effect of pH on the stability of chitinase:

Enzymes were preincubated with buffers at various pH values and at 37°C for 3 hours, and the residual activity was measured under standard conditions. Citrate-phosphate buffer (pH 3, 5 and 7) and glycine-NaOH buffer (pH 9 and 11) were used.

3.14.9. Effect of various chemicals on the stability of chitinase:

2 mM solution of various inorganic chemicals were prepared. 1 ml of enzyme solution and 1 ml of the chemical solution were mixed and individually incubated at 37°C for 30 minutes. Afterwards the residual activity was measured under standard conditions.

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4. RESULTS.

4.1. Occurrence and distribution of total heterotrophic and chitinoclastic bacterial population in waters and sediments:

As pointed out earlier under materials and method section a total of 8 stations were selected for the present investigation. All these stations are subjected to a variety of human activities. In these stations surface water, bottom water and sediment samples were analysed for various physico-chemical factors, for total heterotrophic and chitinoclastic bacterial population and the results are given in Table 3 and Fig. 3.

Station 1 - Aroor:

In this station only two collections were made during the period of study (29.1.80 and 9.5.80). The temperature of the surface water was found to be slightly higher than the bottom water and sediment on both the occasions. Otherwise the temperature was almost uniform (29°C or 32°C) throughout the water column of 2.5 m deep. The pH values also did not vary much (7.5 - 7.85) except the surface water sample collected

on 9.5.1980 which was moderately high (pH 8.4). The dissolved oxygen content of surface water was considerably higher (8.11 and 9.12 ml/l) than the bottom water (3.15 and 2.39 ml/l). The salinity of the bottom water was significantly higher than the surface water at this station. However, the maximum salinity recorded was only 26.30‰. The turbidity of the water remained almost same during the period of observation (k = 1.75). The sediment in this station was silty and brownish to black in colour. Highest total heterotrophic bacterial population was recorded in sediments (80.80 x 10^3/g). However the population was considerably low (2.46 x 10^3/g) on another occasion. Such wide fluctuations were also noticed in the overlying water. Out of the two collections made the samples collected during January 1980 contained chitinoclastic bacteria constituting 21.42% of the total bacterial population only in the surface waters. Interestingly no chitinoclastic bacterial population was recorded in bottom water and sediment samples. However in the second collection made during May, 1980 the water and sediment samples harboured as much as 60 - 70% of chitinoclasts.

Station 2 - Fisheries Harbour:

In this station totally five collections were made during the course of study (i.e. April, May, July and September) only in May two collections were made i.e. 9.5.1980 and 23.5.1980. The temperature of surface waters was found to be always higher than the bottom water and sediment on all occasions. Maximum temperatures were recorded during May in surface waters (32.25°C), bottom waters (31.75°C) and sediment (31.50°C) while the minimum temperatures were observed in September in bottom water (26°C) and sediment (25°C) except for the surface water (29°C). pH varied much in this station during the period of study. The pH in surface water fluctuated from 6.95 (July) to 8.30 (May) and in bottom water from 7.05 (July) to 8.0 (May). Compared to overlying water the sediment always showed higher pH values ranging from 7.35 (April) to 8.55 (July). The dissolved oxygen content of surface water was always considerably higher (2.99 - 7.45 ml/l) than the bottom water (2.20 - 3.63 ml/l) and maximum values were recorded during the month of May. The salinity of bottom water was consistently higher than the surface water throughout the period of investigation suggesting

the exposure of sediments to almost a constant saline water. The salinity values of both surface and bottom waters dropped to 0.41‰ and 0.51‰ respectively in the month of July because of the monsoon and heavy inflow of fresh water into the station. The maximum salinity recorded for surface water was 25.78‰ (April) and for bottom water it was 27.32‰ (April). The turbidity of water remained almost constant (k = 1.59 - 1.75) throughout the year except during July when the value increased to 2.33 possibly due to the heavy inflow of rain water from the adjoining areas carrying lot of suspended load. The sediment in this station was clayey in nature and brownish to black in colour. Higher total heterotrophic bacterial populations were recorded in sediments when compared to overlying water. In surface water the total bacterial population varied from 2.06×10^3/100 ml (April) to 23.40×10^3/100 ml (July). In bottom water the population was always higher than the surface water and it varied from 25.50×10^3/100 ml (April) to 35.60×10^3/100 ml (September). In sediments higher population (239.63×10^3/g) was recorded in May and lowest population (1.60×10^3/g) in April. Sediments always harboured more chitinoclasts than the overlying water. The percentage of chitinoclasts in surface water

ranged from 3.41 (April) to 50.56 (May) and in bottom water it varied from 5.88 (April) to 38.83 (May). In July and September the chitinoclastic population in both the waters decreased. In sediments highest chitinoclastic bacterial populations (67.13 and 74.44% of total bacteria) were recorded in the month of May. The percentage of chitinoclasts in sediments varied from 4.76 (September) to 74.44 (May). These observations clearly indicate that the chitinoclastic bacterial population increased with an increase in temperature of the environment.

Station 3 - Mattancherry Channel:

In this station totally five collections were made during the period of study (i.e. April, May, July and September). Two collections were made in the month of May only. On all occasions the temperature of the sediment was much lower than the overlying water. During April and early May the temperature of the surface water was slightly higher than the bottom water. Maximum temperatures were recorded during May in surface waters (32.50°C), bottom water (31.50°C) and sediment (31.0°C). While the minimum temperatures were observed in July in surface water (27.50°C), bottom water (27.25°C) and

sediment (27.0°C). The pH of the surface water fluctuated from 7.05 (July) to 8.15 (May) and in bottom water from 7.10 (July) to 8.10 (May). Compared to overlying water the sediment always showed higher pH values ranging from 7.50 (April) to 8.85 (July). The dissolved oxygen content of surface water was always considerably higher (3.43 - 6.31 ml/l) than the bottom water (3.48 - 4.74 ml/l) and maximum values were recorded in May. The salinity of the bottom water was consistently higher than the surface water throughout the period of investigation indicating that the sediment was exposed always to a constant saline water. The salinity of both surface and bottom waters dropped to 0.61‰ and 0.80‰ respectively in the month of July because of the monsoon and heavy inflow of freshwater into the station. The maximum salinity recorded for surface water was 25.40‰ (May) and for bottom water it was 28.67‰ (April). The turbidity of water remained almost constant (k = 1.40 - 1.75) throughout the period of study except during July, when the value increased to 3.50 possibly due to the heavy inflow of rain water from the adjoining areas carrying lot of suspended load. The sediment in this station was silty

to clayey in nature and grey to black in colour. Higher total heterotrophic bacterial populations were recorded in sediments when compared to overlying waters. In surface water the total bacterial population varied from 3.05 x 10^3/100 ml (April) to 49.70 x 10^3/100 ml (July). In bottom water the population was always higher than the surface water and it varied from 24.0 x 10^3/100 ml (July) to 201.60 x 10^3/100 ml (May). In sediments highest population (131.56 x 10^3/g) was recorded in May and lowest population (1.94 x 10^3/g) in April. Generally sediments harboured more chitinoclasts than the overlying water. The percentage of chitinoclasts in surface water ranged from 5.40 (September) to 42.10 (May) and in bottom water it varied from 14.28 (September) to 64.29 (May). In April and September the chitinoclastic population in both the waters decreased. In sediments highest chitinoclastic bacterial population (62.55 and 71.42% of total bacteria) were recorded in the months of May and July. The percentage of chitinoclasts in sediments varied from 6.10 (later part of May) to 71.42 (July). A marginal increase in the chitinoclastic population in the month of July when compared to May, may possibly be attributed to heavy suspended matter settling on the surface of the sediments. In other words, the population

encountered might have been of allochthonous in nature.

Station 4 - Barmouth:

In this station totally 4 collections were made during the period of study (i.e. April, May, July and September). On all occasions the temperature of the sediment was much lower than the overlying water. Temperatures of the surface waters were slightly higher than the bottom waters. Maximum temperatures were recorded during April in surface waters (31.50°C) and bottom waters (31.0°C) and during April and May in sediments (30.5°C). While the minimum temperature was observed in July in surface water (25.50°C), in bottom water and sediment minimum temperatures (25.50°C) were recorded in September. The pH in surface water fluctuated from 7.10 (July) to 7.80 (May) and in bottom water from 7.00 (July) to 7.80 (April and May). Compared to overlying water the sediment most often showed higher pH values which ranged from 7.10 (April) to 8.75 (July). The dissolved oxygen content of surface water was always higher (3.35 - 8.68 ml/l) than the bottom water (2.38 - 7.87 ml/l) and maximum values were recorded during the month of July. The salinity of bottom water was consistently higher than the surface water throughout the

period of investigation, suggesting continuous exposure of sediments to higher saline water. The salinity values of both surface and bottom waters dropped to 0.31‰ and 0.51‰ respectively in the month of July because of the monsoon and heavy inflow of freshwater into the station. The maximum salinity recorded for surface water was 24.63‰ (April) and for bottom water it was 30.97 ‰ (April). The turbidity of water fluctuated to a limited extent (k = 1.25 - 2.33) throughout the year except in the month of July when the value increased substantially to 7.0. Like other stations, it might have been due to the heavy inflow of rain water from the adjoining areas carrying lot of suspended load. The sediment in this station was clayey in nature and grey to black in colour. Higher total heterotrophic bacterial populations were recorded in sediments when compared to overlying water. In surface water the total bacterial population varied from 2.98 x 10^3/100 ml (April) to 67.60 x 10^3/100 ml (July). In bottom water the population was higher than the surface waters in April and September and it varied from 4.90 x 10^3/100 ml (May) to 65.60 x 10^3/100 ml (September). In sediments highest population (91.53 x 10^3/g) was recorded in the month of May and lowest

population (6.25×10^3/g) in April. Sediments harboured more chitinoclasts than the overlying water in July and September. However in other months overlying water harboured more chitinoclasts than the sediment. The percentage of chitinoclasts in surface water ranged from 10.81 (September) to 28.55 (July) and in bottom water it varied from 2.43 (September) to 50.00 (July). In sediments highest chitinoclastic bacterial population (51.72 % of total bacteria) was recorded in the month of July. The percentage of chitinoclasts in sediments varied from 10.11 (April) to 51.72 (July).

Station 5 - Tanker Jetty:

In this station totally four collections were made during the period of investigation (i.e. April, May, July and September). On all occasions the temperature of the sediment was much lower than the overlying water. Maximum temperatures were recorded during April in surface waters (32.15°C), bottom waters (31.0°C) and sediment (31.0°C). While the minimum temperatures were observed in July in surface waters (27.0°C) and bottom waters (27.25°C). For sediments minimum temperature

was observed in the month of September (26.50°C). The pH in surface water fluctuated from 7.0 (July) to 7.80 (September) and in bottom water from 7.05 (July) to 7.85 (April). In sediments pH fluctuated from 7.30 (April) to 8.0 (July). The dissolved oxygen content of surface water was always considerably higher (2.08 - 8.29 ml/l) than the bottom water (2.73 - 4.17 ml/l) and maximum values were recorded during the month of July. The salinity of bottom water was consistently higher than the surface water throughout the period of investigation indicating that the sediment was exposed to higher saline water always. The salinity values of both surface and bottom waters dropped to 0.13 ‰ and 0.32 ‰ respectively in the month of July because of the monsoon and heavy inflow of freshwater into the station. The maximum salinity recorded for surface water was 27.90 ‰ (April) and for bottom water it was 30.59 ‰ (April). The turbidity of water remained almost constant (k = 1.75) throughout the year except during July when the value increased considerably to 7.0 possibly due to heavy inflow of rain water from the adjoining areas carrying lot of suspended load. The sediment in this station was clayey in nature and brownish to black in colour. Higher total heterotrophic bacterial populations were recorded

in sediments when compared to overlying waters. In surface water the total bacterial population varied from 4.90 x 10^3/100 ml (May) to 25.40 x 10^3/100 ml (September). In bottom water the population was most often higher than the surface water and it varied from 4.50 x 10^3/100 ml (May) to 40.80 x 10^3/100 ml (July). In sediments highest population (111.56 x 10^3/g) was recorded in May and lowest population (5 x 10^3/g) in September. The percentage of chitinoclasts in surface water ranged from 0.0 (May) to 20.0 (April) and in bottom water it varied from 3.83 (September) to 52.50 (July). In sediments highest chitinoclastic bacterial population (28.63 % of total bacteria) was recorded in the month of May. The percentage of chitinoclasts in sediments varied from 1.96 (April) to 28.63 (May).

Station 6 - Narakkal Channel:

In this station only two collections were made during the period of study (27.3.80 and 9.5.80). The temperature of the surface water was found to be slightly higher than the bottom water and sediment on both occasions. Otherwise the temperature was almost uniform (31 or 33°C) throughout the water column of 2.5 m deep. The pH also did not vary much (7.3 to 8.25),

except the surface water sample collected on 9.5.80 which was marginally high (8.6). The dissolved oxygen content of surface water was considerably higher (3.96 and 8.80 ml/l) when compared to bottom water (2.34 and 6.33 ml/l). Salinity of the bottom water was slightly higher at this station compared to surface water and the maximum salinity recorded was 24.63 ‰. The turbidity of the water remained almost same during the period of study (k = 1.75). The sediment in this station was silty to clayey in nature and black in colour. Highest total heterotrophic bacterial population was recorded in sediments (72.04 x 10^3/g). However the population was considerably low (4.89 x 10^3/g) on another occasion. Such wide fluctuation was also noticed in the overlying water. In this station higher number of chitinoclasts were encountered in overlying water when compared to sediment. Highest chitinoclastic bacterial populations were noted only in surface water on both occasions. Out of the two collections made, the surface water sample collected during May, 1980 showed the maximum chitinoclasts (66.66 % of the total bacterial population). Similarly bottom water samples collected during May 1980 contained more chitinoclastic bacteria (44.52 % of the

total bacterial population). The sediment harboured about 43.81 % of chitinoclasts during the month of May 1980.

Station 7 - Narakkal Pond:

In this station totally three collections were made during the period of study (i.e. March, May and July). Since this was a prawn culture pond and the depth of water column was only 1.0 m , surface water samples were collected during the period of study. On all occasions the temperature of the sediment was much lower than the overlying water. Maximum temperature was recorded during May in water (36.50°C), and in sediment (35.50°C). Minimum temperatures were registered in July in both water (30.0°C) and sediment (29.50°C). The pH of water fluctuated from 8.55 (July) to 9.20 (May). Compared to overlying water sediment always showed lower pH values ranging from 7.35 (July) to 8.25 (May). The dissolved oxygen content of surface waters fluctuated from 7.04 - 8.75 ml/l. The salinity of the water varied between 9.74 and 23.48 ‰. Maximum salinity (23.48 ‰) was recorded in the month of March. Throughout the

period of investigation the sediment was exposed, to almost constant saline water except during July when the salinity dropped to 9.74 ‰. This was due to the heavy monsoon. The turbidity of the water remained almost constant (k = 1.75) throughout the year. The sediment in this station was silty in nature and black in colour. Higher total heterotrophic bacterial populations were recorded in sediments when compared to overlying water. In water the total bacterial population varied from 6.20 x 10^3/100 ml (March) to 34.80 x 10^3/100 ml (July). In sediments highest population (66.89 x 10^3/g) was recorded in July and lowest population (3.23 x 10^3/g) in March. Water always harboured more chitinoclasts than the sediment. The percentage of chitinoclasts in water ranged from 19.35 (March) to 48.27 (July). In sediments highest chitinoclastic bacterial population (33.33 % of total bacteria) was recorded in the month of May. The percentage of chitinoclasts in sediments varied from 13.04 (March) to 33.33 (May).

Station 8 - Eloor:

In this station totally three collections were made during the course of study (i.e. January, March and May).

On all occasions the temperature of the sediment was slightly less than the overlying water. Temperature of the bottom water was also slightly less than the surface water. While maximum temperatures were recorded during March in surface water (34°C), bottom water (34°C) and sediment (34°C), minimum temperatures were observed in January for surface water (32.0°C), bottom water (31.0°C) and sediment (30.0°C). The pH of the surface water fluctuated from 5.40 (March) to 6.70 (January) and the bottom water from 6.45 (January) to 7.0 (May). Compared to overlying water the sediment always showed higher pH ranging from 7.0 (January) to 7.20 (March and May). The dissolved oxygen content of surface water was always considerably higher (3.49 - 5.86 ml/l) than the bottom water (3.12 - 3.86 ml/l) and maximum values were recorded during the month of May. The salinity of the bottom water was considerably higher than the surface water throughout the period of investigation except May when the difference was only marginal. As in other stations, the sediments of this station were also exposed to a constant saline water eventhough the salinity of water was much less. The maximum salinity recorded for surface water was 4.26 ‰ (March) and for bottom water it was 12.33 ‰ (March). The turbidity of water

remained constant (k = 1.75) throughout the period of investigation. The sediment in this station was silty in nature and black in colour. Higher total heterotrophic bacterial populations were recorded in surface water during different collections. In surface water the total bacterial population varied from 0.60 x 10^3/100 ml (January) to 125.0 x 10^3/100 ml (May). In bottom water the population was always lower than the surface water and it varied from 1.0 x 10^3/100 ml (January) to 90.0 x 10^3/100 ml (May). In sediments highest population (8.74 x 10^3/g) was recorded in May and lowest population (4.33 x 10^3/g) in March. Interestingly no chitinoclasts were recorded in surface water samples collected on two occasions (January and March). However during the third time (May) the percentage of chitinoclasts was found to be 46.0 % of the total heterotrophs. Similarly no chitinoclasts were encountered in the bottom water during the first collection (January). During the second and third time the chitinoclasts were present and it varied from 2.43 % (March) and 35.55 % (May). Like the water samples there was no chitinoclasts in the sediment samples collected during January. However in the subsequent collections they were found and they varied from 46.15% (March) to 28.14 % (May) of the total population.

4.2. Taxonomy of chitinoclastic bacteria:

During the present study a total of 210 strains of chitinoclastic bacteria (sediment - 52; water - 25; prawns - 78; fishes - 55) were isolated and their taxonomy was studied. The source details are shown in Table 9 and Fig. 4. Based on the Schewan's classification (Schewan, 1963) out of 210 strains, 206 were identified to the generic level. The genera represented were Vibrio, Aeromonas, Alcaligenes, Pseudomonas, members of Enterobacteriaceae, Bacillus and Micrococcus. Vibrio was found to be the most common genus in sediment, water, prawns and fishes. In sediment 86% of the isolates belong to Vibrio. While Aeromonas and Micrococcus represented only 6% each, the Alcaligenes constituted as low as 2 %. Other genera were not encountered in sediments.

An almost similar distribution pattern was noticed in water also. In addition to the four genera encountered in sediment members of Enterobacteriacea exhibiting chitinolytic properties were also isolated from water and it constituted nearly 4 % of the chitinoclastic population. 72 % of the water isolates were

found to be Vibrio. Aeromonas, Micrococcus and Alcaligenes constituted 12, 8, and 4 % respectively. Compared to sediment the last three genera were found to be almost double in number in water.

Out of the 76 prawn isolates identified, 29 isolates were from the gut of the animal and 47 were from the shell surface. Out of the 29 prawn gut isolates, 20 were identified as Vibrio. Similarly out of the 47 shell surface isolates as many as 34 isolates were found to be Vibrio. The preponderance of Vibrio was seen both on the shell surface and inside the gut. The distribution of other genera were found to be almost uniform both on the shell surface and in the gut except Aeromonas which was isolated only from the gut samples. Considering the prawn isolates as a whole, Vibrio constituted as much as 71.05 %. Members of Enterobacteriaceae, Micrococcus and Aeromonas accounted for 9.21, 6.57, and 5.26 % of total chitinoclasts. The genera Alcaligenes, Pseudomonas and Bacillus were least represented (2.43 % each). One of the interesting observations was the genus Pseudomonas was found to be associated only with the prawns.

A total of 55 chitinoclastic strains were isolated from the gut samples of 17 species of fin fishes

(Table 9). As with other samples, Vibrio was found to be the most predominant genus in the fish also. It constituted nearly 65% of the total chitinoclasts examined. Except Pseudomonas all other genera were encountered. Micrococcus, constituting 14.5 %, was found to be the next common genus. This is followed by Aeromonas (12.7%), members of Enterobacteriaceae (3.6%), Alcaligenes (1.8%) and Bacillus (1.8%).

4.3. Chitinoclastic bacteria associated with prawns and fishes:

Some of the most common penaeid prawns were examined for the presence of chitinoclastic bacteria. Totally seven species of penaeids (P. indicus, P. monodon, P. semisulcatus, Metapenaeus monoceros, M. affinis, M. dobsoni, Parapenaeopsis stylifera), and one brackish water species, Machrobrachium rosenbergii (Palaemonidae) were examined for the distribution of chitinoclastic bacteria on the shell surface and in the digestive tract and the data are presented in Table 4. Chitinoclastic bacteria were found to be present both on the shell surface and in the contents of digestive tract of prawns examined. In general, the digestive tract harboured more chitinoclasts when compared to shell

surface, except Metapenaeus affinis. This observation can be accepted only with some reservations. In the present investigation, the shell surface bacterial population was estimated for the entire animal and not on unit basis. The total heterotrophic bacterial population on the shell surface varied from 1.15×10^6 to 64.20×10^6/animal. The chitinoclastic bacterial population also fluctuated widely. In case of M. affinis as much as 75 % of the total bacterial population was found to be chitinoclasts whereas in P. monodon it was only 4.76 % . In the digestive tract also total heterotrophic population widely fluctuated from 1.30×10^6 to 241×10^6/g of gut contents. The chitinoclastic bacterial population in the digestive tract varied from 3.0 (M. affinis) to 86.2 % (P. indicus). No definite relationship was observed between total heterotrophic bacterial population and chitinoclasts. The gut content analysis of P. monodon, P. semisulcatus, M. monoceros, M. affinis showed that atleast 20 to 30 % of the gut contents were of chitinous nature (Table 5).

In addition, 24 species of fish belonging to 15 families were examined for the occurrence and distribution of chitinoclastic bacteria in their digestive

tracts. Out of these 24 species, 14 were obtained from trawl collections of the Integrated Fisheries Project vessels which normally operate in offshore regions. The remaining 10 species were collected mainly by Chinese dip net and cast net in the estuary. Chitinoclastic bacteria were present atleast in 20 species of fish examined (Table 6). Maximum heterotrophic bacterial population in the digestive tract was recorded in Carangoides malabaricus (542.20×10^6/g) and the minimum (2.61×10^6/g) was recorded in Rastrelliger kanagurta. More than 90 % of the total bacterial population in Leiognathidae fishes were fourd to be chitinoclastic. The chitinoclastic bacteria were also found to be predominant in fishes belonging to Sciaenidae and Mugillidae. In general, species of Carangidae harboured low levels of chitinoclastic bacteria except Selar mate and Chorinemus tala where the percentage of chitinoclasts were 34 and 20 % respectively. No chitinoclastic bacteria could be recognised atleast in 4 species (Selar kalla; Rastrelliger kanagurta, Etroplus maculatus and E. suratensis) examined. In order to understand the feeding behaviour of fishes, gut contents of 6 species were examined and the results are given in Table 7. Most of the fishes were found to

contain some chitinous material in their digestive tracts.

4.4. Chitinase activity in sediments and digestive tract of prawns and fishes:

4.4.1. Sediments:

In an attempt to measure the free chitinase activity in sediments a total of 15 samples were collected from different parts of the estuary right from Thattampally in the south to Munampam in the north. All the sediments were almost clayey in nature and the salinity of the overlying water varied from almost fresh water to 12.2 ‰ (Table 8). In all the sediment samples no detectable levels of chitinase activity was noted (Table 8). Various combinations of enzyme assay mixture were tried and still no chitinase activity could be detected in sediments tested. In addition, the sediments were also analysed for total bacterial content and the chitinoclastic population. The total bacterial population varied from 1.13×10^5/g to 134×10^5/g. The percentage of chitinoclasts also fluctuated from 1.13 to 13.94 %.

4.4.2. Digestive tracts of prawns:

All species of prawns examined had some level of chitinase activity associated with their digestive tracts (Table 4). Highest activity was recorded in Penaeus indicus (150.51 µg NAG produced/g dry weight/hr). Levels of activity greater than 100 µg NAG/g/hr were found in P. monodon, P. semisulcatus, Parapenaeopsis stylifera and Macrobrachium rosenbergii. The lowest activity (43.50 µg NAG/g/hr) was recorded in M. affinis.

