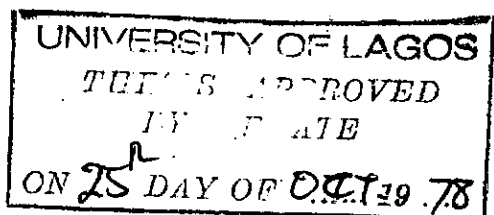MICROBIOLOGICAL STUDIES OF SHRIMP SPOILAGE
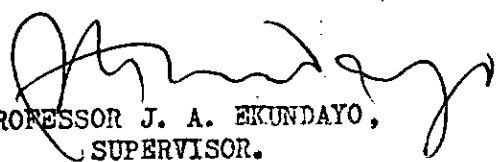
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ABSTRACT

Bacterial counts of samples of freshly caught shrimps, Penaeus duorarum and of sea water from Nigeria, were carried out for a period of ten months, from April 1975 to January 1976. A monthly variation in the size of the bacterial populations of shrimps and sea water was observed.

Gram-positive bacteria were generally more abundantly isolated than Gram-negative bacteria from shrimps and sea water. Coryneforms predominated the isolates. Next in abundance were micrococci, followed by staphylococci. Other genera occurring on shrimps and in sea water were Acinetobacter spp, Bacillus spp, Flavobacterium spp, Pseudomonas spp, some members of Enterobacteriaceae, Aeromonas spp and Moraxella spp. The flora of the shrimp after spoilage at chilled temperature consisted mainly of Pseudomonas spp, and at room temperature consisted mainly of Aeromonas spp.

Aerobic bacterial flora of smoked shrimps obtained from markets in Lagos and Port Harcourt was examined. The bacterial loads ranged from $\log_{10} 3.3$ to $\log_{10} 9.4$. The isolates were mostly Gram-positive, micrococci and coryneforms.

Chitinolytic Vibrio alginolyticus, Bacillus cereus, Pseudomonas spp, Aeromonas spp, Flavobacterium spp. were isolated from sea water, Chitinolytic Vibrio alginolyticus, Pseudomonas spp

and _Bacillus cereus_ were also isolated from the surface and
gut of freshly caught shrimps.

The production and properties of chitinase by _V. alginolyticus_
was studied using 3, 4-dinitrophenyl-tetra N-acetyl-chitotetraoside
(3, 4 - DNP-TNAC) as substrate. The chitinase had a pH optimum
of 5.5 to 6.0 and temperature optimum of $56^{\circ}C$. Chitinase
activity on 3, 4 - DNP- TNAC was inhibited by chitin. The Km
value for the chitinase was about 7.5µM and V max was 0.020u mol
min^{-1} of 3,4-DNP-released.

Chitinase production by _V. alginolyticus_ was highly
inducible. The peak of chitinase activity was recorded from
the culture broth of _V. alginolyticus_ on the 6th day of incuba-
tion. A percentage of 90 - 96 of the total chitinase activity
was recovered by partially purifying the enzyme with ammonium
sulphate (80% saturation) and a sixty-fold increase in specific
activity was obtained, by further purification on G-200 Sephadex,
as compared with culture supernatant.

CONTENTS

INTRODUCTION

Over the past few years, fishing for shrimps (Penaeus
duorarum) has been increasing steadily in importance in Nigeria.
A total of 1942.3 metric tons were landed in Nigeria in 1971
(Report, 1975). The catch has been increasing steadily from
1971 values to 2267.48 metric tons in 1975 (Report, 1975).

Shrimps are caught almost throughout the year except for
three off-season months, December - February. The catch from
artisan fishermen is usually landed whole, uniced. Shrimps
caught by trawlers are either beheaded or boxed whole. They
are either covered with ice or frozen directly in a freezer
after catch. The time interval between catching and landing
by trawlers varies between 2 - 40 days. The time between capture
and receipt at the depot is therefore longer than desirable for
the optimum quality of the product. Furthermore, before entry
into markets or delivery at the processing plant, organoleptic
changes which render the shrimps unsuitable for use as food may
occur. The method of preservation by means of ice, although
partially effective, allows spoilage to take place.

The spoilage of marine products is generally attributed
to three main causes (Williams, Campbell and Rees, 1952b).

   (1) the presence of autolytic enzymes which are in part

        responsible for gradual softening of the tissues;

(2) the oxidant changes such as the development of rancidity;

(3) bacterial activity which brings about very noticeable changes in the flavour, odour and general appearance of the product.

Of the three, the last is generally considered the most important. For this reason a knowledge of the types of bacteria present in the products, and of their characteristics is important in developing and improving methods of handling and preservation of marine products.

In 1944, Shewan postulated that fish in their natural environment carry a commensal bacterial load. The composition of the bacterial flora is determined by external factors, among which are the feeding habits of the fish, the method of catching, the quality of the waters in which they exist, and the geography of the areas in which they are caught. For example, if shrimps are caught in the deep sea, the bacterial load can be expected to consist largely of marine bacteria, whereas if collected from zones subject to pollution, the naturally occurring flora may be contaminated by other types introduced by the presence of pollutants in the water. Also during catch and handling procedures, further contaminants are introduced as a result of contact with man. In this process,

the mesophilic organisms _Escherichia_ _coli_, faecal streptococci
and the common food pathogens, _Salmonella_ and _Staphylococcus_
aureus, may be introduced. The bacterial load of shrimps
therefore varies considerably and the quality of the catch will
depend on the numbers and types of bacterial contaminants and
how the contaminants are controlled during processing and marketing.

The results of investigation by Reay and Shewan (1949) into
the numbers of bacteria on the skin of haddock caught by trawl
off Aberdeen, at various times of the year indicated that there
was a seasonal variation in the quantitative load of bacteria on
the fish. Seasonal variation of the number of bacteria in sea-
water has been observed by workers in different parts of the
world (Lloyd, 1930; Zobell and McEwan, 1935; Wood, 1953).

Reay and Shewan (op.cit.) observed that of the various
types of bacteria found on fish the most important genera were
Achromobacter, _Micrococcus_, _Flavobacterium_, _Pseudomonas_ and
Bacillus. Although members of the coli-aerogenes group have
been found on fish their presence is regarded as of little
significance in spoilage.

The bacterial flora of shrimps has been investigated by
several workers. Green (1949) showed that the total viable counts
at $20^{\circ}C$ for freshly landed Gulf shrimps ranged between 10^{4}/gm and
10^{7}/gm. It was also found during the study that out of 41 samples

of freshly caught shrimps, 49% contained members of the coli-aerogenes group.

The main groups of bacteria present on freshly caught Gulf shrimps were the same as those found on other marine products; these included Achromobacter, Micrococcus, Pseudomonas, and Bacillus (Williams, Campbell and Rees, 1952b). Headed Gulf shrimps contained a bacterial flora consisting of Micrococcus, Achromobacter, Pseudomonas and Flavobacterium (Campbell and Williams, 1952), while the intestinal flora comprised Bacterium, Achromobacter and Micrococcus in equal frequency (Williams and Rees, 1952).

Magar and Shaikmahmud (1956) reported that the main groups of bacteria on Bombay prawns were Achromobacter and Micrococcus. Other genera less frequently isolated included Bacillus, Bacterium, Flavobacterium, Sarcinia, Pseudomonas, Serratia, Kurthia and Aerobacter. Sreenivasan (1959) reported total viable counts of 10^4/gm and 10^7/gm for freshly caught prawns in India. He also found that Micrococcus and Corynebacterium were the genera frequently isolated from the shrimps.

De Silva (1976) reported that the bacterial flora of prawns off shore Sri-Lanka consisted of coryneforms, Micrococcus, Flavobacterium, Cytophaga, Achromobacter, Pseudomonas, Aeromonas, some members of the Enterobacteriaceae and Bacillus.

Cann, Hobbs, Wilson and Horsley (1971) found that over 50%
of the microbial flora of tropical shrimps comprised of coryneforms
and Micrococcus species. Sreenivasan (1959); Velankar and
Govindam (1959); Pillai, Sastri and Nayar (1961); Velankar,
Govindam, Appukutan and Iyer (1961); Jacob, Iyer, Nair and Pillai
(1962); and Lekshmy, Govindam and Pillai (1962) reported on
the different aspects of spoilage of prawns, preservation of
prawns in ice, assessment of quality by objective standards,
chemical assessment and estimation of total bacterial loads of
the iced and uniced shrimps in India.

The microbiology of the West African Coastal waters, and
of Nigerian waters in particular has not so far been studied.

Most of the shrimps landed in Nigeria are marketed whole
either as fresh or frozen shrimp. If processed, processing
usually consists of the removal of the head, deveining and
freezing of the tail meats. Although there are many variations
in the details of processing, throughout the whole process,
considerable manual handling of the product occurs.

The increasing world demand for shrimp from Nigeria has
opened up export markets which have become important sources of
foreign exchange. There is now an appreciable export most
particularly to America and Japan.

As with many other foods, quality standards are being
introduced in the shrimp industry to satisfy international trade require-
ments. The shrimp must meet any bacteriological and chemical

standards required by the importing country. It therefore becomes necessary that a preshipment quality-grading scheme be established based on bacteriological data obtained through research. Such bacteriological standards would serve in quality control in shrimp processing factories. Bacteriological quality control during processing is essential because fish deteriorate rather fast in the tropics owing to the high relative humidity of the atmosphere and the high ambient temperature which enhance deteriorative microbial activities.

The bacteriology of the spoilage flora of crustacea has been extensively studied, Tobin, Alford and McClesky, 1941; Alford, Tobin and McCleskey, 1942; Green, 1949b; Campbell and Williams, 1952; Fellers, Gagnon, and Khatchikian, 1956; Khatchikian, Fellers and Litsky, 1959; Sreenivasan, 1959; Walker, Cann and Shewan, 1970; Hobbs, Cann, Wilson and Horsley, 1971; and Cann, 1973. From the literature cited above, spoilage of fishery products at ice temperatures results from the growth of Gram-negative bacteria of the Pseudomonas and Achromobacter genera. At higher temperatures, the Gram-positive coryneforms and Micrococcus and some members of the Enterobacteriaceae contribute significantly to deterioration of the products. Cann (1973) showed that at the chilling temperatures usually employed for low-temperature spoilage experiments, species of Pseudomonas are more frequently isolated from storage specimens, next in abundance are Achromobacter spp. Frazier (1958) on the other hand showed

that at higher temperatures <u>Micrococcus</u> spp. and <u>Bacillus</u> spp are frequently isolated.

Campbell and Williams (1952); Walker, Cann and Shewan (1970); Hobbs, Cann, Wilson and Horsley (1971); and Cann (1973) studied changes in bacterial populations of iced shrimps. For non-tropical shrimps, <u>Pandalus</u> and <u>Nephrops</u> species, they found that <u>Micrococcus</u>, <u>Bacillus</u> and <u>Flavobacterium</u> populations decreased steadily while <u>Achromobacter</u> increased. For tropical shrimps, Cann (1971) in Thailand found the flora of the shrimps during spoilage in ice to consist mainly of <u>Pseudomonas</u>.

Further work on spoilage of tropical shrimps has mainly been carried out in India by Sreenivasan (1959). Velanker and Govindam (1959), Pillai, Sastri and Nayar (1961); Velankar, Govindam, Appukutan and Iyer (1961); Jacob, Iyer, Nair and Pillai (1962); LeKshmy, Govindam and Pillai (1962).

Two principal types of decomposition in raw shrimps were postulated by Duggan and Strasburger (1946). One of these, the putrefactive type, occurs when shrimps, prior to icing, are exposed for a time at a temperature favourable for bacterial multiplication. The subsequent breakdown is characterized by the appearance of indole, presumably formed from tryptophan through bacterial action. Once started, this type of decomposition proceeds fairly rapidly, even though the shrimps are subseqnetly well-iced. The second type of decomposition, the ammoniacal type is slow and it is characterized by an odour of free ammonia in addition to other odours associated with protein

decomposition.

Indole is a reliable indication of spoilage of raw shrimps prior to freezing and canning (Duggan and Strasburger, 1946). Its production during processing has also been accepted as an indication of inferior sanitation in processing plants or deterioration in storage prior to final processing (Barry, Weeks and Duggan, 1956).

The spoilage of shrimps during refrigerated storage has been found to be due to the metabolic activities of psychrophilic bacteria, e.g. Pseudomonas. The production of trimethylamine (T.M.A.) during storage and its contribution to the easily detectable pungent spoilage odours has led to the widespread use of T.M.A. production as an index of quality. The relation between T.M.A. and bacterial spoilage, however, is not clear. It has been found (Tarr, 1939), and Shaw and Shewan (1968) that the characteristic odours of spoilage can occur independently of T.M.A. production, depending on the nature of the bacterial inoculum. It is also possible that T.M.A. accumulates in shrimps' muscle first as an odourless salt which is converted to the "fishy" smelling base only in the later stages of spoilage.

Shrimps have a high content of non-protein nitrogen, and autolytic changes caused by the enzymes present in the tissues of shrimps may increase the supply of nitrogenous substrates (e.g., amino acids and amines) and simple sugars for bacterial

growth. From these hydrolytic products, the bacteria may synthesise T.M.A., ammonia, amines (e.g., putrescine and cadaverine), lower fatty acids and aldehyde and eventually hydrogen and other sulfides, mercaptan and indole, which   are indicative of putrefaction (Shewan, 1961).

The production of T.M.A. from trimethylamine oxide (T.M.A.O.) is considered to be due to bacterial action - the activities of one group, the Gram-negative, non-pigmented rods with peritrichous flagella (Watson, 1939). No fungi (except a few yeasts) have been associated with shrimp spoilage. In those instances in which yeasts were isolated, it was not possible to show that they were responsible for spoilage (Wood, 1953). Most previous workers - Reed and Spencer (1929) Proctor and Nickerson (1935), Stewart (1932) and Shewan (1944) - did not isolate anaerobes from spoiling fish muscle and slime. Shaw and shewan (1968) found out that Pseudomonas group 4 brought about the greatest organoleptic changes in sterilized fish flesh. Achromobacter, although present as a part of the spoilage flora, appeared to play a minimal role in the production of organoleptic changes (Nickerson and Sinkey, 1972). Investigation on cod and haddocks (Castel, Richards and Wilmot, 1949; Chai, Chen, Rosen and Len, 1968) have shown that Pseudomonas putrefasciens produced hydrogen sulphide from protein thus causing spoilage in water-fish from North Atlantic. It is also known that certain bacteria

produce an enzyme triaminooxidase which activiates T.M.A.O.
thus making it susceptible to reduction by dehydrogenase enzymes
of bacteria.

A variety of chemical compounds may be added to foods to
prevent microbial spoilage. In some instances this is done to
extend the storage life of foods which are held at refrigeration
temperatures. Salting, for example, is used to preserve shrimps.
In some cases limited amounts of salt, usually 2 - 5%, are used.
This, together with refrigerated storage, is sufficient to
prevent the growth of the psychrophilic organisms which would
otherwise grow and spoil the product.

Bacteria vary in their capacity to grow in the presence of
sodium chloride. Some of them are not able to grow in materials
containing less than 3 per cent salt, while others grow well even
in concentrations higher than 10%. Salted shrimps are spoilt
by salt-tolerant or halophilic bacteria of the genera Serratia,
Micrococcus, Bacillus, Achromobacter, Pseudomonas and others
(Frazier, 1958).

Constituents of the exoskeleton of crustaceans are chiefly
chitin and calcium carbonate. Seki (1965) described calcified
and uncalcified layers in the exoskeleton of crustaceans, the
latter of which is favourable for the decomposition of chitin
after the death of the crustaceans. Both layers contain chitin
which amounts to about 60 to 80 per cent of the dry weight of the

organic fraction of shrimps. During spoilage of shrimps, changes have been found in the exoskeleton of shrimps ranging from the softening and weakening of the shells to the gradual decomposition of the shells. There are few animals which can attack chitin and it is generally agreed that its degradation in nature is largely due to microbial action (Seki, op.cit.).

Shelling and beheading of shrimps form part of the processing. The carapace and shell of shrimps which are very rich sources of chitin are continuously dumped back into the sea as waste during processing. The chitin from this shrimp waste (and from other coelentrates, annelids, molluscs and arthropods) is probably utilised by biological agents, because little accumlates in marine sediments. If the chitin was not decomposed, its accumulation would soon cause a serious drain on the marine carbon and nitrogen (Zobell and Rittenberg, 1938). Research has continued to find new uses for chitin as a resource, and practical recovery methods are being investigated. Chitin is available in industrial quantity and is utilized in pharmaceuticals, food processing, paper formulation, cement factories, agriculture, and waste treatment (First International Conference, Chitin Chitosan, 1977).

Chitin is a tough, leathery substance. It is insoluble in the usual organic solvents, and requires strong mineral acid for its solution. Chitin is a continuous homopolymer of $B - D \ (1 \ 4) - N -$ acetylglucosamine (NAG) residues (Fig 1). It is extremely resistant to ordinary chemical or physical
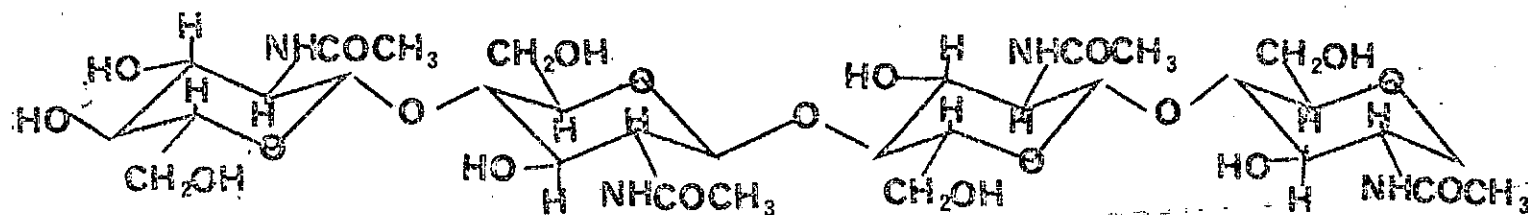
FIG 1   CHITIN

degradation under conditions found in the marine environment, requiring the enzyme chitinase to catalyse its breakdown.

.As part of the study of spoilage of shrimp, it is necessary to know which of the spoilage flora is chitinolytic. Also, the ability to degrade chitin can be used to characterise the organisms on the surface and in the gut of shrimps. The important role of these chitinolytic bacteria in decomposing the chitin of the exoskeleton of shrimps during spoilage and also in cycling nutrients in the biological economy of the sea necessitates further investigation into various parameters governing the isolation and characterization of these bacteria.

Since Benecke (1905) reported the isolation of Bacillus chitinovorous from the polluted waters of Kiel harbour, there have appeared numerous reports of the isolation of micro-organisms capable of decomposing chitin. Stormer (1908) observed chitin decomposition by a Streptomyces sp. Folpmers (1921) isolated two chitinolytic bacteria from water of the harbour of Bergen off Zoom and chitinolytic actinomycetes from the soil. Rammelberg (1931) isolated an identical organism to B. chitinovorous from manured garden soil. Stanier (1931) reported the presence of chitinolytic bacteria in lake water and showed that chitin was decomposed both aerobically and anaerobically. Johnson (1931) isolated a chitinolytic Myxococcus, which was antibiotic to

several <u>Ustilago</u> species. The same author (1932) reported the presence of chitinolytic micro-organisms from decaying exoskeletons of the hard shell crab (<u>Cancer magister</u>). Jensen (1932) reported an increase in the population of bacteria and actinomycetes in soil to which chitin was added. Among the fungi in this soil, <u>Mycogne nigra</u> and <u>Fusarium</u> species were prevalent. These fungi, as well as two actinomycetes, were isolated. These were able to grow and produce ammonia on chitin.

Benton (1935) isolated and classified 250 chitinolytic bacteria from mud, water and plankton of lakes. All of these bacteria were motile aerobes and most were Gram-negative. Benton (op.cit) listed a variety of sources from which chitinolytic bacteria were isolated: intestine of frogs, bats, snipe, speckled trout, mud from stagnant pools, sand under running water, soil, compost heaps, water of different lakes, decaying crayfish, and Mayfly nymph shells.

Bucherer (1935) isolated two chitinolytic bacteria from soil, one of them being <u>Bacillus chitinobacter</u>, a spore-forming rod. Several strains of chitinolytic actinomycetes isolated by Bucherer were identified by Waksman as <u>Streptomyces griseolus</u>, <u>S</u>. <u>exfoliatus</u> Waksman and Curtis, <u>S</u>. <u>fradiae</u> Waksman and Curtis, <u>S</u>. <u>aureus</u> Waksman and Curtis, and <u>S</u>. <u>griseus</u> (Krainsky) Waksman and Curtis. Stuart (1936) isolated eight halophilic chitinolytic

micro-organisms from solar salts of different parts of the world; five of them resembled <u>Serratia</u> <u>salinaria</u> Harrison and Kennedy, whereas the other three strains were similar to <u>Sarcinia</u> <u>littoralis</u> Paulsen. Hess (1937) isolated chitinolytic bacteria from live lobsters (<u>Homarus</u> <u>americanus</u>) that showed a disease of the exoskeleton. These bacteria were facultatively aerobic and did not digest cellulose or agar.

