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 \(10^3 \text{ cells/g}\) 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 \(1.3 \times 10^2 \text{ cells/g}\) 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 \(1.5 \times 10^2 \text{ cells/ml}\) 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 $2.4 \times 10^4$ 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 $2.0 \times 10^4$ cells/g, $6.8 \times 10^4$ cells/g and $3.7 \times 10^3$ 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 $2.5 \times 10^2$ cells/ml while bottom waters contained fewer cells, $2 \times 10^2$/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. incolithetica 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 chitonoclast \((10^2 - 10^3 \text{ cells/ml})\) was observed in the water column. A concomitant rise in the chitonoclastic 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 chitonoclast. Large concentrations of chitonoclast were found to be associated with intact exoskeleton and the digestive tract of penaeid shrimp.

2.1.2. Association of chitonoclastic bacteria with aquatic animals:

A number of reports show that chitonoclastic 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 chitonoclasts.

Jones (1958) demonstrated that the surface of the radiolarian Castanidin longispinum contained at least 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 i.e. *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.

<table>
<thead>
<tr>
<th>Name of animal</th>
<th>Region</th>
<th>Chitinoclastic bacterial population (Cells/g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octopus</td>
<td>Intestine</td>
<td>$1.5 \times 10^5$</td>
<td>Seki &amp; Taga (1963d)</td>
</tr>
<tr>
<td>Squid</td>
<td>&quot;</td>
<td>$4.3 \times 10^5$</td>
<td>&quot;</td>
</tr>
<tr>
<td>Swell fish</td>
<td>&quot;</td>
<td>$4.4 \times 10^3$</td>
<td>&quot;</td>
</tr>
<tr>
<td>Japanese Sea bass</td>
<td>Stomach</td>
<td>$1.7 \times 10^4$</td>
<td>Okutani (1966)</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>$5.4 \times 10^7$</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Pyloric caeca</td>
<td>$2.3 \times 10^7$</td>
<td>&quot;</td>
</tr>
<tr>
<td>Yellow tail Sea bass</td>
<td>Stomach</td>
<td>$2.0 \times 10^4$</td>
<td>Okutani et al. (1967a)</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>$1.4 \times 10^5$</td>
<td>&quot;</td>
</tr>
<tr>
<td>Variety of estuarine</td>
<td>Intestine</td>
<td>$1.0 \times 10^7$</td>
<td>Chan (1970)</td>
</tr>
</tbody>
</table>
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; Converse 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). \textit{V. \text{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 \textit{V. \text{parahaemolyticus}} (Balakrishnan Nair, 1981). He attributed the higher incidence/level of \textit{V. \text{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 \textit{V. \text{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, \textit{Bacillus chitinovorosus}, which exhibited chitinoclastic properties. Numerous reports are available on the non-marine chitinoclastic microorganisms representing a variety of genera such as \textit{Flavobacterium}, \textit{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. Hook (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. alopris* 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. omhus*, *V. labrakos*, *Aeromonas sklaira*, *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. pierantoni, 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 $\alpha$, $\beta$, and $\gamma$ types. Each chitin is composed of chains of the N-acetyl-glucosamine unit linked in the 1-4 $\beta$ glucosidic manner but arranged in different physical structure (Richards, 1951). The chitin from the insects and crustaceans is of $\alpha$ type and contains two helical polysaccharide chains oriented in opposite directions with screw axes along the chain direction. The bonding phenomena has been explained by Dweltz (1960), Carlstrom (1962) and Ramakrishnan and Prasad (1972). The $\beta$ type is produced by members of Coelentrata, Annelida, Mollusca and Brachiopoda and is associated with collagen cuticle (Hackman, 1964). The $\gamma$ 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 histidyl 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)$_2$O$_3$, MgO, CaO, P$_2$O$_5$ 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:
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 \textit{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 \textit{Streptomyces griseus} with two chitinases with similar activities and a chitobiase. While working with chitinase extracted from a snail Kimura \textit{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 chitinophthra*) 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 (CH₁) 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 marnescens 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; Dobrogox, 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 Chytrmyces 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 CaCO₃ 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/10¹⁰ 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 (Enrophys 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); Chrytriumyces hyalinus (Reisert, 1972); Verticillum 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 Streptomyces, 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 (Canidae: 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 (Lundblad 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 quinqueraadiata, Hippoglossoides dubius, Stichaeus grigorjewi, Gadus macrocephalus, Mustelus manazo, Polypterus dofleini, 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 quinqueraadiata), 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 caeca 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, 1962a). 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, 1983). 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, 1977a). 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, 1977a, 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 (1977a) 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 (1977a) 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 2.9% chitin. However for the
fungus *Chytromyces*, 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-acetylg glucosamine units).

Maximum enzyme yield of *B. neptuna* was at 25 – 27°C at 4 to 5 days, with 0.5% chitin concentration (Hood and Meyers, 1977 a). The initial H 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-acetylg glucosamine, 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 3.2% 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 focusing 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 (Tomina 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-$\beta$-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. Even though N-acetylglucosamine and glucose did not inhibit the activity, Cu²⁺ and Cd²⁺ caused total inhibition. Co²⁺, 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 \( \sim 0.23 \text{ mM} \). Higher concentration of the substrate inhibit the reaction. Double reciprocal Lineweaver-Burke plots indicated higher activity than expected in substrate concentration \( \sim 5 \text{ mM} \); the apparent Km value obtained by linear extrapolation was \( \sim 7.3 \text{ mM} \). Colloidal chitin and \( \alpha \) 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 \( \sim 40°C \).

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. CaCl₂ was the best co-factor among the chemicals tested. Using CaCl₂ 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 CaCl₂. The enzyme preparation from Enterobacter lost its activity at 50°C, and the Vibrio preparation did not lose 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 16.5 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\textsuperscript{++}, Cu\textsuperscript{++} and Ag\textsuperscript{+} and completely inhibited by Hg\textsuperscript{++}. Monoiodoacetic acid and \textit{P}-chloromercuribenzoate did not inhibit the enzyme but \textit{EDTA} showed slight inhibition of chitinase activity.

Crude enzyme preparation from \textit{Bacillus} R-4 digested cell walls of \textit{Rhizopus}, \textit{Mucor}, \textit{Trichoderma} and \textit{Piricularia} (Tominaga, 1977). Column chromatographic studies showed that the \textit{Bacillus} enzyme preparation consisted of protease and chitosanase. The chitosanase had a molecular weight of 31,000, an 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 \textit{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 \textit{Beneckea neptuna} isolated from gut of \textit{Penaeus setiferus} was found to be a moderately actively inducible one (Hood and Meyers, 1977a). Examination of the cell-free enzyme of the bacterium revealed an optimal pH of 7.0 and a temperature optimum of 40°C. When \textit{N}-acetylglucosamine was assayed (Reissing \textit{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 (1965), 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 quinquergiata*) the rainbow trout (*Salmo arideus*) 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 acantbias* (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 $\approx 26,000$ 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 $\approx 26,000$ was divided into two chitinase active regions with pI at 5.7 and 3.8. The second peak with molecular weight $\approx 13,000$ had a pI at 7.3.

The chitinase in the gastric juices of American lobster *Homarus 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 *Homarus 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.