4.4.3. Digestive tracts of fishes:

All species of fishes which were examined for the chitinoclastic population were also tested for chitinase activity. Out of the 24 species tested, 21 species exhibited some levels of chitinase activity in their digestive tracts (Table 6). Chitinase activity ranged from no detectable level to as high as 725 µg NAG/g/hr. In general, members of Carangidae exhibited significantly higher levels of chitinase activity in their digestive tracts. Very low levels of activity were recorded with members of Mugillidae and Drepanidae. Two species (Etroplus maculatus and E. suratensis) of

Cichlidae were examined and none of them exhibited any detectable level of chitinase activity.

4.5. Growth and physiology of chitinoclastic bacteria:

4.5.1. Screening of chitinoclasts for chitinase activity:

A preliminary screening was made to select most potent chitinase producing strains for further detailed investigation. Accordingly one species from each genus was arbitarily selected from the collection of chitinoclasts isolated from various sources. Totally five species viz. Aeromonas sp. (C.7), Vibrio sp. (C.25), Bacillus sp. (C.33), Micrococcus sp. (C.53) and Alcaligenes sp. (C.73) were selected, grown in chitin medium and their chitinases were partially purified and assayed under standard conditions. The results are presented in Table 10. Out of the five species tested Aeromonas sp. (C.7) and Vibrio sp. (C.25) exhibited highest chitinase activity (58.8 and 50.4 µg NAG/ml/2 hr respectively). These two strains were used for further studies. The Alcaligenes sp. (C.73) exhibited very low chitinase activity (1.04 µg NAG/ml/2 hr) when compared to Aeromonas and Vibrio.

4.5.2. Effect of temperature on the growth of chitinoclastic bacteria:

The effect of temperature on the growth of chitinoclastic Aeromonas sp. and Vibrio sp. was investigated by growing them at different temperatures (4°, 10°, 15°, 20°, 25°, 30°, 37° and 40°C) and estimating their final biomass. The results are given in terms of growth index. Growth index is the percent of average turbidity of cultures grown for a specific period and at specific temperature. As it can be seen from the results (Table 11) both the cultures exhibited maximum growth at 30°C. However almost uniform growth was observed between 25° and 37°C. At 40°C the growth of both species was very much affected and it dropped to nearly 20% of the maximum growth (100%) at 30°C. With decrease in temperature a corresponding decrease in growth was observed and at 4°C the growth was very much limited.

4.5.3. Effect of pH on the growth of chitinoclastic bacteria:

The effect of pH on the growth of chitinoclastic bacteria was studied by growing them in media adjusted to various levels of pH values (pH 2 - 11). The results

are expressed in terms of growth index and given in Table 12. Aeromonas sp. exhibited maximum growth at pH 6, while Vibrio sp. showed maximum growth at pH 5. However, the optimum range was found to be pH 5.0 - 6.0 for Aeromonas sp. and pH 5.0 - 8.0 for Vibrio sp. Beyond these ranges the growth reduced considerably and Vibrio sp. could not grow at pH 11.0. At pH 2.0 Aeromonas sp. grew better than Vibrio sp. eventhough the overall growth was very poor.

4.5.4. Effect of sodium chloride on growth of chitinoclastic bacteria:

The effect of sodium chloride on the growth of chitinoclastic bacteria was studied by growing the cultures in Campbell's basic medium with various concentrations of sodium chloride (0 - 15%) and the results in terms of growth index are given in Table 13. Both the species grew well in medium containing 1 % NaCl. However both strains grew equally well in the absence of NaCl also. Concentrations higher than 5 % NaCl adversely affected the normal growth of the bacteria.

4.5.5. Effect of temperature on survival of chitinoclastic bacteria:

The temperature tolerance of bacteria was tested by exposing them to different temperatures (40°, 50° and 60°C) for varying periods and estimating the survival percentage. The results are presented in Table 14. Both Aeromonas sp. and Vibrio sp. tolerated very well 40°C even upto 60 minutes exposure. 93.7 % cells survived an exposure of 40°C for 60 minutes, in the case of Aeromonas sp. and it was 98.7 % survival in Vibrio sp. When the temperature was increased the survival percentage of both the species decreased with time. 60°C exposure for 60 minutes considerably affected the viability of the cells and the survival percentages were 5.1 and 2.6 for Aeromonas sp. and Vibrio sp. respectively.

4.5.6. Effect of pH on survival of chitinoclastic bacteria:

The effect of pH on survival of chitinoclastic bacteria was examined by incubating a known concentration of viable cells in buffers having different pH values (pH 2.6 to 10.6) and estimating the survival percentage at predetermined intervals upto 30 hours.

As it can be seen from the results (Table 15) Aeromonas sp. survived better at pH 5.6 and Vibrio sp. at pH 7.0. However, Vibrio sp. tolerated a wide range of pH from 4.6 to 8.6. The strains were found to be more sensitive towards acidic conditions than alkaline conditions. Both the species failed to survive beyond 12 hours at pH 3.6 and 2.6. However, at pH 10.6 nearly 60 % survival was noticed even after 30 hours exposure.

4.5.7. Effect of sodium chloride on survival of chitinoclastic bacteria:

The survival of chitinoclastic bacteria at various sodium chloride concentrations was tested by incubating a known quantity of actively growing cells with different concentrations of sodium chloride and estimating the survival percentage at predetermined intervals upto 30 hours. The results are presented in Table 16. Both the species survived well at 1.0 and 3.0 % of NaCl upto a maximum period of 18 hrs. Concentrations below 0.1 % and above 10.0 % drastically affected the survival of Aeromonas sp. and at the end of 30 hr incubation practically 100 % of the cells were killed. Moreover this species was found to be more tolerant towards lower concentrations of NaCl than higher concentrations

i.e. above 5 %. 15 % NaCl was found to be extremely lethal for Aeromonas sp. as only 0.7 % of the cells survived at 12 hr. In contrary, the same species survived upto 18 hrs to an extent of 70.9 % in the absence of NaCl. However, the survival percentage dropped drastically to 0.7 % at the end of 24 hr incubation.

The Vibrio sp. tolerated a wide variation of NaCl concentration. The percentage of survivors reduced with increasing incubation time and reached almost to a constant level after 30 hrs of incubation with 0 - 3.0 % NaCl concentration. Further increase in NaCl concentration drastically affected the survival rate as 80 % of the cells were killed at concentration of 8.0 and 10.0 % NaCl at 30 hr. Like Aeromonas sp., Vibrio sp. also found to be highly susceptable to 15.0 % NaCl concentration as 100 % cells were killed after 30 hr incubation. However, Vibrio sp. was found to be comparatively resistant to 15 % NaCl concentration as 1.0 % of survivors was noticed after 24 hrs incubation.

4.6. Cultural conditions and chitinase production:

The effect of various culture conditions such as the nature of substrate, concentration, size of particles, certain added chemicals, temperature, pH and sodium chloride on chitinase production by both Aeromonas sp. and Vibrio sp. was studied in detail and the results are reported in the following pages.

4.6.1. Effect of chitin type and initial concentration on chitinase production:

The effect of different chitin types such as powdered chitin, colloidal chitin and powdered chitosan and their initial concentrations on chitinase production of both the species of bacteria was investigated as outlined under Materials and Methods Section - 3.10.1 and the results are given in Tables 17, 18 and 19.

All the chitin types favoured chitinase production in both Aeromonas sp. and Vibrio sp. The initial concentration of chitin and chitosan did not affect the growth to any appreciable extent but a marked influence was seen in the chitinase production. When powdered chitin was used as the substrate, an increase in initial concentration resulted in increased pro-

duction of chitinase in both the species (Table 17). In Aeromonas sp. the specific activity of chitinase increased from 6.46 to 10.22 when the concentration of ~~increased~~ chitin powder was increased from 0.1 to 3.0 %. Similarly, in Vibrio sp. the specific activity increased from 4.86 to 6.91. Varying levels of N-acetyl-glucosamine (NAG), glucosamine, glucose and ammonia were detected in culture filtrates of both the species irrespective of the concentration of chitin powder. More ammonia accumulated in cultures of Aeromonas sp. when compared to Vibrio sp. There was no significant differences in levels of other components (NAG, glucosamine and glucose) between the two species except at 3.0 % concentration of chitin powder.

When colloidal chitin was used as the substrate, a decrease in initial concentration resulted in increased production of chitinase in both the species (Table 18). In Aeromonas sp. the specific activity showed a maximum of 7.43 at 0.1 % chitin (colloidal). The activity decreased (7.19 to 5.62) as the concentration of the initial colloidal chitin was increased from 0.1 to 3.0 %. A similar situation i.e. an inverse relationship between specific activity and initial concentration of colloidal chitin was noticed with Vibrio sp. also. Compared to

Aeromonas sp., Vibrio sp. showed a marked increase in chitinase production at 0.1 % colloidal chitin as indicated by increased specific activity. The specific activity increased from 7.73 to 35.64 as the concentration of colloidal chitin decreased from 3.0 to 0.1%. Varying levels of N-acetylglucosamine (NAG), glucosamine, glucose and ammonia were detected in culture filtrates of both species irrespective of the concentration of colloidal chitin. Like chitin powder medium more ammonia was found to be accumulated in cultures of Aeromonas sp. when compared to Vibrio sp. There was no significant difference in levels of other components (NAG, glucosamine and glucose) between the two species. However, unlike powdered chitin glucose levels were very much reduced in colloidal chitin medium. Maximum growth was recorded at 1.0 % concentration and the minimum was observed at 3 % level for both the species.

When chitosan powder was used as the substrate, an increase in initial concentration resulted in increased production of chitinase in both species indicating a direct relationship between the substrate concentration and chitinase production (Table 19). In Aeromonas sp. the specific activity increased from

6.05 to 12.72 when the concentration of chitosan powder was increased from 0.1 to 1.0 % . At 3 % concentration a slight reduction in specific activity noticed. Similarly in Vibrio sp. the specific activity increased from 5.74 to 9.93 when the concentration of chitosan powder increased from 0.1 to 3.0 % . Varying levels of N-acetylglucosamine (NAG), glucosamine, glucose and ammonia were detected in culture filtrates of both the species irrespective of the concentration of chitosan powder. A very significant amount of glucose (997.50 µg/ml) accumulated in cultures of Aeromonas sp. when compared to Vibrio sp. and more ammonia, glucosamine and NAG accumulated in cultures of Vibrio sp. compared to Aeromonas sp. The growth of both the species appeared unaffected by the variation of chitosan concentration.

4.6.2. Effect of chitin particle size on chitinase production:

Effect of different size particles of chitin powder on chitinase production and growth of chitinoclastic bacteria was studied by incorporating chitin particles of two sizes (0.5 mm and 1.0 mm) into liquid medium separately and growing the cultures under standard conditions. The growth and chitinase production were

estimated and the results are given in Table 20. Different size particles exhibited marked difference in the chitinase production of cultures eventhough the effect was not much pronounced on their growth. Higher chitinase production was noticed in Aeromonas sp. when smaller particles (0.5 mm) were used. However, in case of Vibrio sp. higher production was found to be associated with larger particles (1.0 mm). In other words a direct relationship between chitinase production and size particles of chitin was observed with Vibrio sp. whereas it was an inverse relationship with Aeromonas sp. NAG, glucosamine, glucose and ammonia were present in culture filtrates of both the species irrespective of the particle size. NAG and glucosamine levels in culture filtrates of both the species increased with increase in particle size. With reference to Aeromonas sp. the residual concentration of glucose in the culture filtrate slightly increased with increase in particle size. However a reverse trend was noticed with Vibrio sp. more ammonia accumulated in culture media of both the species supplemented with 0.5 mm size chitin particles compared to 1.0 mm particles.

4.6.3. Effect of other substrates on chitinase production:

In order to ascertain whether the chitinase systems of *Aeromonas* sp. and *Vibrio* sp. were of induced one or constitutive one, certain enzymatic break down products of chitin were incorporated into the medium replacing chitin and their chitinase production was tested. As it can be seen from the results (Table 21), both the species produced chitinase system in the absence of chitin or chitosan. Out of the break down products tested NAG induced more chitinase production when compared to glucosamine and glucose. When the concentration of glucosamine was increased from 0.25 % to 0.5 % the chitinase production also increased from 19.90 µg NAG/ml to 53.13 µg NAG/ml in *Aeromonas* sp. and from 22.90 µg NAG/ml to 27.36 µg NAG/ml in *Vibrio* sp. In the case of glucose lower concentration favoured chitinase production than the higher concentrations. These results indicate that the chitinase system in both the species was a constitutive one and it can be stimulated to a greater extent with N-acetylglucosamine.

4.6.4. Effect of temperature on chitinase production:

Effect of temperature on chitinase production of chitinoclastic bacteria was assessed by growing them at different temperatures (4° - 40°C) and estimating their chitinase production. Maximum chitinase production was noticed at 37°C for both the species (Table 22). In general, increase in temperature resulted in increased chitinase production indicating a direct relationship between temperature and chitinase production. In Aeromonas sp. the specific activity increased from 11.08 to 39.07 when temperature was raised from 4° to 37°C. However a further increase of 3°C i.e. 40°C resulted in a drastic reduction of chitinase production. Aeromonas sp. showed maximum production (S.A. = 39.07) at 37°C. Similarly in Vibrio sp., the specific activity increased from 2.82 to 22.98 when the temperature increased from 4° to 37°C. Further increase in temperature to 40°C resulted a greater reduction in specific activity. Vibrio sp. also showed maximum production (S.A. = 22.98) at 37°C. Varying levels NAG, glucosamine, glucose and ammonia were detected in culture filtrates of both the species

irrespective of the temperature. More NAG, glucosamine, glucose and ammonia were found to be accumulated in cultures of Vibrio sp. when compared to Aeromonas sp. The residual concentration of NAG, glucosamine, glucose and ammonia in the culture filtrates increased with increasing temperature upto 37°C. The maximum production of NAG, glucosamine, glucose and ammonia were noticed at 30°C and 37°C. In Aeromonas sp. maximum glucose (137.37 µg/ml) accumulation in culture filtrate was at 4°C. Similarly Vibrio sp. also exhibited a slight increase in glucose level at 4°C when compared to 30°C. Maximum growth was recorded at 37°C and the minimum was observed at 4°C for both the species.

4.6.5. Effect of pH on chitinase production:

The effect of pH on chitinase production of chitinoclastic bacteria was studied and the results are presented in Table 23. Both Aeromonas sp. and Vibrio sp. produced chitinase in all pH levels i.e. 5, 7 and 9 tested. Both species produced maximum chitinase at pH 7.0 although maximum growth was recorded in pH 5. At pH 9.0, not only the growth was affected but also the chitinase production was affected as indicated by the drop in specific activity when compared to pH 7.0. The

final pH values were around 7.0 irrespective of the initial pH values. In Aeromonas sp. the specific activity increased from 17.16 to 18.86 when the pH was raised from 5 to 7. A further increase in pH reduced the specific activity to a level of 3.98. Vibrio sp. also responded in the same manner and the specific activity increased from 22.96 to 39.71 when the pH was raised from 5 to 7. When the pH was adjusted to 9 the specific activity decreased considerably to a level of 9.13. Varying levels of NAG, glucosamine, glucose and ammonia detected in culture filtrate of both the species irrespective of the initial pH. Maximum levels of NAG, glucosamine and glucose were recorded at pH 7 for both the cultures. In the culture filtrate of Aeromonas sp. ammonia levels were found to be almost uniform irrespective of the initial pH whereas in Vibrio sp. more ammonia was detected in cultures adjusted to pH 5 and 7 than pH 9.

4.6.6. Effect of sodium chloride on chitinase production:

The effect of sodium chloride on chitinase production of chitinoclastic bacteria was investigated and the results are given in Table 24. From the results

it is evident that sodium chloride concentration in the culture medium considerably influenced the production of chitinase. Both Aeromonas sp. and Vibrio sp. produced chitinase at all NaCl concentration tested and also in the absence of NaCl. Highest chitinase production was noticed at 3.0 % NaCl concentration in both Aeromonas and Vibrio cultures. A wide fluctuation in the specific activity i.e. from 6.18 to 41.02 was noticed in culture of Vibrio sp. In Aeromonas sp. the chitinase production reduced considerably (SA = 1.24) when NaCl concentration was increased to 5.0 %. In Vibrio cultures also a similar trend was noticed Varying levels of NAG, glucosamine, glucose and ammonia were detected in the culture filtrates of both the species irrespective of the NaCl concentration. The residual concentration of NAG and glucosamine in the culture filtrate increased with increasing NaCl concentration upto 1.0 % level in both the cultures. Further increase in NaCl level resulted in decreasing levels of NAG and glucosamine. Maximum levels of glucose were detected at 0 % NaCl in both the cultures. The glucose level gradually decreased with increasing concentration of NaCl. No definite relationship between NaCl concentration and ammonia was noticed. However,

the ammonia level increased steadily upto 3.0 % NaCl and decreased with higher concentrations of NaCl in Aeromonas sp. In Vibrio sp. an almost similar situation was noticed but maximum ammonia accumulation was noted at 1.0 % NaCl level. Maximum growth was observed at 1.0 % NaCl concentration in both the cultures even-though the maximum chitinase production was at 3.0 %. 7.0 % NaCl affected the growth of both the cultures considerably and only minimum growth was recorded at that concentration.

4.7. Generation time of chitinoclastic bacteria:

The generation time of both Aeromonas sp. and Vibrio sp. was worked out using nutrient broth with and without chitin. The generation time was found to be slightly more when the cultures were grown in medium with chitin compared to medium without chitin (Table 25). The generation time for Aeromonas sp. was 1.50 $\pm$ 0.10 hr and for Vibrio sp. it was 1.0 $\pm$ 0.17 hr in chitin medium. The generation time reduced to 1.25 $\pm$ 0.10 and 0.70 $\pm$ 0.10 hr for Aeromonas sp. and Vibrio sp. respectively when they were grown in medium without chitin.

4.8. Rate of chitin degradation by chitinoclastic bacteria:

An attempt was made to study the rate of degradation of pure chitin by pure cultures of chitinoclastic bacteria under laboratory conditions. In the nutrient medium colloidal chitin was added at the rate of 325 mg/100 ml. Known quantities of actively growing cells of Aeromonas sp. and Vibrio sp. were added to the chitin medium and the bacterial biomass and the residual chitin were estimated at 24 hours interval upto a period of 96 hours. The results are given in Table 26 and Fig. 5. The percentage of chitin degraded increased steadily upto 96 hours in both the cultures however the increment after 72 hrs. was comparatively low (3.08 % for Aeromonas sp. and 8.61 % for Vibrio sp.). At 72 hrs. nearly 50% of the added chitin were degraded by the bacteria. The total bacterial biomass increased from 2.60×10^5 at 0 hr. to 30.02×10^5 at 96 hrs. for Aeromonas sp. and 2.65×10^5 to 21.54×10^5 for Vibrio sp. The chitin degradation rates were calculated on the basis of unit biomass as well as unit time. Among the two cultures, Vibrio sp. was found to be more active than Aeromonas sp. Maximum degradation rates were

recorded at 48 hrs. (0.088 mg/hr/10^5 cells) for Aeromonas sp. and at 72 hrs. (0.111 mg/hr/10^5 cells) for Vibrio sp. At 96 hrs., the degradation rates reduced to a level of 0.061 and 0.083 mg/hr/10^5 cells for Aeromonas sp. and Vibrio sp. respectively. When chitin degradation rate was calculated on the basis of amount of chitin supplied, maximum degradation rates (172.32 and 148.80 mg/g/day for Aeromonas sp. and Vibrio sp. respectively) were noticed only at 72 hrs. In this method of calculation higher levels of degradation rates were noticed in Aeromonas cultures compared to Vibrio cultures. This is possible because the total bacterial biomass was more in Aeromonas cultures than the Vibrio cultures.

4.9. Properties of microbial chitinases:

The enzyme was prepared for this study as outlined under Materials and Methods Section 3.9.1 and assayed by following the general procedure given in Section 3.9.2.

4.9.1. Time course of the activity:

Time courses of enzyme reactions for Aeromonas sp. and Vibrio sp. are shown in Fig. 6. The end product formation was rapid in both the cases and reached maximum (100%) at 40 min. and 60 min. for Aeromonas sp. and Vibrio sp. respectively. After this time there was no further increase even upto 120 min.

4.9.2. Effect of temperature on the activity:

The effect of temperature on the chitinase activity is shown in Fig. 7. Enzymes from both the sources exhibited maximum activity at 37°C. When the temperature was varied further the chitinase from Vibrio sp. lost its activity completely at 55°C. In the case of Aeromonas sp. at 55°C, only 65% of the total activity was found to be lost. At 5°C Aeromonas chitinase showed 60% of the total activity whereas Vibrio chitinase exhibited only 34% of the total activity.

4.9.3. Effect of pH on the activity:

The effect of pH on chitinase activity is shown in Fig. 8. The optimum pH for Aeromonas chitinase was

5.6 and for Vibrio sp. it was 7.0. Both chitinases exhibited another peak of activity at pH 3.5. Even at pH 10.5 chitinase from Aeromonas sp. exhibited 45% of the total activity and that from Vibrio sp. had much higher activity (60%). Both chitinases had almost 55% of the total activity at pH 2.0.

4.9.4. Effect of substrate concentration on the activity:

For chitinase from Vibrio sp. maximum activity was registered at a substrate concentration of 0.21 mg colloidal chitin/ml (Fig. 9). In the case of Aeromonas chitinase the maximum activity was recorded at a concentration of 0.04 mg colloidal chitin/ml. Further increase in the substrate concentration (upto 0.5 mg/ml) resulted in marginal decrease of the activity of both the enzymes.

4.9.5. Effect of enzyme concentration on the activity:

The effect of various amounts of enzyme on the activity was tested and the results are shown in Figs. 10, 11, and 12. The extent of reaction by Aeromonas chitinase was not linear with times (Fig.10) but the assay was proportional to the amount of added

enzyme solution (Fig. 12). However the Vibrio chitinase exhibited a more or less linear relationship with time (Fig. 11).

4.9.6. Effect of buffer molar concentration on the activity:

The activity of chitinases from both Aeromonas sp. and Vibrio sp. was not affected upto a molar concentration of 0.1 of the buffer (Fig. 13). The Aeromonas chitinase was almost incentive to buffer molar concentration and 100% activity was registered even upto 0.5 M. On the other hand the Vibrio chitinase was found to be very sensitive to the buffer molar concentration and any increase above 0.1 M resulted in decreased activity. At 0.5 M buffer concentration, nearly 42% of the total activity was found to be lost.

4.9.7. Effect of temperature on the stability of the enzyme:

The heat stability of chitinases both Aeromonas sp. and Vibrio sp. was examined and the results are shown in Fig. 15. Both enzymes were stable upto 40°C and further increase in temperature gradually inactivated

the enzyme. When Aeromonas chitinase was completely inactivated at 70°C the Vibrio chitinase exhibited atleast 25% of the total activity at that temperature.

4.9.8. Effect of pH on the stability of the enzyme:

The stability of the enzyme at various pHs was examined and the results are shown in Fig. 14. Both chitinases were found to be stable at pH 5.0. Any increase or decrease in pH value beyond this level affected the enzyme stability. Both the enzymes were found to be relatively more acid tolerant than alkaline conditions. When the enzymes were exposed to pH 3.0, 97% and 75% of the total activity were recorded for Aeromonas and Vibrio chitinases respectively. When the enzymes were exposed to pH 11.0, only 72% and 57% of the total activity were noted.

4.9.9. Effect of various chemicals on the stability of the enzyme:

The effects of various chemicals on the chitinase activities are presented in Table 27. Eventhough the stability of both the chitinases was affected by the various metal ions tested the pattern of influence was

different for each enzyme. Hg^{++}, Cu^{++}, Zn^{++}, Ca^{++} and Ag^{++} affected the stability of Vibrio chitinase to a greater extent when compared to Aeromonas chitinase. On the other hand Na^{++} and Mg^{++} affected the stability of Aeromonas chitinase to a larger extent than the Vibrio chitinase.

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Table: 3. PHYSICO-CHEMICAL PARAMETERS, TOTAL HETEROTROPHIC BACTERIAL AND CHITINOCLASTIC BACTERIAL POPULATIONS IN WATER AND SEDIMENTS.