Aleshina (1938) reported chitin degradation by a mixture of sulphate-reducing organisms (but he probably had mixed cultures). Zobell and Rittenberg (1938) isolated 31 strains of chitinolytic bacteria from marine sources. Most of the strains were Gram-negative rods; one coccus and two species of <u>Vibrio</u> were also present. The authors reported the occurrence of bacteria which derived their nitrogen from chitin when other energy sources were not available, but no non-nitrogenous breakdown product of chitin was isolated. Other bacteria were found to degrade chitin in the presence of other carbon and nitrogen sources.

Schmidt-Lange and Bucherer (1938) inoculated a wide variety of micro-organisms pathogenic to man and animals on chitin agar plates. All of the chitinolytic organisms isolated were actinomycetes. Hock (1940) isolated two Gram-negative asporogenous rods from marine sources, <u>Bacterium</u> <u>chitinophilum</u> and <u>Bacterium</u> <u>chitinochroma.</u> Erickson (1941) studied ten strains

of actinomycetes of the species group Micromonospora chalceae (Foulerton) Ørskov isolated from lake mud. Stanier (1947) isolated a number of chitinolytic bacteria from enrichment cultures. All of the isolated strains were non-fruiting and belong to the Cytophaga johnsonae sp. Campbell and Williams (1951) isolated twenty strains of chitinolytic bacteria from marine mud. These strains comprised four new species of Achromobacter, two new species of Pseudomonas, one new species of Flavobacterium and one new species of Micrococcus.

VeldKamp (1955) did a review of chitinolytic micro-organisms and also isolated fifty strains of chitinolytic bacteria from the soil; among these organisms were Achromobacter, Flavobacterium, Chromobacterium, Bacillus, Cytophaga and Pseudomonas. Okutani (1966) isolated sixty-two strains of chitinolytic bacteria from the digestive tract of Japanese sea-base fish, Lateolabrax japonicus. The strains were Gram-negative asporogenous motile rods and were classified as Vibrio spp. and Aeromonas spp. Timmis, Hobbs and Berkeley, (1974) isolated anaerobic chitinolytic bacteria from chitin-enriched marine sediments. All isolates were species of Clostridia.

For a long time it was believed that the higher animals did not digest chitin. Abderhalden (Reference from Benton, 1935) called attention to the fact that the thinning of chitinous shells in the digestive tracts of fish is due to the acidity of the

gastric juice. The chitin is not destroyed, and can be recovered from the lower intestine. Some workers claimed to have detected chitinase in the alimentary tract of certain animals, but other investigators find no evidence that animals can digest chitin. However, the possibility of symbiotic bacteria aiding animals in the digestion of chitin should not be overlooked. Chitinolytic bacteria were isolated from the intestinal contents of several common marine animals. Zobell and Rittenberg (1937) found from a hundred to more than a thousand chitinolytic bacteria per cubic centimetre in the stomach contents of squids and other cephalopods, which ingest chitinous food. Okutani (1966) also reported chitinolytic bacteria from the digestive tract of the Japanese sea-base fish, Lateolabrax japonicus. These suggest the possibility that many bacteria play an important role as symbionts which aid animals in the digestion of chitin.

Lysozyme with chitinase activity have been reported from the blood and mucous of Plaice (Fletcher and White, 1937). Chitinase activity has been reported from the gastric mucosa of insectivorous fish (Okutani, 1966), the tissue, plasma and lymph of fishes (Fange, Lundbhad and Lind, 1976). The latter group of workers suggested that the high chitinase activity found in the pancreas of Chimaera monstrosa may be connected with the habit of the fish feeding on invertebrates such as shrimps, which possess chitin exoskeleton. The possibility of the chitinase

having a defensive function in fishes, offering protection against invading chitin-containing organisms (parasitic crustaceans and fungi) was suggested by Fange, Lundbhad and Lind (op.cit.).

The degradation of chitin by micro-organisms involves random hydrolyses of 1, 4-B-acetamido-2-deoxy-D-glucoside linkages in chitin by the enzyme chitinase (EC 3.2.1.14). Jeuniaux (1963) suggested that the complete enzymatic hydrolysis of chitin to free N-acetyl-D-glucosamine is performed by two
are
hydrolases, the actions of both/ consecutive, the chitinase and the chitobiase. The chitobiase hydrolyses diacetyl-chitobiose (the dimer of N-acetyl-D-glucosamine) and triacetyl-chitotriose. This enzyme chitobiase (EC 3.2.1.29) has now been included with B-N-acetylglucosaminidase (EC 3.2.1.30). The enzyme B-N-acetylglucosaminidase hydrolyses the terminal-non-reducing-2-acetamido-2-deoxy-B-glucose residues in chitobiose and glycoproteins.

Some lysozymes have been found to exhibit chitinase activity (Berger and Weiser, 1957). Lysozyme (EC 3.2.1.17) breaks down the membranes of certain bacteria; this process is a hydrolysis of B-1-4-links between N-acetylmuraminic acid and N-acetylglucosamine (Salton and Ghysen, 1960). The enzyme is also called N-acetyl-muramide-glycanohydrolase or Muramidase.

Chitinases have no muramidase activity and are thus distinguished from most lysozymes which possess muramidase as well as chitinase activity (Wadstrom and Hisatsune, 1970).

Jolles, Bernier, Berthou, Charlemagne, Faure, Hermann, Jolles, Perlin and Saint-Blanchard (1974) found that turnip lysozyme has weak chitinase activity while the hen lysozyme has none. Lysozyme activity was generally estimated from the bacteriolytic effect of suspensions of Micrococcus lysodeikticus (Shugar, 1952). Ballardie and Capon (1972) synthesized a new substrate for lysozyme, 3, 4-Dinitrophenyl-tetra-N-acetyl-chitotetraoside (3, 4-DNP-TNAC) (Fig. 2). This substrate was considered appropriate for investigation of the studies of chitinase activity, because it was prepared from acetylated chitin (NAG-4) reacted with sodium 3, 4-dinitrophenolate in dimethyformamide.

Chitinolytic bacteria grown on chitin agar show a clear zone around their colonies, indicating a breakdown of chitin by extracellular chitinase. Reynolds (1954) reported extracellular chitinase produced by a species of Streptomyces in agitated submerged culture. The enzyme was found to be inducible and its pH optimum value was between 6.5 and 7.5. Clarke and Tracey (1956) found a surprising number of human pathogenic bacteria producing chitinase and suggested that the chitinase may be a constitutive enzyme in many species of bacteria. Jeuniaux (1958) reported an optimum pH of 6.3 for Streptomyces griseus chitinase and an inhibition at a pH below 4.5. The value for Michealis constant for Streptomyces antibioticus chitinase was 0.010-0.011 gm/100 ml (Jeuniaux, 1957). Berger and Reynolds (1958) worked with a cell-free enzyme preparation of a strain of Streptomyces griseus grown in chitin liquid medium. The chitinase

<u>Fig. 2.</u>   <u>The chemical structure of the chromogenic</u>
<u>substrate 3, 4-DNP-TNAC</u>

The order of bond hydrolysis could be one of the
following:

     (a)   1, 3

           1, 2, 4

           1, 4, 2

     (b)   2, 4

           4, 2

     (c)   3, 1

The amount of colour released depended on the type
of cleavage of bonds.

       3, 1   Rapid colouration

       1, 3   Slower colouration

       2, 4   No colouration.
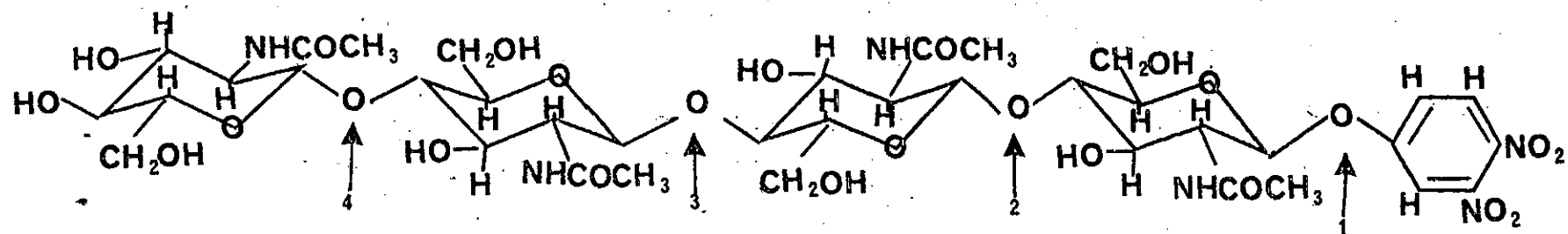
system was found to consist of two enzymes, chitinase and chitobiase.

Okutani (1966) reported an extracellular chitinase from Aeromonas chitinophthora and Vibrio gerris. The optimum pH values were 7.0 and 5.5 to 6.0 respectively. Reissert (1972) reported an optimum temperature of 25°C and pH 5.5 for chitinase from the fungus Chytriomyces hyalinus, Karling. The Michaelis Constant was 5.7 μ moles of reducing sugar released per mg of protein. Sundarraj and Bhat (1972) investigated chitin decomposition by Cytophaga johnsonii and found that, unlike other chitinolytic bacteria, some strains of this bacterium did not liberate chitinase extracellularly. Instead, the cells of such strains had need for close contact with the chitin particles in order to hydrolyse them. The partially purified chitinase was found to be most active at between pH 6.3-6.6 and at 40°C. Morrisey, Duggan and Koths (1976) reported chitinase production by an Arthrobacter spp. The enzyme was found to be inducible and optimal pH and temperature for enzyme activity were found to be 4.9 and 50°C respectively. Chigalenchik and Piriera (1976) reported extracellular chitinase from Aeromonas liquefaciens in the culture liquid containing demineralized crab shell as carbon and nitrogen source. The chitinase had maximum activity at pH 6.5 and 40°C.

A study of the bacteriology of the pink shrimp (<u>Penaeus</u> <u>duorarum</u>) from the time of catching through processing up to the time the product gets to consumers was undertaken. The information obtained from the study, is hoped, would make it possible to formulate bacteriological standards for this commercially important shrimp.

The aspects of the bacteriology of the shrimp that would be studied include:

(a) Quantitative and qualitative observations of the flora of freshly caught shrimps.

(b) The bacterial groups responsible for spoilage and their relative importance in the spoilage process.

(c) Determination of the physiological properties of the spoilage organisms.

(d) Isolation and characterization of chitinolytic bacteria from shrimps and sea water and the production, properties and purification of their chitinases.

MATERIALS AND METHODS

A. <u>Sources and collection of samples</u>

Fresh shrimps were collected from artisan fishermen at Ilubirin village, Lagos, within 12 hours after catch and taken to the laboratory in sterile insulated containers chilled with ice. The shrimps were caught in waters around Lagos and landed whole, uniced.

Between April 1975 and January 1976, the shrimps were caught off shore at fishing positions described below, using the Kiara fishing trawler of the Federal Department of Fisheries. The fishing positions which are off Lagos Bar included:

1st position, Lat 6° 19.5N - Long. 3°.28^E,

Depth 27 metres

Warp length, 180 metres, course 090° True.

The 2nd position was Lat 6° 16.7^N; Long. 30 369^E; Depth 45 metres; warp length 225 metres and course 270° True. The shrimps were emptied from the trawl unto the deck and were selected at random by means of sterile forceps, into sterile cans which were held in ice.

Examination of specimens was done between 8 - 10 hours after catch. The shrimps generally were given no wash other than

that incident to lifting the loaded trawl through the water.
Sea water samples for bacteriological examination were collected
at the same time as the shrimp samples.

Frozen shrimps (Plate 1) were supplied by Osadjere
Fishing Company, Lagos. Smoked shrimps were bought from local
processors at Adeniji Adele road, Ilubirin village, Elemankiri
village and Port Harcourt markets.

B. **Media.**

(1) **Brilliant green agar (modified) (BGA)**

Fifty-two gm of Oxoid brilliant green agar (modified)
(1973) were dissolved in 1 litre sea water, by boiling.
This was cooled to $50^{\circ}C$, mixed well and poured into plates.

(2) **Chitin Agar**

Yeast extract	–	0.1 gm
Bacteriological peptone	–	5.0 gm
Agar	–	1.5 gm
Colloidal chitin	–	1.0 gm
Filtered sea water	–	1000 ml
pH	–	6.0

Yeast extract, peptone and agar were dissolved in
sea water by steaming for 30 minutes.
The colloidal chitin was added to the molten medium.
The medium was sterilized at 1.1 $KgCm^{-2}$ for 15 minutes,
allowed to cool to $45^{\circ}C$, and poured into 9 cm diameter
Petri dishes.

Plate 1

(a) Whole shrimp

(b) Dissected shrimp exposing the gut.
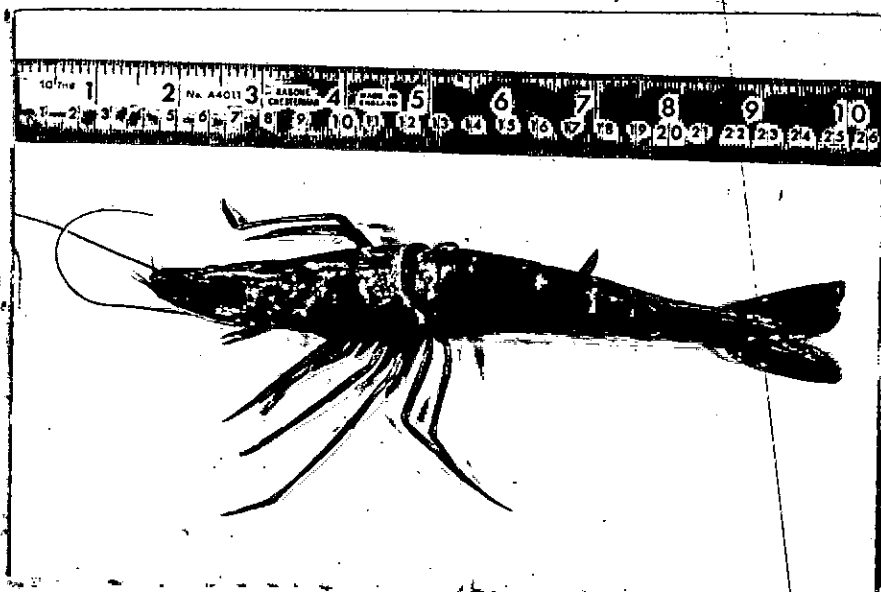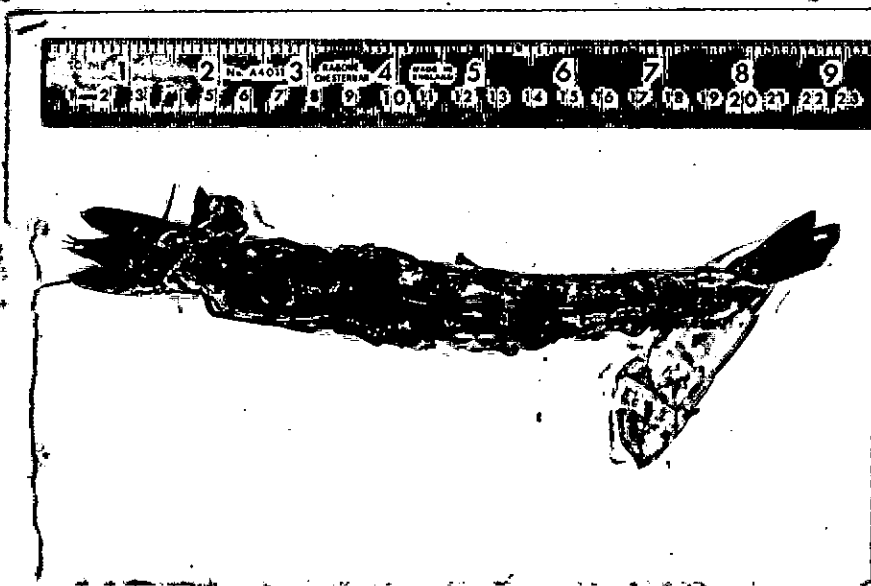
3. <u>Chitin enrichment medium</u>

K_2HPO_4 -   1.0 gm

$MgSO_4 \cdot 7H_2O$ -   0.5 gm

$CaCl_2$ -   0.1 gm

$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ - 0.005 gm

NH_4Cl -   0.1 gm

Filtered sea water - 1000 ml.

The mineral salts listed above were dissolved

in the sea water, and the pH of the medium adjusted

to 6.0. Strips of purified chitin from exoskeleton

of shrimps were added before autoclaving at 1.1 $kgCm^{-2}$

for 15 minutes.

4. Deoxychocolate citrate agar $(D.C.A)$ agar were dissolved

in 1 litre sea water. The suspension was boiled to

dissolve completely, and poured into plates.

5. <u>Glucose yeast extract agar</u>

Glucose   -   1.0 gm

Yeast extract   -   0.1 gm

Agar   -   1.5 gm

Filtered sea water   -   1000 ml

pH   -   6.0

Yeast extract and agar were dissolved in sea water by

steaming for 30 minutes and then sterilized at 1.1 $kgCm^{-2}$

for 15 minutes. Membrane-filtered glucose solution was added before pouring the medium into plates.

6. Hugh and Leifson's medium (1953)

Base:
Tryptone	2.0 gm
NaCl	5.0 gm
K_2HPO_4	0.3 gm
Agar	3.0 gm
Bromo-cresol purple (2% aqueous)	1.0 ml
Distilled water	1000 ml

The various components were dissolved and dispensed in 5 ml amounts in tubes and sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 10 minutes. Half a millilitre of a 10 per cent sterile glucose solution was added, mixed and allowed to set.

7. Hugh and Leifson's medium
Recommendations (1965)

Base:
Tryptone	1.0 gm
Yeast extract	0.1 gm
Glucose	1.0 gm
Bromocresol – purple	0.004 gm
Agar	0.2 gm
Distilled water	1000 ml.

The components were dissolved by steaming, and dispensed in 5 ml aliquots into test tubes and autoclaved at 0.73 $kgCm^{-2}$ for 20 minutes.

8. Litmus milk

One hundred gm of Oxoid litmus milk were stirred in 1 litre distilled water to dissolve. The suspension was filtered through cotton wool and dispensed in 5 ml amounts into tubes and sterilized by autoclaving at 0.73 $kgCm^{-2}$ for 5 minutes. The indicator was reduced when the medium was hot, but the mauve colour was restored on cooling.

9. MacConkey broth (purple)

	Single strength MacConkey broth (SS)	Double strength MacConkey broth (D.S.)
MacConkey broth powder	40 gm	80 gm
Distilled water	1000 ml	1000 ml.

To rehydrate the commercial preparation of the medium recommended quantities were dissolved in the distilled water and dispensed in 10 ml aliquots into test tubes fitted with Durham's tubes. The broth was sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes.

10. <u>Nitrate broth</u>

Potassium nitrate (AR grade)	0.2 gm
Tryptone	10.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml.

The potassium nitrate was dissolved along with the other chemicals in the water and dispensed in 5 ml aliquots in test tubes, each provided with an inverted Durham tube and sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes.

11. <u>Nutrient agar (Cm_3)</u>

Twenty eight gm of Oxoid nutrient agar powder was dissolved by boiling in 1 litre sea water. The suspension was sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes, and poured into 9 cm diameter Petri dishes.

12. <u>Nutrient broth (Cm_1)</u>

Thirteen gm of Oxoid 'Cm_1' powder were dissolved in 1 litre sea water and dispensed in 10 ml amounts into universal bottles. The suspension was sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes.

13. <u>Nutrient gelatin</u>

One hundred and twenty eight gm of Oxoid nutrient gelatin powder were dissolved in 1 litre distilled water by boiling and 10 ml were dispensed into each tube. The tubes were sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes.

14. <u>Peptone phosphate buffer</u>

$Na_2 HPO_4 12H_2O$	1.5 gm
KH_2PO_4	0.3 gm
Evans peptone	0.1 gm
Distilled water	1000 ml

The $Na_2 HPO_4$ and KH_2PO_4 were dissolved one after the other in water, before peptone was added. Four hundred ml aliquots of the suspension were dispensed into flasks and 9 ml aliquots into universal bottles for preparation of serial dilutions. Both the flasks and the dilution bottles were sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes.

15. <u>Salt mannitol agar $(S.M.A.)$</u>

One hundred and eleven gm of Oxoid S.M.A. powder were dissolved in 1 litre sea water by boiling. The solution was sterilized by autoclaving at 1.1 $kgCm^{-2}$ for 15 minutes and poured into 9 cm diameter Petri dishes.

16. <u>Sea water agar</u>

Fifteen gm of Oxoid agar were dissolved by steaming for 30 minutes in 1 litre of filtered sea water. The pH of the medium was adjusted to 6.0 and sterilized at 1.1 $kgCm^{-2}$ for 15 minutes.