Stn. No.	Particulars	Sample	Date of collection	Temperature (°C)	Salinity (‰)	Dissolved O_2 (ml/l)	pH	Depth (m)	Attenuation coefficient (k)	Nature of sediment	Total heterotrophic bacteria (x 10^3/ 100ml or x 10^3/g)	Chitinoclastic bacteria (% of total)
1	Aroor	Surface water	29.1.80	29.20	7.42	8.11	7.50		1.75		7.00	21.42
			9.5.80	32.50	8.48	9.12	8.40		1.75		21.05	64.53
		Bottom water	29.1.80	29.00	22.41	3.15	7.75				4.00	0.00
			9.5.80	32.00	26.30	2.39	7.85				9.35	70.08
		Sediment	29.1.80	29.00			7.50	2.50		Blacksand	2.46	0.00
			9.5.80	32.00			7.65	2.50		"	80.80	60.39
2	Fisheries Harbour	Surface water	29.4.80	31.00	25.78	2.99	7.30		1.59		2.06	3.41
			9.5.80	32.25	21.17	7.45	8.30		1.75		11.00	48.42
			23.5.80	32.00	11.18	5.01	8.20		1.75		9.90	50.56
			3.7.80	27.50	0.41	4.45	6.95		2.33		23.40	13.56
			2.9.80	29.00	2.54	4.74	8.00		1.59		7.70	20.77
		Bottom water	29.4.80	30.10	27.32	2.87	7.65				25.50	5.88
			9.5.80	31.75	25.40	3.40	8.00				32.70	38.83
			23.5.80	31.50	22.52	2.20	8.00				32.20	25.46
			3.7.80	27.00	0.51	3.63	7.05				27.10	24.35
			2.9.80	26.00	20.98	2.56	7.80				35.60	11.53

Table: 3 (Contd.)

Stn. No.	Particulars	Sample	Date of collection	Temperature (°C)	Salinity (‰)	Dissolved O_2 (ml/l)	pH	Depth (m)	Attenuation coefficient (k)	Nature of sediment	Total heterotrophic bacteria (x 10^3/ 100ml or x 10^3/g)	Chitinoclastic bacteria (% of total)
2	Fisheries Harbour	Sediment	29.4.80	31.00			7.35	6.00		Black clay	1.60	41.66
			9.5.80	31.50			7.90	3.50		-do-	150.18	67.18
			23.5.80	30.00			7.70	3.50		-do-	239.63	74.44
			3.7.80	27.25			8.55	5.00		Brownish black clay	62.79	53.67
			2.9.80	25.00			7.90	5.00		Grey clay	43.07	4.76
3	Mattancherry Channel	Surface water	29.4.80	31.50	24.05	3.43	7.30		1.40		3.05	6.82
			9.5.80	32.50	25.40	6.31	8.15		1.75		47.50	42.10
			23.5.80	31.50	15.39	5.02	8.15		1.75		24.30	20.50
			3.7.80	27.50	0.61	5.41	7.05		3.50		49.70	35.61
			2.9.80	28.50	4.74	5.41	7.75		1.75		14.80	5.40
		Bottom water	29.4.80	28.00	28.67	4.31	7.60				28.00	16.21
			9.5.80	31.00	28.28	3.71	7.80				201.60	64.29
			23.5.80	31.50	18.10	3.61	8.10				32.30	32.81
			3.7.80	27.25	0.80	3.48	7.10				24.00	40.00
			2.9.80	28.50	5.51	4.74	7.80				28.00	14.28

Table: 3 (Contd.)

Stn. No.	Particulars	Sample	Date of collection	Temperature (°C)	Salinity (‰)	Dissolved O_2 (ml/l)	pH	Depth (m)	Attenuation coefficient (k)	Nature of sediment	Total heterotrophic bacteria (x 10^3 / 100ml or x 10^3/g)	Chitinoclastic bacteria (% of total)
3	Mattancherry Channel	Sediment	29.4.80	30.50			7.50	6.00		Grey clay	1.94	32.81
			9.5.80	31.00			7.65	2.00		Black "	131.56	62.55
			23.5.80	30.50			7.90	2.00		-do-	37.90	6.10
			3.7.80	27.00			8.85	3.00		Black sand	97.92	71.42
			2.9.80	27.50			7.90	3.00		-do-	35.86	6.15
		Surface water	29.4.80	31.50	24.63	5.11	7.30		1.75		2.98	20.10
			23.5.80	31.00	16.36	3.35	7.80		2.33		16.80	14.60
			3.7.80	25.50	0.31	8.68	7.10		7.00		67.60	28.55
			2.9.80	28.00	3.52	4.74	7.55		1.25		18.50	10.81
4	Barmouth	Bottom water	29.4.80	31.00	30.97	3.43	7.80				32.00	15.60
			23.5.80	30.50	25.98	2.38	7.80				4.90	14.00
			3.7.80	25.75	0.51	7.87	7.00				64.00	50.00
			2.9.80	25.50	21.82	2.65	7.65				65.60	2.43
		Sediment	29.4.80	30.50			7.10	6.00		Grey clay	6.26	10.11
			23.5.80	30.50			7.70	3.50		Black "	91.53	13.77
			3.7.80	26.00			8.75	5.00		Black silty clay	78.64	51.72
			2.9.80	25.50			7.75	3.50		Blackclay	10.95	11.63

Table: 3 (Contd.)

Stn. No.	Particulars	Sample	Date of collection	Temperature (°C)	Salinity (‰)	Dissolved O_2 (ml/l)	pH	Depth (m)	Attenuation coefficient (k)	Nature of sediment	Total heterotrophic bacteria (x 10^3/ 100ml or x 10^3/g)	Chitinoclastic bacteria (% of total)
		Surface water	29.4.80	32.15	27.90	3.08	7.50		1.75		20.00	20.00
			23.5.80	31.50	9.83	4.40	7.50		1.75		4.90	0.00
			3.7.80	27.00	0.13	8.29	7.00		7.00		17.00	4.83
			2.9.80	29.00	1.17	5.59	7.80		1.75		25.40	3.14
5	Tanker Jetty	Bottom water	29.4.30	31.00	30.59	3.96	7.85				19.60	4.08
			23.5.80	31.00	22.13	2.73	7.70				4.50	12.12
			3.7.80	27.25	0.32	4.17	7.05				48.80	52.50
			2.9.80	28.50	2.82	3.02	7.65				41.70	3.83
		Sediment	29.4.30	31.00			7.30	9.90		Black clay	13.24	1.96
			23.5.80	30.00			7.60	5.00		-do-	111.56	28.63
			3.7.30	27.00			8.00	6.00		-do-	9.15	25.58
			2.9.80	26.50			7.70	3.00		Grey clay Greyish black	5.00	7.84
		Surface water	27.3.80	33.00	23.86	3.96	7.60		1.75		44.50	26.96
			9.5.80	33.75	18.09	8.80	8.60		1.75		12.00	66.66
6	Narakkal	Bottom water	27.3.80	31.40	24.63	2.34	7.70				12.10	14.70
			9.5.80	31.00	18.67	6.33	8.25				39.00	44.52
		Sediment	27.3.80	32.50			7.30	2.50		Black silt	4.89	2.00
			9.5.30	32.50			7.60	2.50		Black clay	72.04	43.81

Table: 3 (Contd.)

Stn. No.	Particulars	Sample	Date of collection	Temperature (°C)	Salinity (‰)	Dissolved O_2 (ml/l)	pH	Depth (m)	Attenuation coefficient (k)	Nature of sediment	Total heterotrophic bacteria (x 10^3/ 100ml or x 10^3/g)	Chitinoclastic bacteria (% of total)
		Water	27.3.80	35.00	23.48	7.04	8.65		1.75		6.20	19.35
			8.5.80	36.50	20.40	8.36	9.20		1.75		13.06	33.23
			2.7.80	30.00	9.74	8.76	8.55		1.75		34.80	48.27
7	Narakkal Pond	Sedi-	27.3.80	33.50			7.70	1.00		Black silt	3.23	13.04
		ment	8.5.80	35.50			8.25	1.00		Black sand	23.63	33.33
			2.7.80	29.50			7.35	1.00		-do- -do-	66.89	32.43
		Surface	28.1.80	32.00	3.25	3.49	6.70		1.75		0.60	0.00
		water	27.3.80	34.00	4.26	3.78	5.40		1.75		28.00	0.00
			9.5.80	33.75	1.37	5.86	6.65		1.75		125.00	46.00
8	Eloor	Bottom	28.1.80	31.00	11.21	3.12	6.45				1.00	0.00
		water	27.3.80	34.00	12.33	3.51	6.60				4.00	2.43
			9.5.80	33.50	1.95	3.86	7.00				90.00	35.55
		Sedi-	28.1.80	30.00			7.00	4.00		Black silt	4.61	0.00
		ment	27.3.80	34.00			7.20	4.00		Black sand	4.33	46.15
			9.5.80	33.50			7.20	2.50		-do- -do-	8.74	28.15

Table: 4. CHITINOCLASTIC BACTERIAL POPULATION IN THE DIGESTIVE TRACT AND ON SHELL SURFACE OF PRAWNS AND ASSOCIATED CHITINASE ACTIVITY IN THE DIGESTIVE TRACT.

S. No.	Specimen	Source	No. of specimens examined*	Size: Length (cms)	Size: Wt. (gms)	Shell surface: total heterotrophic bacteria ($x10^6$/g)	Shell surface: Chitinoclastic bacteria (%)	Digestive tract: Total heterotrophic bacteria ($x10^6$/g)	Digestive tract: Chitinoclastic bacteria (%)	Average chitinase activity (ug NAG/g/hr)
	PENAEIDAE:									
1.	Penaeus indicus	Cast net	2	10.00 10.00	5.00 5.00	2.24	25.00	5.72	86.20	150.51
2.	P. monodon	Trawl	2	20.00 21.50	55.00 59.00	2.40	4.76	147.00	67.21	120.00
3.	P. semisulcatus	-do-	2	14.00 15.00	21.00 25.00	20.50	12.19	42.30	38.42	115.01
4.	Metapenaeus monoceros	-do-	2	12.00 14.00	11.00 20.00	1.15	13.04	1.30	8.64	75.20
5.	M. affinis	-do-	1	13.00	22.00	64.20	75.07	21.00	3.00	43.50
6.	M. dobsoni	-do-	3	6.00 6.20 6.00	1.63 1.90 1.93	N.A.**	N.A.	5.10	9.80	66.43
7.	Parapenaeopsis stylifera	-do-	2	10.00 7.00	6.50 4.00	14.50	24.82	241.00	78.89	105.00
	PALAEMONIDAE:									
8.	Macrobrachium rosenbergii	Cast net	2	19.00 11.00	135.00 56.00	N.A.	N.A.	38.00	17.39	118.51

* Samples were pooled before assay.
** Not analysed.

Table: 5. GUT CONTENT ANALYSIS OF PRAWNS.

S.No.	Name of the specimens	No. examined	Total length (Range in cm)	Percentage composition of gut contents	
1.	*Penaeus monodon*	4	20.00 - 21.50	Detritus	- 35
				Shell pieces	- 30
				Crustacean appendages	- 17
				Diatoms	- 3
				Unidentified	- 15
2.	*P. semisulcatus*	2	14.00 - 15.00	Detritus	- 30
				Crustacean appendages	- 30
				Diatoms	- 10
				Unidentified	- 30
3.	*Metapenaeus monoceros*	5	12.00 - 14.00	Detritus	- 40
				Crustacean appendages	- 30
				Diatoms	- 10
				Unidentified	- 20
4.	*M. affinis*	3	13.00 - 14.00	Detritus	- 40
				Crustacean appendages	- 20
				Algae	- 10
				Diatoms	- 10
				Unidentified	- 20

Table: 6. CHITINOCLASTIC BACTERIAL POPULATION AND CHITINASE ACTIVITY IN THE DIGESTIVE TRACT OF FISHES.

S.No.	Specimens	Method of collection	No. of specimens examined	Size		Digestive tract		Average chitinase activity (µg NAG/g/hr)
				Length (cms)	Wt. (gms)	Total heterotrophic bacteria ($.x10^6/g$)	Chitinoclastic bacteria (%)	
	TACHYSURIDAE:							
1.	Tachysurus maculatus	Trawl (IFP)*	1	24.0	250.00	21.80	50.00	725.00
	SYNODONTIDAE:							
2.	Saurida tumbil	IFP	1	24.0	160.00	37.11	7.21	225.00
	LATIDAE:							
3.	Lates calcarifer	IFP	2	24.0 21.0	320.00 225.00	151.60	58.90	717.00
	BELONIDAE:							
4.	Tylosurus strongylurus	Chinese dip net	1	26.0	52.00	115.00	15.21	70.26
	MUGILLIDAE:							
5.	Mugil cephalus	Chinese dip net	1	14.0	50.00	85.20	73.46	7.00
6.	Liza macrolepis	Chinese dip net	1	12.0	30.00	36.80	43.47	0.00
	CARANGIDAE:							
7.	Carangoides malabaricus	IFP	1	15.5	92.00	542.20	8.19	201.00
8.	Atropus atropus	IFP	1	19.5	200.00	32.30	8.53	715.00
9.	Selar kalla	IFP	1	18.5	160.00	7.50	0.00	600.00
10.	Selar mate	IFP	1	21.0	160.00	32.50	34.61	150.00

Table: 6 (Contd.)

S.No.	Specimens	Method of collection	No. of specimens examined	Size		Digestive tract		Average chitinase activity (µg NAG/g/hr)
				Length (cms)	Wt. (gms)	Total heterotrophic bacteria (x10^6/g)	Chitinoclastic bacteria (%)	
11.	*Megalaspis cordyla*	IFP	1	29.0	355.00	37.01	3.19	720.00
12.	*Chorinemus tala*	Cast net	2	14.0 11.5	25.00 13.00	200.00	20.20	240.00
	LUTIANIDAE:							
13.	*Lutianus* sp.	IFP	1	18.0	170.00	27.30	6.60	175.00
	SCIAENIDAE:							
14.	*Sciaena russelli*	Cast net	1	8.8	13.00	312.00	89.75	251.00
15.	*S. albida*	Cast net	1	9.2	14.00	320.00	62.50	98.00
	NEMIPTERIDAE:							
16.	*Nemipterus japonicus*	IFP	1	22.5	55.00	109.80	4.37	250.50
	LEIOGNATHIDAE:							
17.	*Leiognathus brevirostris*	Chinese dip net	2	7.0 7.0	10.00 10.00	60.80	92.10	56.25
18.	*L. equulus*	Chinese dip net	2	6.5 6.0	10.00 8.00	340.00	97.00	84.61

Table: 6 (Contd.)

S.No.	Specimens	Method of collection	No. of specimens examined	Size		Digestive tract		Average chitinase activity (µg NAG/g/hr)
				Length (cms)	Wt. (gms)	Total heterotrophic bacteria ($\times 10^6$/g)	Chitinoclastic bacteria (%)	
	STROMATEIDAE:							
19.	Parastromateus niger	IFP	1	25.0	435.00	230.70	50.00	650.70
	THUNNIDAE:							
20.	Katsuwonus pelamis	IFP	1	35.5	730.00	230.70	22.11	625.00
	SCOMBRIDAE:							
21.	Rastrelliger kanagurta	IFP	1	16.5	70.00	2.61	0.00	720.00
	CICHLIDAE:							
22.	Etroplus maculatus	Cast net	1	7.5	12.50	28.50	0.00	0.00
23.	E. suratensis	Cast net	1	12.0	81.00	34.30	0.00	0.00
	DREPANIDAE:							
24.	Drepane punctata	IFP	2	18.0 19.0	320.00 345.00	45.26	45.74	7.50

* Integrated Fisheries Project.

Table: 7. GUT CONTENT ANALYSIS OF FISHES.

S.No.	Name of the specimen	No. examined	Total length (cms)	Percentage composition of stomach content	
1.	Parastromateus niger (Pomfret)	20	10.00 - 23.00	Fish scales Crustacean shells Semidigested material	- 20 - 20 - 30
2.	Drepane punctata (Spotted batfish)	5	11.00 - 19.00	Semidigested material	- 90
3.	Atropus atropus (Vatta)	20	13.50 - 21.00	Crab flesh & legs Semidigested food	- 50 - 40
4.	Selar mate (Vatta)	5	18.50 - 20.00	Sepia Fishes Semidigested food	- 60 - 20 - 10
5.	Rastrelliger kanagurta (Mackerel)	5	13.00 - 20.00	Zoea larvae Semidigested food material	- 20 - 60
6.	Nemipterus japonicus (Parrot fish)	25	10.00 - 25.00	Semidigested crab parts Prawn larvae Other digested materials	- 50 - 10 - 20

Table: 8. CHITINOCLASTIC BACTERIAL POPULATION AND CHITINASE ACTIVITY IN SEDIMENTS.

S.No.	Name of the Location	Nature of sediment	Salinity (‰)	Total bacteria (No./g) ($1x10^3$)	Chitino-clasts (%)	Chitinase activity (µg NAG/g)
1	Munampam	Clay	7.0	94.00	13.94	ND**
2	Narakkal	Clay	1.4	72.00	1.66	ND
3	Vaduthala	Clay	0.6	61.00	2.12	ND
4	Barmouth	Clay	12.2	95.00	5.27	ND
5	Vypeen	Sandy clay	12.1	1.50	9.33	ND
6	Arookutty	Clay	NTS*	116.00	4.29	ND
7	Panavally	Clay	NTS	84.00	3.26	ND
8	Thycattussery	Clay	NTS	134.00	11.25	ND
9	Nedumbrakkad	Silty clay	NTS	7.20	3.12	ND
10	Vechoor	Clay	NTS	128.00	10.33	ND
11	Muhamma	Clay	NTS	132.00	11.35	ND
12	Karyil	Sand	NTS	1.13	1.13	ND
13	Manachery	Clay	NTS	96.00	4.13	ND
14	Ariad	Clay	NTS	15.20	2.11	ND
15	Thattampally	Clay	NTS	116.00	1.56	ND

* NTS = No traceable salinity
** ND = Not detected.

Table: 9. SHOWING THE DETAILS OF CHITINOCLASTIC BACTERIAL CULTURES ISOLATED AND IDENTIFIED.

S.No.	Sample	Number of chitinoclastic bacteria identified/total	Species
1	Sediment (Stn. 2)	7/7	Vibrio (7)
2	Sediment (Stn. 3)	2/2	Vibrio (2)
3	Sediment (Stn. 4)	7/7	Vibrio (6), Alcaligenes (1)
4	Sediment (Stn. 5)	2/2	Vibrio (2)
5	Sediment (Stn. 6)	3/3	Vibrio (3)
6	Sediment (Stn. 7)	5/5	Vibrio (5)
7	Sediment (Stn. 8)	1/1	Vibrio (1)
8	Sediment (Vaduthala)	2/2	Vibrio (2)
9	Sediment (Vypeen)	7/8	Vibrio (5); Aeronomas (1); Micrococcus (1)
10	Sediment (Kadakkara)	1/1	Vibrio (1)
11	Sediment (Cherai)	0/1	--
12	Sediment (Munampam)	13/13	Vibrio (9); Aeromonas (2); Micrococcus (2)
13	Water (Stn. 1)	5/5	Vibrio (4); Alcaligenes (1)
14	Water (Stn. 2)	9/9	Vibrio (5); Micrococcus (1); Aeronomas (3)

Table: 9 (Contd.)

S.No.	Sample	Number of chitinoclastic bacteria identified/total.	Species
15	Water (Stn. 3)	2/2	Vibrio (2)
16	Water (Stn. 4)	2/2	Vibrio (2)
17	Water (Stn. 5)	1/1	Vibrio (1)
18	Water (Stn. 7)	6/6	Vibrio (4); Enterobacteriaceae (1); Micrococcus (1)
	Prawns:		
19	Penaeus indicus (gut)	8/8	Vibrio (7); Alcaligenes (1)
20	P. indicus (surface)	4/4	Micrococcus (2); Vibrio (2)
21	Metapenaeus monoceros (gut)	1/1	Pseudomonas (1)
22	M. monoceros (surface)	4/4	Vibrio (3); Bacillus (1)
23	M. dobsoni (gut)	5/6	Micrococcus (1); Bacillus (1) Vibrio (3); Unidentified (1)
24	M. dobsoni (surface)	19/19	Vibrio (15); Alcaligenes (1) Pseudomonas (1); Micrococcus (2)
25	M. affinis (surface)	4/4	Vibrio (4)
26	Macrobrachium rosenbergii (gut)	1/1	Enterobacteriaceae (1)
27	M. rosenbergii (surface)	6/7	Aeromonas (4); Vibrio (1) Enterobacteriaceae (1)

Table: 9 (Contd.)

S.No.	Sample	Number of chitinoclastic bacteria identified/total.	Species
28	Parapenaeopsis stylifera (surface)	10/10	Vibrio (9); Enterobacteriaceae (1)
29	Palaemon sp. (gut)	14/14	Vibrio (10); Enterobacteriaceae (4)
	Fishes:		
30	Nemipterus japonicus (gut)	13/13	Vibrio (4); Aeromonas (4) Micrococcus (4); Bacillus (1)
31	Leiognathus sp. (gut)	5/5	Vibrio (5)
32	Sardinella longiceps (gut)	2/2	Micrococcus (2)
33	Scomberomorus commersoni (gut)	7/7	Vibrio (7)
34	Parastromateus niger (gut)	2/2	Vibrio (2)
35	Rastrelliger kanagurta (gut)	1/1	Vibrio (1)
36	Megalaspis cordyla (gut)	3/3	Vibrio (2); Enterobacteriaceae (1)
37	Tachysurus maculatus (gut)	3/3	Vibrio (3)
38	Katsuwonus pelamis (gut)	1/1	Micrococcus (1)
39	Lates calcarifer (gut)	1/1	Enterobacteriaceae (1)
40	Lutianus sp. (gut)	2/2	Aeromonas (1); Vibrio (1)

Table: 9 (Contd.)

S.No.	Sample	Number of chitinoclastic bacteria identified/total	Species
41	*Atropus atropus* (gut)	1/1	*Alcaligenes* (1)
42	*Carangoides malabaricus* (gut)	1/1	*Vibrio* (1)
43	*Mugil* sp. (gut)	7/7	*Vibrio* (5); *Aeromonas* (1); *Micrococcus* (1)
44	*Sciaenid* sp. (gut)	4/4	*Vibrio* (4)
45	*Carangoides* sp. (gut)	1/1	*Vibrio* (1)
46	*Caranx* sp. (gut)	1/1	*Aeromonas* (1)
	Sediment	50/52	
	Water	25/25	
	Prawns	76/78	
	Fisnes	55/55	

Table: 10. CHITINASE ACTIVITY OF SELECTED ISOLATES.

S.No.	Culture No.	Species	Source	Chitinase activity (µg NAG/ml/2 hrs)
1	C.7	*Aeromonas* sp.	Sediment	58.8
2	C.25	*Vibrio* sp.	Fish gut (*Leognathus* sp)	50.4
3	C.33	*Bacillus* sp.	Prawn (surface) (*M.dobsoni*)	11.4
4	C.53	*Micrococcus* sp.	Prawn (gut) (*M. donsoni*)	16.2
5	C.73	*Alcaligenes* sp.	Prawn (gut) (*P. indicus*)	1.04

Table: 11. EFFECT OF TEMPERATURE ON THE GROWTH OF CHITINOCLASTIC BACTERIA.

S.No.	Organism	Index of Growth*							
		Temperature (°C)							
		4	10	15	20	25	30	37	40
1.	*Aeromonas* sp.	23.80	41.50	56.00	71.20	89.50	100.00	92.60	21.40
2.	*Vibrio* sp.	11.40	31.60	51.20	66.50	93.00	100.00	99.70	17.48

* Growth index is the percent of average turbidity of cultures observed in tubes.

Table: 12. EFFECT OF pH ON THE GROWTH OF CHITINOCLASTIC BACTERIA.

S.No.	Organism	Index of Growth* pH									
		2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0
1.	*Aeromonas* sp.	6.2	12.7	60.1	98.2	100.0	86.8	85.9	52.1	50.7	8.2
2.	*Vibrio* sp.	2.3	5.2	34.9	100.0	99.1	97.5	90.1	43.2	42.0	0.0

* Growth index is the percent of average turbidity of cultures observed in tubes.

Table: 13. EFFECT OF SODIUM CHLORIDE ON THE GROWTH OF CHITINOCLASTIC BACTERIA.

S.No.	Organism	Index of Growth* NaCl concentration (%)									
		0	0.1	1.0	2.0	3.0	5.0	7.0	8.0	10.0	15.0
1.	*Aeromonas* sp.	92.0	97.4	100.0	80.1	55.3	15.5	4.5	3.8	1.8	0.9
2.	*Vibrio* sp.	88.5	92.9	100.0	67.6	34.4	15.8	9.2	5.5	5.1	3.3

* Growth index is the percent of the average turbidity of cultures observed in tubes.

Table: 14. EFFECT OF TEMPERATURE ON SURVIVAL OF CHITINOCLASTIC BACTERIA.*

S.No.	Organism	Temperature (°C)	Exposure time (minutes)				
			10	20	30	40	60
1.	*Aeromonas* sp.	40	100.0**	97.8	95.1	94.2	93.7
		50	99.2	96.6	77.6	64.7	50.0
		60	80.9	52.8	11.6	5.2	5.1
2.	*Vibrio* sp.	40	100.0	99.2	98.9	98.7	98.7
		50	96.6	90.1	86.7	77.4	66.9
		60	96.1	14.1	3.7	3.3	2.6

* Values are expressed in percentage.

** Maximum survival.

Table: 15. EFFECT OF pH ON SURVIVAL OF CHITINOCLASTIC BACTERIA*

S.No.	Organism	Time (hrs)	pH 2.6	3.6	4.6	5.6	7.0	8.6	9.6	10.6
1.	*Aeromonas* sp.	6	0.7	0.8	70.3	100.0	85.1	81.7	76.7	69.6
		12	0.2	0.5	67.2	92.1	84.5	78.4	72.0	68.5
		18	0.1	0.0	66.3	91.1	82.2	76.7	71.7	66.9
		24	0.0	0.0	65.3	87.9	77.3	74.3	66.3	63.8
		30	0.0	0.0	63.7	80.5	72.0	71.8	64.3	63.6
2.	*Vibrio* sp.	6	0.2	0.2	99.3	98.3	100.0	96.4	86.6	83.1
		12	0.0	0.0	98.0	97.7	97.8	92.1	83.8	82.8
		18	0.0	0.0	94.9	95.9	97.4	91.6	82.8	81.8
		24	0.0	0.0	93.5	94.9	96.6	88.3	72.8	71.7
		30	0.0	0.0	90.5	93.5	96.3	82.0	66.8	66.2

* Values are expressed in percentage.

Table: 16. EFFECT OF SODIUM CHLORIDE ON SURVIVAL OF CHITINOCLASTIC BACTERIA*.

S.No.	Organism	Time (hrs)	NaCl (%)							
			0	0.1	1.0	3.0	5.0	8.0	10.0	15.0
1.	*Aeromonas* sp.	6	94.2	98.8	100.0	98.3	91.4	84.6	57.3	1.1
		12	85.2	88.2	96.7	97.7	89.8	57.4	51.7	0.7
		18	70.9	78.9	95.2	97.7	81.7	46.7	2.3	0.0
		24	0.7	78.4	92.0	88.0	79.1	38.8	1.3	0.0
		30	0.0	76.7	82.8	87.5	77.1	20.8	0.7	0.0
2.	*Vibrio* sp.	6	94.8	95.6	100.0	99.9	86.6	66.2	37.2	29.4
		12	94.1	94.1	99.0	99.8	82.2	58.5	34.0	8.0
		18	81.1	88.7	96.1	89.2	53.4	32.0	25.2	3.3
		24	67.4	67.9	92.7	83.0	46.6	25.4	23.1	1.0
		30	67.4	67.4	87.9	71.3	38.1	20.0	19.3	0.0

* Values are expressed in percentage.

Table: 17. EFFECT OF POWDERED CHITIN CONCENTRATION ON CHITINASE PRODUCTION AND GROWTH OF CHITINOLYTIC BACTERIA.

S.No.	Organism	Conc. of chitin powder (%)	Growth ($O.D_{450}$)	Residual concentration in culture filtrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
				NAG (µg/ml)	Glucosamine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
1.	*Aeromonas* sp.	0.1	1.60	52.50	96.25	127.11	0.62	4.17	26.95	6.46
		1.0	1.63	68.95	98.37	121.41	0.58	4.22	32.90	7.79
		3.0	1.64	57.40	87.44	132.81	0.52	10.78	110.25	10.22
2.	*Vibrio* sp.	0.1	1.63	52.50	86.73	155.61	0.32	3.38	16.45	4.86
		1.0	1.62	52.15	86.38	127.11	0.31	3.85	23.45	6.09
		3.0	1.10	31.15	42.31	132.81	0.36	3.29	22.75	6.91

Table: 18. EFFECT OF COLLOIDAL CHITIN ON CHITINASE PRODUCTION AND GROWTH OF CHITINOCLASTIC BACTERIA.

S.No.	Organism	Conc. of colloidal chitin (%)	Growth ($O.D_{450}$)	Residual concentration in culture filtrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
				NAG (µg/ml)	Glucosamine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
1.	Aeromonas sp.	0.1	1.09	51.80	104.01	14.82	0.70	2.87	21.35	7.43
		1.0	1.58	65.10	118.12	50.73	0.68	1.46	10.50	7.19
		3.0	0.78	97.30	164.66	21.66	0.73	5.48	30.80	5.62
2.	Vibrio sp.	0.1	1.18	56.00	113.18	40.29	0.34	2.64	94.15	35.64
		1.0	1.78	78.75	123.05	18.84	0.35	3.57	33.60	9.13
		3.0	0.84	100.80	135.75	67.26	0.80	3.57	27.65	7.73

Table: 19. EFFECT OF CHITOSAN POWDER ON CHITINASE PRODUCTION AND GROWTH OF CHITINOLYTIC BACTERIA.

S.No.	Organism	Conc. of chitosan powder (%)	Growth ($O.D_{450}$)	Residual concentration in culture filtrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
				NAG (µg/ml)	Gluco-samine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
		0.1	1.58	63.70	86.55	997.50	0.29	3.99	24.15	6.05
a.	*Aeromonas* sp.	1.0	1.15	57.75	58.06	712.50	0.42	3.96	50.40	12.72
		3.0	1.34	57.50	58.94	62.70	0.38	5.55	64.05	11.54
		0.1	1.58	68.95	76.06	119.70	0.41	3.71	20.30	5.74
2.	*Vibrio* sp.	1.0	1.56	77.35	77.57	39.90	0.59	3.86	32.90	8.52
		3.0	1.54	77.70	79.53	39.90	0.46	3.77	37.45	9.93

Table: 20. EFFECT OF DIFFERENT SIZE PARTICLES OF CHITIN ON CHITINASE PRODUCTION AND GROWTH ON CHITINOLYTIC BACTERIA.

S.No.	Organism	Chitin size (mm)	Growth ($O.D_{450}$)	Residual concentration in culture fultrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
				NAG (µg/ml)	Glucosamine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
1.	*Aeromonas* sp.	0.5	1.38	26.95	27.85	151.05	0.29	4.49	39.20	8.73
		1.0	1.13	65.90	67.34	182.40	0.21	4.94	21.70	4.39
2.	*Vibrio* sp.	0.5	1.81	22.35	22.56	296.40	0.20	3.75	26.95	7.18
		1.0	1.82	31.80	34.20	142.50	0.12	3.60	37.10	10.30

Table: 21. SUBSTRATE EFFECT ON THE PRODUCTION OF CHITINASE BY CHITINOCLASTIC BACTERIA.

Substrate	Concentration (%)	Chitinase activity*	
		Aeromonas sp.	Vibrio sp.
Chitin (Powder)	0.10	26.95	16.45
	1.00	32.90	23.45
	3.00	110.25	22.75
Chitosan (Powder)	0.10	24.15	20.30
	1.00	50.40	32.90
	3.00	64.05	37.45
N-Acetylglucosamine	0.10	79.00	98.00
Glucosamine	0.25	19.90	22.90
	0.50	53.13	27.36
Glucose	0.10	47.60	30.45
	1.00	50.75	15.05
	3.0	19.60	15.05

* $\mu g\text{-}NAG\ ml^{-1}$ of enzyme assay mixture under optimal conditions.

Table: 22: EFFECT OF TEMPERATURE ON CHITINASE PRODUCTION GROWTH OF CHITINOLYTIC BACTERIA.

S.No.	Organism	Temp. (°C)	Growth ($O.D_{450}$)	Residual concentration on culture filtrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
				NAG (µg/ml)	Gluco-samine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
1.	*Aeromonas* sp.	4	0.08	1.05	4.23	137.37	0.07	1.69	18.79	11.08
		30	1.49	38.35	69.46	71.25	0.63	2.76	78.40	28.40
		37	1.55	53.90	106.48	74.67	0.65	3.09	120.75	39.07
		40	0.08	9.10	11.28	40.47	0.09	5.55	44.10	7.94
2.	*Vibrio* sp.	4	0.02	1.75	5.99	95.19	0.18	2.97	8.40	2.82
		30	1.52	66.50	106.48	80.94	0.79	3.87	44.45	11.43
		37	1.60	71.75	134.69	104.31	0.86	4.05	93.10	22.98
		40	0.27	11.90	11.92	8.55	0.09	9.90	50.05	5.05

Table: 23. EFFECT OF pH ON CHITINASE PRODUCTION AND GROWTH OF CHITINOLYTIC BACTERIA.

S. No.	Organism	Initial pH	Growth ($O.D_{450}$)	Final pH	Residual concentration in cultural filtrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
					NAG (µg/ml)	Gluco-samine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
1.	*Aeronomas* sp.	5.0	0.73	6.5	25.20	31.02	33.63	0.59	3.06	52.50	17.16
		7.0	0.53	7.0	27.65	50.42	19.38	0.60	6.31	119.00	18.86
		9.0	0.32	7.5	23.80	35.61	11.97	0.62	5.72	22.75	3.98
2.	*Vibrio* sp.	5.0	0.67	6.5	46.55	53.59	49.59	0.73	1.91	43.25	22.96
		7.0	0.36	7.0	49.35	59.23	34.20	0.77	1.53	60.76	39.71
		9.0	0.13	7.5	36.75	37.61	23.94	0.57	1.73	15.75	9.13

Table: 24. EFFECT OF SODIUM CHLORIDE ON CHITINASE PRODUCTION AND GROWTH OF CHITINOLYTIC BACTERIA.

S.No.	Organism	Conc. of Na Cl (%)	Growth ($O.D_{450}$)	Residual concentration in culture filtrate				Enzyme protein (mg/ml)	Chitinase activity (µg NAG)	Specific activity
				NAG (ug/ml)	Gluco-samine (µg/ml)	Glucose (µg/ml)	Ammonia (mg/ml)			
		0.0	1.09	77.00	76.40	128.25	0.11	8.13	27.65	3.40
		1.0	1.52	108.15	108.79	78.09	0.46	8.58	59.85	6.98
1.	Aeromonas sp.	3.0	1.48	46.20	48.14	63.84	0.66	3.59	33.60	9.37
		5.0	1.31	23.45	24.06	58.14	0.47	7.91	9.80	1.24
		7.0	0.83	2.10	3.87	11.97	0.45	3.21	5.95	1.85
		0.0	1.17	65.80	92.73	113.43	0.11	3.28	20.30	6.18
		1.0	1.57	104.30	107.89	104.88	0.57	9.64	87.85	9.11
2.	Vibrio sp.	3.0	1.47	75.25	77.57	62.70	0.49	0.87	35.70	41.02
		5.0	1.52	19.95	22.65	58.14	0.28	0.46	6.65	14.45
		7.0	0.89	3.85	6.34	14.25	0.10	0.30	2.10	7.00

Table: 25. GENERATION TIME OF CHITINOCLASTIC BACTERIA.

S.No.	Organism	Generation time (h)	
		Nutrient broth with chitin	Nutrient broth without chitin
1.	*Aeromonas* sp.	1.50 ± 0.10	1.25 ± 0.10
2.	*Vibrio* sp.	1.00 ± 0.17	0.70 ± 0.10

Table: 26. RATE OF CHITIN DEGRADATION BY CHITINOLYTIC BACTERIA.

S.No.	Organism	Bacterial number inoculated (cells/100ml)	Amount of chitin decomposed (mg)*			
			24 hrs.	48 hrs.	72 hrs,	96 hrs.
1.	Aeromonas sp.	2.60×10^5	(16.01×10^5) 25.0	(21.10×10^5) 90.0	(28.03×10^5) 168.0	(30.02×10^5) 178.0
2.	Vibrio sp.	2.65×10^5	(10.56×10^5) 24.0	(15.04×10^5) 78.0	(18.04×10^5) 145.0	(21.54×10^5) 173.0
			Chitin degradation (%)			
1.	Aeromonas sp.		7.69	27.69	51.69	54.77
2.	Vibrio sp.		7.38	24.00	44.62	53.23
			Degradation rates			
1.	Aeromonas sp.	mg/hr/10^5	0.065	0.088	0.083	0.061
		mg/day/10^5	1.560	2.112	1.992	1.464
		mg/day/10^{10}	3.120	4.224	3.984	2.928
		mg/g/day	77.040	138.480	172.320	137.040
2.	Vibrio sp.	mg/hr/10^5	0.094	0.108	0.111	0.083
		mg/day/10^5	2.256	2.592	2.664	1.992
		mg/day/10^{10}	4.512	5.184	5.328	3.984
		mg/g/day	73.920	120.000	148.800	132.960

* Mean of duplicate.

Table: 27. EFFECT OF VARIOUS CHEMICALS ON THE STABILITY OF CHITINASE ACTIVITY.

S.No.	Organism	Residual activity (%)						
		Control (No chemical)	$HgCl_2$	$CuSO_4$	$ZnSO_4$	NaCl	$CaCl_2$	$MgCl_2$	$AgNO_3$
1.	*Aeromonas* sp.	100.00	75.00	52.77	89.81	63.88	70.37	61.11	37.96
2.	*Vibrio* sp.	100.00	50.00	41.66	38.88	75.00	25.00	94.44	36.11

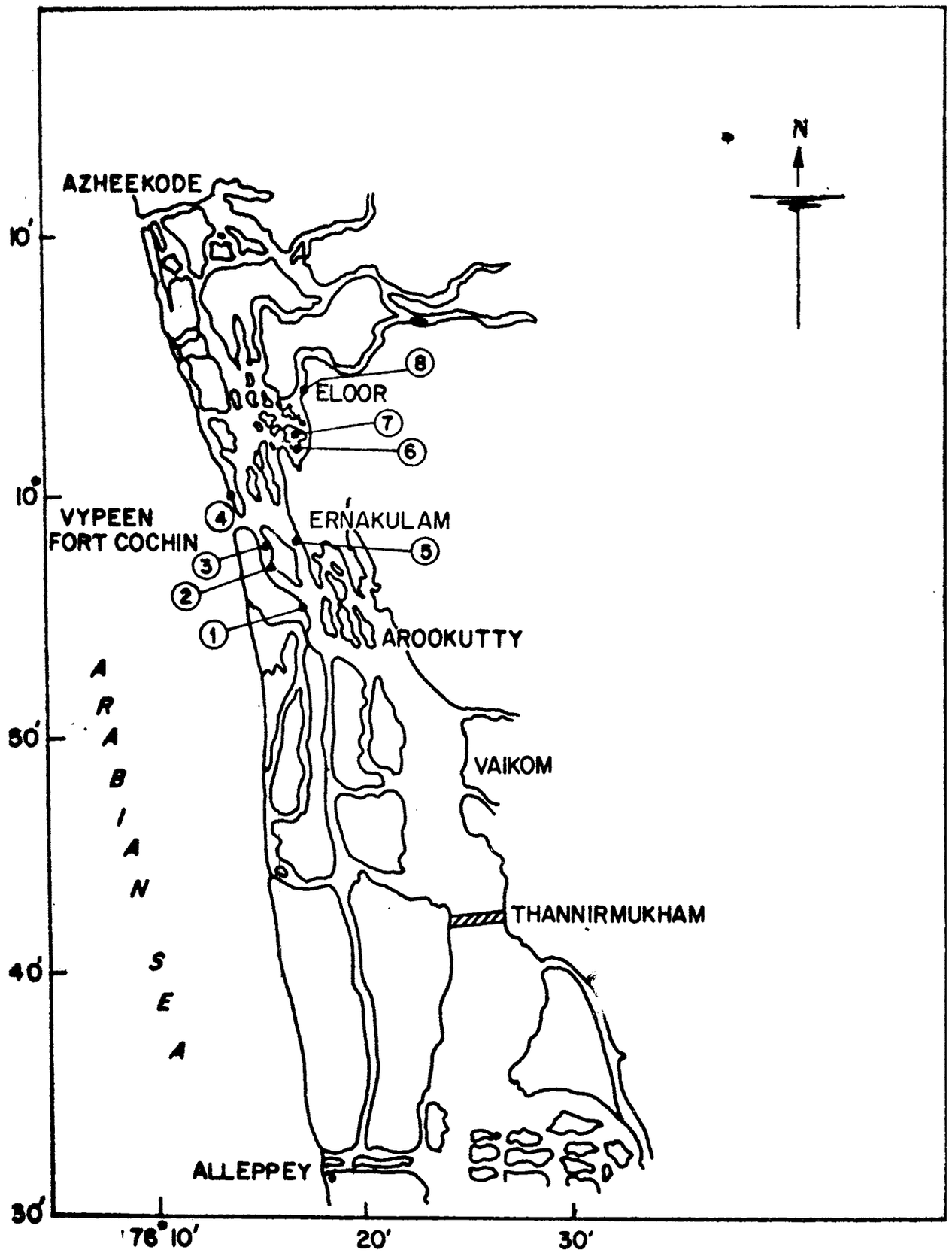
N
AZHEEKODE
10'
ELOOR
8
7
6
10°
VYPEEN
FORT COCHIN
4
ERNAKULAM
5
3
2
1
AROOKUTTY
50'
ARABIAN SEA
VAIKOM
THANNIRMUKHAM
40'
ALLEPPEY
30'
76°10'
20'
30'

FIG. 2

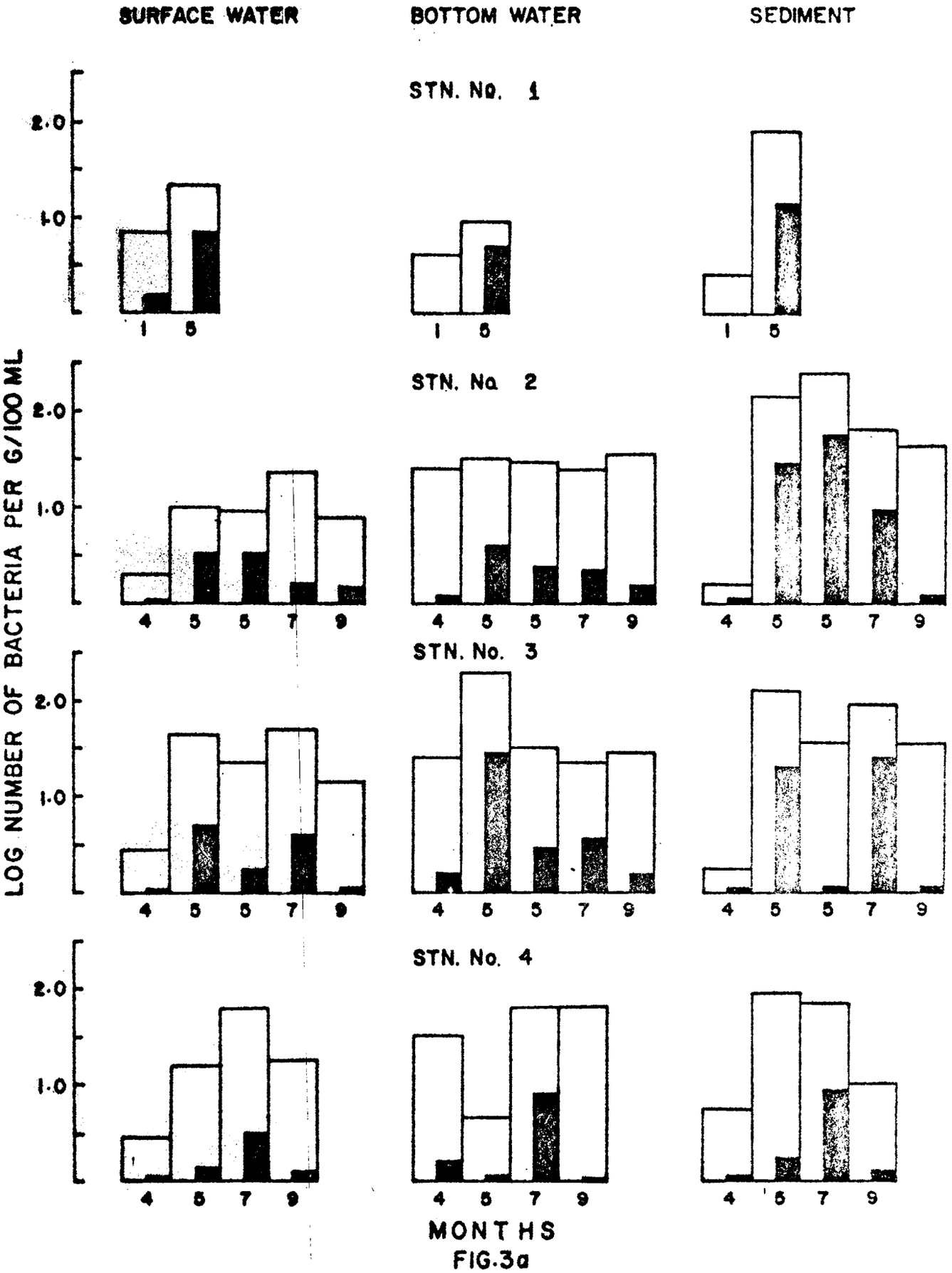
SURFACE WATER
BOTTOM WATER
SEDIMENT
STN. No. 1
STN. No. 2
STN. No. 3
STN. No. 4
LOG NUMBER OF BACTERIA PER G/100 ML
2.0
1.0
1 5
4 5 5 7 9
4 5 7 9
MONTHS

FIG.3a

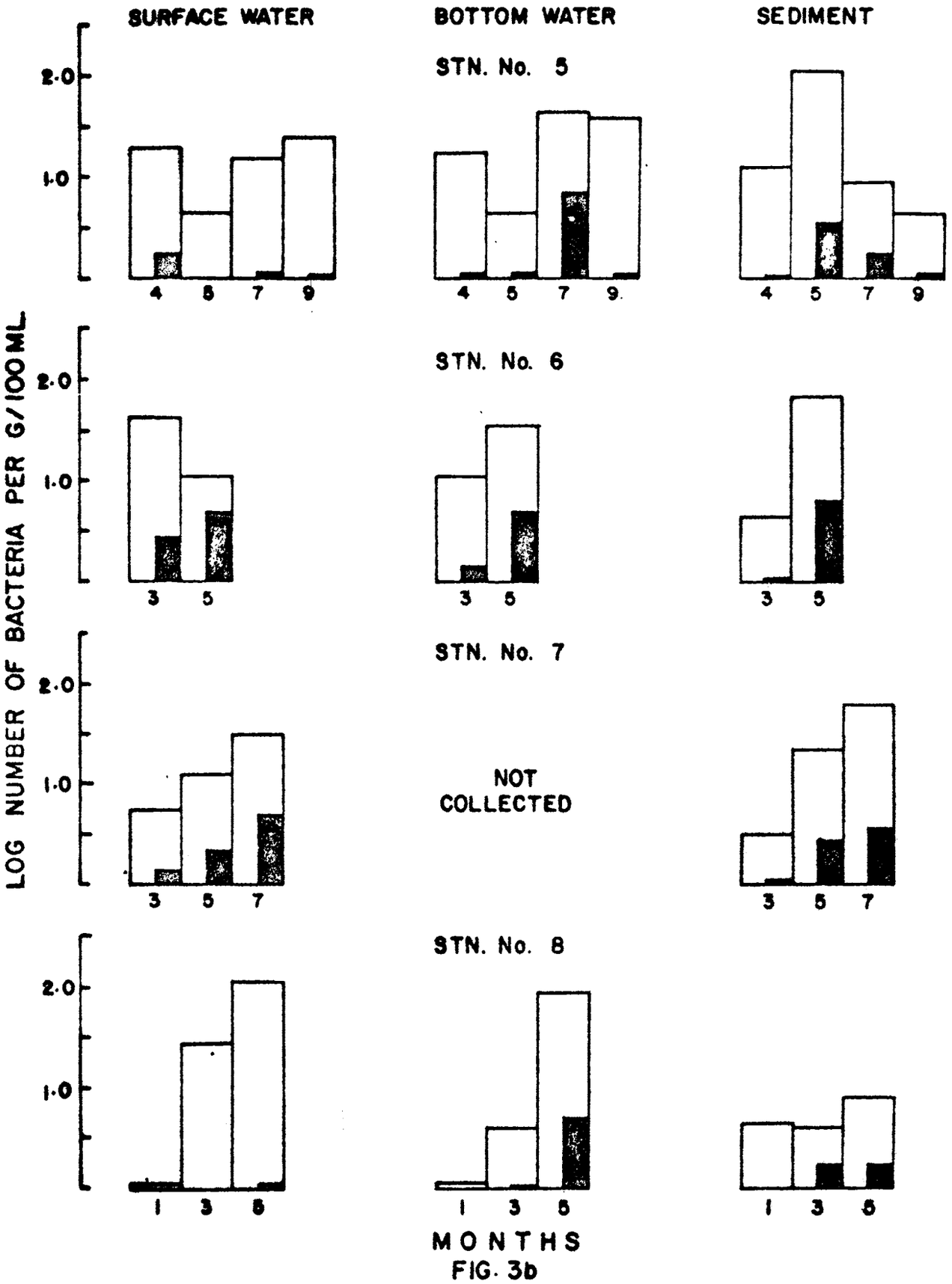
SURFACE WATER
BOTTOM WATER
SEDIMENT
STN. No. 5
STN. No. 6
STN. No. 7
STN. No. 8
NOT COLLECTED
LOG NUMBER OF BACTERIA PER G/100 ML
2·0
1·0
4 5 7 9
3 5
3 5 7
1 3 5
MONTHS