17. <u>Sea water chitin medium</u>

Bacteriological peptone	-	5.0 gm
Yeast extract		0.1 gm
Chitin (Sigma)	-	1.0 gm
Filtered sea water	-	1000 ml
pH	-	6.0

Two hundred and fifty ml of medium were dispensed in each 500 ml conical flask. The flasks were incubated at $25^{o}C$ on a rotary shaker at a speed of 138 r.p.m.

18. <u>Sea water peptone yeast extract agar (SWPYA)</u>

Peptone	-	5.0 gm
Yeast extract	-	0.1 gm
Agar	-	1.5 gm
Sea water	-	1000 ml

The components were dissolved in sea water by steaming for 30 minutes, and then sterilized at 1.1 $kgCm^{-2}$ for 15 minutes. The medium was allowed to cool to $45^{o}C$ and then poured into 9 cm diameter Petri dishes.

19. <u>Selenite broth</u>

Nineteen gm of Oxoid Selenite broth base were dissolved in 1 litre distilled water to which 4 gm of sodium biselenite (Oxoid) powder have been added. The mixture was dispensed in 200 ml into flasks and sterilized by boiling in a water bath for 10 minutes.

20. <u>Sodium azide medium (Hannay and Norton) 1947</u>

	<u>Single strength</u> (<u>S.S.</u>)	<u>Double strength</u> (<u>D.S.</u>)
Peptone	10.0 gm	20.0 gm
NaCl	5.0 gm	10.0 gm
K_2HPO_4	5.0 gm	10.0 gm
KH_2PO_4	2.0 gm	4.0 gm
Glucose	5.0 gm	10.0 gm
Yeast extract	3.0 gm	6.0 gm
Sodium azide	0.25 gm	0.5 gm
Bromocresol purple (1.6% ethanolic solution)	2.0 ml	4.0 ml
Distilled water	1000 ml	1000 ml

The components were dissolved in sea water by boiling.
The pH was adjusted to 7.3. The mixture was dispensed
in 10 ml amounts into tubes and autoclaved for 15 minutes
at 1.1 kgCm^{-2}.

21. <u>Sulphide indole motility medium (S.I.M.)</u>

Beef extract	3.0 gm
Trypticase	30.0 gm
Sodium thiosulphate	0.05 gm
Cysteine hydrochloride	0.02 gm
Ferric ammonium citrate	0.5 gm
Sodium chloride	30.0 gm
Agar	5.0 gm
Sea water	1000 ml.

All the components were dissolved in sea water by boiling. The pH was adjusted to 7.3. The mixture was dispensed in 10 ml amounts into tubes and autoclaved for 15 minutes at 1.1 kgCm^{-2}.

22. <u>Starch agar medium</u>

Soluble starch   0.2 gm

Molten nutrient agar (Cm$_3$)   100 ml

The soluble starch component was dissolved in the molten nutrient agar and sterilized by autoclaving at 1.1 kgCm^{-2} for 15 minutes. The mixture was poured into 9 cm diameter Petri dishes.

23. <u>Thiosulfate citrate bile salt agar (TCBS)</u>

Eighty-eight gm of Oxoid TCBS agar powder were dissolved in 1 litre sea water by boiling. The mixture was poured into 9 cm diameter Petri dishes, without further heating.

24. <u>Tryptone water</u>

Twenty gm of Oxoid tryptone powder were dissolved in 1 litre sea water, dispensed into tubes in 10 ml amounts and sterilized by autoclaving at 1.1 kgCm^{-2} for 15 minutes. For salt tolerant tests, distilled water was used for preparing the tryptone water.

C. Reagents

- 1. Acetylacetone reagent

    To 0.1 ml of pure acetylacetone (2, 4-
pentanedione) was added 50 ml of sodium carbonate
solution (27 $gm\,l^{-1}$) in a stoppered flask. The compo-
nents were mixed and used within 15 minutes of
preparation.

2. Copper reagent

    Five gm of copper sulphate ($CuSO_4.5H_2O$) were
dissolved in 1 litre solution of 1% (2/v)
tartaric acid.

3. Dinitrosalicylic acid reagent (DNS Reagent)

    Stock solutions required:

    1. 4.5% sodium hydroxide

    2. 10% sodium hydroxide

    3. 1% dinitrosalicyclic acid (DNS).

    Eight hundred and eighty ml of 1% DNS acid
solution were added to 255 gm of Rochelle salt
(potassium sodium tartarate) and 300 ml of 4.5%
sodium hydroxide solution. Twenty-two ml of 10%
sodium hydroxide solution were added to 10 gm of
crystalline phenol, and diluted to 100 ml.
To 69 ml of the latter was added 6.9 gm

of sodium bisulphite and to this mixture was added

the dinitrosalicylic acid solution. The reagents were

mixed and shaken until all the Rochelle salt had

dissolved. The preparation was kept in tightly stoppered

bottles.

4.   Dimethyl-alpha napthylamine solution.

To 100.0 ml of glacial acetic acid and 250 ml

of distilled water was added 2.1 ml of pure dimethyl-

alpha-napthylamine in a stoppered flask.

5.   Ehrlich's reagent (for colorimetric estimation of indole)

To 0.4 gm of purified p-dimethyl-amino-benzaldehyde

was added 5 ml of acid and 3 ml of a 5% hydrochloric

acid solution. The mixture was stored in a dark

bottle in the refrigerator.

6.   Erhlich's reagent (for chitin estimation)

A quantity of 0.80 gm p-dimethylaminobenzal-

dehyde was dissolved in a mixture of 30 ml of 95%

ethanol and 30 ml of concentrated hydrochloric acid.

This solution was stored in the refrigerator in a

well-stoppered glass bottle, and was stable for two

weeks.

7. <u>Ehrlich's - Bohme's reagent (for indole test)</u>

Four gm of para-dimethyl-amino-benzaldehyde were dissolved in 380 ml of 96 per cent ethanol by gentle warming in a 50°C water bath. The mixture was cooled before adding 80 ml of concentrated hydrochloric acid. The reagent was stored in a dark bottle in the refrigerator.

8. <u>Folin and Ciocalteu's phenol reagent</u>

Folin and Ciocalteu's phenol reagent was diluted 1 to 5 with distilled water immediately before use.

9. <u>Kovacs Oxidase reagent (1956)</u>

One gm tetra-methyl-p-phenylenediamine was dissolved in 100 ml of distilled water and stored in a dark bottle in the refrigerator.

10. <u>Sodium carbonate solution (4%)</u>

Forty gm of analytical reagent quality anhydrous sodium carbonate were dissolved in 1 litre distilled water.

11. <u>Sulphanilic acid solution</u>

To 2.8 gm of sulphanilic acid were added 100 ml of glacial acetic acid and 250 ml of distilled water. The mixture was stored at room temperature.

D. Analytical procedures

1. Homogenisation

Hundred gm of the shrimps were homogenised ascoptically in a sterile steel blender, in 400 ml sterile chilled peptone phosphate buffer solution. The homogenate was placed at $4^{o}C$ for 30 minutes to allow the particulate matter to settle and the bubbles to disperse. To obtain gut homogenate, guts of shrimps were ascoptically teased out into a flask containing sterile chilled peptone phosphate buffer solution. Sterile beads were added into the flask which was shaken vigorously for 5 minutes to homogenise the gut.

2. Total aerobic viable count (TVC)

Serial dilutions of homogenate were prepared using the peptone phosphate buffer as the diluent (Cann, Hobbs, Wilson and Horsley, 1971). Triplicate aerobic counts were made on Oxoid nutrient agar ___ (Cm_3) using the modified Miles and Misra (1938) technique of Appleman, Bain and Shewan, (1964). A sample of 0.1 ml of each dilution was pipetted and dispensed in drops on to nutrient agar (Cm_3) plates. Triplicate plates were incubated for 3 days at $30^{o}C$ or $37^{o}C$ and for five days at $20^{o}C$. Total bacterial counts were enumerated from the Cm_3 plates.

3. Flora analysis (isolation and identification)

For each sample, 100 colonies were picked from TVC plates. The picked colonies were selected by means of random number tables (Lindley and Miller, 1962), and purified for further study. The isolates were identified by means of the following characteristics suggested by Hendrie, Mitchell and Shewan, (1968):

Medium	Observations
Cm_3	Colony appearance
	Gram stain
	Morphology
	Oxidase test (Kovacs 1956).
Nutrient broth (Cm_1 (Oxoid)	Motility
Hugh and Leifson's medium (1953)	Dissimilation of carbohydrate.

The scheme is outlined in Fig.3. The modifications introduced in the scheme during the present study are shown with broken lines. The modifications permit the differentiation of Staphylococcus and Micrococcus by means of their reaction in Hugh and Leifson's medium. The tests outlined above are described in detail below:

DIAGNOSTIC KEY FOR THE IDENTIFICATION OF CERTAIN ISOLATES.

FIGURE: 3

Gram Reaction

Morphology

Morphology Rod

Coccus.

Rod: displaying club shaped, chinese characters and 'V' pairs.

Rod: Sporing

Motile.

Non motile.

"Coryneform organisms"

Bacillus

Polar flagella kovacs oxidase positive.

Peritrichous flagella stain

No pigment

Yellow or orange pigment.

Fermentative in Hugh and Leifsons medium (glucose)

Oxidative or alkaline in Hugh and Leifsons medium (glucose)

Oxidative or alkaline in Hugh & Leifson's medium (glucose)

Kovacs oxidase negative Fermentative in Hugh & Leifson's medium (glucose)

Kovacs oxidase.

Staphylococcus.

Micrococcus.

Moraxella*

Acinetobacter?

Flavobacterium cytophaga.

Fermentative in Hugh and Leifson's medium (glucose)

Not fermentative in Hugh and Leifson's medium (glucose)

Enterobacteriaceae

Kovacs oxidase positive Not fermentive in Hugh and Leifson's medium (glucose)

Kovacs oxidase negative Fermentative in Hugh and Leifson's medium (glucose)

Enterobacteriaceae

Acid only in glucose Insensitive to compound O/129.

Acid only in glucose insensitive to compound O/129.

Acid and gas in glucose medium. insensitive to compound O/129.

Vibrio

Aeromonas Formicans

Aeromonas

3-Ketolactose not pro-duced in Bernaerts & Delay's medium.

3-Ketolactose produced in Bernaerts & Delay's medium.

Alcaligenes

Agrobacterium.

Oxidative in Hugh & Leifson's medium (glucose)

No acid in Hugh & Leifson's medium (glucose).

Pseudomonas Gp. III and IV.

Green fluorescent pigment produced

No fluorescent pigment produced

Pseudomonas Gp. I.

Pseudomonas Gp. II.

*Formerly classified as Achromobacter.

(a) Colonial appearance:

     After inoculation of the medium and after incubation, the colonial appearance of the isolates was observed and recorded. The features of growth considered when recording the colonial appearance were:

(i) Size

(ii) Margin or edge (smooth, rough or thread-like).

(iii) Elevation (thin, flat or raised colonies).

(iv) Pigmentation (various shades of yellow, orange, pink).

(v) Optical features (opaque or transparent).

(b) Morphology:

     The morphology of the isolates was determined from the Gram stain preparations. The shape and arrangement of bacterial cells were noted.

(c) Oxidase test (Kovacs 1956):

     A loopful of Kovac's oxidase reagent was added to a filter paper in a Petri-dish. By means of a platinum loop the colony from the isolate was smeared across the paper. Positive reaction was indicated by a purple colony appearing across the streak in 10 seconds.

(d) <u>Motility</u>:

Motility was observed by a phase contrast microscope and by the study of growth in sulphide indole motility (S.I.M.) medium. Stab inoculation was used to detect motility. With motile bacteria, growth proceeds away from the line of inoculation, whereas non-motile bacteria grow only along the lines of inoculation.

4. <u>Dissimilation of carbohydrates (oxidation or fermentation of sugar using Hugh and Leifson's medium 1953)</u>

The medium was steamed for 10-15 minutes to expel dissolved oxygen, and allowed to solidify by placing the tubes in cold water. Two tubes of Hugh and Leifson's medium were inoculated from each isolate. The surface of one of the tubes was covered with a 1-inch layer of sterile liquid paraffin to maintain anaerobic condition. Oxidation of glucose, indicated by acid production, was recorded from tubes without paraffin. Fermentation of glucose was recorded as positive/if acid production occurred, (with or without gas) formed from the content of the two tubes. If no change in the colour of the medium occurred, or the medium became alkaline, it was recorded as inability to utilize glucose.

5. <u>Anaerobic utilization of glucose by Staphylococcus</u>
   <u>and Micrococcus, using Hugh and Leifson's medium</u>
   <u>modified.</u> (Recommendations, 1965)

   Methods of inoculation and interpretation of tests
   were the same as described above for the dissimilation
   of carbohydrate.

6. <u>Determination of coliform count</u>

   Presumptive coliform count was obtained by the
   standard 'Most Probable Number' technique and confirmed
   as described in the Bacteriological examination of water
   supplies (Department of Environment (1969) - Report on
   public health and medical subjects, No. 71).
   The following sets of experiments were set up:

   (a) Five tubes containing 10 ml double strength
       MacConkey broth and equal volume of shrimp
       homogenate.

   (b) Five tubes containing 10 ml single strength
       MacConkey broth and 1.0 ml of shrimp homogenate.

   (c) Five tubes containing 10 ml single strength MacConkey
       broth and 1.0 ml of a 1:10 dilution of shrimp
       homogenate.

   The inoculated broths were incubated at $37^{o}C$ and
   examined after 24 hours and 48 hours for acid and
   gas production. Using 'The Most Probable Number'
   tables, the MPN of coliforms/100 gm was estimated
   from the number of positive reactions.

7. Determination of Escherichia coli

Each positive tube of the coliform count experiment was subcultured into a preheated $(44^{o}C)$ tryptone water broth and 'SS' MacConkey broth. The broths were incubated at $44^{o}C$ for 24 hours. Acid and gas production in the MacConkey broth and indole production in the tryptone broth were generally regarded as diagnostic for E. coli. E. coli count/100 gm was derived from the number of positive tubes obtained using the MPN tables.

8. Determination of faecal streptococci count

The standard 'MPN' method was employed using the 'DS' sodium azide broths. The presence of faecal Streptococcus was indicated by an acid reaction. All tubes positive for Streptococcus test at $37^{o}C$ were confirmed by subculturing into more 'SS' sodium azide broths and incubated at $45^{o}C$ for 48 hours. Positive tubes giving an acid reaction at this temperature are regarded as being true faecal Streptococcus and MPN was estimated, using the 'MPN' tables.

9. Detection of Vibrio cholera, Vibrio paraheamolyticus and Vibrio alginolyticus

Each sample after homogenisation was directly plated out on commercial preparation of T.C.B.S. agar. On this medium, V. paraheamolyticus colonies were bluish green, V. cholera and V. alginolyticus were yellowish. Colonies with the typical appearance of any of these two organisms were further identified using the biochemical tests.

10. Detection of Salmonella

Two hundred ml. of the shrimp homogenate were added to 200 ml of double strength Selenite broth, and incubated at $37^{\circ}C$. After 48 hours incubation, a loopful of the culture was plated out on to D.C.A. and Brillant green agar. These plates were incubated at $37^{\circ}C$ for 24 hours. On these media Salmonella gave the following characteristic reaction:

D.C.A.   Colourless colonies surrounded by an orange-yellow zone of medium.

B.G.A.   Pink colonies surrounded by a bright red medium.

11. Detection of Staphylococcus aureus (S. aureus)

A 0.1 ml sample of shrimp homogenate was pipetted on to Salt mannitol agar (S.M.A.). To allow adequate

development of the colonies and their characteristic reaction, the S.M.A. plates were incubated at 37°C for 48 hours. On this medium <u>Staphylococcus</u> <u>aureus</u> produced colonies with bright yellow zones, while coagulase negative <u>Staphylococcus</u> was surrounded by a red or purple zone. Suspect colonies of <u>S</u>. <u>aureus</u> were inoculated on to blood agar plates and incubated at 37°C for 24 hours.

Coagulase test:

A loopful of sterile distilled water was placed on a slide. The suspect colony from the blood agar plate was emulsified on the slide with a loopful of human plasma added to it. A positive reaction was denoted by the coagulation of the cells within 10 seconds.

12. ## Thermal inactivation of bacteria

Nutrient broth was inoculated with isolates to be tested, and incubated at 37°C for 72 hours. The broth cultures were then plated out on Cm_3 plates to check for growth. The broth cultures showing growth were subsequently heated at seven different temperatures: 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C for 24 hours in a regulated water bath. The heated cultures were plated out and incubated at 37°C and observed for growth every 24 hours for 72 hours.

13. <u>Investigation of flora of frozen shrimps</u>

Shrimps caught on board Kiara by trawling were held
in sterile containers in a cooler (filled with crushed
ice) and brought to the laboratory. The interval between
catching and the start of the experiment was at most
10 hours. One hundred gm of shrimps were examined for
their bacterial load in the laboratory before freezing
the rest. Shrimps for freezing were packed in sterile
polythene bags which were sealed up with a tape. The
bags were kept in a freezer at $0^{o}C$ to $3^{o}C$. The shrimps
were brought out at intervals of 7 days, and left on
the bench to thaw before carrying out bacteriological
analysis.

14. <u>Investigation of spoilage flora of shrimps</u>

One hundred gm of whole frozen shrimps were weighed
out into eight sterile flasks. Four of the flasks were
kept in a cooler filled with crushed ice to maintain a
chilled temperature. The cooler was drained daily and
refilled with crushed ice. The remaining four flasks
were kept on the bench at room temperature. Samples
were taken as follow from each batch: a flask each was
removed from the cooler after 3, 6, 9, 12 days for
bacteriological analysis. A total of eight hundred

isolates were picked from the spoilage flora and tested
for their spoilage potentiality using proteolysis and
indole production as chemical indices.

15. Test for proteolysis

Test for the ability of the bacteria to hydrolyse
proteins e.g. gelatin was carried out. Inoculation into
nutrient gelatin was by a stab with a heavy growth from
a 12-hour old Cm_1 culture. Incubation was at $35^{\circ}C$ for
3 days after which tubes were immersed in iced water
and liquefaction recorded. Tubes with the cultures
remaining fluid were recorded as positive.

16. Test for indole production

Two per cent tryptone water was inoculated with a
12-hour nutrient broth culture and incubated at $30^{\circ}C$ for
2 days. Five ml ether were added to the tube to extract
the indole, and 0.5 ml of Ehrlich's-Bohmes reagent was
then added by running it down the side of the tube. A
positive result was indicated by development of rose
purple colour.

17. Inoculation experiments for indole and trimethylamine (T.M.A.) production.

Pure colonies of known bacteria were inoculated into
universal bottles each containing 10 ml nutrient broth and

incubated for 12 hours. Homogenised shrimps were
distributed in 20 gm amount into previously weighed
and labelled glass Petri dishes. All the Petri dishes
were wrapped up with aluminium foil and autoclaved at
1.1 $kgCm^{-2}$ for 15 minutes to sterilise the homogenates.
The dishes were left to cool at room temperature and
inoculated with 12-hour broth cultures of the following
bacteria:

(1) _Bacillus_ sp. A.

(2) _Bacillus_ sp. B.

(3) _Pseudomonas_ II

(4) _Aeromonas_ sp. A

(5) _Aeromonas_ sp. B.

(6) _Aeromonas_ sp. C

(7) _Aeromonas_ sp. D

(8) _Aeromonas_ sp. E

(9) _Aeromonas_ sp. F

(10) Enterobacteriaceae sp. A

(11) Enterobacteriaceae sp. B

(12) Enterobacteriaceae sp. C

(13) Enterobacteriaceae sp. D

(14) Enterobacteriaceae sp. E

(15) _Moraxella_ sp.

(16) Coryneform sp. A

(17) Coryneform sp. B

(18) Coryneform sp. C

(19) Coryneform sp. D

(20) Coryneform sp. E

(21) Coryneform sp. F

(22) Coryneform sp. G

(23) Coryneform sp. H

(24) Coryneform sp. I

(25) Coryneform sp. J

(26) Coryneform sp. K

(27) _Staphylococcus_ sp. A

(28) _Staphylococcus_ sp. B

(29) _Staphylococcus_ sp. C

(30) _Staphylococcus_ sp. D

(31) _Staphylococcus_ sp. E

(32) _Staphylococcus_ sp. F

(33) _Micrococcus_ sp.

(34) All Isolates (1 gm unsterilized) shrimp
　　　　　　　　homogenate).

Two sterilized shrimp homogenate samples were inoculated with 1 ml from each 12-hour broth culture. Also two sterilized shrimp homogenate samples were inoculated each with 1 gm of unsterilized shrimp

homogenate. Two sterilized homogenate samples were left uninoculated and used as the control, one for T.M.A. and the other for indole.

All samples were incubated at $37^{\circ}C$ for 44 hours after which the quantities of T.M.A. and indole produced by each bacteria were determined.