FIG. 3b

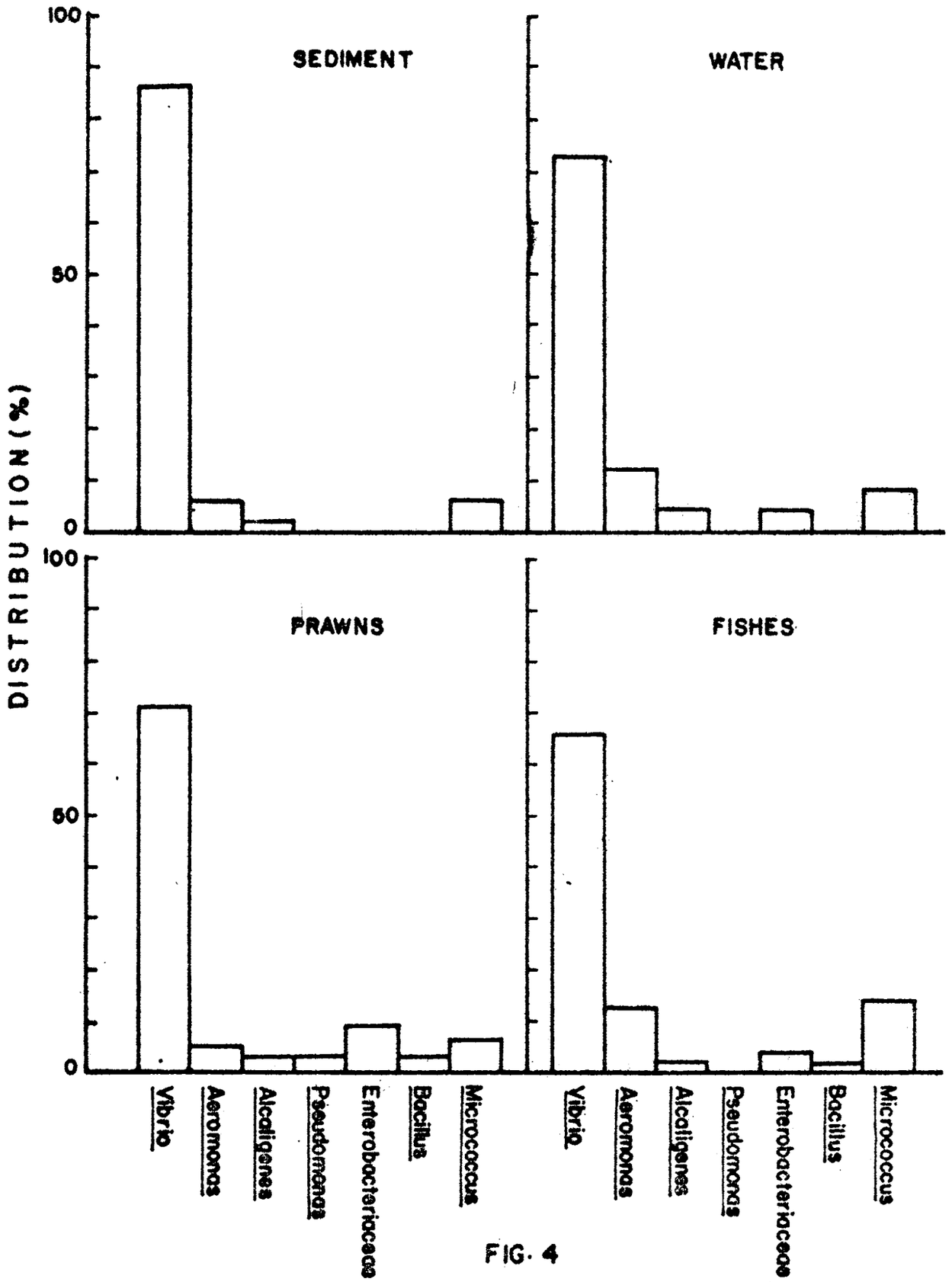
SEDIMENT
WATER
PRAWNS
FISHES
DISTRIBUTION (%)
100
50
0
Vibrio
Aeromonas
Alcaligenes
Pseudomonas
Enterobacteriaceae
Bacillus
Micrococcus

FIG. 4

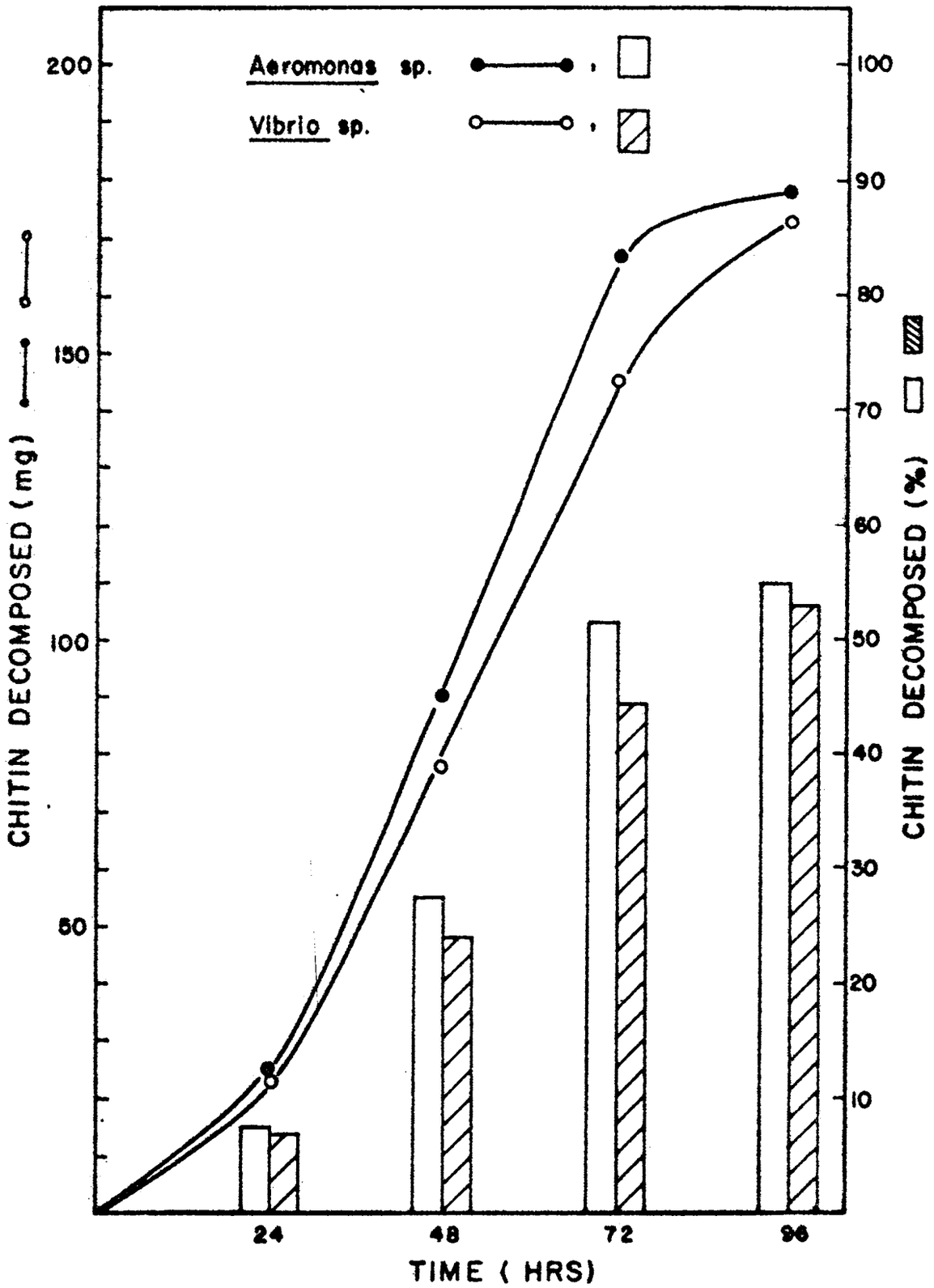
Aeromonas sp.
Vibrio sp.
CHITIN DECOMPOSED (mg)
CHITIN DECOMPOSED (%)
200
150
100
50
100
90
80
70
60
50
40
30
20
10
24
48
72
96
TIME (HRS)

FIG. 5

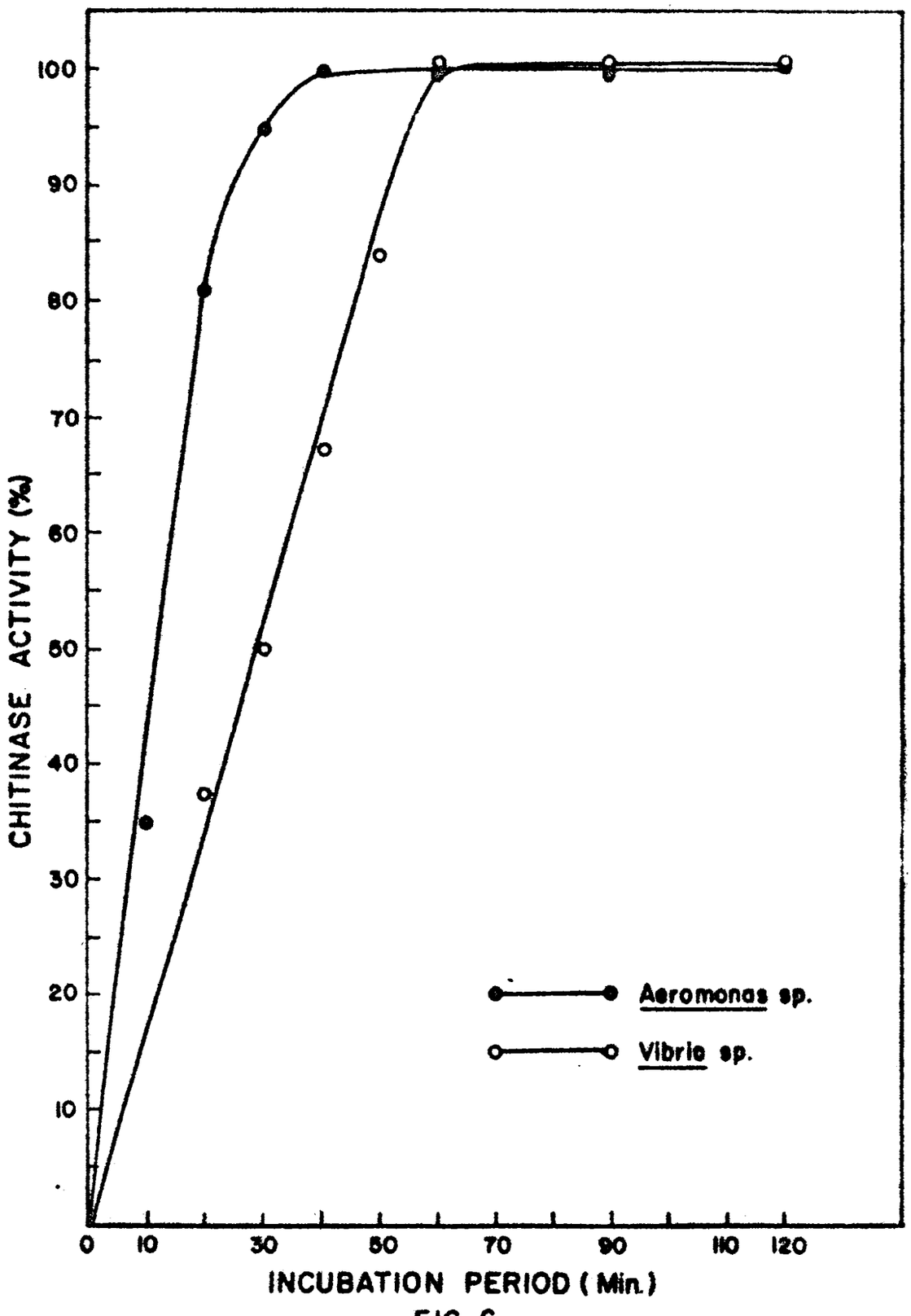
CHITINASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
10
30
50
70
90
110
120
INCUBATION PERIOD (Min.)
Aeromonas sp.
Vibrio sp.

FIG. 6

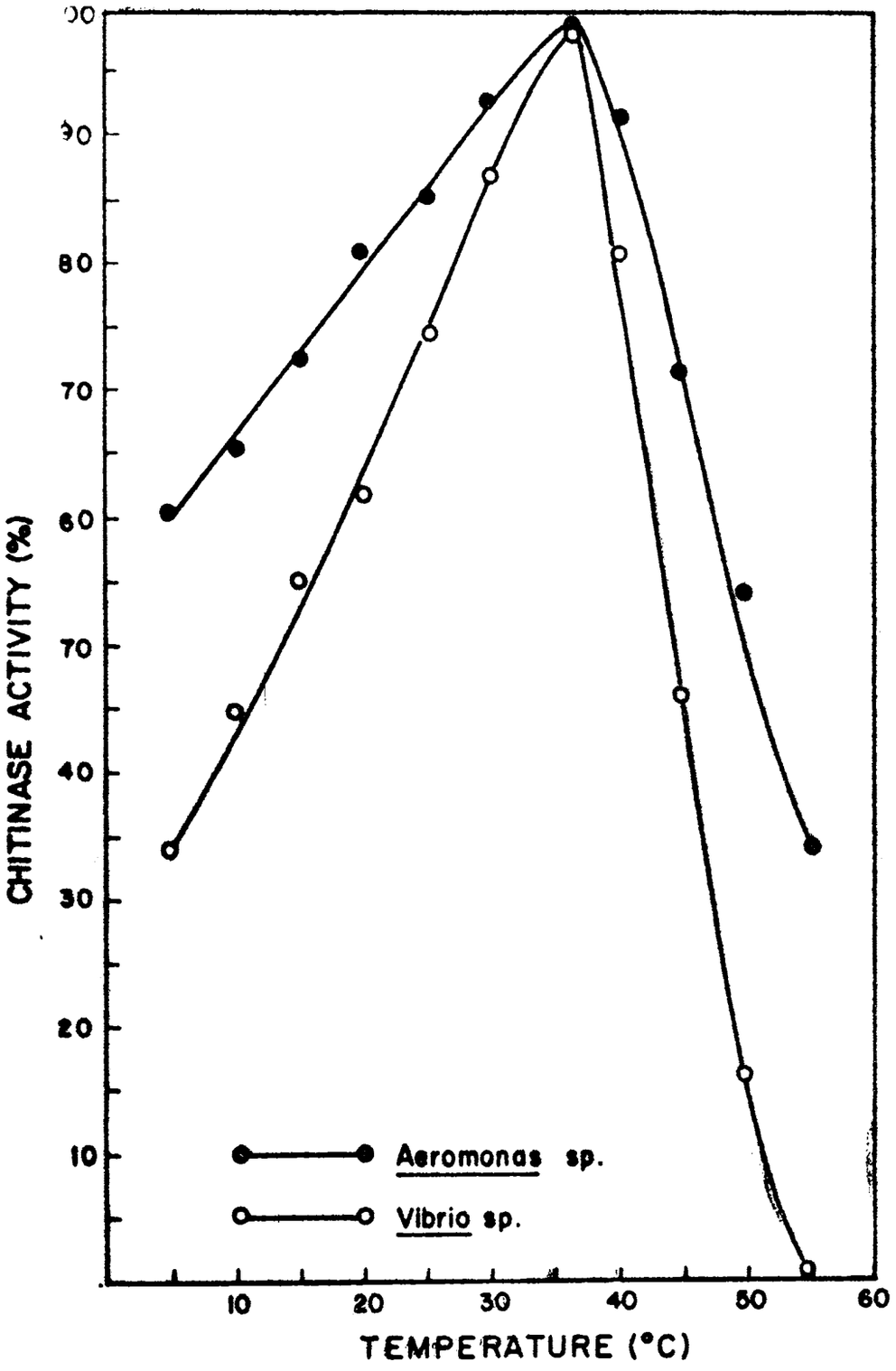
CHITINASE ACTIVITY (%)
90
80
70
60
70
40
30
20
10
10
20
30
40
50
60
TEMPERATURE (°C)
Aeromonas sp.
Vibrio sp.

FIG. 7

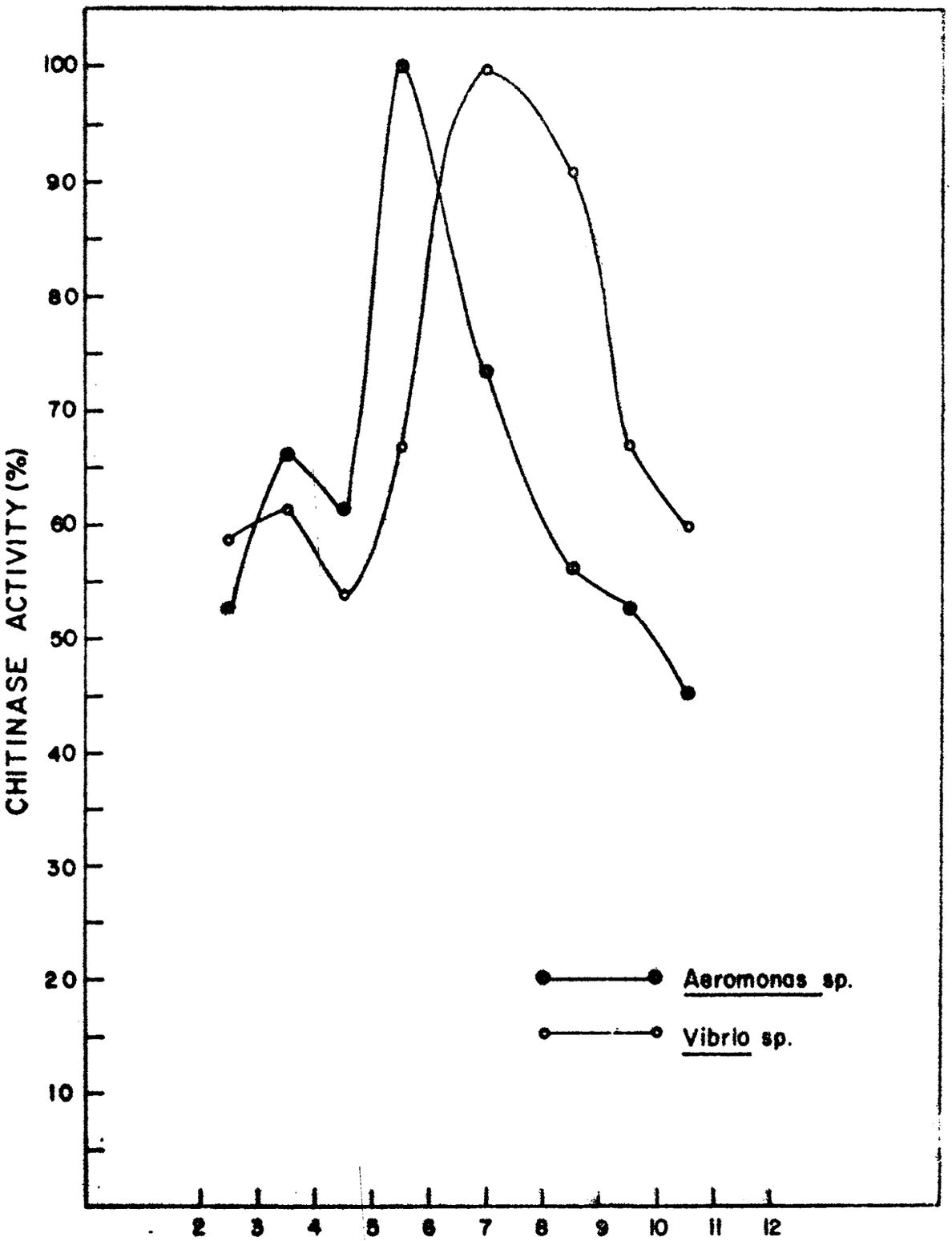
CHITINASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
2
3
4
5
6
7
8
9
10
11
12
pH
Aeromonas sp.
Vibrio sp.

FIG. 8

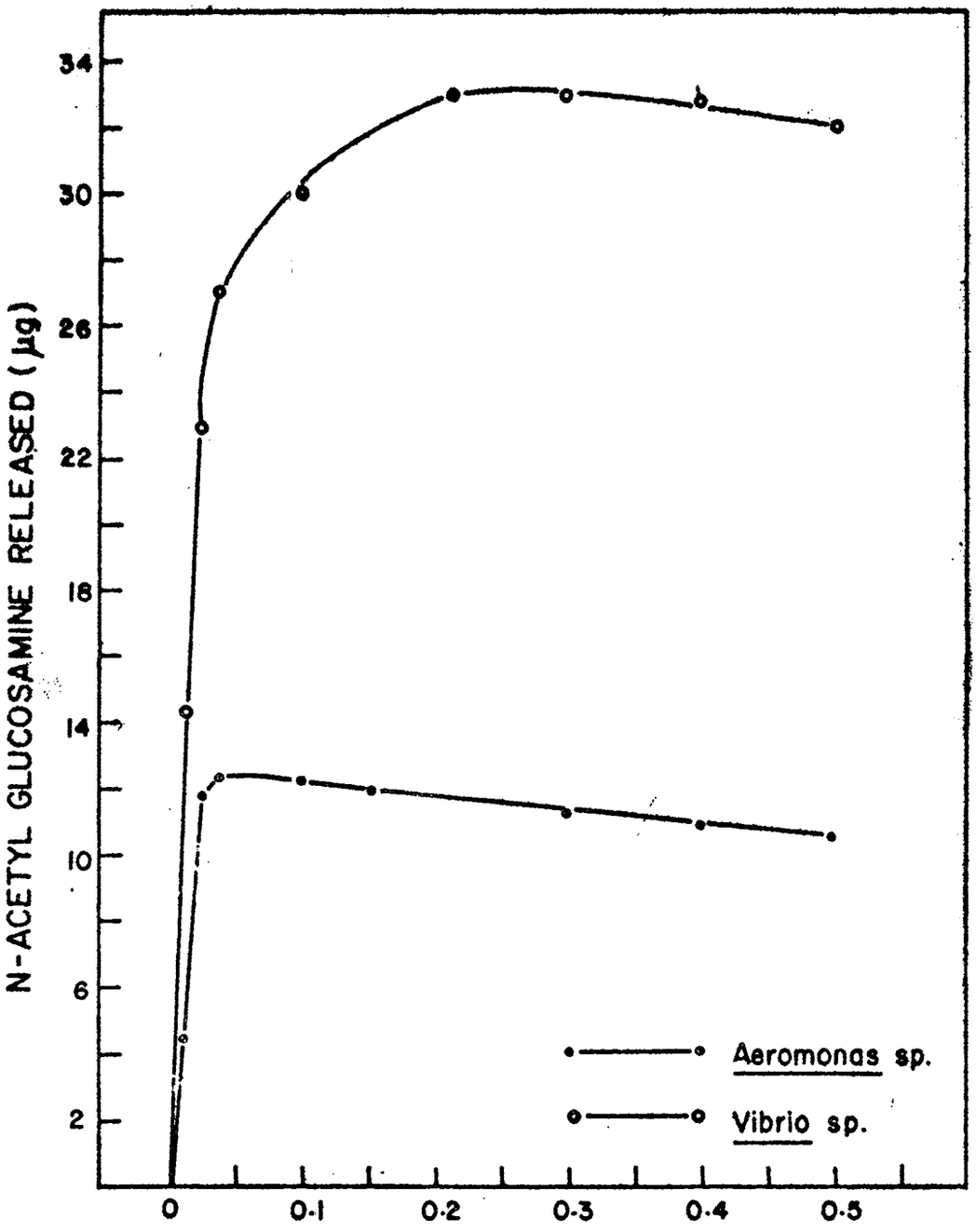
N-ACETYL GLUCOSAMINE RELEASED (µg)
34
30
26
22
18
14
10
6
2
0
0·1
0·2
0·3
0·4
0·5
SUBSTRATE CONCENTRATION (mg/ml)
Aeromonas sp.
Vibrio sp.