18. <u>Determination of trimethylamine nitrogen (Okolo's 1977 modification of Hart and Fischer (1971) method)</u>

<u>Materials:</u>

(1) <u>Toluene</u>:

Toluene was shaken with 0.5 M sulphuric acid, distilled and dried over anhydrous sulphate.

(2) <u>Picric acid stock solution:</u>

0.02 gm of dry picric acid was dissolved in 100 ml of dry toluene.

(3) <u>Picric acid working solution:</u>

Dilute 1 ml of the stock solution to 100 ml with dry H_2O free toluene.

(4) <u>Formaldehyde solution:</u>

Forty per cent formaldehyde was shaken with magnesium carbonate and filtered. 10 ml of the filterate was diluted to 100 ml with water.

(5) <u>Trimethylamine stock solution</u>:

    0.682 gm of trimethylamine hydrochloride was dissolved in 1 ml of 25 per cent hydrochloric acid and diluted to 100 ml with water. This contained 100 gm T.M.A.N per ml.

(6) <u>Trimethylamine standard solution</u>:

    One ml of the stock solution was added to 1 ml hydrochloric acid and diluted to 100 ml with water. The solution contained 0.01 gm T.M.A. per ml.

<u>Procedure</u>:

    Ten gm of inoculated shrimp homogenate were weighed and added to 30 ml of 5 per cent trichloracetic acid solution (T.C.A.). The suspension was filtered and 2 ml of the filtrate were pipetted into a test tube and diluted to 7 ml. Forty per cent formaldehyde reagent, 10 ml of toluene and 3 ml of 10 per cent potassium carbonate solution, were all added to the filtrate. The tube was shaken vigorously and allowed to settle. Ten ml of the toluene layer were then transferred gently into another tube through a funnel lined with cotton wool containing about 0.3 gm of anhydrous sodium sulphate to dry the toluene extract. Five ml of the toluene extract were

pipetted into a dry test tube and 5 ml of picric acid working solution were added. The absorbance of the solution was determined at 410 nm on a S.P. 600 spectrophotometer using (1) a reagent blank of 5 ml of picric acid working solution and 5 ml of toluene, (2) a mixture from the control substrate set up from an uninoculated sterilized shrimp homogenate. The results were expressed in absorbance units per 100 gm of shrimp muscle. The data obtained using the control as blank was used for the histograms. This would take care of chemical changes that might have occurred during the sterilization of the shrimp homogenate by autoclaving.

19. Determination of indole (Jacobs, 1965)

Materials:

(1) Colour reagent: Ehrlich's reagent.

(2) Chloroform

(3) Ammonium sulphate

(4) Phosphoric acid

(5) Glacial acetic acid

(6) Sodium hydroxide.

Procedure:

Ten gm of inoculated shrimp homogenate were mixed with 10 ml of 10 per cent NaoH in a 400 ml beaker. One hundred ml of chloroform were added. The homogenate was transferred to a conical flask and mixed thoroughly with 10 gm of ammonium sulphate. The lower chloroform layer was passed through a folded filter paper into a clean dry container and 25 ml of the chloroform extract were pipetted into a 250 ml separating funnel. To this was added 1 ml of Ehrlich's reagent and 5 ml of phosphoric acid. The mixture was shaken vigorously for 30 seconds; and allowed to stand for 5 minutes. Fifteen ml of glacial acetic acid were added, the funnel was shaken and the contents allowed to settle. The lower layer was tapped into a 50 ml volumetric flask and diluted to mark with acetic acid. The absorbance of the solution was read at 560 nm on a SP 600 spectrophotometer against a reference consisting of (1) a mixture of 1 ml Ehrlich's reagent, 5 ml of phosphoric avid and 15 ml of glacial acetic acid and (2) a mixture from the control substrate set up from uninoculated sterilized shrimp homogenate. The results were expressed in absorbance units per 100 gm of shrimp muscle.

20. Detection of nitrate reduction

Twelve-hour old broth cultures of bacteria were inoculated into nitrate broths and incubated at $37^{\circ}C$ for 3 days. The

cultures were tested for the presence of nitrate by adding a drop each of sulphanilic acid solution and dimethyl-alpha-naphtalmine solution. The development of a red or brown colour denoted the presence of nitrite.

21. Detection of Litmus reduction and clotting

Sterile litmus milk broth was inoculated with a 12-hour nutrient broth culture and incubated at $35^{o}C$ for 4 days. Results were recorded under the following headings:

(A) Breakdown of lactose which was indicated by:

    (1) acid production (A) shown by a change in the colour of the litmus from mauve to pink,

    (2) acid clot (AC) if sufficient acid was produced to make the milk clot,

    (3) reduction of litmus (R) and loss of colour,

    (4) gas production (G).

(B) Hydrolysis of casein which was indicated by:

    (1) Coagulation of the milk

    (2) peptonisation

    (3) utilisation of citrate shown by a change of colour to deep blue.

22. Test for hydrolysis of starch

Starch agar plates were inoculated by streaking once across the surface of each plate the isolate, and incubated for 4 days at $35^{o}C$. At the end of incubation period, the

surface of the plate was flooded with 10 ml of Gram-iodine solution. Hydrolysed starch appeared as a clear zone as a result of B amylase activity of the isolates.

23. Test for salt tolerance

Isolates were picked by means of using sterile tooth-picks into nutrient broths and incubated for 24 hours at 37°C. Two per cent tryptone water was supplemented with various concentrations of NaCl (1 per cent to 30 per cent concentration of NaCl were used). The solutions were inoculated with 0.1 ml. of the broth cultures of selected organisms. The cultures were incubated for four days at 37°C and growth checked by means of turbidity determination and recorded.

24. Chitin preparation.

(a) Preparation of chitin from exoskeleton of shrimps

Five gm of the exoskeleton of shrimp, Penaeus duorarum, was thoroughly washed in 100 ml warm water and decalcified in the refrigerator in 1 per cent (v/v) hydrochloric acid which was changed every day for 4 days. The preparation was then washed and placed in a 2 per cent (w/v) potassium hydroxide for 10 days, and on 4 occasions brought to boil and allowed to cool. This was washed free from alkali 10 times by changes of distilled water, and cut in strips and extracted repeatedly with

boiling ethanol 4 times for 1 hour. The extracted chitin was dried in the oven at 70°C (Benton, 1935).

(b) Preparation of colloidal chitin

Crude unbleached chitin (Calbiochem Ltd.) was washed 8 times alternately for 24 hours with 500 ml of 1N sodium hydroxide and 500 ml of 1N HCl for every 100 gm This was held at 4°C during washings, and rinsed 5 times with distilled water. This was later washed with 95% (v/v) ethanol 4 times. Fifteen gm of the cleaned material were moistened with acetone and dissolved in 100 ml of cold concentrated hydrochloric acid by stirring for 20 minutes in an ice bath (to arrest hydrolysis). This was filtered through a thin glass wool pad in a Buchner funnel into 2 litres of stirred ice-cold distilled water. The treatment precipitated the material as a fine colloidal suspension. The original extracted residue was re-dissolved and refiltered until no more chitin was precipitated (usually three times). The precipitated chitin was washed in distilled water and centrifuged until the pH was that of distilled water. The precipitated chitin was preserved against bacterial action by storing in the refrigerator at 4°C (Lingappa and Lockwood, 1962).

(c) Determination of dry weight of colloidal chitin

A sample of precipitated chitin paste was freeze-dried overnight in an Edwards Freeze-Drier. One gm of precipitated chitin paste treated as above when freeze-dried yielded about 30 mg of chitin powder.

25. Isolation and classification of chitinolytic bacteria from shrimps

Frozen shrimps (P. duorarum) were left in polythene bags to thaw. A sample of 100 gm of shrimps was washed thoroughly in 400 ml of sterile chitin enrichment liquid medium in 1 litre conical flask by shaking the flask continuously by hand for 5 minutes. The liquid medium was decanted into a sterile 500 ml flask and the shrimps discarded.

The shells of another batch of 100 gm shrimps were ascceptically removed and added to a second 500 ml flask containing 400 ml of sterile chitin enrichment medium.

The guts from 50 gm of shrimps (Fig. 3) were teased out into a sterile Petri dish and both the gut wall and gut contents were added to another flask of sterile chitin enrichment medium.

Samples from the three different flasks were streaked on to chitin agar plates in triplicates and one plate from each preparation was incubated at $20^{\circ}C$, 30° or $37^{\circ}C$.

This procedure was repeated for freshly caught shrimps. The inoculated flasks containing chitin enrichment medium and shrimp washing were incubated at 22°C for 14 days on a rotary shaker at a speed of 138 r.p.m. Serial dilutions using peptone phosphate buffer were made from the chitin enrichment cultures after incubation. A sample of 0.1 ml of each dilution was pipetted on to each of three chitin agar plates and incubated at 20°C, 30°C or 37°C until haloes appeared around the bacterial colonies.

To isolate chitinolytic bacteria from sea water samples of freshly collected sea water were streaked on chitin agar plates, and incubated at 20°C, 30°C or 37°C. Hundred ml of sea water were added to 100 ml of the sterile chitin enrichment medium, and incubated at room temperature for 14 days. Serial dilutions were made from the chitin enrichment culture. A sample of 0.1 ml of each dilution was pipetted on to a chitin agar plate. The inoculation was also done in triplicate. Colonies of bacteria which formed clear zones on the chitin plates were isolated and purified. Pure isolates were obtained by successive subculturing on chitin agar. The isolates were maintained on sea water agar and chitin agar slants.

(b) Identification and classification of isolated bacteria

Colonies were picked from the mixed cultures and purified by repeated subculturing for further study. The morphological and biochemical characteristics of the bacterial species were examined. Pure cultures grown on sea water agar for 24 hours were used for the test listed below:

(a) Cell form and Gram-reaction

(b) Colonial characteristics

(c) Motility

(d) Indole production

(e) Methyl-red test

(f) Voges - Proskauer tests

(g) Nitrate reduction

(h) Oxidation and fermentation of glucose.

(i) Hydrogen sulphide production

(j) Catalase production

(k) Gelatin hydrolysis

(l) Hydrolysis of starch

Some isolates were sent to the National Collection of Marine Bacteria, Torry Research Station, Aberdeen, and some Gram-positive sporing rods were sent to Dr. R.C.W. Berkeley in Bristol for confirmation of identification.

26. <u>Optimum conditions for production of chitinase on solid media</u>

(a) <u>Effect of pH value of media on the growth and chitinase</u>
<u>production of Vibrio alginolyticus</u>

During preparation of chitin agar plates the pH
was adjusted to the required pH (ranging from 5.0-8.0)
by the addition of either 0.1NHCL or 0.1N NaOH.
A loopful of broth culture of <u>V. alginolyticus</u> was
streaked on the chitin agar plates which were incubated
at $20^{\circ}C$ for 10 days.

(b) <u>Effect of temperature of incubation on growth and</u>
<u>chitinase production of V. alginolyticus</u>

A loopful of broth culture of <u>V. alginolyticus</u>
was streaked on the following solid media:

(i) SWPYA agar plates with 1 per cent colloidal chitin.

(ii) SWPYA agar plates with 1 per cent colloidal chitin.
and 0.1 per cent glucosamine hydrochloride.

(iii) SWPYA agar plates with 1 per cent glucose.

(iv) SWPYA agar plates with 0.1 per cent glucosamine
hydrochloride.

The pH of the SWPYA media was adjusted to 6.0 before
sterilization. Glucosamine hydrochloride and glucose
were sterilised by means of a Millipore filter (0.45 µm pore),
and added to the other constituents of the medium. A
check was carried out on the pH of the medium after
adding glucose or glucosamine hydrochloride, and the
changes in pH values were less than 0.2 units. The
plates were incubated at $20^{\circ}C$ and examined daily for

growth and production of extra-cellular chitinase,

shown by zones of clearing around the colonies.

27. Extraction and purification of enzyme

After the required time of incubation, the broth

culture was centrifuged at 7000 r.p.m. for 30 minutes in

a refrigerated centrifuge. The culture supernatant was

concentrated and salted out by adding ammonium sulphate

(80% saturation). To 250 ml of culture supernatant was

added 140.24 gm of ammonium sulphate. This was left in the

refrigerator overnight and the precipitated fraction was

obtained by centrifugation at 7000 r.p.m. for 30 minutes at

4°C. The precipitate was dissolved in 15 ml of distilled water.

This solution was centrifuged at 15,000 r.p.m. for 30 minutes

and the supernatant solution was used as the stock enzyme

solution. The use of ammonium sulphate for separation and

purification of proteins was described by Nozaki and Hayashi

(1971).
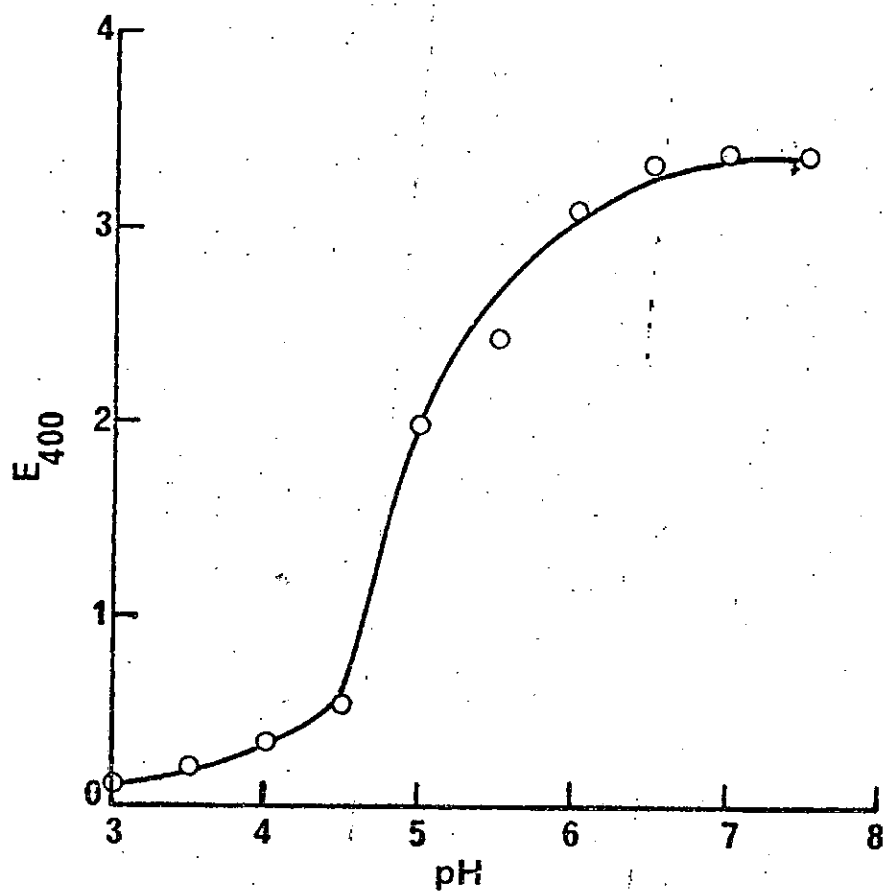
28. Substrate for enzyme assays

The substrate used for the colorimetric assay of

chitinase was 3, 4-dinitrophenyl-tetra-N-acetyl-B-D-

chitotetraoside (3, 4-DNP-TNAC) which was developed by

Ballardie and Capon (1972) and produced in research quantities

by Koch-light Laboratories Ltd. Fig. 2 shows the chemical

structure of this nearly colourless compound. Degradation of this soluble substrate by the enzyme released the coloured 3, 4-dinitrophenol which was measured on an SP 1800 Unicam Spectrophotometer at 400 nm. The molecular weight of the substrate is 997.

The compound 3, 4-dinitrophenol (3, 4-DNP) has an extinction coefficient value of 7325 $cm^{-1}mol^{-1}$ at 400 nm at pH 5.0 (Capon, B. Personal Communication). This value is very much dependent on pH and to determine correction factors, for the experiments with varying pH values, a sample (kindly given by B. Capon originally from Fluka Ltd.) was dissolved in distilled water. A sample of 0.1 ml of this solution was diluted with 10 ml of buffer at pH values ranging from 3.0 to 7.5. The buffer solution used was 0.1 M phosphate (McIllvaine, 1921). The colour production was read with a Unicam SP 30 at 400 nm, which is very close to the absorption maximum for this compound in aqueous solution, and a curve for the pH values of the compound was drawn (Fig. 4). The correction value of extinction coefficient at pH 6.0 using the equation $7325 \times \dfrac{EpH6.0}{EpH5.0} = 11580 \ cm^{-1}mol^{-1}$.

FIG.4

The effect of pH on 3, 4-dinitrophenol. The
compound was dissolved in distilled water. A
sample of 0.1 ml of this solution was diluted with
10 ml of McIlvaine buffer at pH values ranging
from 3.0 to 7.5. The colour production was read
at 400 nm on an SP 30.

29. Enzyme assays

(a) Using 3, 4-DNP-TNAC

The standard assay system consisted of 0.5 ml McIlvaine buffer pH 6.0, plus 0.5 ml appropriate enzyme solution, plus 0.1 ml of an 0.5 mg ml^{-1} aqueous solution of substrate 3, 4-DNP-TNAC in a semi-micro glass cuvette of 1 cm pathlength. This brought the concentration of the substrate to 45.45 μg ml^{-1}. The reaction and rate of activity was recorded and measured by means of a Unican SP 1800 Spectrophotometer at 400 nm at 37°C against a cuvette made up of 0.5 ml buffer, 0.5 ml distilled water, and 0.1 ml substrate solution. The rate of activity was measured for 10 minutes for each assay. The activity obtained was calculated as E_{400}min^{-1}.

(b) Reducing sugar test

Ten mg alpha chitin (Sigma) was weighed into a 250 ml flask and dispensed in 4 ml of McIlvaine buffer pH 6.0, and 0.2 ml toluene was added each time. The reaction was started by adding 1 ml of enzyme stock solution. The flask was incubated at 37°C in a shaking water bath. After 48 hours, the reaction was stopped by heating the flask in a boiling water bath for 5 minutes. The reaction mixture was cooled and centrifuged at 15,000 r.p.m. for 10 minutes, and the supernatant thus obtained was used for the dinitrosalicyclic acid test (DNS) (Summer and Somers, 1949).

One ml of the supernatant was added to 0.5 ml of
DNS reagent in a test tube. The tube was mixed with a
"Whirlimix" and placed in a boiling water bath for
5 minutes. The tube was cooled in an ice bath for 5
minutes. and colour production read at 520 nm on a
Unicam SP 30 Spectrophotometer. The sample was read
against a blank which was run the same way as the
sample but with 1 ml of distilled water instead of
enzyme. From the reading the μg glucose equivalents
per ml was calculated from the prepared glucose
curve (Fig.5). A calibration curve was prepared
from 0-200 μg glucose in 40 steps. A calibration
curve was prepared with each test. The sample and
the blank were scanned on a Unicam SP 1800 spectro-
photometer at scan speed 0.2 nm sec^{-1}, chart speed
50 seconds per cm, against water as the control
over the absorbance range 480-600 nm to check that the
maximum absorbance was at 520 nm (Fig. 6).

(c) Protein determination modification of Lowry, Rosebrough, Farr, and Rundall (1951).

For routine protein estimation, 1 ml of stock enzyme
solution was diluted with 20 ml of distilled water. One
ml of this dilution was added to 3 ml of a freshly pre-
pared mixture of 50 ml 4% sodium carbonate, 3 ml 2 N

FIG.6

Calibration curve for glucose.

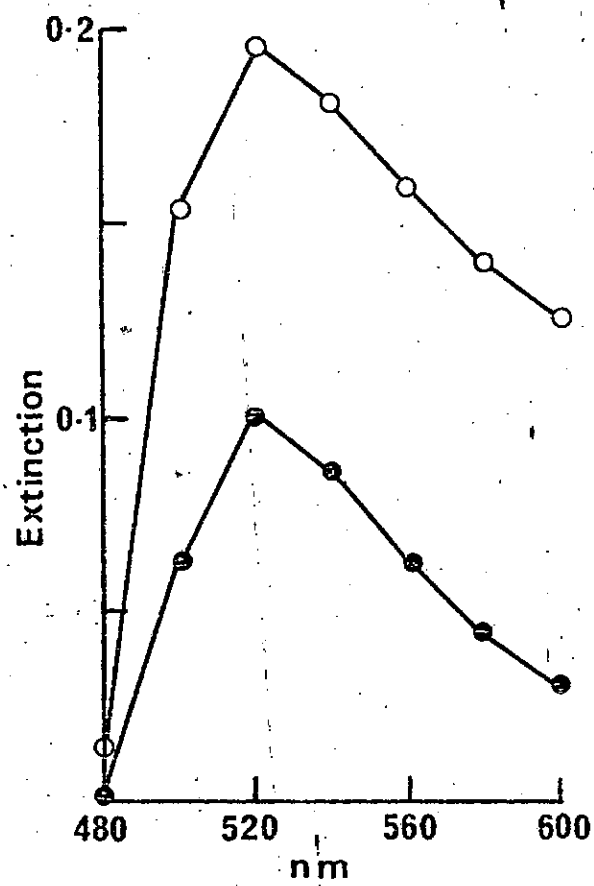All values were read against water as

the blank.
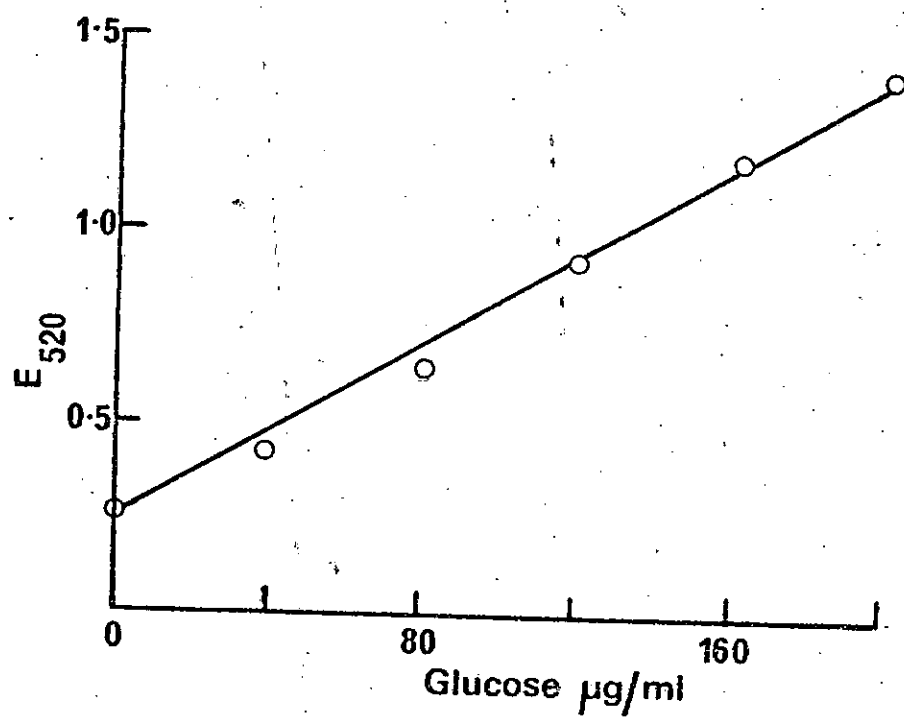
FIG.6

Absorption spectra of the control ●————●(1 ml
distilled water boiled for 5 minutes with 0.5 ml
DNS reagent) and 1 ml of supernatant of reaction
mixture ○————○ (1 ml enzyme, 10 mg chitin, 4 ml
of McIlvaine buffer pH 6.0) boiled for 5 minutes
with 0.5 ml DNS reagent. Distilled water was used
as the blank. The spectra were measured on SP 1800
Spectrophotometer over the absorbance range 480 nm-
600 nm. Notice characteristic maximum at about
520 nm.

sodium hydroxide and 2 ml copper reagent. The mixture
was incubated at 37°C for 7 minutes in a water bath
before adding 1 ml of freshly prepared Folin and
Ciocalteu's phenol reagent. This was instantly mixed
on a "Whirlimix" and the mixture incubated for a further
15 minutes at 37°C. The resulting blue colour was
read at 745 nm on a Pye Unicam SP 30 spectrophotometer.
A calibration curve for protein was always simultaneously
prepared from 0-200 µg bovine serum albumen (B.S.A.).
The amount of protein in the enzyme solution was read
off the standard curve (Fig.7).

30. General properties of chitinase enzyme

(a) Effect of temperatures on enzyme activity

The 3, 4-DNP-TNAC was used for assaying the effect
of temperature on the rate of degradation of substrate
by enzyme. The semi-micro cuvette containing the buffer
and substrate was left in a thermostat block to heat up
to the required temperature. Heat was supplied to the
spectrophotometer from a C.100 Circulator (Technae
Cambridge Ltd.). The correct temperature was recorded
by inserting a Digitron electronic probe thermometer into
the control cuvette. A sample of the enzyme solution
was added to the cuvette when the required temperature
was reached. This started off the enzyme reaction.

<u>FIG. 7</u>

Calibration curve for protein.

All values were read against water as the

blank on SP 30 at 745 nm.
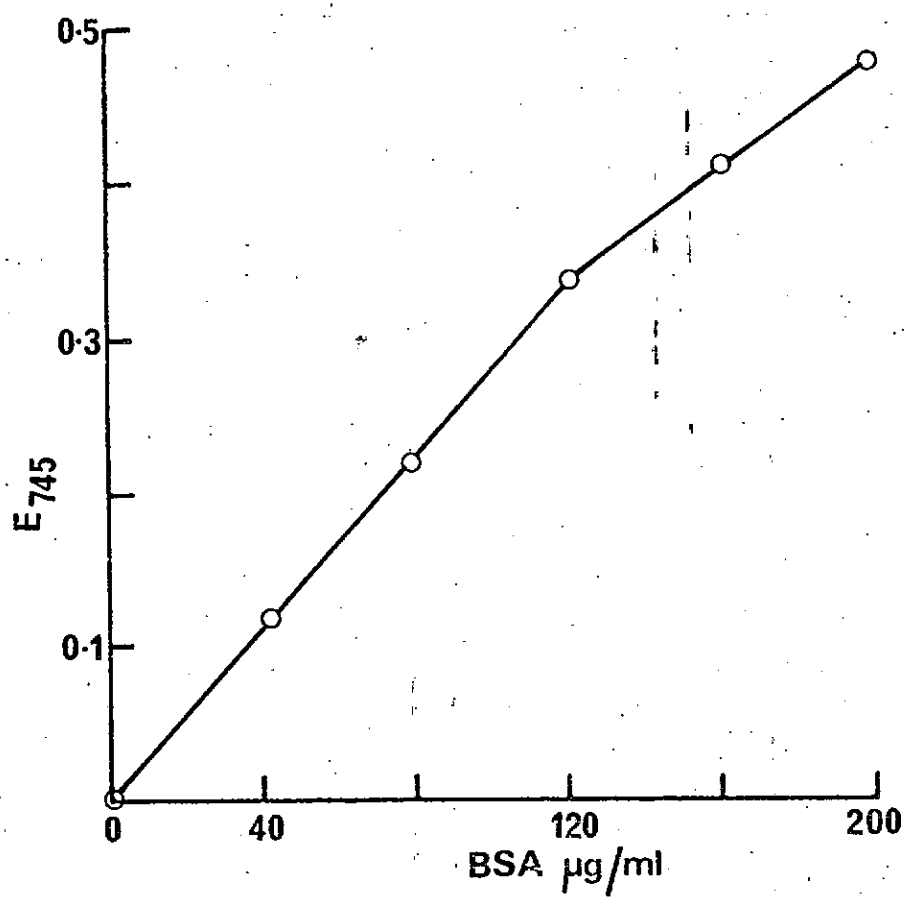
The rate of activity was recorded for 10 minutes. The temperature range tested was from $15^{\circ}C-70^{\circ}C$.

(b) Effect of pH on enzyme activity

The pH optimum for enzyme activity was determined by varying the pH of the buffer solution under otherwise standard assay conditions, using both 3, 4-DNP-TNAC assay and the reducing sugar test.

(i) Using the 3, 4-DNP-TNAC assay the pH of the buffer solution was varied from 3.0-7.5. A sample of the required pH buffer solution was substituted for the buffer used in the standard assay. The 3, 4-DNP correction curve was used to construct the pH curve.

(ii) Using the reducing sugar test, a sample of the required pH buffer solution was substituted for the buffer used to set up the reducing sugar test. The reducing sugar per ml was estimated using the DNS test.

(c) Effect of different buffers on enzyme activity

Different Zwitterionic biological buffers were tested for their effect on enzyme activity, using the 3, 4-DNP-TNAC assay. 0.1 molar solutions were prepared from TES (N-tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid) and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) at pH 7.0. The buffer was substituted for the buffer in the standard assay.

(d) <u>Effect of different molar concentrations of buffer solution</u>
<u>on enzyme activity</u>

A pH 6.0 McIlvaine buffer solution was prepared in
different molar concentrations, 0.1, 0.01, 0.001, and
0.0001. These were each substituted for the buffer in
the 3, 4-DNP-TNAC assay.

(e) <u>The effect of pre-incubating enzyme solution before</u>
<u>enzyme assay</u>

Enzyme solutions were heated in an electrical heating
block at $20^{\circ}C$, $30^{\circ}C$, $40^{\circ}C$, $50^{\circ}C$ $60^{\circ}C$ and $80^{\circ}C$ for
15 minutes. The enzyme solutions were cooled down
to $37^{\circ}C$ and the rate of activity recorded using the 3,
4-DNP-TNAC assay.

31. <u>Enzyme kinetic properties</u>

(a) <u>Effect of enzyme concentration on enzyme activity</u>

The stock enzyme solution was diluted logarithmetically
from 1 to 1/32 dilution. A sample of each dilution was
substituted for the enzyme solution in the 3, 4-DNP-TNAC
assay. The protein content of each dilution was also
determined.

(b) <u>Effect of substrate concentration on enzyme activity</u>

Ten mg of the substrate 3, 4-DNP-TNAC were dissolved
in 1 ml of distilled water and used as the stock substrate

solution.   The stock substrate solution was diluted
logarithmetically from 10 mg/ml to 0.015 mg/ml, and
0.1 ml of each dilution was substituted for the substrate
using the 3, 4-DNP-TNAC assay.

(c)   <u>Enzyme activity on various substrates</u>

Hydrolysis of the synthetic substrate p-Nitrophenyl-
B-N-acetylglucosaminide (Koch-Light Ltd.) and dried
<u>Micrococcus lysodeikticus</u> cells (Sigma) were tested
using the enzyme solution from $\underline{V}$. <u>alginolyticus</u>.

(i)   <u>Assay for N-acetylglucosaminidase (EC 3.2.1.30)</u>

Four mg of the substrate p-Nitrophenyl-B-N-
acetylglucosaminide were dissolved in 1 ml of water
and used as the stock substrate solution.   A
sample of 0.1 ml of substrate solution was incubated
with 0.5 ml of enzyme solution and 0.5 ml of buffer
at pH 6.0 for 10-40 minutes, at $37^{\circ}C$.   The reaction
was stopped by adding 2 ml of 0.1 molar sodium
borate buffer pH 9.8 and the intensity of the
colour produced was estimated at 400 nm using a
Pye Unicam SP 30 Spectrophotometer.   A control
was set up as the sample except that 0.5 ml of
water was used instead of enzyme solution

(Frohwein and Gatt, 1967). A sample of B.N-acetylglucosaminidase (Sigma) was substituted for the enzyme solution in the 3, 4-DNP-TNAC assay and tested for ability to degrade the substrate. A sample of 0.01 ml of enzyme (4.4 μg protein/ml) with a minimum activity of 74 units/mg was used as the enzyme solution.

(ii) <u>Assay for lysozyme (EC 3.2.1.17)</u>

A suspension of 0.3 mg ml^{-1} in McIlvaine buffer pH 6.0 was prepared from the substrate (dried (<u>M. lysodeikticus</u> cells). A sample of 0.1 ml of enzyme solution was added to 2.9 ml of substrate stock solution in a 3 ml cuvette. Absorbancy and rate of activity were recorded on Pye Unicam SP 1800 Spectrophotometer at 450 nm using a water blank. This method is that of Shugars (1952).

A control was prepared by using a commercial lysozyme from hen egg white (Sigma). A suspension of 2.5 mg ml^{-1} in distilled water was prepared from lysozyme powder with a minimum activity of 24,000 units/mg. A sample of 0.1 ml of lysozyme solution was added to 2.9 ml of substrate stock solution and reacted as above.

A sample of 0.5 ml of the lysozyme solution was substituted for enzyme solution using the 3,

4-DNP-TNAC assay, and tested for ability to degrade the substrate.

(d) Effect of additional compounds on the enzyme activity and degradation of 3, 4-DNP-TNAC

One mg each of the following soluble compounds was separately dissolved in 0.5 ml buffer solution, pH 6.0 in a 1 ml cuvette. To each was added 0.5 ml enzyme and 0.1 ml substrate stock solution (3, 4-DNP-TNAC). The various compounds were:

(a) M. lysodeikticus dried cells.

(b) N-acetyl-D-mannosamine.

(c) N-acetyl-D-glucosamine

(d) N-acetyl-B-galactosamine

(e) N-acetyl-neuraminic acid

(f) Agglutinin, wheat germ (Sigma)

(g) Colloidal chitin

(j) ∝ chitin (Sigma).

After 10 minutes incubation at 37°C, the absorbancy at 400 nm was read. The tubes containing M. lysodeikticus, ∝-chitin, and colloidal chitin were centrifuged before reading the absorbancy. For the tubes containing the other soluble materials, the rate of activity was monitored continuously as for standard 3, 4-DNP-TNAC assay.

(e). <u>Possible inhibitors of enzyme activity on 3,</u>
<u>4-DNP-TNAC</u>

(1) <u>Method 1</u>

Quantities of 2, 4, 8, 16 mg of colloidal
chitin (dry weight) were weighed into separate
1 ml centrifuge tubes. To each of the tubes
was added 0.5 ml McIlvaine buffer pH 6.0, 0.1 ml
substrate stock solution (3, 4-DNP-TNAC) and
0.5 ml enzyme solution. The tubes were mixed
with a "Whirlimix" and incubated in a heating
block at 37°C for 10 minutes. The tubes were
mixed every 2 minutes to ensure complete enzyme
reaction. A control tube made up of buffer,
enzyme and substrate solution was reacted at
the same time. The reaction was stopped after
10 minutes by heating in a heating block at
100°C for 5 minutes. The tubes were cooled
for 1 minute and centrifuged in an Eppendorf
mini centrifuge for 2 minutes. The supernatant
was carefully poured into a semi-micro
cuvette and absorbancy read at 400 nm on the
SP 1800. The 2 substrate concentrations used
were 0.045 mM and 0.0225 mM. The experiment
was repeated using 2, 4, 8 and 16 mg alpha
chitin (Sigma) at these 2 substrate concentrations.

(ii)   Quantities of 4 mg colloidal chitin
(dry weight) were weighed into 5 centrifuge tubes.
To each of the tubes was added 0.5 ml enzyme
solution, 0.5 ml buffer pH 6.0, and 0.1 ml
of the different substrate concentration solution.
The following substrate concentrations were
used:

(i)   0.227mM

(ii)   0.113 mM

(iii)   0.056 mM

(iv)   0.028 mM

(v)   0.014 mM

All the substrate concentrations were previously
used for the 3, 4-DNP-TNAC assay to ensure that
the enzyme activity was linear with substrate
concentration for 10 minutes.

A sample of 0.1 ml of each substrate concentration
was added to the various tubes and the tubes
incubated and treated as in Method 1.

The experiment was repeated using 4 mg $\propto$ chitin
with the 5 different substrate concentrations.

The control was a set of 5 tubes, each with 1 ml
enzyme solution, and 0.5 ml buffer, but no added
chitin. The control tubes were incubated and treated
as in Method 1.

(f) Enzyme-chitin complex

Four mg of chitin were weighed into a centrifuge tube. To the tube was added 0.5 ml enzyme solution and 0.5 ml buffer solution. A control tube was set up containing 0.5 ml enzyme solution and 0.5 ml buffer. After 5 minutes incubation at 37°C the 2 tubes were centrifuged in an Eppendorf mini centrifuge for 2.5 minutes. The supernatant was carefully poured out and used as the enzyme solution. This was assayed for enzyme activity using the 3, 4-DNP-TNAC assay.

32. Chitinase production

To determine when maximum chitinase activity was reached in liquid medium, a 500 ml flask containing 300 ml of sea water chitin medium was inoculated with 1 ml of a 24 hour _V. alginolyticus_ broth culture. At zero hour, 25 ml of the inoculated sea water medium were precipitated with ammonium sulphate (80 per cent saturation) and the enzyme preparation tested for protein and enzyme activity using 3, 4-DNP-TNAC assay and reducing sugar test. The medium was then incubated on a rotary shaker at 25°C at a speed of 138 r.p.m. Twenty-five ml of the culture were asceptically removed every 2 days, from the 2nd day to the 14th day of incubation. Enzyme solution was prepared from

the 25 ml by partial purification using ammonium sulphate
(80 per cent saturation). The enzyme solution was tested
for chitinase activity, using reducing sugar test and
3, 4-DNP-TNAC assay. The protein content was also estimated.

A control flask containing 300 ml of sea water medium
(without chitin) was similarly inoculated and sampled at
0 hour and every 2 days for 14 days for protein and chitinase
activity.

33. Estimation of chitin in gut of shrimps

(a) The gut of shrimps was put into a 1 ml centrifuge tube
and freeze-dried for 4 hours. The preparation was
weighed out with a 1 ml "Reactivial" (Pierce Chemical
Company) and extracted with 80 per cent methanol
overnight at 45°C. The mixture was centrifuged at
3,000 r.p.m. for 5 hours at 45°C. The dried residue
was extracted with 2 per cent potassium hydroxide for
15 minutes in a boiling water bath. This was cooled
and centrifuged at 3,000 r.p.m. for 5 minutes. The
residue was again dried in the oven for 3 hours at 45°C
before adding 0.75 ml of 6N-HcL. This was mixed with a
"Whirlimix" and left soaking overnight at room temperature.
The sample was then hydrolysed for 8 hours at 100°C in a

heating block and the contents mixed every hour to ensure
complete hydrolysis. The vial was cooled down and
centrifuged for 5 minutes at 3,000 r.p.m. A sample of
0.2 ml of the suspension was dried in a vacuum dessicator
over potassium hydroxide pellets. The dry residue was
made up to 1 ml with distilled water and the glucosamine
estimated (Tracey, 1955). Chitin hydrolysed with hot
acid gives D-glucosamine and acetic acid in equimolar
amounts. Gut wall and gut content of shrimps were
separated and treated as above.

(b) Estimation of chitin.

Acetylacetone reagent (0.5 ml) was mixed with a
0.5 ml sample in a tube and sealed with a silicone rubber
septum that had been pierced with a syringe needle to
equalise the pressure. The tube was heated in a boiling
water bath for 45 minutes. It was cooled for 5 minutes
and 5 ml ethanol plus 0.5 ml Ehrlich's reagent were
added, and left for 1 hour at room temperature (Gardell,
1953). The colour yield was read at 530 nm on a Unicam
SP 30 spectrophotometer. A standard of 1 mg chitin
(BDH, pure Grade A) accompanied every hydrolysis and
dilutions of this provided a calibration curve from
0-200 μg in 40 μg steps (Fig. 8).

Fig. 8.   Calibration curve for chitin (BDH pure Grade

A).   All values were read against water as the

blank.
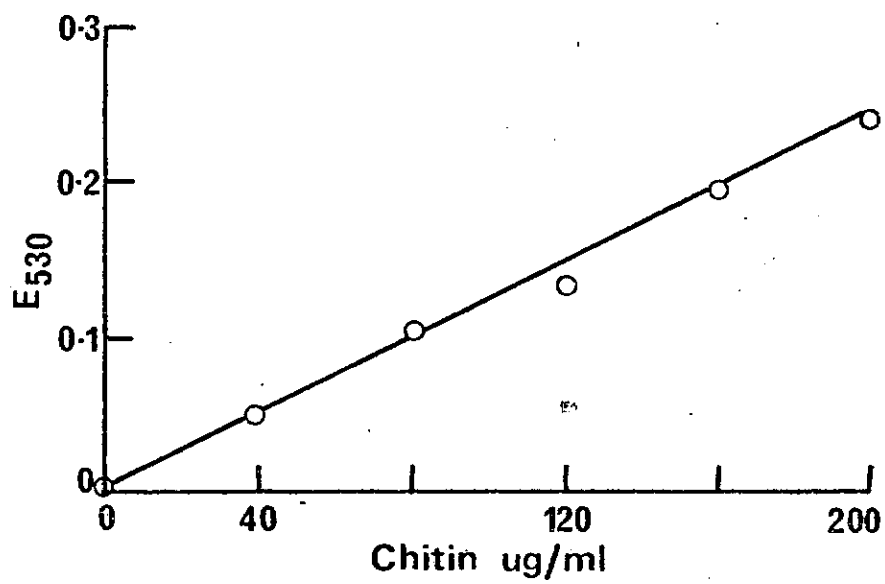
34. Enzyme purification

(a) Preparation of column

Sephadex Superfine G-75 of particle size 10-40
(Pharmacia Uppsala, Sweden) was swollen with 0.1
McIlvaine buffer pH 6.0 for 24 hours. This was de-
gassed under reduced pressure and packed under
gravity in a short column (35 cm long by 0.9 cm in
diameter). Several bed volumes of the starting buffer
were run through until the pH of the column was the
same as the buffer. Sephadex G-200 (fine Grade Pharmacia)
were treated the same way as G-75. The size of the
column for G-200 was 60 cm long by 1.5 cm in diameter.
The operating pressure head for the G-200 column was
kept within 10-20 cm water, as a higher pressure would
cause this gel to compress and so reduce the flow rate.
All columns were run at 0-4°C.