FIG. 9

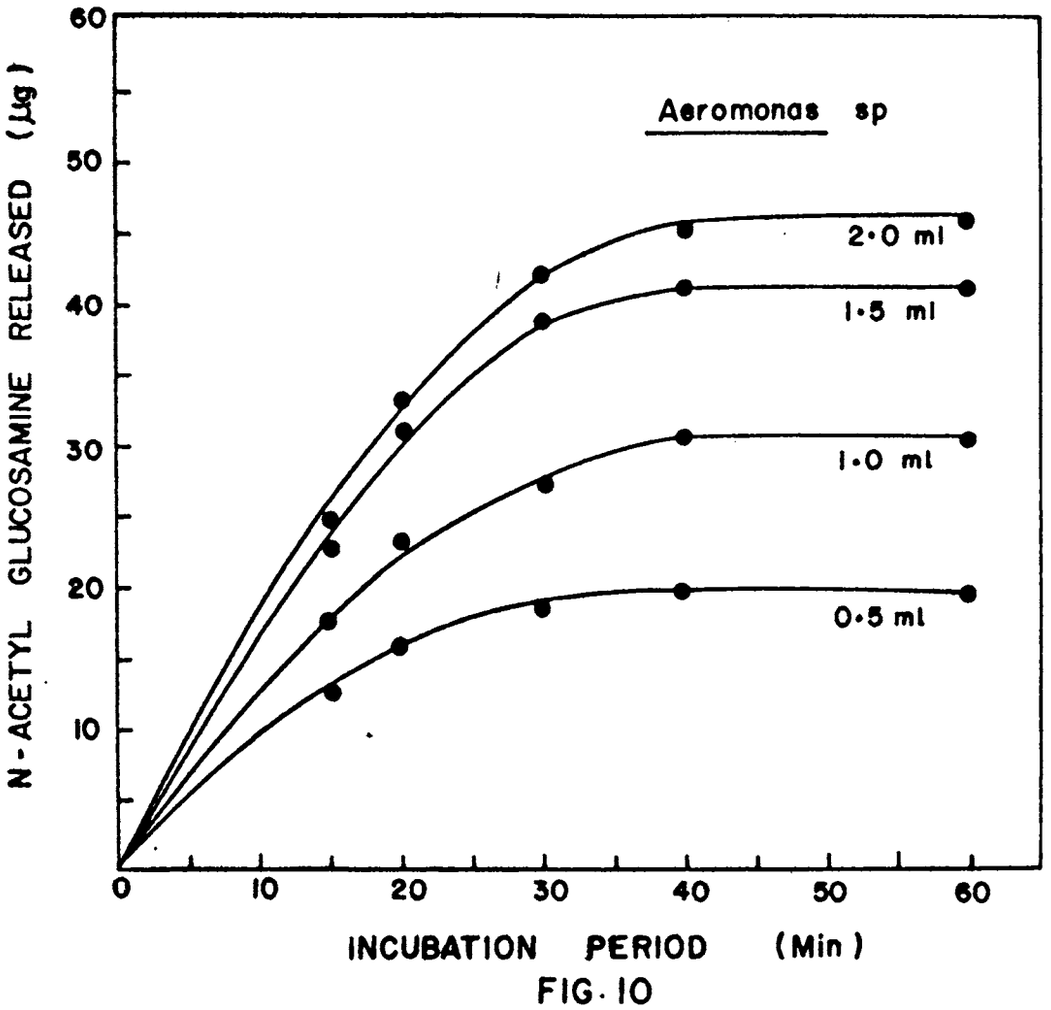
Aeromonas sp
2·0 ml
1·5 ml
1·0 ml
0·5 ml
N-ACETYL GLUCOSAMINE RELEASED (µg)
INCUBATION PERIOD (Min)
0
10
20
30
40
50
60

FIG. 10

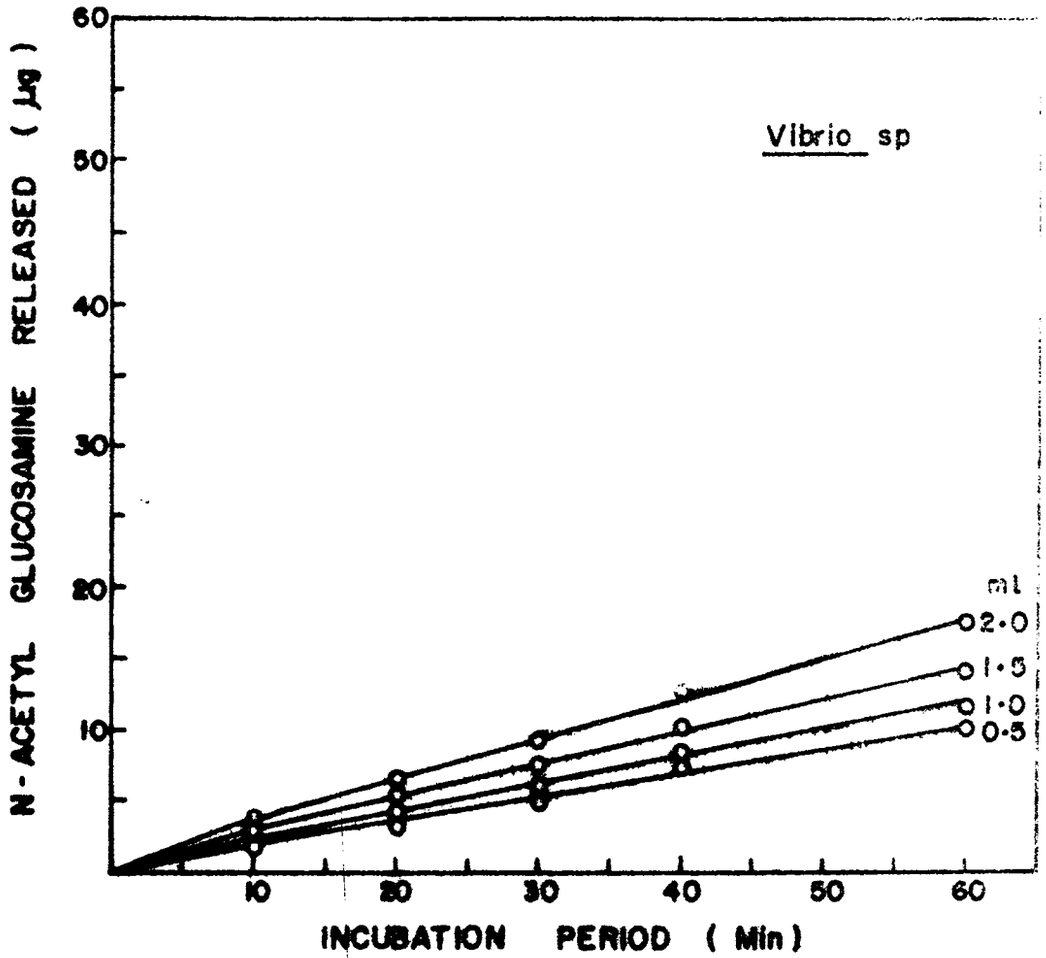
N-ACETYL GLUCOSAMINE RELEASED (µg)
60
50
40
30
20
10
Vibrio sp
ml
2·0
1·5
1·0
0·5
10
20
30
40
50
60
INCUBATION PERIOD (Min)

FIG. II

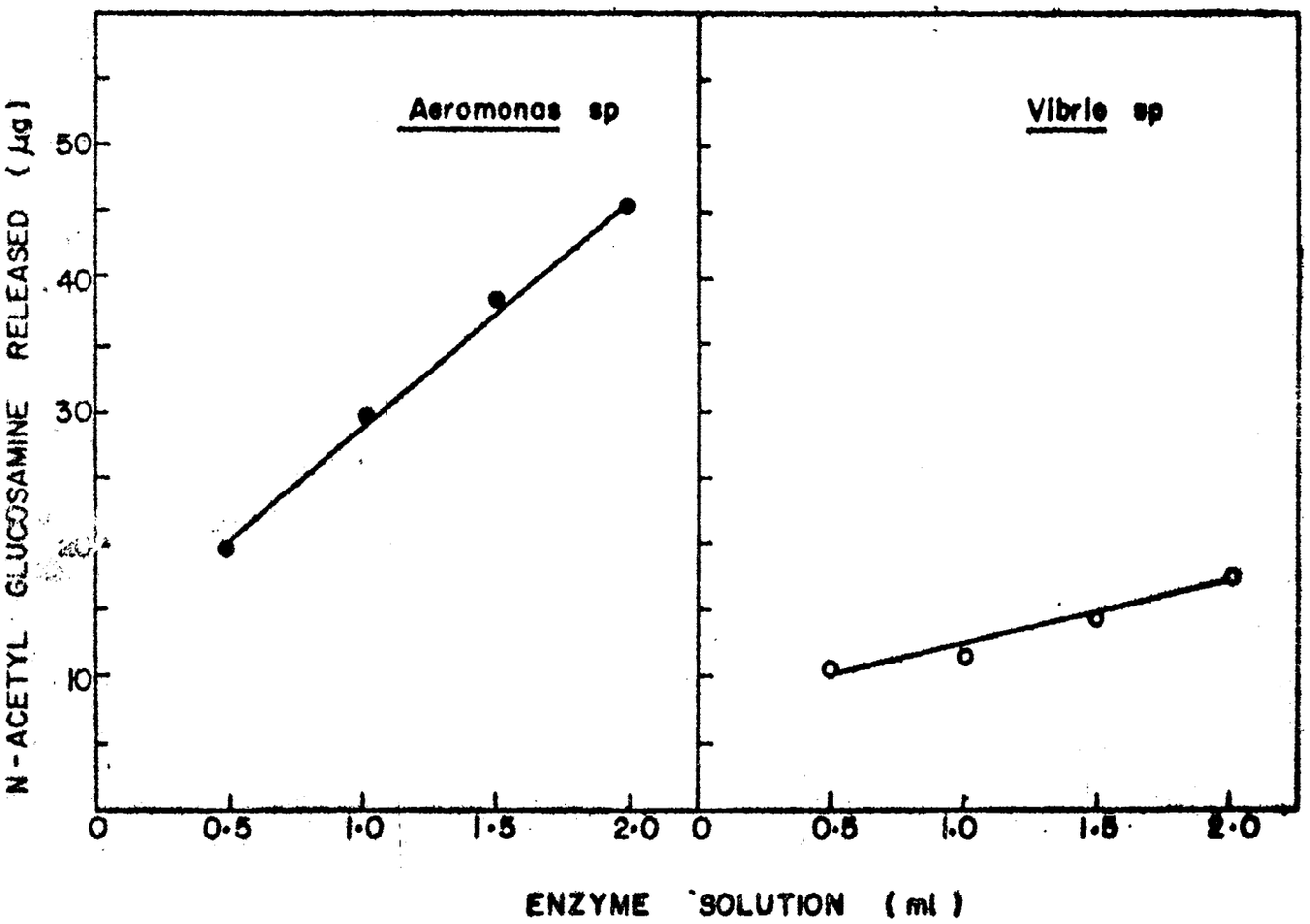
Aeromonas sp
Vibrio sp
N-ACETYL GLUCOSAMINE RELEASED (µg)
50
40
30
20
10
0
0·5
1·0
1·5
2·0
ENZYME SOLUTION (ml)

FIG. 12

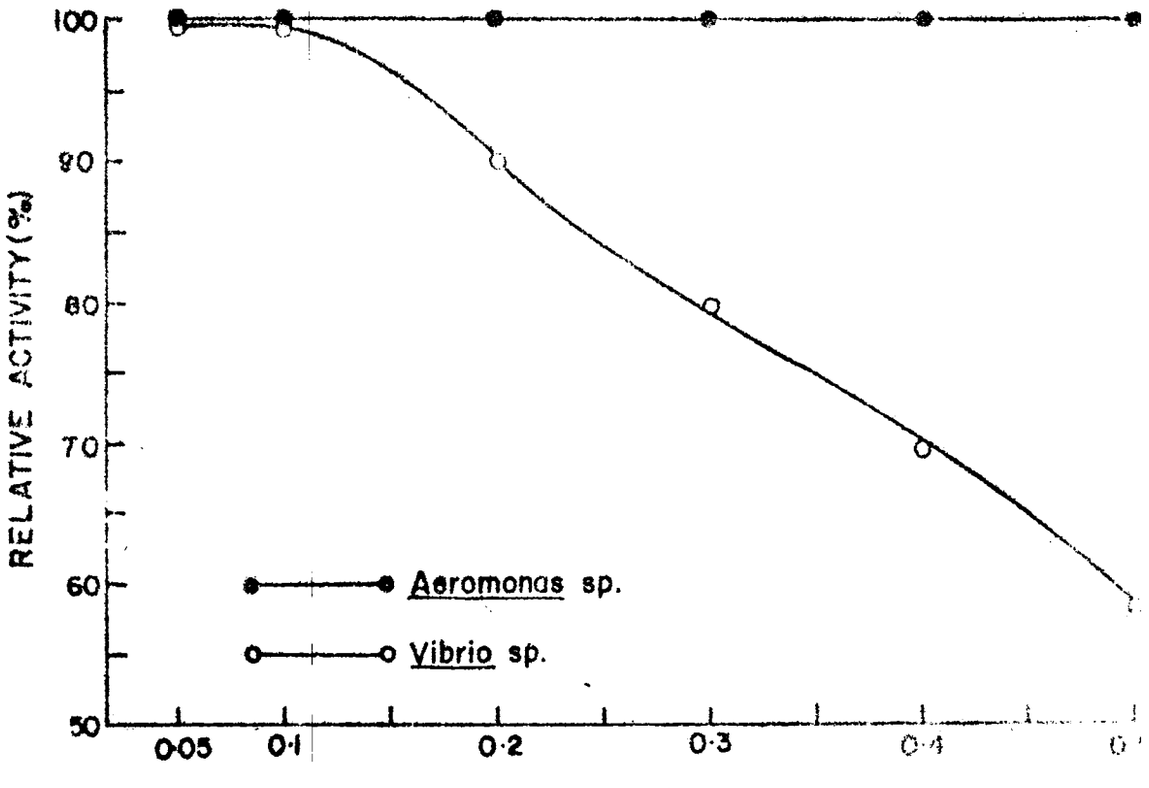
100
90
80
70
60
50
RELATIVE ACTIVITY (%)
Aeromonas sp.
Vibrio sp.
0·05
0·1
0·2
0·3
0·4
BUFFER CONCENTRATION (M)

FIG. 13

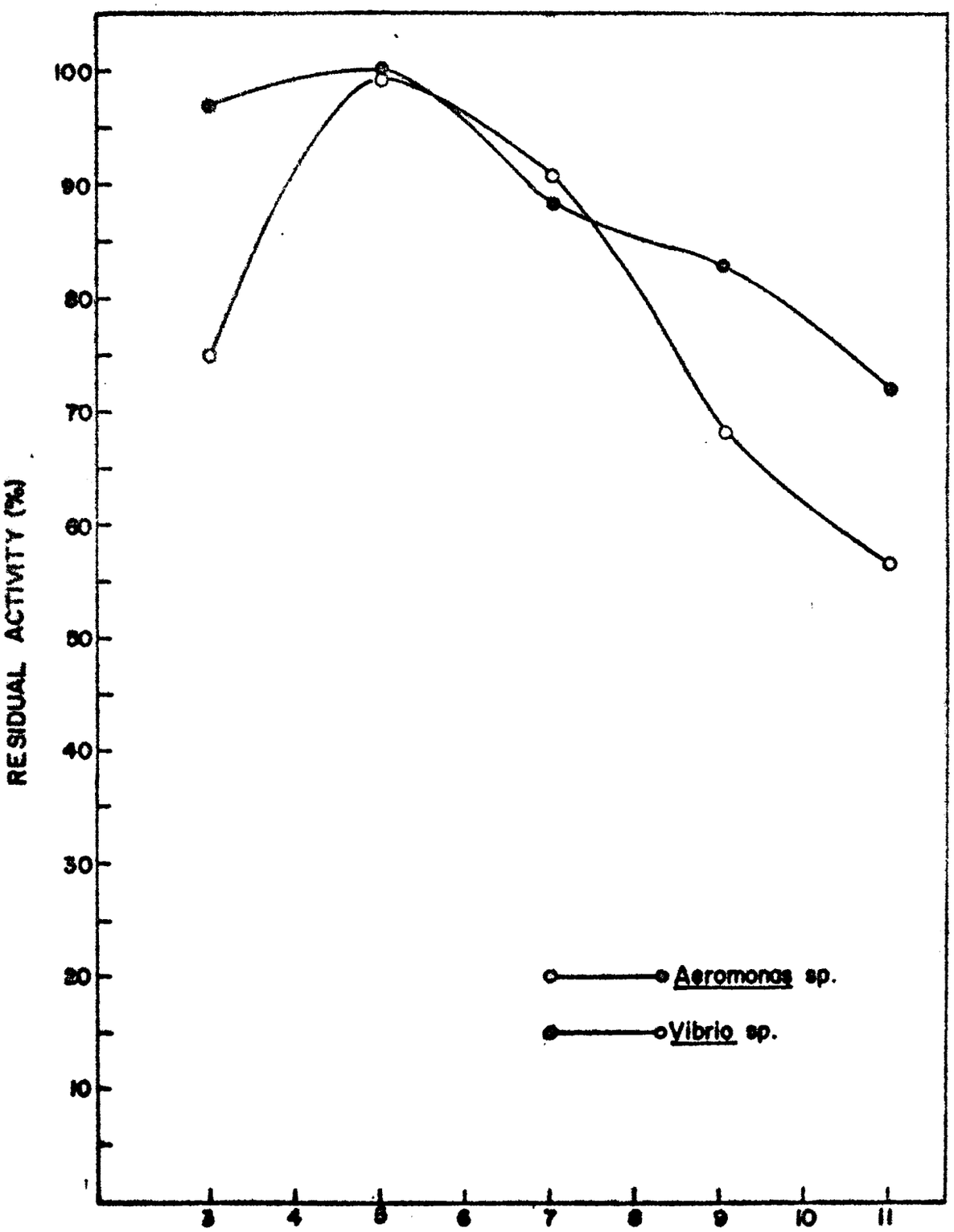
RESIDUAL ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
3
4
5
6
7
8
9
10
11
pH
Aeromonas sp.
Vibrio sp.

FIG. 14

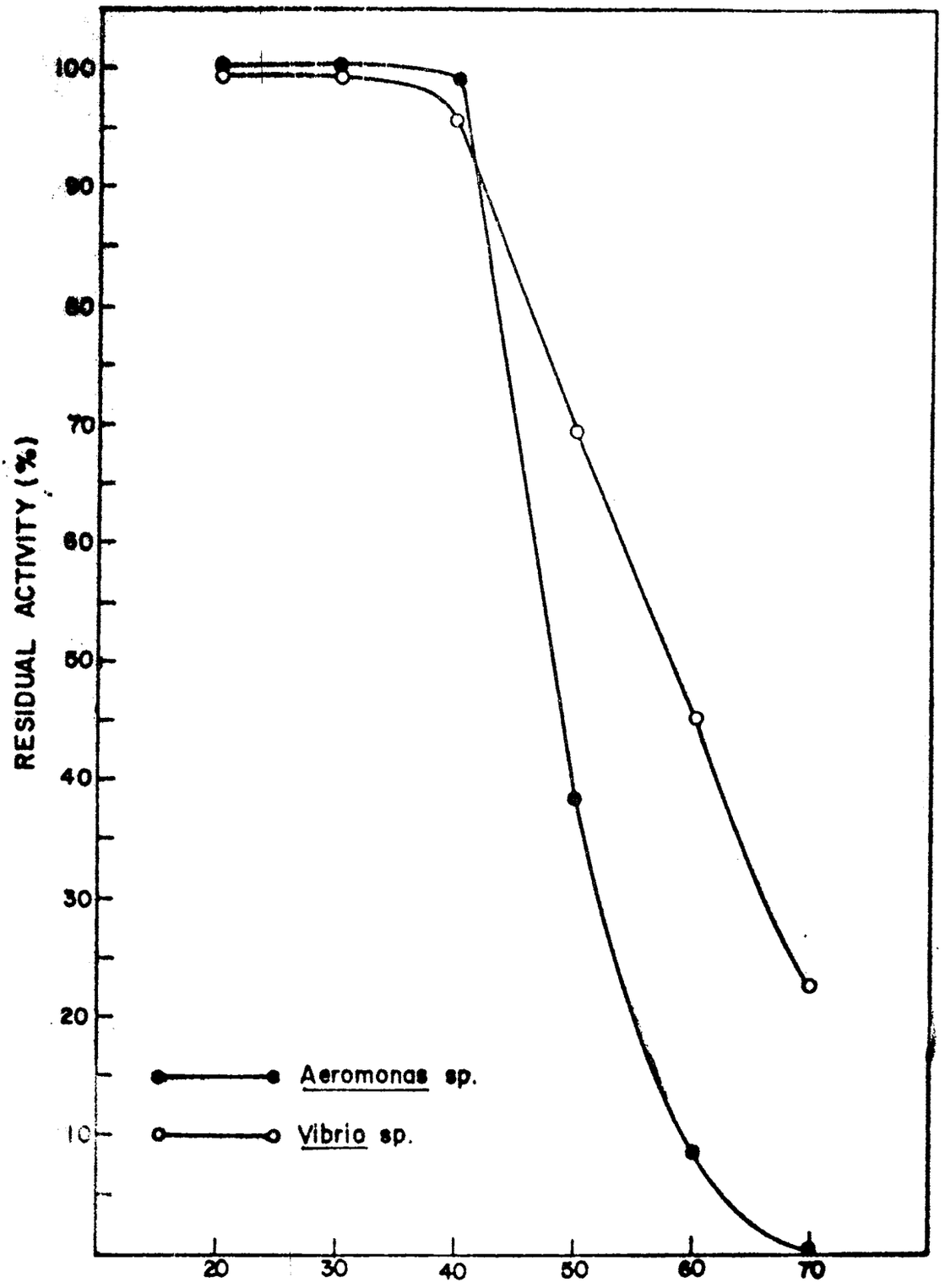
RESIDUAL ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
Aeromonas sp.
Vibrio sp.
20
30
40
50
60
70
TEMPERATURE (°C)

FIG. 15

5. DISCUSSION.

The results of the present investigation indicate that chitinoclastic bacteria are widely distributed in the inshore marine environments of Cochin and adjacent waters. In the present study a total of 28 sediment samples, 53 water samples (28 surface water samples and 25 bottom water samples), 8 species of prawns, 24 species of fish were examined over a period of one year for the occurrence and distribution of chitinoclastic bacteria. Except few samples nearly all the fauna samples and majority of water and sediments were found to contain chitinoclastic bacteria. These findings agree in general with other reports which showed the occurrence of chitinoclastic bacteria in almost all marine samples. The chitinoclastic bacterial population in sediment and water fluctuated widely from undetectable levels to as high as 74% of the total heterotrophic bacterial population. It is generally believed by most of the investigators that there exists a close

correlation between the chitinoclastic bacterial population and the presence of chitin (Lear, 1961; Seki and Taga, 1965c). However, such relationship was not consistently reported. For example, Chan (1970) failed to observe such positive correlation between chitinoclast abundance and chitin when he examined microscopically and macroscopically numerous subtidal and intertidal sediments from Puget Sound.