Purification using G-75 column of enzyme stock
solution (1 ml) prepared as described previously
(partially purified with ammonium sulphate, 80 per cent
saturation), was added directly to the top of the bed
of the Sephadex G-75. Fractions (5 ml) were collected
using a LKB Uvicord fraction collector. The actual
volume of each fraction was checked by weighing the

tubes. The fractions were assayed for chitinase
activity using 3, 4-DNP-TNAC assay, and protein content
was also estimated.

(b) Concentration and dialysis of samples

Two and a half ml samples were taken from each of
the fraction tubes 4, 5, 6, showing chitinase activity
and pooled. The 7.5 ml purified enzyme solution were
concentrated and dialysed against 0.1 McIlvaine
buffer pH 6.0. The dialysis tubes were previously
soaked in distilled water for 2 hours. The enzyme
solution was pipetted into the tube which was in turn
fitted into a vacuum flask containing cold McIlvaine
buffer. The flask was kept under reduced pressure and
left in a cold room overnight. The 7.5 ml volume was
concentrated to 1.5 ml. A sample 0.5 ml of the dialysed
enzyme solution was assayed for chitinase activity,
using the 3, 4-DNP-TNAC assay.

(c) Purification using G-200 column

A sample (0.5 ml) of the dialysed enzyme solution
was added directly to the top of the bed of the Sephadex
G-200 column. Fractions (80 drops), equivalent to 3.7 ml
were collected using a Golden Retriever fraction collector.
The volume was again checked by weighing the tubes. the
fractions were assayed for chitinase activity and protein
content.

35. <u>The recovery of activity from enzyme preparation after</u>
<u>partial purification with ammonium sulphate (80% saturation)</u>

A 6th day broth culture of <u>V. alginolyticus</u> grown
in sea water chitin medium was centrifuged at 7,000 r.p.m.
for 30 minutes at 4°C. A sample of the culture supernatant
was assayed for chitinase activity using the 3, 4-DNP-TNAC
assay.

After partial purification overnight with ammonium
sulphate (80% saturation) the supernatant was centrifuged
at 7,000 r.p.m. for 30 minutes at 4°C and the supernatant
obtained from this was assayed for chitinase activity.
The precipitate was dissolved in distilled water and centri-
fuged at 15,000 r.p.m. for 30 minutes. The supernatant
thus obtained was assayed for chitinase activity.

RESULTS

1. Quantitative estimation of the microflora of freshly caught

shrimps and of sea water

The results of the bacterial counts carried out each month on shrimp or sea water samples are shown in tables 1 and 2. For convenience of presentation, the values are recorded as the logarithm to the base ten of the number of bacteria per gm of the shrimp tissue or per ml of sea water.

The bacterial counts obtained on incubation at $20^{o}C$ ranged between $\log_{10} 3.1$ and $\log_{10} 8.7/gm$ of shrimp tissue, while at $37^{o}C$ counts ranged between $\log_{10} 3.1$ and $\log_{10} 6.1/gm$ of shrimp tissue. For sea water incubated at $20^{o}C$, the range of bacterial count was between $\log_{10} 3.2$ and $\log_{10} 7.6/ml$; at $37^{o}C$ the range was between $\log_{10} 3.3$ and $\log_{10} 8.4/ml$.

During most months, the bacterial counts obtained per gm shrimp tissue incubated at $20^{o}C$ were somewhat higher than values obtained at $37^{o}C$, but in a few instances, the counts at $20^{o}C$ were lower than values obtained at $37^{o}C$. Figures 9 and 11 show that there were some fluctuation also in the bacterial load of sea water from which the shrimps were caught. The reason for the apparent correlation between the patterns of the bacterial load of the shrimps and the bacterial load of the sea water sample was not investigated in this study and cannot therefore

Table 1.

Bacterial counts of freshly caught shrimp samples incubated at $20^{\circ}C$ or $37^{\circ}C$

Month and year	$\log_{10}$ count/gm at $20^{\circ}C$	$\log_{10}$ count/gm at $37^{\circ}C$
April, 1975	5.6	5.7
May, 1975	4.2	4.1
June, 1975	4.5	4.8
July, 1975	5.9	5.7
August, 1975	3.1	3.1
September, 1975	8.7	6.8
October, 1975	3.2	3.5
November, 1975	5.2	3.7
December, 1975	4.4	4.3
January, 1976	3.2	3.3

Table 2.

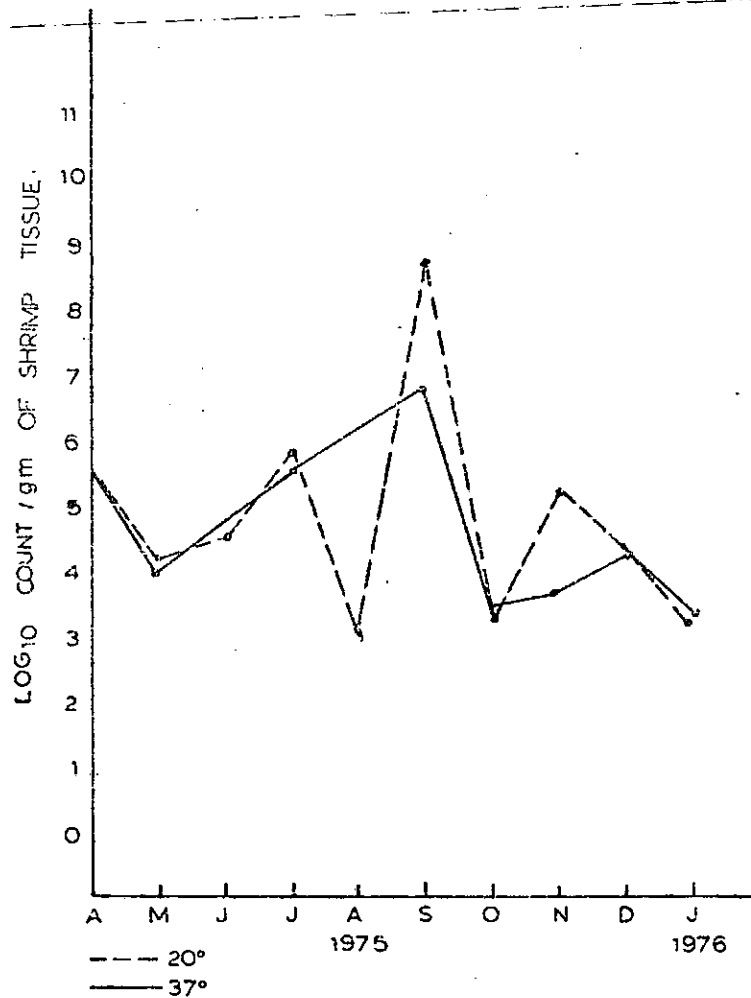| Month and year	Log_{10} count/gm at 20°C	Log_{10} count/gm at 37°C
April, 1975	5.2	5.2
May, 1975	4.3	4.1
June, 1975	4.0	4.1
July, 1975	6.0	6.9
August, 1975	3.5	3.2
September, 1975	7.6	6.7
October, 1975	6.2	8.4
November, 1975	3.4	6.6
December, 1975	3.2	3.3
January, 1976	7.2	7.1

Fig 9. Viable bacterial counts from shrimp samples (Cultures were incubated at 20° or 37°C)
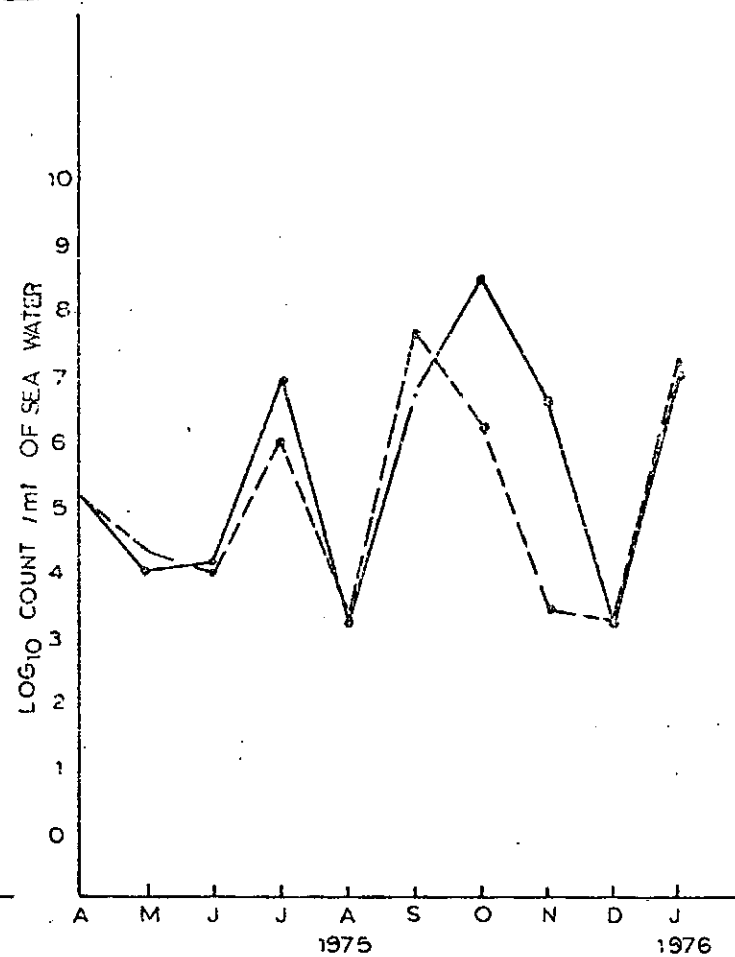
Fig 11. Viable bacterial counts from water samples (Cultures were incubated at 20° or 37°C)

be fully accounted for in the present investigation, but it must be emphasized that freshly caught shrimps derive their microflora from the environment which serves as their habitat.

2. Qualitative observation of the microflora of freshly caught shrimps and sea water.

Tables 3 and 4 show the genera or families of bacteria found on freshly caught shrimps and in the sea water from which they were caught. The bacteria present on freshly caught shrimps and in sea water were predominantly coryneforms. Four hundred and sixty-six isolates from shrimps and 420 isolates from sea water (which together represented 46.6% of the total isolates) were classified as Corynebacteriaceae. Many genera of the Corynebacteriaceae have been reported as present in fresh, spoiling fish and in sea water (Wood, 1940, 1950 and 1953; Dyer, 1947; Liston, 1957; Georgala, 1958 and Cann, 1971).

The bacteria isolated from freshly caught shrimps were predominantly coryneform. Fewer numbers of Micrococcus spp, Staphylococcus spp, Bacillus spp, Aeromonas spp, Acinetobacter spp and some members of Enterobacteriaceae were isolated. There were several isolates of the Enterobacteriaceae (about 46 isolates from freshly caught shrimps and sea water) and it was not considered necessary to classify them into their generic level, since their importance in the present studies is only in the

Table 3.

The types of bacteria present on freshly
caught shrimps

Bacteria	Percentage composition
1. Coryneforms	46.6
2. Micrococcus	16.5
3. Staphylococcus	14.0
4. Bacillus	4.2
5. Aeromonas	4.0
6. Acinetobacter	3.7
7. Enterobacteriaceae	3.5
8. Pseudomonas	2.7
9. Flavobacterium	2.6
10. Moraxella	2.2

Table 4.

The types of bacteria present in sea water

Bacteria	Percentage composition
1. Coryneforms	42.0
2. Staphylococcus	22.2
3. Micrococcus	12.5
4. Enterobacteriaceae	11.6
5. Aeromonas	3.3
6. Acinetobacter	3.2
7. Bacillus	2.4
8. Flavobacterium	0.8
9. Pseudomonas	0.6

fact that they may be indicators of faecal pollution.

The same genera and family of bacteria (except for <u>Moraxella</u> which was isolated from shrimp tissue only) were isolated from the sea water in which the shrimps were caught. There were however differences in the abundance of the various bacterial groups in sea water and on the shrimps (Fig. 14). In addition to the foregoing observations, the following points of interest were noted:

(a) Above 45% of the isolates from shrimp tissue and 31% from sea water were markedly pigmented. They were generally chromogenic, producing yellow, orange, buff, pink and green pigments on agar media.

(b) The asporogenous non-motile Gram-positive rods which were classified as coryneforms (Jensen, 1952) appeared to fall into distinct groups. The first group consisted of two types: type (a) formed minute slender rods that stained intensively with Gram stain; type (b) formed short stout rods which were pear-shaped and on superficial examination, might be confused with cocci. The second group showed coryneform morphology and branching. The third group consisted of club-shaped bacterial cells which after Gram-stain normally looked empty.

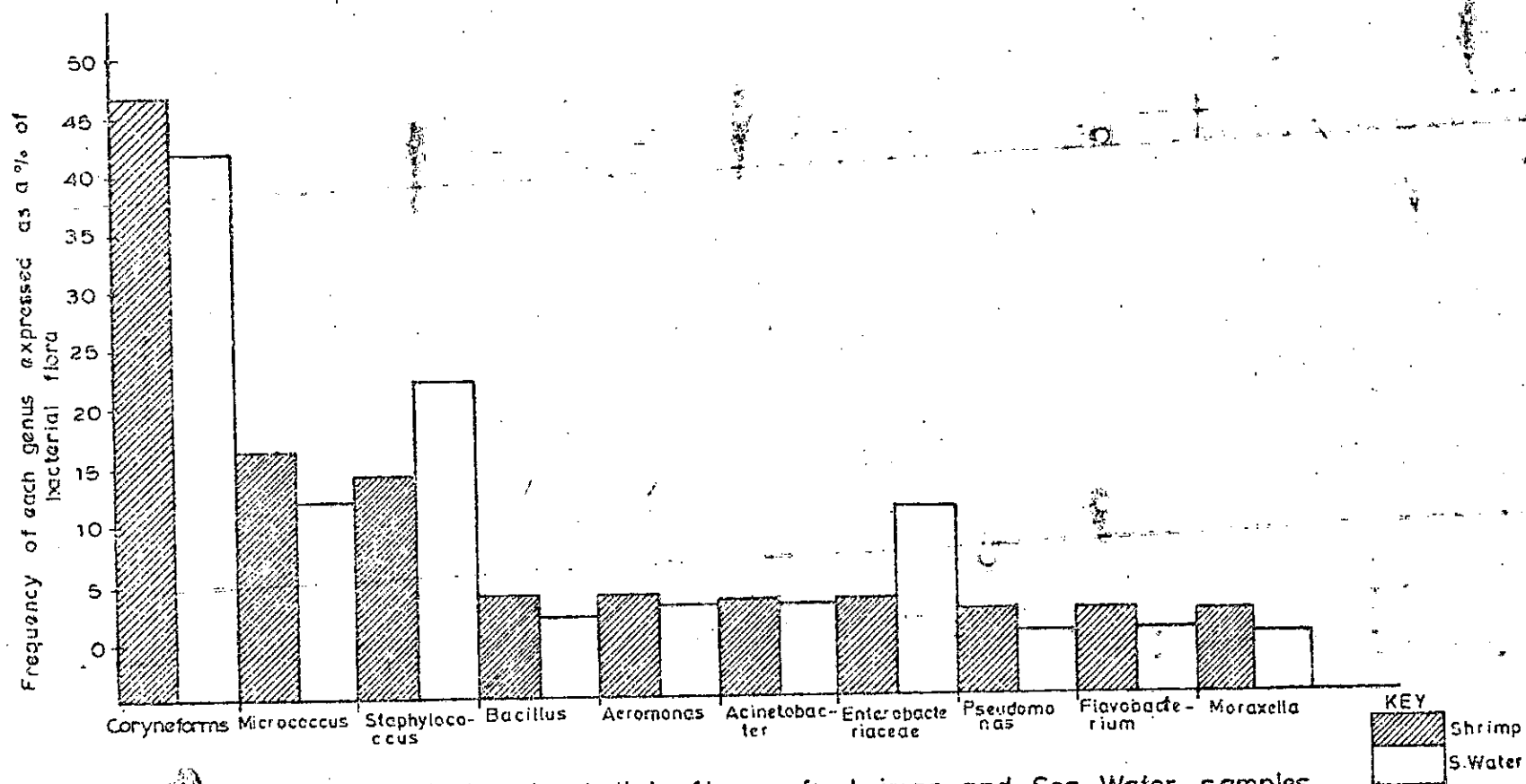(c) Some of the coryneforms were pleomorphic and formed coccoid elements during growth.

Fig. 11. Comparison of the bacterial flora of shrimps and Sea Water samples

(d) Most of the micrococci isolated were Gram-positive
most being chromogenic, producing white, yellow,
orange, buff or pink colours.

Coliforms and faecal streptococci on shrimps and in sea water

Tables 5 and 6 show that coliforms including E. coli, and
faecal streptococci were detected on shrimps and in sea water.
Coliforms were always isolated and the counts ranged between
550 - 1800/100 gm shrimp tissue. Escherichia coli with a count
of 0 - 21/100 gm shrimp tissue was only isolated occasionally.
Faecal streptococci   was not frequently isolated
and the count was 0 - 40/100 gm shrimp tissue. In sea water
sample coliform counts ranged between 550 - 1800/100 ml sea
water, while E. coli and faecal streptococci ranged between
0 - 24/100 ml sea water and 0 - 50/100 ml sea water respectively.
Of the food poisoning bacteria, Staphylococcus aureus was
isolated and a count of 1 - 10/100 gm shrimp tissue was obtained.
Vibrio cholera, Vibrio paraheamolyticus and Salmonella were
not isolated.

Proteolytic and indole producing ability of isolates

Table 7 shows that out of 200 isolates from freshly caught
shrimps and 100 isolates from sea water, 57 per cent and 30 per
cent respectively were proteolytic. Table 8 shows that out of
100 isolates from freshly caught shrimps and 100 isolates from

Table 5.

'MPN' values for coliform bacteria and faecal
streptococci in shrimps

Months	Coliform MPN/100 gm shrimp tissue	E. coli MPN/100 gm shrimp tissue	Faecal streptococci MPN/100 gm shrimp tissue
April, 1975	1600	1.8	-
May, 1975	1600	2.0	1800
June, 1975	1800	2.0	11
July, 1975	1600	0	14
August, 1975	1800	0.2	0
September, 1975	550	0	1600
October, 1975	-	-	-
November, 1975	1800	21.0	170
December, 1975	1800	2.0	130
January, 1976	1800	0.0	40

Table 6.

'MPN' values for coliform bacteria and faecal
streptococci in sea water.

Months	Coliform MPN/100 ml seawater	E. coli MPN/100 ml seawater	Faecal streptococci MPN/100 ml seawater
April, 1975	1800	24	-
May, 1975	1600	11	1800
June, 1975	1600	0	5
July, 1975	1800	0	0
August, 1975	1800	0	2
September, 1975	1800	0	350
October, 1975	-	-	-
November, 1975	1800	9	350
December, 1975	350	4	5
January, 1976	1800	0	80

Table 7.

Percentages of proteolytic bacteria among the
different groups of isolates from freshly caught
shrimps and sea water.

Bacteria	Nos. tested		No (%) proteolytic	
	sea water	shrimps	sea water	shrimps
Coryneforms	42	100	30.9	53.0
Staphylococcus	20	30	50.0	60.0
Micrococcus	13	32	46.1	71.8
Bacillus	6	7	33.3	85.7
Aeromonas	11	10	18.1	80.0
Pseudomonas	0	3	0	55.5
Enterobacteriaceae	0	9	0	0
Acinetobacter	3	6	0	0
Moraxella	5	3	0	0
Total	100	200	30.0	57.0

Table 8.

Percentages of indole producers among the
different groups of isolates from 'freshly
caught' shrimps and sea water.

Bacteria	Nos. tested		No (%) producing indole	
	sea water	shrimps	sea water	shrimps
Coryneforms	42	57	22.5	3.5
Micrococcus	13	25	46.1	4.0
Staphylococcus	20	15	20.0	6.6
Moraxella	5	0	60.0	0
Bacillus	6	0	33.3	0
Aeromonas	11	0	0	0
Acinetobacter	3	1	0	0
Enterobacteriaceae	0	3	0	0
Total	100	100	24.0	14.0

sea water, 4 per cent and 24 per cent respectively were indole

producers. Proteolytic bacteria were therefore more abundantly

isolated from shrimp tissue than from sea water; indole-producers

were however isolated in larger numbers from sea water than from

shrimp tissue. The apparent preference for shrimp tissue by

proteolytic bacteria, and of indole-producers for sea water cannot be

explained.

(3) The intestinal bacterial flora of shrimps.

Table 9 shows that the main groups of bacteria in the

intestine of freshly caught shrimps were Micrococcus spp,

coryneforms and Acinetobacter spp. A few isolates of

Moraxella, Staphylococcus and of Enterobacteriaeceae were

obtained.

(4) The bacterial flora of shrimps during spoilage.

Tables 10 and 11 show a slight increase in total

viable bacterial counts when shrimps were stored at either

chilled or room temperature. Bacteria of public health

significance (mainly coliforms, E. coli, and faecal

streptococci) decreased in populations as period of storage

increased (Table 10).

Table 9.

The intestinal bacterial flora of shrimps.

Bacterial types	% of total isolates
Micrococcus	55.0
Coryneforms	19.0
Acinetobacter	16.0
Moraxella	6.0
Staphylococcus	3.0
Enterobacteriaceae	1.0

Table 10.

Bacterial counts of shrimp samples during spoilage

at chilled temperature ($6^{\circ}C - 8^{\circ}C$). Isolations were

made at $20^{\circ}C$ or $37^{\circ}C$.

Days storage	TVC/gm $\log_{10}$ count		MPN/100 gm		
	$20^{\circ}C$	$37^{\circ}C$	Coliform	E. coli	Faecal streptococci
3	11.5	8.9	430	20	350
6	12.5	3.7	22	1.8	350
9	12.5	9.7	17	0	250
12	12.5	10.5	17	0	90

Table 11.

Bacterial counts of shrimps samples during
spoilage at room temperature ($22^{\circ}C - 25^{\circ}C$).
Isolations were made at $20^{\circ}C$ or $37^{\circ}C$.

Days stores	TVC/gm $\log_{10}$ count		Coliform MPN/100 gm	E. coli MPN/100 gm	Faecal streptococci MPN/100 gm
	$20^{\circ}C$	$37^{\circ}C$			
3	8.9	11.5	1800	72	540
6	9.9	12.5	1800	72	540
9	12.5	12.5	1800	81	540
12	12.5	12.5	1800	95	920

(b) Qualitative observations:

The following bacterial changes were noted in shrimps in storage:

At 6°C to 8°C initially there was a large population of coryneforms and small populations of Acinetobacter, Aeromonas, Bacillus, Micrococcus, Enterobacteriaceae and Staphylococcus. As the period of storage increased the populations of coryneforms, Acinetobacter, Bacillus Staphylococcus and Micrococcus decreased drastically, and populations of Pseudomonas, Aeromonas, Enterobacteriaceae, Streptococcus and Moraxella became the dominant flora. (Table 12).

Similar changes in flora were observed in shrimps stored at 22°C to 25°C, except that at this temperature, the populations of Enterobacteriaceae increased. (Table 13).

Tables 14 and 15 showed that a high percentage of the spoilage bacteria were proteolytic. One hundred per cent of the bacteria isolated by the 12th day from the shrimps undergoing spoilage at room temperature were proteolytic. The indole producers among the isolates from shrimps stored at chilled or room temperature were few compared to the number of proteolytic bacteria.

Table 12.

Days/ storage	Coryne- forms	Enterobac- teriaceae	Acineto- bacter	Moraxella	Aeromonas	Pseudo- monas	Bacillus	Micro- coccus	Staphy- lococcus	Strep- tococcus
3	77.0	2.0	11.0	0	4.0	0	3.0	2.0	1.0	0
6	31.0	3.0	6.0	2.0	5.0	47.0	3.0	0	0	3.0
9	21.0	10.0	4.0	2.0	11.0	48.0	1.0	0	0	3.0
12	9.0	18.0	2.0	2.0	11.0	55.0	1.0	0	0	2.0

Table 13.

Percentage composition of successive bacterial flora during spoilage of shrimp samples at room temperature (22°C – 25°C).

Days/ storage	Enterobac- teriaceae	Acineto- bacter	Moraxella	Aeromonas	Pseudo- monas	Bacillus	Micro- coccus	Staphy- lococcus	Coryne- forms	Strepto- coccus
3	25.0	2.0	2.0	14.0	0	2.0	0	2.0	53.0	0
6	22.0	1.0	2.0	35.0	5.0	2.0	0	2.0	31.0	0
9	20.0	1.0	2.0	47.0	11.0	0	0	0	18.0	1.0
12	10.0	0	3.0	63.0	17.0	0	0	0	6.0	1.0

Table 14.

<u>Percentage of indole producers and proteolytic</u>
<u>bacteria on shrimp samples during spoilage at</u>
<u>chilled temperature</u> $(6^{\circ}C - 8^{\circ}C)$

Days storage	Percentage of indole producers	Percentage of proteolytic bacteria
	Out of a total of 100 isolates	
3	14.0	80.0
6	22.0	71.0
9	30.0	68.0
12	16.0	64.0

Table 15.

<u>Percentage of indole producers and proteolytic</u>
<u>bacteria during spoilage at room temperature</u>
<u>$(22^{\circ}C - 25^{\circ}C)$</u>

Days storage	Percentage of indole producers	Percentage of proteolytic bacteria
	Out of a total of 100 isolates	
3	67.0	80.0
6	40.0	13.0
9	65.0	48.0
12	21.0	100.0

(5) The aerobic bacterial flora of smoked shrimps.

Table 16 shows the bacterial counts of smoked samples examined. The counts at $37^{\circ}C$ which represent pathogenic populations were lower than the counts at $20^{\circ}C$ which represent the total bacterial load of the shrimps. The shrimps processed in the laboratory by drying had a lower bacterial load and coliform count than the smoked shrimps purchased from local processors. High counts were recorded for smoked shrimps from Lagos markets. Smoked shrimps from Elemankiri Village, had a lower bacterial count and lower 'MPN' values for coliforms and faecal streptococci than smoked shrimps obtained from Port Harcourt markets.

The differences in counts for shrimps from different sources were probably due to various factors including the microflora of the areas from where the shrimps were obtained and also on the precaution taken by the traders during processing and marketing.

The qualitative analysis of the bacterial flora of the smoked shrimps is presented in Table 17. The most frequently isolated bacteria were Gram-positive cocci. Coryneforms, Bacillus, Pseudomonas and Acinetobacter were also isolated in large numbers.

Table 16.

Bacterial counts of smoked shrimp samples
from various sources. Isolations were
made at $20^{o}C$ or $37^{o}C$.

Source of sample	Log_{10} count/gm at $20^{o}C$	Log_{10} count/gm at $37^{o}C$
Adeniji Adele Road	9.0	7.5
Ilubirin Village	9.0	7.5
Elemankiri Village	5.0	3.3
Port Harcourt Market	9.4	8.0
*NIOMR.	3.2	2.6

*Nigerian Institute of Oceanography and Marine Research.

Table 17.

Percentage composition of aerobic bacteria
flora of smoked shrimps

Bacteria	Adeniji Adele Road	Ilubirin Village	Elemankiri Village	Port Harcourt Market
Micrococcus	45.0	58.0	27.0	54.0
Staphylococcus	14.0	8.0	2.0	4.0
Bacillus	2.0	13.0	2.0	0
Coryneforms	38.0	20.0	44.0	27.0
Acinetobacter	1.0	0	16.0	5.0
Pseudomonas	0	3.0	9.0	8.0

Salt and heat tolerance of some bacterial isolates.

Some isolates including Micrococcus, Staphylococcus
and coryneforms were tested for their potential for growth
and survival in different salt concentrations and for
heat tolerance. Table 18 showed that Micrococcus,
the coryneform spp, Bacillus cereus and Staphylococcus spp
could tolerate high salt concentration. The least salt
tolerant was Bacillus cereus and the most salt tolerant
was Micrococcus spp which tolerated up to 23% salt solution.

Presence of coliforms and faecal streptococci on smoked shrimps.

Table 19 showed that shrimps purchased at Adeniji Road
and Ilubirin markets in Lagos were rich in coliforms
including E. coli, and faecal streptococci whereas samples
from Elemankiri Village, Port Harcourt market and N.I.O.M.R.
contained few populations of the bacterial species.
Escherichia coli was not detected in the samples.

(6) The aerobic bacterial flora of frozen shrimps.

Table 20 shows that there was a rapid increase in
the bacterial load after freezing for 7 days and up to
14 days, after which period a decrease in numbers occurred
The initial increase in the numbers was probably due to
the multiplication of the bacteria on the shrimp at the
time of freezing but the decrease after 14 days of freezing

Table 18.

<u>Some characteristics of Gram-positive</u>
<u>bacteria isolated from smoked shrimps</u>

Bacterial types	Highest percentage of Nacl tolerated	Inactivation temperature ^{o}C
<u>Micrococcus</u> sp	23.0	50
<u>Staphylococcus</u> sp	12.0	40
Coryneforms sp	20.0	50
<u>Bacillus</u> <u>cereus</u>	11.0	Spores not inactivated at $100^{o}C$

Table 19.

'MPN' values for coliforms and faecal
streptococci on smoked shrimps from
various sources

Places	Coliform MPN/100 gm smoked shrimp	E. coli MPN/100 gm smoked shrimp	Faecal streptococci MPN/100 gm smoked shrimp
Adeniji Adele Road	350	1.8	1800
Ilubirin Village	300	1.5	1600
Elemankiri Village	10	0	16
Port Harcourt Market	17	0	17
NIOMR	5.4	0	6.8

Table 20.

Bacterial counts of shrimp samples
after freezing. Isolations were
made at 20°C or 37°C.

Duration (in days) of freezing of shrimps	Log_{10} count/gm shrimp tissue at 20°C	Log_{10} count/gm shrimp tissue at 37°C
0	5.9	5.7
7	9.9	8.1
14	9.7	9.9
21	8.2	8.4

would result from the lethal effect of low temperature
on the bacteria.

Table 21 shows that the MPN/100 gm values for
coliforms were reduced from 1600/100 gm shrimp tissue
obtained for the freshly caught shrimps before
freezing to 10/100 gm after freezing for 21 days. The
values for faecal streptococci increased by the end of
21 days. The percentage composition of the bacterial
types throughout the freezing period was similar to that
on the freshly caught shrimps with Gram-positive bacteria
e.g. coryneforms, micrococci, and streptococci predomina-
ting. (Table 22).

(7) Role of bacteria in spoilage of shrimps, using
trimethylamine (T.M.A.) and indole production
as chemical indices.

All the isolates tested for T.M.A. production were
capable of reducing trimethylamine oxide (T.M.A.O) to
T.M.A. The histogram in figure 12 shows that Aeromonas sp
C. produced more T.M.A./100 gm shrimp tissue than the
other bacterial isolates.

Staphylococcus and Micrococcus species did not produce
indole. Aeromonas sp. C that produced the largest quantity
of indole produced only 4.2 T.M.A. N/100 gm shrimp tissue

Table 21.

'MPN' values for coliform and faecal
streptococci on shrimps after freezing
and thawing.

Days	Coliform MPN/100 gm	E. coli MPN/100 gm	Faecal streptococci MPN/100 gm
0	1600	0	10
7	225	0	10
14	16	0	12
21	10	0	17

Table 22.

Percentage composition of successive bacterial flora of
shrimp samples during freezing and thawing.

Days	Percentage composition of bacteria							
	Coryne-forms	Micro-coccus	Strepto-coccus	Bacillus	Aeromonas	Enterobac-teriaceae	Pseudo-monas	Acineto-bacter
0	46.0	20.0	25.0	6.0	1.0	1.0	1.0	0
7	44.0	20.0	23.0	6.0	1.0	1.0	5.0	0
14	40.0	20.0	18.0	6.0	2.0	8.0	6.0	0
21	25.0	18.0	16.0	8.0	3.0	17.0	9.0	4.0

FIG. 12   KEY: TMAN / 100gm SHRIMP-TISSUE   AND   INDOLE / 100gm SHRIMP-TISSUE   PRODUCTION   BY   SPOILAGE   ORGANISMS
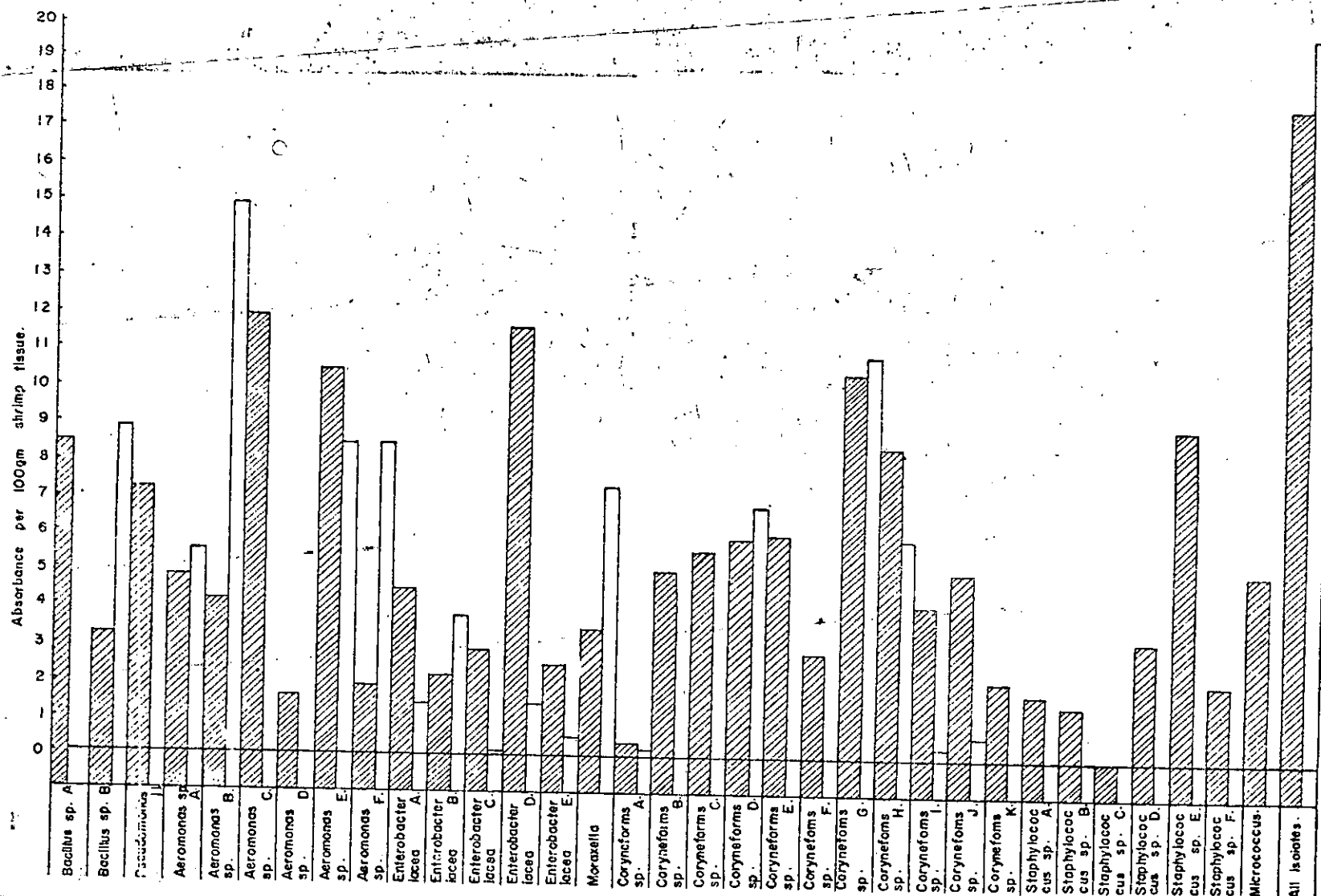
T. M. A.——Absorbance at 410 nm.
INDOLE——Absorbance at 560nm.

(Fig. 12). Of the 33 bacterial isolates tested, only 17 were indole-producers. It is significant that the indole-producers were also able to reduce T.M.A.O. to T.M.A. (Fig. 12).

(8) The physiological properties of bacterial isolates from freshly caught shrimps and spoilt shrimps.

The physiological characteristics of the bacterial isolates from freshly-caught shrimps and from shrimps in the process of spoilage are given in tables 23 and 24 respectively. It is significant to note that all the coryneform species isolated from shrimps undergoing spoilage were all proteolytic, while only two out of the four coryneform species from freshly caught shrimps were proteolytic. All the bacteria isolated from freshly caught shrimps were non-indole producers.

Salt tolerance tests on the isolates showed that most grew best in 2 - 4 per cent salt solutions. Table 25 shows the maximum salt tolerance for some of the isolates. Pseudomonas II tolerated up to 4 per cent salt solutions.

(9) Isolation and characteristics of chitinolytic bacteria.

Direct plating out of shrimp tissue or sea water samples on chitin agar was found not to be suitable for isolation of chitinolytic bacteria probably because they occur in small numbers both in sea water and on shrimps.

Table 23.

Physiological properties of some of the bacteria isolated from 'freshly caught' shrimps.

Bacteria	Gelatin liquefition	Indole production	Nitrate reduction	Starch hydrolysis	Action in litmus milk	Fermentation of glucose	Oxidation of mannitol	Oxidation of lactose	Oxidation of sucrose
Aeromonas sp. A	-	-	-	-	Ac	+	-	-	-
Aeromonas sp. B	+	-	-	+	Ac	+	+g	+g	+g
Aeromonas sp. D	-	-	-	+	Ac	+	-	-	+
Aeromonas sp. F	+	-	-	+	R	+	-	-	-
Enterobacteriaceae sp. B	+	-	-	-	Ac	+	-	-	-
Enterobacteriaceae sp. C	-	-	-	-	R	+	-	-	-
Coryneform sp. B	+	-	-	+	Ac	+	-	+g	+g
Coryneform sp. C	-	-	-	-	R	-	+g	-	-
Coryneform sp. I	+	-	+	+	R	+	-	-	-
Coryneform sp. K	-	-	-	+	Ac	+	-	-	-
Staphylococcus sp. A	-	-	-	+	Ac	+	-	-	+g
Staphylococcus sp. B	-	-	-	+	Ac	+	+g	+g	+g
Staphylococcus sp. E	-	-	-	-	R	+	-	+	+

KEY   A = acid   Ac = acid clot   R = reduction   g = gas.

Bacteria	Gelatin liquefi- tion	Indole production	Nitrate reduction	Starch hydrolysis	Action in litmus milk	Fermentation of glucose	Oxida- tion of mannitol	Oxida- tion of sucrose	Oxida- tion of lactose
Bacillus sp. A	+	-	-	-	Ac	+	-	-	-
Pseudomonas II	+	-	+	+	Ac	-		-	-
Aeromonas sp. C	-	-	+	+	A	+	+g	=g	+g
Aeromonas sp. E	+	-	-	-	Ac	+	-	-	-
Enterobacteriaceae sp. D	+	-	-	-	R	+	+g	+g	+g
Moraxella sp	+	-	-	+	Ac	-	-	-	-
Coryneform sp. D	+	-	-	+	Ac	+	+g	+g	+g
Coryneform sp E	+	-	-	+	R	-	-	+	-
Coryneform sp. C	+	+	+	+	R	+	-	-	-
Coryneform sp. J	+	-	-	+	R	+	-	-	-
Staphylococcus sp. D	+	-	-	-	R	+	-	-	-
Staphylococcus sp. F	+	-	-	-	R	+	-	-	-

KEY: A = acid   Ac = acid clot   R = reduction   g = gas.

Table 25.

Effect of salt concentration on the survival of bacteria

Bacteria	Max. Nacl concentration (% w/v) permitting growth of bacterial isolates.
Bacillus sp. A	8.0
Bacillus sp. B	10.0
Pseudomonas II	4.0
Aeromonas sp. A	11.0
Aeromonas sp. B	6.0
Aeromonas sp. C	6.0
Aeromonas sp. D	3.0
Aeromonas sp. E	9.0
Aeromonas sp. F	7.0
Enterobacteriaceae sp. A	6.0
Enterobacteriaceae sp. B	15.0
Enterobacteriaceae sp. C	10.0
Enterobacteriaceae sp. D	10.0
Enterobacteriaceae sp. E	10.0
Moraxella sp	5.0
Coryneform sp. A	20.0
Coryneform sp. B	4.0
Coryneform sp. C	9.0
Coryneform sp. D	9.0
Coryneform sp. E	9.0
Coryneform sp. F	9.0
Coryneform sp. G	6.0
Coryneform sp. H	8.0
Coryneform sp. I	9.0
Coryneform sp. J	5.0
Coryneform sp. K	12.0
Staphylococcus sp. A	5.0
Staphylococcus sp. B	5.0
Staphylococcus sp. C	12.0
Staphylococcus sp. D	12.0
Staphylococcus sp. E	6.0
Staphylococcus sp. F	10.0
Micrococcus sp.	23.0

Chitinolytic bacteria were therefore isolated from samples
by enriching for 14 days in a chitin enrichment medium.
The observed length of time for the appearance of haloes on
chitin agar plates, subcultured from enrichment medium
inoculated with gut contents of shrimps, or with sea water
samples was 6 days. The visible dissolution of colloidal
chitin on the chitin agar plates was taken as evidence of
extracellular production of chitinase by the mixed culture
of bacteria. The chitin agar plates subcultured from
enrichment medium inoculated with the shell and surface
washings of shrimps showed incipient decomposition of chitin
after 20 days. Although bacteria which were unable to
decompose chitin grew well on the chitin agar plate, they
did not produce any clearing and were therefore easily
distinguished from chitinolytic bacteria by means of tests
suggested by Hendrie, Mitchell and Shewan (1968), for
identification of marine isolates, colonies showing haloes
around them on the chitin agar plates were picked and
purified from the mixed cultures and identified to generic
level. The chitinolytic bacteria isolated from sea water
were <u>Vibrio alginolyticus</u>, <u>Bacillus cereus</u>, <u>Aeromonas</u> spp.
The chitinolytic bacteria isolated from sea water were <u>Vibrio
alginolyticus</u> (Table 26), <u>Bacillus cereus</u> (Table 27) <u>Flavo-
bacterium</u> spp and <u>Aeromonas</u> spp. The chitinolytic bacteria
isolated from the gut of freshly caught and frozen shrimps
were <u>Vibrio alginolyticus</u>, <u>Pseudomonas</u> spp, and <u>Bacillus cereus</u>
whilst <u>Vibrio alginolyticus</u>, <u>Acinetobacter</u> spp and <u>Bacillus
cereus</u> were isolated from the shells and surface

Table 26.