5.1. Occurrence and distribution of chitinoclastic bacteria in sediments, water and fauna:

Out of 28 sediment samples analysed atleast 2 samples (one collected from Station 1 on 29.1.80 and the other from Station 8 collected on 28.1.80) showed no detectable levels of chitinoclastic bacterial population. It is interesting to note that samples collected subsequently from the same station contained chitinoclasts which accounted for 60% of the total heterotrophic population. In sediments the total bacterial population varied from 1.60×10^3/g to 239.63×10^3/g. The minimum and maximum values recorded in each station were taken and the averages were worked out. Accordingly the

average minimum population was 3.71×10^3/g and average maximum population was 100.34×10^3/g. The chitinoclastic population in these samples varied from undetectable levels to a maximum of 74.44% of the total bacterial population. However the average minimum was 4.73% and average maximum was 51.24%. The overall range of chitinoclastic counts in sediments observed in this study is in general, of the same magnitude as reported by other investigators in other parts of the world employing different techniques. Hock (1940) reported a chitinoclastic population of as low as 125 cells/g in the sediment from the Atlantic coast, whereas Seki (1965b) found as many as 10×10^6/g in the mud of the coastal areas of Japan. It is evident from the results of present investigation and from others findings, that chitinoclastic bacteria greatly vary in numbers from area to area and most often in different samples collected from the same area. The work of Chan (1970) also confirms this observation when he worked with sediments from Puget Sound, California, Alaska and Florida. He reported that the chitinoclastic population in those sediments varied from less than 100 to over 1×10^6/ml or g.

The results of the present study indicate clearly the uneven distribution of chitinoclasts in the sediments. ZoBell and Rittenberg (1938) also reported such uneven distribution of chitinoclasts in sediments of the coast of southern California. These authors suggested that the uneven distribution of chitinoclasts were due to the heterogenous distribution of suitable substrate - possibly chitin - which resulted in a microzonation of these bacteria. It may also be pointed out here that a similar trend was also noticed for heterotrophic bacterial population also. Wiebe (1965) in his detailed quantitative study of heterotrophic bacteria of the Washington-Oregon area indicated inshore heterotrophic populations were much more variable than offshore populations which were markedly stable. This high variation in inshore populations were partly attributed to the inherant instability of these inshore areas. In the present study vertical distribution of chitinoclasts in the sediments was not investigated. However, earlier studies indicate a similar uneven distribution of chitinoclasts in core samples of the sediment (Chan, 1970). It is also an established fact that total heterotrophs and chitinoclasts were always highest in the top layers of sediment core and the counts decreased with

increasing depth. Chan (1970) could record the presence of chitinoclasts even upto a depth of 45 cm. ZoBell and Rittenberg (1938) also reported a sharp decrease in chitinoclasts numbers with core depth and they could confirm the presence of chitinoclasts even at 60 cm deep. In the present study all the sediment samples were mostly clayey in nature indicating rich organic matter content.

The study of chitinoclastic bacterial distribution in waters was limited to surface waters and bottom waters. As the depth of water column varied from station to station (Table 3) no constant depth was maintained for collection of bottom waters. The depth was as shallow as 2.5 m in Station 1 and as high as 9.0 m in Station 5. As described earlier these waters are all heavily polluted with sewage, city waste, oil,etc. In surface waters the total heterotrophic bacterial population varied from 0.6 x 10^3/100 ml to 125.0 x 10^3/100 ml. In bottom water the variation was still wide i.e. from 1.0 x 10^3/ 100 ml to 201.6 x 10^3/100 ml. When the average values were compared for surface waters the variation was from 4.85 to 48.93 x 10^3/100 ml and in bottom water it was from 10.86 to 69.99 x 10^3/100 ml. These observations

clearly indicate that bottom water always contained higher bacterial population when compared to surface waters. This may be attributed to the constant contact of bottom water with the sediment. As in sediment a wide fluctuation in chitinoclastic population was noticed both in surface waters and bottom waters collected during different parts of the year. In surface waters the percentage of chitinoclasts varied from nil to 66.66% of the total bacterial population and in bottom waters it varied from nil to 70.05% . Earlier reports indicate that inshore waters, such as Cochin backwater, always harbour more chitinoclasts than the nearby neritic and pelagic waters. The surface and bottom waters of Puget Sound (Chan, 1970) contained chitinoclastic populations 2 to 3 order of magnitude greater than the neritic and pelagic waters of Aburatsubo Inlet and Sagamy Bay in Japan as reported by Seki and Taga (1963a). In some of the heavily polluted area of Burley Lagoon chitinoclast number reached as high as 2×10^5/ml of water (Chan, 1970). These findings suggest that the number of chitinoclasts and total heterotrophic bacteria may be closely related to organic

nutrient content of the water. In the present study water samples from different depths were not collected and analysed. Hence it is not possible to indicate any definite vertical distribution of chitinoclasts in the water column.

The results of the present study show that generally the sediments harboured more bacterial population than the overlying water. There are a number of reports supporting this observation. The high organic content in sediments of shallow water system when compared to overlying waters is a virtually undisputed fact. The bacterial population either in water or in sediment is known to be controlled by a number of physico-chemical and biological factors. One of the important factors which is known to determine the bacterial load in water is the plankton population. With an increase in the plankton concentration a concomitant increase in the bacterial population is also seen because of the released nutrients by the plankton. In the present study no attempt was made to estimate the standing crop of plankton in different water samples. Hence it is difficult to correlate the variations observed in the bacterial population with plankton concentration.

Velankar (1955) observed spikes in mud bacterial population subsequent to plankton bloom in the inshore environments of Mandapam (S. India) and contented that variation in plankton abundance would be expected to be influenced by level of available nutrients and consequently the bacterial population in mud. Since the source of food for mud bacteria is the plant and animal residue that sink down from overlying waters. In the same study Velankar (1955) attributed the prevalence of lower bacterial population in sediments during 1952 as compared to 1951 to a lesser standing crop of plankton 1952.

Another important factor which is known to influence the bacterial population in sediments is the adsorption phenomena. It has been reported that adsorption of bacteria onto different soil fractions varies considerably and in this context Waksman and Vartiovaara (1938) observed strong adsorption of pure cultures of marine bacteria inoculated into marine muds whereas little or no adsorption occurred on sand. Sandy types of substrate have been reported to harbour very low microbial populations when compared to clayey sediments (Ayyakkannu and Chandramohan, 1971). In the present

study sediments collected from Stations 1 and 8 were of silty to sandy types and sediments from other stations were mostly clayey in nature. Hence in general higher bacterial populations were recorded in sediments collected from Stations 2 to 7.

It is clear from the present investigation that a correlation exists between the chitinoclastic bacterial population and environmental temperature. Higher percentages of chitinoclasts were recorded in most of the water and sediment samples collected during the month of May when the temperature was also found to be comparatively more than other months. It is also true that the primary and secondary productions are comparatively higher in summer months in tropical waters. A number of reports showed that the chitinoclastic bacteria are increasingly associated with live and dead plankton. Seki and Taga (1963 a) reported that chitinoclastic bacteria predominated in planktonic crustaceans. They also observed considerable numbers of bacteria attached to living copepods and a correlation was postulated between the percentage of chitin decomposers and planktonic crustaceans. These planktons when they sink to the sediment may encourage the proliferation of chitinoclastic bacteria.

Proportion of chitinoclastic bacteria in a tiny Artemia was only 4.9% but proportion on the dead plankton increased rapidly to 25% within 4 days (Seki and Taga, 1963 a). These observations suggest that organisms with chitinous material sink to the bottom and may contribute to a larger extent to the chitinoclastic bacterial population in sediments.

The results of the present study indicate that no definite relationship may occur between chitinoclastic population and salinity of the water.. For example, even when the salinity dropped below 1‰ level the percentage of chitinoclastic population were nearly 50% of total population. Except during the month of August when the salinity of the water dropped below 1‰ level because of the monsoon, the sediments in all stations were exposed to almost constant salinity throughout the period of investigation. The fact that wider fluctuations of chitinoclastic bacterial population observed during this period of study strongly suggest that salinity may not be a major deciding factor on the chitinoclastic bacterial population. Probably salinity may contribute to the adsorption of chitinoclasts to chitin. Extensive experimental studies conducted by Kaneko and Colwell (1975 a) on factors affecting the adsorption of Vibrio

parahaemolyticus to chitin and copepods clearly indicate that adsorption effect was found to be markedly influenced by salinity with almost 100% adsorption at 4‰ and 70-80% at 10-16‰. Further increase in salinity lowered the adsorption efficiency indicating the adverse effect of salinity beyond certain level. Moreover, the chitinoclastic population encountered in the present study might have been mostly euryhaline in nature an observation which supports the earlier findings of Chan (1970). Other factors such as dissolved oxygen and pH do not seem to exert any effect on the chitinoclastic bacterial population as there was no definite relationship between them.

Chan (1970) has reported that a very definite positive correlation existed between the total heterotrophic bacteria and chitinoclastic bacteria and he attributed the increase of heterotrophic population in summer to the increased nutrient availability. Contrary to his observation, in the present investigation no such positive correlation was observed between the heterotrophic bacterial population and the number of chitinoclasts. Perhaps the chitinoclastic bacteria may not have an absolute requirement for chitin but behave as non-exacting heterotrophs. Chan (1970) has also showed that

chitinoclastic bacteria are able to increase in numbers without the utilization of chitin when other substrates are available. Further he also suggested that the presence and utilization of ancillary substrates like proteins, amino acids etc. appears to be very important to chitin decomposition since pure chitin itself is a relatively poor substrate for growth of most chitinoclasts. These observations indicate that not only the chitin concentration but also the concentration of other nutrients are important factors indetermining the chitinoclastic population in a given environment.

A high incidence of chitinoclastic bacteria in the gastrointestinal tract of marine and estuarine prawns and fishes was observed during the study. Out of the 24 species of fishes examined, 20 species harboured chitinoclastic bacteria in their digestive tract and their percentage occurrence varied from 3.19 to 97.0 % . In the case of prawns, both the shell surface and digestive tracts were examined and chitinoclastic bacteria were found to occur in both the regions of all the species examined. On the shell surface the chitinoclastic population varied from 4.76 to 75.07% of the total and in the digestive tract the population varied from 3.02 to 86.2 % . Prawns are predominantly benthic dwellers

and hence are in constant contact with sediments which harbour appreciable levels of chitinoclastic bacteria throughout the year. Such continuous contact with sediments is likely to result in transfer of chitinoclastic bacteria from the sediment to the exoskeleton of prawns. Secondly, the food of prawns chiefly consists of the remains of tiny animals and large portion of detritus matter. The unrecognizable materials which constitute the main component of prawn diet is believed to be derived from the surface layers of the sediment (Dall, 1968). Similarly fishes which feed on detritus may also harbour a good amount of chitinoclastic bacterial populations. The gut contents of representative fishes and prawns were analysed in order to ascertain whether there was any relation between the presence of chitinous material and chitinoclastic bacterial population. As it can be seen from the results (Tables 5 and 7) the gut contents of prawns and fishes showed the presence of chitinous material. These observations positively indicate that chitinoclastic bacteria are increasingly associated with the fauna. In general, gut microflora contained a large number of chitinoclastic bacteria. Similar observations have already been reported by a number of investigators (Lear, 1961; Seki and Taga,

1963 d; Okutani, 1966; Chan, 1970; Hood and Meyers, 1973 b; Goodrich and Morita, 1977 b; Okutani, 1978).

The results of the present study and the findings of other investigators have led to some general conclusions regarding the occurrence and distribution of chitinoclastic bacteria:

1. Chitinoclasts appeared to be a normal component of the microflora in estuarine and nearshore marine environment. They occur in water, sediment, on the surface of animals and in gastro-intestinal tracts of the fauna.

2. The concentrations of chitinoclastic bacteria vary greatly within the marine ecosystem and their number is often controlled by atleast more than one factor.

3. All chitinoclastic bacteria thus far studied seem to be non-exacting heterotrophs which can utilize a number of other organic substrates.

4. The increase in chitinoclastic abundance in a given ecosystem need not correspond to an increase in chitin concentration. In other words the increase may result from the utilization of organic substrates other than chitin.

Since chitin degradation is mainly enzymatic under natural conditions, free chitinase activity in sediments and in digestive tracts of prawns and fishes was estimated in the present study. All the sediment samples examined showed no free chitinase activity in them. This observation substantiates the earlier report by Goodrich and Morita (1977 a) that all sediment and water samples from Yaquina Bay exhibited no detectable levels of chitinase activity. The methods followed in the present study for the estimation of free chitinase in sediment are essentially the same as employed by Goodrich and Morita (1977 a). They tested the method by incorporating a bacterial chitinase from a marine Vibrio into the reaction mixture and proved that the sediment had no detrimental effect on the assay sensitivity. With the same method they could register the chitinase activity in the offshore sediment (400 m) which suggests that the method is adequate for detecting chitinase from this environment. These observations indicate that free chitinase activity exists in offshore sediments and not in inshore sediments. The possible explanations which can be ascribed to such a situation can be either absence of substrate (chitin) in sediments or chitin degradation process occurs to a greater extent in digestive tracts of fauna of that area.

A considerable number of chitinoclastic bacteria (10^6 to 10^7/g dry weight) are already attached to the exoskeleton of zooplankton and the chitin degradation proceeds even when planktonic crustaceans are dead. This may be one of the main reasons why almost all chitin is decomposed in water column and little accumulate in marine sediment (Seki and Taga, 1965 c). The results of Goodrich and Morita (1977 a) indicate that a major portion of chitin decomposition may occur in the digestive tracts of the sediment dwelling animals including both fish and invertebrates. It is possible that both the phenomena may operate individually or simultaneously in a given ecosystem, especially in inshore areas resulting in undetectable levels of chitinase activity in both water and sediments.

All species of prawns and 21 species of fishes examined had some levels of chitinase activity in their gastro-intestinal tract. These fishes and prawns also harbour varying levels of chitinoclastic population in their digestive tracts. However, there was no relationship between the chitinase activity and chitinoclastic bacterial population. For example, in Tachysurus maculatus highest chitinase activity (725 µg NAG/g/hr)

was recorded in the digestive tract when the chitinoclastic population was 10.9 x 10^6/g and in Mugil cephalus the chitinase activity was very low (7.0 µg NAG/g/hr) even when the chitinoclastic population was 62.59 x 10^6/g (Table 6). There are several similar observations in the present study. Interestingly in Liza macrolepis no chitinase activity was seen in the digestive tract inspite of the presence of considerable number of chitinoclastic bacteria (16.0 x 10^6/g). The possible explanations which can be given are:

1. The generic composition of chitinoclastic bacteria may possibly affect the chitinase production and activity inside the gut.

2. The animal can produce its own chitinase to digest the chitin material.

It was earlier thought that chitinase activity in the digestive tracts of animals was contributed by the chitinoclastic bacteria present (Goodrich and Morita, 1977 b). However, a number of subsequent report have shown that the chitinase can be produced by the animal itself and need not be necessarily contributed by the gut microflora (Hood and Meyers, 1977 a). More often

the chitinase pool in the gastrointestinal tract of animals may be contributed by both bacteria and the animals themselves (Sera, 1968; Hood and Meyers, 1977 a). This hypothesis is further supported by the results of the present investigation. In atleast two fishes Selar kalla and Rastrelliger kanagurta, very high chitinase activity (600 and 720 µg NAG/g/hr respectively) were observed when the chitinoclastic bacterial population was almost nil.

Observations on enteric tract microflora of starved fishes and those fed with sterile food have revealed a remarkable decline in counts of chitinoclasts or their total absence (Goodrich & Morita, 1977 b) indicating that the portal of entry of chitinoclastic bacteria is probably along with the food source. Additional evidence more specifically along this line has been recently furnished by O'Brien and Sizemore (1979) who noticed a pronounced increase in intestinal populations of chitinoclasts Beneckea harvei when fishes were fed with a chitinous diet. These observations suggest beyond doubt that a considerable amount of chitin is being degraded in the digestive tracts of estuarine and marine fauna. The

importance of this chitinase activity inside the gut may be visualized better if we look into the estimates given by Goodrich and Morita (1977 a). According to them if a population of 1 x 10^5 fish (Enophrys bison) in Yaquina Bay, a chitinase activity rate of 100 µg NAG produced/g/ dry weight/hour and an average dry weight stomach content of 50 g were used for calculation, this single species population would be responsible for the production of approximately 14 metric tons of NAG/year or the decomposition of approximately 16 metric tons of chitin.

5.2. Taxonomy of chitinoclastic bacteria:

The present study indicate that Vibrio sp. is the most predominant genus among chitinoclasts in water, sediment and those associated with prawns and fishes. This is followed by Aeromonas, Micrococcus and Alcaligenes. Earlier reports also show that the genus Vibrio was the dominant form among chitinoclasts followed by Pseudomonas. Vibrio has been observed as a normal part of the total heterotrophic microflora of Puget Sound sediment, water and fauna by previous investigators (Colwell, 1962; Colwell and Liston, 1962 and Wiebe, 1965). Investigators in other areas have also found the dominance of Vibrio,

Pseudomonad and Aeromonad type flora in a variety of marine environments (Simidu et al., 1971; Dhevendran et al., 1978; Sochard et al., 1979). Liston (1957) and Colwell (1962) found numerous "gut group" vibrios as part of the gut microflora in a variety of marine fishes. Chan (1970) has also found that most of the chitinoclast isolated from fish gut have been pleomorphic vibrios resembling the "gut group" vibrios described by Liston. Okutani (1966) also observed the predominance of chitinoclastic vibrios in the gastrointestinal tract of Japanese Sea Bas. In the present study also the dominance of chitinoclastic vibrios in the gut of prawns and fishes was recorded. The dominance of Vibrio sp. in gut microflora is ascribed to their ability to withstand bile salts even upto a concentration of 10% and their facultative anaerobic nature (Davis and Park, 1962; Aiso et al., 1968). These vibrios are also capable of responding to increased levels of organic nutrients. Enrichment studies with relatively high levels of organic substrate yielded a microflora dominated by vibrio type (Chan, 1970). Perhaps this may be the reason in encountering higher number of chitinoclastic vibrios in water and sediments. In view of this wide spread distribution, abundance and

versatile metabolic capabilities it can be suggested that chitinoclastic bacteria of the vibrio type play a major role in the recycling of chitin in the marine and estuarine environments.

5.3. Effect of some environmental factors on the growth and survival of chitinoclastic bacteria:

In the present investigation one strain of Aeromonas sp. (C.7) and one strain of Vibrio sp. (C.25) were tested for their reaction to the most common environmental factors i.e. temperature, pH and sodium chloride concentration. These two genera were selected because they form the dominant group among chitinoclasts and exhibited higher chitinolytic activities. From the results it is clear that both cultures preferred a temperature of 30°C for their maximum growth. However, the growth was not affected to any considerable extent when they were grown between 25 and 37°C. Eventhough the growth was found to be very much affected at 40°C, they survived very well at this temperature when they were exposed to 60 min. Temperatures higher than 60°C may be necessary to kill the cells. Seki and Taga (1963 b) also reported similar observations. Their

results indicate that chitinoclastic bacteria cease to develop at 40°C and develop preferentially at 30°C. Their data also showed that exposure to a temperature of 50°C for 20 or 30 min. was needed for bactericidal effect. These observations reveal the strong heat resistance of chitinoclastic bacteria as compared to other marine bacteria and their mesophilic character.

The Aeromonas sp. exhibited maximum growth at pH 6.0 and Vibrio sp. at pH 5.0. However, the optimum pH range for the former was 5.0 - 6.0 and for the latter it was 5.0 to 8.0. Aeromonas sp. survived better at pH 5.6 and Vibrio sp. at pH 7.0. In fact Vibrio sp. tolerated a wide pH range of 4.6 - 8.6. Both the strains were found to be more sensitive towards acidic conditions than alkaline conditions. Seki and Taga (1963 b) reported that chitinoclastic bacteria ceased to grow at pH 4.0 and grew well at pH values ranging from 7.0 to 9.0. The Aeromonas sp. which exhibited maximum growth at pH 6.0 was isolated from sediment. It is interesting to note that a species which exhibits maximum growth at pH 6.0 was isolated from an environment where the pH was between 7.0 and 8.0. It is possible that micro-environments which are different from general

environment are more likely to exist in stable habitats (e.g. sediments) rather than in dynamic or unstable habitats prone to frequent mixing (e.g. water) whose pH value may be slightly varying from that of the surrounding area. Further, the pH can decrease in subsurface sediment, especially when reduced conditions are present (Chan, 1970). These observations suggest that chitinoclastic bacteria can very well adjust to the changing pH in the environment and pH may not be a limiting factor.

Both species exhibited maximum growth at 1% sodium chloride concentration and even in the absence of sodium chloride, nearly 90% of the maximum growth was observed. At 3% NaCl concentration only about 50% of the maximum growth was seen and concentrations above 5% drastically inhibited the growth. However both species survived better at 1% and 3% NaCl concentrations. Both species were found to be more tolerant towards lower concentration of NaCl than higher concentrations. All the above observations suggest that chitinoclastic bacteria may be chiefly euryhaline in nature. This supports the earlier report by Seki and Taga (1963 b) that chitinoclastic bacteria grew well in medium where NaCl concentration ranged from 0.5 to 5.0 % . They also reported

that eventhough most of the chitinoclasts were euryhaline in nature, few strains were stenohaline. Based on these reports it is logical to conclude that the two strains used in the present study might have originated from fresh water run off and terrestrial situation. It is possible in areas like estuaries both euryhaline and halophobic chitinoclasts occur. Hence it is expected that chitin decomposition occurs in the areas of decreased salinity also, but under the action of chitinoclast different from those of the marine areas.

5.4. Cultural conditions and production of microbial chitinases:

The effect of several cultural conditions on the production of chitinase by chitinoclastic bacteria, was investigated and the results indicate that the production depends on a number of factors. The type and concentration of chitin on which the organisms were grown proved to be important. Both Aeromonas sp. and Vibrio sp. produced chitinases in medium supplamented with chitin or chitosan. The chitin was supplied in the form of powdered purified chitin and colloidal chitin. When

powdered chitin was used an increase in chitinase production was noticed in both the species with an increase in the concentration of chitin. A similar trend was noticed with chitosan also. However, when colloidal chitin was used an increase in chitin concentration resulted in decreased chitinase production. These observations clearly indicate that concentration of chitin in the medium can profoundly affect the chitinase production of the organism. Baxby and Gray (1968) reported that for some soil micro-organisms maximal yields of enzymes occurred on 1.5% to 2% chitin. In the present study maximum chitinase production was noticed at 3% concentration for chitin powder whereas it was at 0.1% concentration for colloidal chitin. For a fungus, Chytriomyces, only 0.2% substrate was required for higher enzyme production (Reisert and Fuller, 1962). These observations show that the concentration of substrate may vary from organism to organism for maximal production of chitinase and the concentration may also depend on the type of chitin used. It can also be noted from the results (Table-17) that the variation in the chitin concentration never affected the growth of the bacteria, but the enzyme production was found to be altered. Almost similar situation was noticed when

chitosan powder was used in place of powdered chitin. In the case of colloidal chitin maximum growth was observed at a medium concentration of 1.0% whereas maximum chitinase production was noticed at 0.1% . In this case higher concentration of colloidal chitin almost reduced the growth rate of bacteria to 50% eventhough such a reduction was not noticed in the chitinase production. From these results it is evident that chitinase production is not necessarily governed by the growth rate of bacteria. Some of the earlier reports indicate that the source of chitin may also affect the chitinase production. For example, little enzyme was elaborated on mushroom chitin or on beetle (Tribolium) chitin while shrimp chitin permitted higher enzyme production (Monreal and Reese, 1969). In the present investigation only prawn chitin was employed and in the absence of data with other chitin sources, it is not possible to check this effect.

Another factor which is known to influence the chitinase production is the substrate particle size. Baxby and Gray (1968) noted that with the reduction of substrate particle size an increase in chitinase activity was observed. Similarly Seki and Taga (1963 c) and Hood and Meyers (1977 b) reported that as particle size

decreased the rate of chitin decomposition increased. This relatively rapid dissolution of smaller particle of chitin was attributed to the larger surface area available for hydrolysis. In the present study an attempt was made to study the effect of chitin particle size on the chitinase production of chitinoclastic bacteria. As it can be seen from the results different size particles caused differences in chitinase production of cultures without any marked effect on the growth. The relationship between the particle size and chitinase production was found to be different in both the cultures. Maximum chitinase production was noticed in Aeromonas sp. when smaller particles (0.5 mm) were used whereas in Vibrio sp. it was found to be associated with larger particles (1.0 mm). Perhaps it might have been possible to arrive at any definite conclusion if more substrate particle sizes were used in the study. However the results definitely show that a change in the particle size can affect the chitinase production of the culture.

The results also suggest that the chitinase systems of both Aeromonas sp. and Vibrio sp. may be constitutive and it was observed that these systems can be stimulated to a greater extent with N-acetylglucosamine, the monomer

of chitin. Leopold and Samsinkova (1970) also reported that the chitinase of the fungus Beauveria bassiana produced chitinase in the chitin free medium indicating that this system is a constitutive one. While Hood and Meyers (1977 a) showed that the enzyme system in Beneckea neptuna and B. nereida is induced by chitin units, Monreal and Reese (1969) have suggested that the probable inducers of chitinase system are short chain units, three or more, of N-acetylglucosamine. The results of the present study also suggest that the chitinase system in bacteria can not only be induced by chitin units but also by the monomer, N-acetyl-glucosamine. It can also be seen that the chitinase production got depressed with increasing concentration of glucose (Table-21) in the medium. This supports the earlier contention of Clarke and Tracey (1956) who found that glucose in the culture medium depressed chitinase production by a factor of 3 to 5. It is known that glucose or its metabolite nonspecifically inhibits the synthesis of enzymes (Magasanik, 1961). Inhibition may also be due to production of lactic and acetic acids as was shown by Okutani and Kitada (1968 a,b). Chan (1970) also observed similar inhibition by glucose of chitinoclastic bacteria in sediments.

The temperature, pH and sodium chloride concentration markedly influenced chitinase production by Aeromonas sp. and Vibrio sp. Both species produced maximum chitinase at 37°C. At 40°C both growth and chitinase production were drastically reduced. In general an increasing chitinase production was noticed with an increase in temperature atleast upto 37°C. For Beneckea neptuna maximum chitinase production was noticed at 25°C and further increase in temperature resulted in decreased production (Hood and Meyers, 1973 b). Monreal and Reese (1969) also reported that optimal temperature for several bacteria and an Aspergillus sp. was 30°C. Uchida et al. (1979) showed that for a Vibrio sp. chitinase production was maximum at 30°C.

Eventhough both species produced chitinase at all pH levels tested maximum production was noticed at pH 7.0. In Aeromonas sp. there was no significant difference in the chitinase production between pH 5.0 and 7.0 but when they were grown at pH 9.0 a drastic reduction was noticed. In Vibrio sp. also a similar pattern was noticed. Hood and Meyers (1973 b) reported very little difference in chitinase yield of Beneckea neptuna at pH 6.0 and 7.0. Bacillus thuringiensis

also produced maximum chitinase at pH 7.2 (Chigaleichik, 1976). The earlier reports also suggest that bacteria showed optimum chitinase production at neutral pH, 7.0 - 7.5 and fungi at pH 4.5 (Monreal and Reese, 1969). These observations suggest that the initial pH for highest enzyme production vary with species type.

Both species produced chitinase at all concentrations of NaCl used and also in the absence of NaCl. However maximum production was noticed at 3% NaCl. The chitinase production increased with an increase in the concentration of NaCl upto 3% level and further increase resulted in reduction of chitinase yield. As pointed out earlier these results confirm the euryhaline nature of the bacteria and also the necessity of NaCl for maximum chitinase yield. In other words the chitinoclastic bacteria may survive the changing salinity conditions in the environment and perhaps maximum chitin degradation occurs at salinities above 10‰ and below 30‰ approximately. Hence it is clear that salinity and temperature are the two important factors in chitin degradation in estuarine and nearshore regions.

In the present study, residual concentration of N-acetyl-glucosamine, glucosamine, glucose and ammonia were estimated in culture filtrates of both the species in all experiments (Tables 17-20; 22-24). All the compounds were detected at varying levels in different experiments. This suggests that both the bacteria may have enzyme systems as suggested by Hood and Meyers (1977 a) (refer page 31). Since these enzymatic breakdown products were seen in culture medium supplemented with chitosan, it is possible that these bacteria also contain chitosanase. Chitosanases (EC. 3.2.1.99) are known to be produced by many micro-organisms including fungi, bacteria and actinomycetes (Monaghan et al., 1972; Dennis and Eveleigh, 1981). One of the interesting observations made during the study is the accummulation of ammonia in the culture medium. In some cases it accumulated even upto a concentration of 0.86 mg/ml. Such accumulation of ammonia in chitin degradation experiments with bacterial cultures has been recognised (Seki and Taga, 1963 c; Warnes and Randles, 1977). The accumulation of ammonia in culture medium may be the result of chitin degradation or decomposition of peptone or both. It has earlier been shown that at a concentration of 0.01% peptone

in sea water the chitinoclastic bacteria converted 40% of organic matter into cell substance and 60% were decomposed to ammonia (Seki and Taga, 1965 b). It may be pointed out here that in the present investigation also the mediun employed for chitinase production studies contained 1.5% peptone. The marine chitinoclastic bacteria can utilize inorganic nitrogen as available nitrogen sources i.e. ammonia, nitrate, nitrite with available carbohydrate (Seki and Taga, 1965 b).

5.5. Generation time of chitinoclastic bacteria:

Among the two bacterial species studied Aeromonas sp. showed a higher generation time (1.25 $\pm$ 0.10 hr) than the Vibrio sp. (0.70 $\pm$ 0.10 hr) at room temperature(28°C). In the presence of chitin the generation time of both the bacteria were slightly higher than in the absence of chitin. Most probably the bacteria may get attached to the chitin particles and multiply on the surface. This adsorption may cause the initial lag in multiplication resulting in slightly higher generation times. It may be recalled that Aeromonas sp. used in these studies was isolated from sediment and the Vibrio sp.

from the gut of Leognathus sp. Generally, isolates from sediment show comparatively a higher generation time than the isolates from animal sources. Hood and Meyers (1973 b) also reported that sediment isolates showed a generation time of 2 hrs at 22°C whereas the isolates from the digestive tract of Penaeus setiferus exhibited a 30 min. generation time. It is a well known fact that the generation time is mostly dependent on temperature and available nutrients. For example, Vibrio parahaemolyticus had a generation time of 13.5 $\pm$ 0.75 hr at 10°C and the time got reduced to 0.8 $\pm$ 0.17 hr at 25°C and 0.1 $\pm$ 0.02 hr at 30°C (Lee and Pfeifer, 1977). So it is reasonable to assume that chitinoclastic bacteria may also behave in the same fashion and the generation time may be slightly modified by the presence of chitin in the environment.

5.6. Rate of chitin degradation by chitinoclastic bacteria:

The omnipresent chitinoclastic bacteria and the absence of chitin accumulated strongly suggest the participation of bacteria in the degradation of chitin in natural environment. A number of reports on field scale studies and laboratory experiments indicate that

the indigenous marine or estuarine microflora takes part in *in situ* decomposition of chitin. Eventhough no attempt was made to study the *in situ* chitin degradation in the present investigation, the ability of pure cultures to degrade chitin under laboratory conditions was tested using both *Aeromonas* sp. and *Vibrio* sp. Both the species differ in their ability to hydrolyse colloidal chitin. Okutani (1966) also reported that 32 strains of chitinoclasts isolated from fish gut varied in their chitinoclastic activity. He also observed in a detailed comparative studies of two chitinoclastic strains that *Vibrio gerris* exhibited a five fold greater decomposition rate than *Aeromonas chitinophthora* strain in broth containing reprecipitated chitin. In the present study also *Vibrio* sp. was found to be more active in chitin degradation than *Aeromonas* sp. Maximum degradation rate for *Vibrio* sp. (5.328 mg/day/10^{10} cells) was observed at 72 hrs. after inoculation. For *Aeromonas* sp. maximum rate (4.224 mg/day/10^{10} cells) was noticed at 48 hrs. The average rate of degradation for the strains in pure culture (*Beneckea* sp.) isolated from Barataria Bay site was 33.8 mg/day/10^{10} cells at 22°C (Hood and Meyers, 1977 b). This value is comparable to that reported by Seki and Taga (1963 c) for strains isolated from Aburatsubo Inlet

(30 mg/day/10^{10}cells at 25°C) and by Chan (1970) for strains isolated from Puget Sound (20.9 - 31.9 mg/day/10^{10} cells at 22°C). Compared to these values, the rates observed in the present study are very low. The main reason which can be attributed to such low levels of chitin degradation rates is the method of incubation followed in the present investigation i.e. static cultures. One factor which is known to affect the growth and activity of chitinoclastic bacteria significantly is the access to air during incubation. It was shown that growth and activity on chitin agar under anaerobic conditions was sparse compared to that under aerobic conditions. Quantitative studies with growing cell culture of Vibrio showed that the dissolution of particulate chitin in shaken cultures was 325% greater than in static cultures (Chan, 1970). These observations are in agreement with that of Okutani (1966) who also found that growth and activity of faculative chitinoclasts to be highest in shaken cultures. With facultative organism the increased cell synthesis and activity concomitant with increased aeration is related to more efficient utilization of substrate i.e. more energy in the form of ATP is available per unit substrate utilized through the oxidative pathways.

The increased growth and cell yield of facultative organisms under aerobic conditions as compared to anaerobic condition have been well documented. Reynolds (1954) and Veldkamp (1955) reported that shaken cultures of Streptomyces and Pseudomonas were much more active than static cultures in decomposing chitin. These findings strongly suggest that decomposition of chitin would proceed most efficiently in well oxygenated areas of the estuarine and coastal environment.

5.7. Properties of Microbial chitinases:

Investigators have used a number of methods like viscometric method, turbidimetric method or the estimation of N-acetylglucosamine or reducing sugar using substrates like native chitin, regenerated chitin, chitodextrin, chitosan etc. to study the activity of chitinase obtained from different sources. Chitosan has been used as substrate for the characterisation of crude chitinase preparations (Tracey, 1955; Vessey and Pegg, 1973). The use of chitosan in these instances is inappropriate since chitinase from Serratia sp., Streptomyces sp. and Streptomyces griseus (Hirano and Yagi, 1980) show no activity towards chitosan. The activities observed

using chitosan as a substrate for chitinase may reflect the presence of specific chitosanase in the crude preparations. It has been now well established that chitosanases are produced by many microorganisms (Monaghan et al., 1972). A crude extracellular preparation from Penicillium islandicum contained chitosanase, chitinase and carboxymethyl cellulase activities (Monaghan et al., 1972). Most of the people have used reprecipitated or colloidal chitin as a substrate for the enzymic assay of chitinase. Some people consider chitinase assay with colloidal chitin is troublesome and difficult to reproduce (Lundblad et al., 1974). Glycol chitin, a water soluble derivative by glycolating chitin with ethylene oxide prepared by Senju and Okimasu (1950) has been employed as a substrate for the viscometric assay of chitinases by many investigators because the assay is simple and rapid. However, glycol chitin also forms the substrate for lysozymes and in many cases especially enzymes of animal origin chitinase activity has to be differentiated from lysozyme activity (Lundblad et al., 1974, 1979 a,b; Fange et al., 1979). It must also be remembered that the properties of chitinase from a given source may change according to the type of substrate employed. For example, the

pancrease chitinase from the fish Chimaera monstrosa exhibited optimum pH values from 8.0 to 10.0 with glycol chitin as a substrate whereas with colloidal chitin the values shifted to 10.0 - 13.0 (Fange et al., 1979). Some other investigators used certain chromogenic substrates like 3, 4 dinitrophenyl-tetra-N-acetyl chitotetraoside as the substrate for bacterial chitinase (Aribisala and Gooday, 1978). Hence it must be borne in mind that while comparing the properties of different chitinases the substrate can markedly influence the enzyme properties.

As pointed out earlier most of the reports on the properties of chitinase have involved crude extracts and it is a well known fact that extraneous protein content of an enzyme extract may cause marked changes in the substrate breakdown rate, effect of pH on the activity and other properties of the enzyme (Tracey, 1955). With these observations in mind some of the chitinase properties studied in the present investigation are discussed here. For the present work reprecipitated chitin or colloidal chitin only was used as the substrate and partially purified enzyme was employed.

Enzymes from both the species exhibited maximum activity at 37°C. When chitinase from Vibrio sp. lost

its activity at 55°C, the Aeromonas chitinase exhibited atleast 35% of the total activity. Most of the other microbial chitinases also showed maximum activity around 40°C - Beneckea neptuna (40°C) (Hood and Meyers, 1977 a); Vibrio sp. from marine mud (37°C) (Ohtakara et al., 1979), and Aeromonas chitinophthora and Vibrio gerris (40°C) (Okutani, 1966). However, chitinases exhibiting higher optimum temperatures have also been recorded - Bacillus thuringiensis (60°C) (Chigaleichik, 1976); Pseudomonas aeruginosa and Vibrio anguillarum (60°C) (Nagahata and Shimahara, 1979) and Vibrio alginolyticus (56°C) (Aribisala and Gooday, 1978). The results of the present study also indicate that both enzymes were stable upto 40°C and they were almost completely inactivated at 70°C. Most of the other chitinases also exhibit similar behaviour (Ohtakara et al., 1979; Nagahata and Shimahara, 1979) except that of Streptomyces antibioticus which was stable even upto 65°C (Skujins, 1970). These results suggest that the chitinase of bacteria withstand a reasonably higher temperature and their activity is unaffected under in situ temperatures.

The optimum pH was found to be 5.6 for Aeromonas sp. and 7.0 for Vibrio sp. Both chitinases exhibited another common peak of activity of pH 3.5. Even at extreme pH

values i.e. 2.0 and 10.5 nearly 50% of the maximum activity was noted for both the chitinases. Chitinases from Beneckea neptuna, Vibrio sp., Vibrio gerris exhibited optimum activity at pH 7.0, 6.0 - 8.0, and 7.0 respectively (Hood and Meyers, 1977 a; Ohtakara et al., 1979; Okutani, 1966). Okutani (1966) reported that the chitinase of Aeromonas chitinophthora exhibited maximum activity of pH 5.5 which is comparable to that of Aeromonas sp. in the present study. It may also be recalled that this Aeromonas sp. exhibited maximum growth at pH 6.0 and Vibrio sp. at pH 5.0. Maximum stability of chitinases of these bacteria were noticed at pH 5.0. However they were found to be equally stable over a range of pH 4.0 - 10.0. Other bacterial chitinases also exhibited similar stability over such a wide range of pH values. Okutani (1966) reported that chitinases of A. chitinophthora and V. gerris were stable over a range of pH 5.0 - 9.0. The enzyme of P. aeruginosa and V. anguillarum were reported to tolerate pH 6.0 to 9.0 without any appreciable loss of activity (Nagahata and Shimahara, 1979). The chitinase of Vibrio sp. isolated from marine mud was found to be more stable in alkaline pH (9.0 - 11.0) (Ohtakara et al., 1979). These

observations clearly indicate that the chitinases produced by the chitinoclastic bacteria are highly pH stable and they can withstand any change in the pH of the environment within these limits without affecting the normal chitin degradation process.

The activity of both the chitinases marginally decreased with increasing concentrations of substrate (colloidal chitin) above to optimum level. Otakara (1961) while studying the chitinase activity of Black-koji mold with glycol chitin as a substrate observed that the activity was not completely independent of the concentration of substrate solution but it became progressively and proportionally low as the latter was increased. However under natural conditions increased decomposition rates were noticed with increased quantity of seeded chitin (Hood and Meyers, 1977 b). The concentration of enzyme in the assay mixture influenced the total activity. The buffer molar concentration exerted no effect on the Aeromonas chitinase even upto 0.5 M. However Vibrio chitinase was found to be very sensitive to any increase in buffer molar concentration above 0.1 M and at 0.5 M only 58% of the total activity was noticed. This suggest that sediment isolate chitinase

may be insensitive to buffer molar concentration. The effects of various chemicals on the stability of the enzymes indicate that both the enzymes are affected by metal ions tested and that too they affected the Vibrio chitinase to a greater extent than the Aeromonas chitinase. This shows that the chitin degradation in a given ecosystem may be significantly affected by the presence and concentration of various metal ions originating from industrial effluents and other sources.

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6. SUMMARY.

This thesis presents a detailed account on (a) occurrence and distribution of total heterotrophic and chitinoclastic bacterial populations in water, sediments and fauna of estuarine and neritic environs of Cochin (Lat. 9°58'N and Long. 76°15'E) over a period of one year; (b) taxonomy of chitinoclastic bacteria; (c) chitinase activity in sediments and digestive tract of prawns and fishes; (d) growth and physiology of selected strains of chitinoclastic bacteria - the effects of temperature, pH and sodium chloride on growth and survival; (e) cultural conditions and microbial chitinase production - effects of chitin type, initial concentration, particle size, chitin derivatives, temperature, pH and sodium chloride; (f) generation time of chitinoclastic bacteria; (g) rate of chitin degradation by chitinoclastic bacteria; (h) certain properties of microbial chitinases.

1) Totally 8 stations were selected for routine sample collection in the coastal and estuarine regions of Cochin. During this study a total of 28 sediment samples, 53 water samples (28 surface water samples and 25 bottom water samples), 8 species of prawns, 24 species of fishes were examined over a period of one year. In addition to these, 15 sediment samples collected from other locations were also examined for chitinase activity.

2) Except few samples nearly all the fauna samples and majority of water and sediment samples harboured varying levels of chitinoclastic bacteria. In general, the bacterial population was more in sediments when compared to the overlying water. The total bacterial population in sediments during the period of study varied from 1.60×10^3/g to 239.63×10^3/g. The chitinoclastic population in these samples varied from undetectable levels to a maximum of 74.44 % of total bacterial population. In surface waters the total heterotrophic bacterial population ranged from 0.6×10^3/100 ml to 125.0×10^3/100 ml. In bottom water the variation was still

wide i.e. 1.0 x 10^3/100 ml to 201.6 x 10^3/100 ml. In surface waters the percentage of chitinoclasts varied from almost nil to 66.66 % of the total bacterial population and in bottom waters it varied from nil to 70.05 %.

3) Out of the various physico-chemical parameters examined temperature seems to have exerted marked effect on the chitinoclastic population. Higher percentage of chitinoclasts were recorded in most of the water and sediment samples collected during the month of May when the temperature was found to be comparatively more than other months. The results also indicate that there is no definite relationship between chitinoclastic bacterial population and salinity of the water. The pH and oxygen content of the waters seem to have no influence on the chitinoclastic population under natural conditions.

4) Totally seven species of Penaeids (P. indicus, P. monodon, P. semisulcatus, Metapenaeus monoceros, M. affinis, M. dobsoni, Parapenaeopsis stylifera) and

one brackish water species, Machrobrachium rosenbergii (Palaemonidae) were examined for the distribution of chitinoclastic bacteria on the shell surface and in the digestive tract of the animals. All the species harboured chitinoclastic bacteria both on the shell surface and in the contents of the digestive tract. In general, the digestive tract harboured more chitinoclast than the shell surface. The total heterotrophic bacterial population on the shell surface varied from 1.15×10^6 to 64.20×10^6 per animal. The chitinoclasts exhibited a marked fluctuation in their distribution (4.76 - 75.0 %). In the digestive tract the heterotrophic population varied from 1.30×10^6 to 241.0×10^6/g of gut contents. Like shell surface the chitinoclastic bacterial population varied from 3.0 to 86.2 % of the total population in the digestive tract also. There was no relation between total heterotrophic bacterial population and chitinoclasts. Random analyses of the gut contents revealed that 20 to 30 % were of chitinous nature.

5) Out of the 24 species of fish belonging to 15 families examined chitinoclastic bacteria were present in the digestive tract of 20 species of fish. The total heterotrophic bacterial population in the digestive tracts varied from 2.61 x 10^6/g to 542.20 x 10^6/g. The percentage distribution of chitinoclastic bacteria also fluctuated widely and in some cases they accounted for more than 90 % of the total bacterial population. Most of the fishes were found to contain some chitinous material in their digestive tracts.

6) No free chitinase activity could be detected in sediments collected from 15 locations in the estuary. The total bacterial population in these samples varied from 1.13 x 10^5/g to 134.0 x 10^5/g. The percentage of chitinoclasts also fluctuated from 1.13 to 13.94.

7) All species of prawns examined had some level of chitinase activity, which varied from 43.50 µg NAG/g/hr to 150.51 µg NAG/g/hr, associated with their digestive tracts. Out of 24 species of fish examined, 21 species exhibited some levels of chitinase activity (7.0 to 725.0 µg NAG/g/hr). In general,

members of Carangidae exhibited higher levels of chitinase activity in their digestive tracts. No relation could be established between the chitinoclastic bacterial population and chitinase activity in the digestive tracts of these animals.

8) During the present study a total of 210 strains of chitinoclastic bacteria (sediment - 52; water - 25; prawns - 78; fishes - 55) were isolated, purified and identification upto generic level could be confirmed for 206 strains. The genera represented were Vibrio, Aeromonas, Alcaligenes, Pseudomonas, Bacillus and members of Enterobacteriacea. Vibrio was found to be the most common genus in sediments (86.0 %), water (72.0 %), prawns (71.05 %) and fishes (65.0 %).

9) Two strains belonging to Aeromonas sp. and Vibrio sp. which exhibited a considerable chitinase producing capacity were used for further detailed investigation. Both cultures exhibited maximum growth at 30°C. However, almost uniform growth was observed between 25 and 37°C. At 40°C the growth of both species was very much affected. Both species survived an exposure of 40°C for 60 min. and

they could not tolerate 60°C for the same period.

10) The optimum range of pH for growth was found to be 5.0 to 6.0 for Aeromonas sp. and 5.0 to 8.0 for Vibrio sp. Aeromonas sp. survived better at pH 5.6 and Vibrio sp. at pH 7.0. In fact Vibrio sp. tolerated a wide pH range of 4.6 to 8.6. Both strains were found to be more sensitive towards acidic conditions than alkaline conditions.

11) Both species grew well in medium containing 1.0 % NaCl. An almost equal growth (90 % of the maximum) was observed in the absence of NaCl also. Both species were found to be more tolerant towards lower concentrations of NaCl than higher concentrations. This study indicated the euryhaline nature of chitinoclastic bacteria.

12) The type and initial concentration of chitin were recognized as important cultural conditions for chitinase production. Both the species produced chitinase in medium supplemented with chitin or chitosan. These cultures produced chitinase even in the presence of certain chitin derivatives, i.e. N-acetylglucosamine (the monomer of chitin),

glucosamine and glucose. The results of the present study indicate that the chitinase system in both the species may be of a constitutive one. The presence of various intermediate degradation products in the culture filtrate of both the cultures positively indicated the presence of complete chitinase system.

13) The temperature, pH and NaCl concentration in the medium markedly influenced chitinase production by both the species. They produced maximum chitinase at 37°C, pH 7.0 and 3.0 % NaCl concentration. Both species produced chitinase even in the absence of NaCl in the medium. No definite relationship between growth and chitinase production could be established.

14) The generation time for Aeromonas sp. was 1.50 ± 0.10 hr and for Vibrio sp. it was 1.00 ± 0.17 hr. in chitin medium. In the absence of chitin a marginal reduction in the generation time of both the cultures was noticed.

15) Rate of chitin degradation under *in vitro* conditions for both the cultures was worked out. Maximum degradation rate for *Vibrio* sp. (5.328 mg/day/10^{10} cells) was observed at 72 hrs. after inoculation. In the case of *Aeromonas* sp. maximum rate (4.224 mg/day/10^{10} cells) was noticed at 48 hrs.

16) The properties of partially purified chitinases from both the species were studied using colloidal chitin as a substrate. The time course study indicated that the end product formation in both the cases was rapid and reached maximum at 40 min. and 60 min. for *Aeromonas* and *Vibrio* sp. respectively. Enzymes from both the sources exhibited maximum activity at 37°C. The optimum pH for *Aeromonas* chitinase was 5.6 and for *Vibrio* it was 7.0. Both chitinases exhibited another common peak of activity at pH 3.5. In *Vibrio* sp. maximum activity was recorded at a substrate concentration of 0.21 mg/ml and in *Aeromonas* sp. at a concentration of 0.04 mg/ml. The extent of reaction of *Aeromonas* chitinase was not linear with time but the assay was proportional to the amount of added enzyme solution. However, the

Vibrio chitinase exhibited a more or less linear relationship with time. Both enzymes were stable upto 40°C and they were almost completely inactivated at 70°C. Maximum stability of chitinases of these bacteria was noticed at pH 5.0. However, they were found to be equally stable over a wide range of pH 4.0 to 10.0 Hg^{++}, Cu^{++}, Zn^{++}, Ca^{++} and Ag^{++} affected the stability of Vibrio chitinase when compared to Aeromonas chitinase. On the other hand Na^{++} and Mg^{++} affected the stability of Aeromonas chitinase than the Vibrio chitinase. The buffer molar concentration exerted no affect on the Aeromonas chitinase even upto 0.5 M. However, Vibrio chitinase was found to be very sensitive to any increase in buffer molar concentration above 0.1 M and at 0.5 M only 58 % of the total activity was noticed.

17) A detailed discussion on the occurrence and distribution of chitinoclastic bacteria, their growth and physiology, production and properties of their chitinases and the rate of chitin degradation under laboratory condition is also given.

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