Characteristics of V. alginolyticus

Test	Reaction
Motility	+
Flagella	Peritrichous and polar flagella at 20°C on solid medium. Polar flagella in liquid media at 37°C.
Gram Stain	−
Morphology on sea water agar at 30°C 24 h.	Medium rod, sides parallel or slightly bulging, ends rounded and tapered, occurring singly and in pairs.
Colony appearance 24 h. sea water agar at 30°C.	Off-white, raised, opaque, very mucoid. 1.0–2.0 mm diameter
Growth in liquid medium sea water. broth 24 h at 30°C	Uniform turbidity
Oxidase	+
Catalase	+
Indole production	+
Methyl red	−
Voges-proskauer test	+
NH_3 from peptone	+ (weak)
Nitrate reduction	+ to NO_2
Urease	−
Citrate utilization	−
Gelatin stab	Stratiform liquefaction
Litmus milk	Complete peptonisation
Arginine dihydrolase	−
Lysine decarboxylase	+
Ornithine decarboxylase	−
Peptone water sugars: Glucose	Acid
Fructose	Acid
Lactose	−
Sucrose	Acid
Maltose	Acid
Mannitol	Acid
Glycerol	Acid
Starch	Acid
OF medium (glucose)	Fermentative
Antibiotic Sensitivity: Penicillin	−
Streptomycin	+
Chloramphenicol	+
Tetracycline	−
Novobiocin	+
Polymyxin B	+
O/129	
TCBS medium (Thiosulphate citrate bile salt	Yellow
Nacl tolerance	No growth at 0%. Growth at 0.5%, 7.0% and 10%.

Table 27.

Characteristics of <u>Bacillus</u> <u>cereus</u>

Test	Reaction
Motility	-
Gram stain	+
Morphology	Rods, 4-6 um in length 1-1.5 nm in width. Cells singly and in chains. Spores elliptical, central or paracentral, not distending the sporangia.
Oxidase	-
Catalase	+
Indole production	-
Methyl red	-
Voges-Proskauer test	+
Nitrate reduction	+ to NO_2
Citrate utilization	+
Propionate utilization	+
Gelatin stab	+
Lecithinase	+
Casein	+
Arginine dihydrolase	+
Tyrosine	+ (weak)
Dihydroxyacetone production	+ (weak)
Phenylalanine deamination	-
Lysozyme resistance	+
Peptone water sugars:	
Glucose	+
Trehalose	+
Glycerol	+
Lactose	-
Arabinose	-
Xylose	-
Rhamnose	-
Mannitol	-
Hydrogen sulphide production	-
Growth in 5% Hacl	+

washings of shrimps. The isolates of

V. alginolyticus were strongly chitinolytic and these were

therefore selected for further study.

10. Optimum conditions for production of chitinase on
     solid media

As shown in table 28, the pH value of the media

affected both the growth and production of chitinase by

V. alginolyticus. Maximum growth occurred on the agar

between pH 5.5 and pH 8.0. Maximum chitinase production

was observed between pH 6.0 and pH 7.0 and there was more

clearing of the chitin in the media between this pH range.

The optimum temperature for chitinase production by

these organisms was between 20°C and 30°C. Although there

was considerable growth at 37°C, there was no visible

clearing on the plates (Table 29).

The effect of the carbon sources, glucose, glucosa-

mine hydrochloride and N-acetyl-glucosamine on the production

of extracellular chitinase is illustrated in plates 2-4.

Plate 2a shows that V. alginolyticus was strongly

chitinolytic. There was not much stimulation of chitinase

production by the addition of glucose and glucosamine

hydrochloride, because the bacterium was strongly chitinolytic

on chitin agar plates without these carbon sources.

Plates 2b and 2d were control plates of glucosamine

hydrochloride and glucose respectively, with no added chitin.

Table 28.

Effect of pH value on the growth and production of chitinase by V. alginolyticus.

pH	Incubation time (days)									
	2		4		6		8		10	
	growth	clearing	growth	clearing	growth	clearing	growth	clearing	growth	clearing
5	+	–	+	–	+	+	+	+	+	+
5.5	+	–	++	–	++	+	+++	++	+++	++
6	+	–	++	+	+++	++	+++	+++	+++	+++
6.5	+	–	++	+	+++	++	+++	+++	+++	+++
7	+	–	++	+	+++	++	+++	+++	+++	+++
7.5	+	–	++	–	+++	+	+++	+	+++	+
8	+	–	++	–	+++	+	+++	+	+++	+

The organisms were grown on chitin agar plates.   Incubation was carried out at 20°C.

-   No growth and no clearing.
+   Slight growth or slight clearing
++   Considerable growth or considerable clearing
+++   Heavy growth or heavy clearing

Table 29.

Effect of temperature of incubation on the growth and chitinase production by V. alginolyticus

Temp. °C	Incubation time (days)									
	2		4		6		8		10	
	growth	clearing	growth	clearing	growth	clearing	growth	clearing	growth	clearing
20	+	−	++	+	+++	++	+++	+++	+++	+++
30	+	−	++	++	+++	+++	+++	+++	+++	+++
37	+	−	++	−	++	−	++	−	++	−

The organisms were grown on chitin agar plates (pH adjusted during media preparation to 6.0).

-    No growth and no clearing
+   Slight growth or slight clearing
++   Considerable growth or considerable clearing
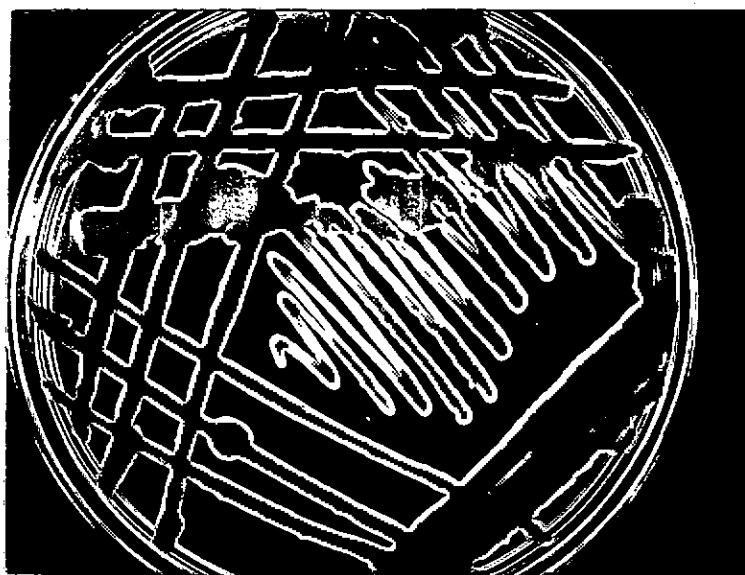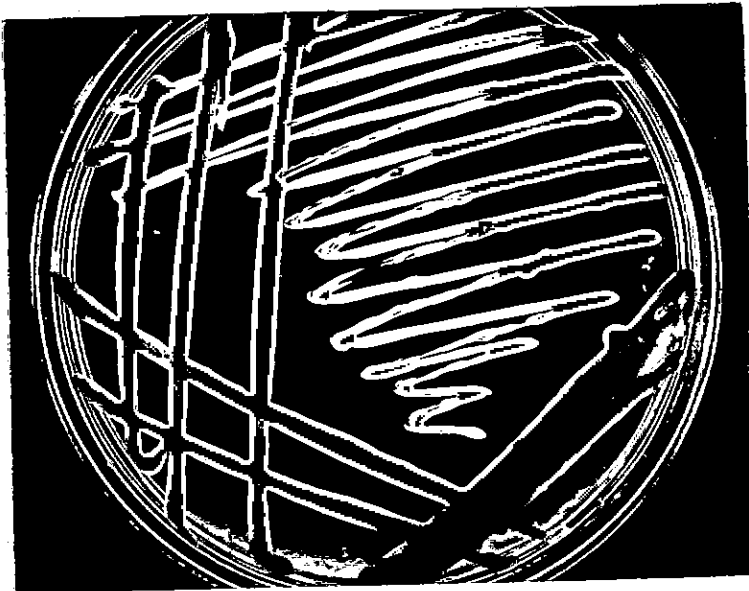+++ Heavy growth or heavy clearing

a
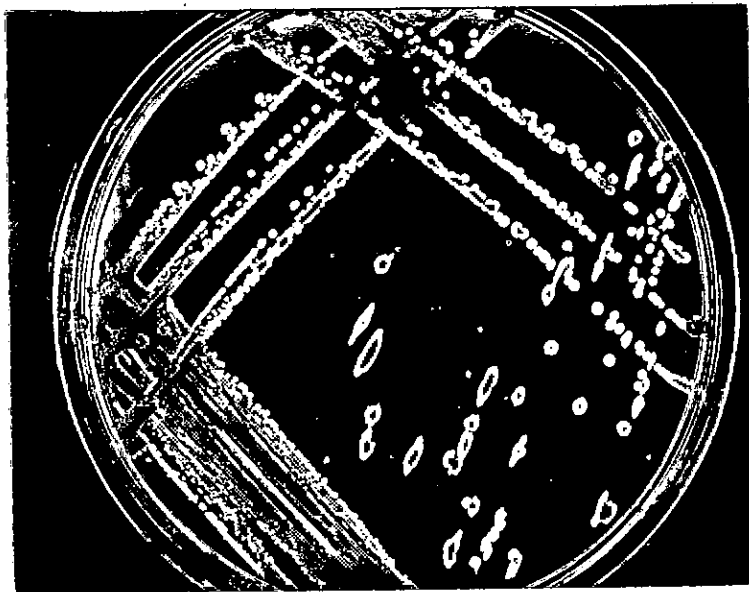
b
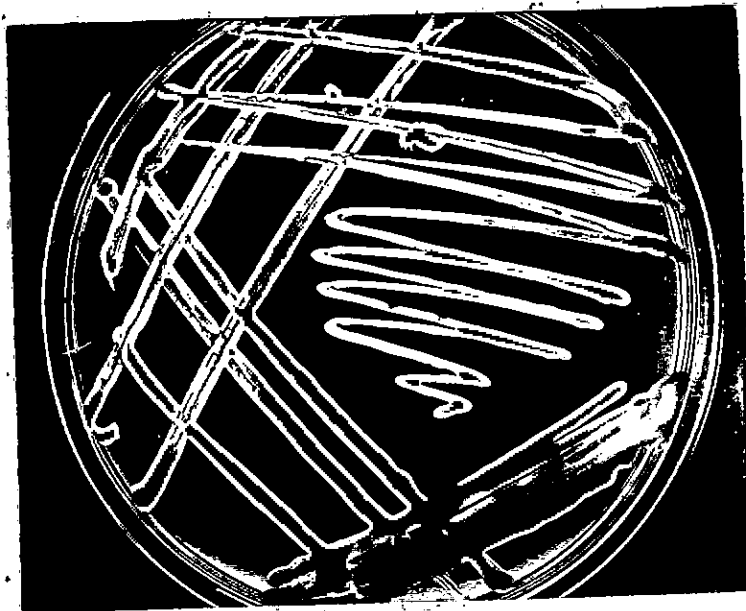
c

Plate 2.   <u>Chitinase production on agar</u>
               <u>plates by V. alginolyticus.</u>

        (a)   Bacterium on chitin agar plate
              Note the heavy clearing of chitin
              by the bacterium.

        (b)   Bacterium on glucosamine hydrochloride
              agar plate. No clearing.

        (c)   Bacterium on chitin glucosamine
              hydrochloride agar plate. Note
              the heavy growth and clearing
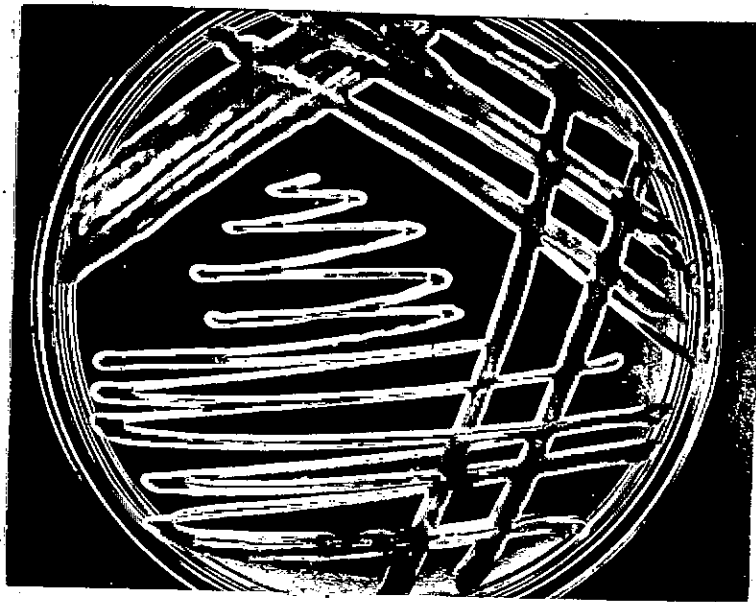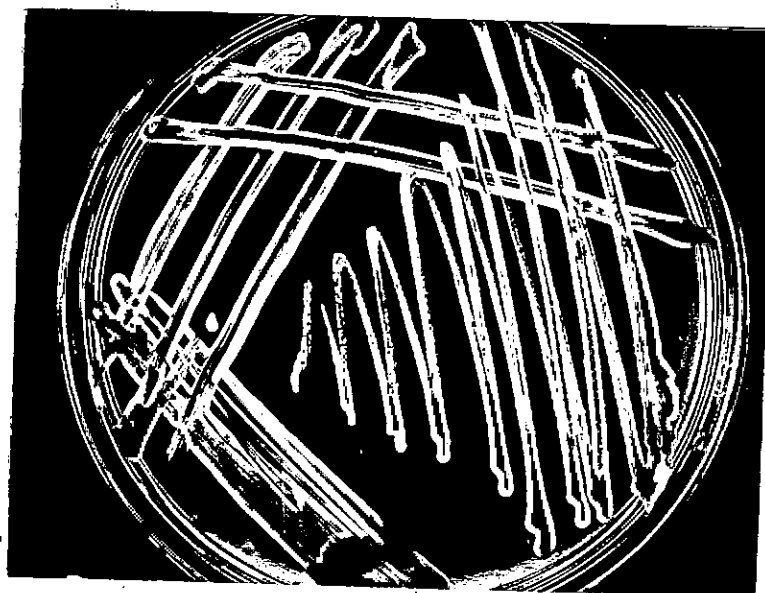
Plate 3. <u>Chitinase production on agar plates by V. alginolyticus.</u>

(a) Bacterium on chitin agar plate. Note the
     heavy clearing of chitin by the bacterium.

(b) Bacterium on glucosamine hydrochloride agar
     plate. No clearing.

(c) Bacterium on chitin glucosamine hydrochloride
     agar plate. Note the heavy growth and
     clearing.

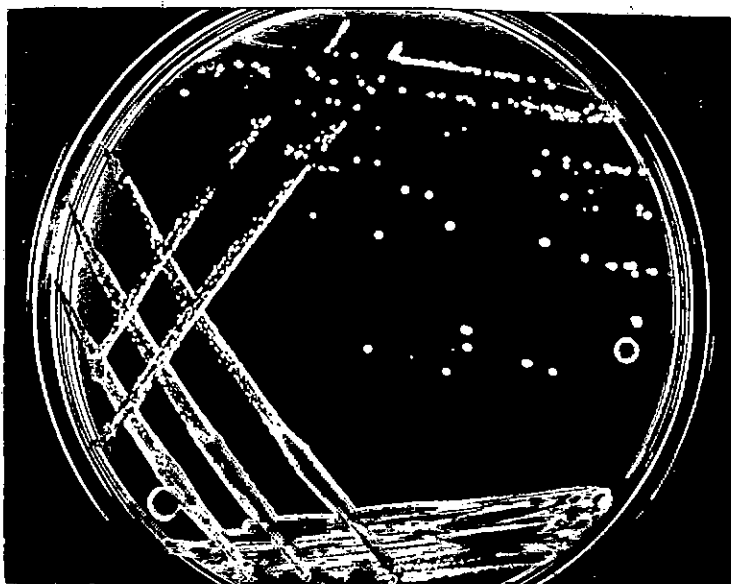(d) Bacterium on glucose agar plate. No clearing.

(e) Bacterium on chitin glucose agar plate.
     Note heavy growth and clearing.

Table 30 shows that there was more growth on chitin N-acetyl-glucosamine agar plates but less clearing occurred.

A similar observation was made on the Pseudomonas sp. (Plate 3). With B. cereus (Plate 4), chitinase production on the solid media was stimulated by glucosamine hydrochloride (Plate 4a) and not glucose (Plate 4c).

11. General properties of V. alginolyticus chitinase.

V. alginolyticus was chosen for further work because it was the most strongly chitinolytic of all the isolates.

(a) Effect of temperature on enzyme activity and stability.

The data presented in figure 13 show an optimum temperature of between 55°C and 57.5°C, with activity rapidly decreasing beyond this point.

The temperature stability of the enzyme was investigated by pre-incubating the enzyme solution for 15 minutes before assay. This decreased the activity at temperatures higher than 40°C (Fig. 14). At 52.5°C, about 50% of the activity was lost, and at 60°C the activity was completely abolished by this treatment. Routine enzyme assays were conducted at 37°C as there would have been technical difficulties at higher temperatures, e.g., bubble formation in cuvettes.

Table 30.

Effect of additional carbon sources on the growth and chitinase production by V. alginolyticus

Media pH 6.0	Incubation time (days)									
	2		4		6		8		10	
	growth	clearing	growth	clearing	growth	clearing	growth	clearing	growth	clearing
CA	+	−	++	+	+++	++	+++	+++	+++	+++
CA + N-acetyl-glucosa-mine	++	−	+++	+	+++	+	+++	++	+++	++
CA + glucosa-mine hydro-chloride	+	+	++	+++	+++	+++	+++	+++	+++	+++

CA = chitin agar

Incubation was carried out at 20°C.

- −   No growth or no clearing
- +   Slight growth or slight clearing
- ++   Considerable growth or considerable clearing
- +++   Heavy growth or heavy clearing

a
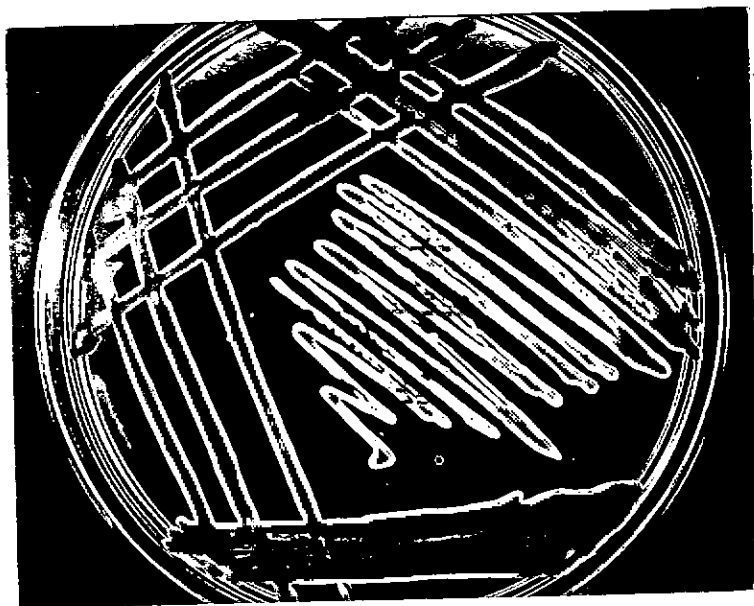
b

Plate 3.   <u>Chitinase production on agar</u>

<u>plate</u>   <u>Pseudomonas aeruginosa</u>

(a) Bacterium on chitin glucose agar
    plate. Heavy growth and clearing.

(b) Bacterium on glucose agar plate.
    Swarming growth, but no clearing.

c

d.
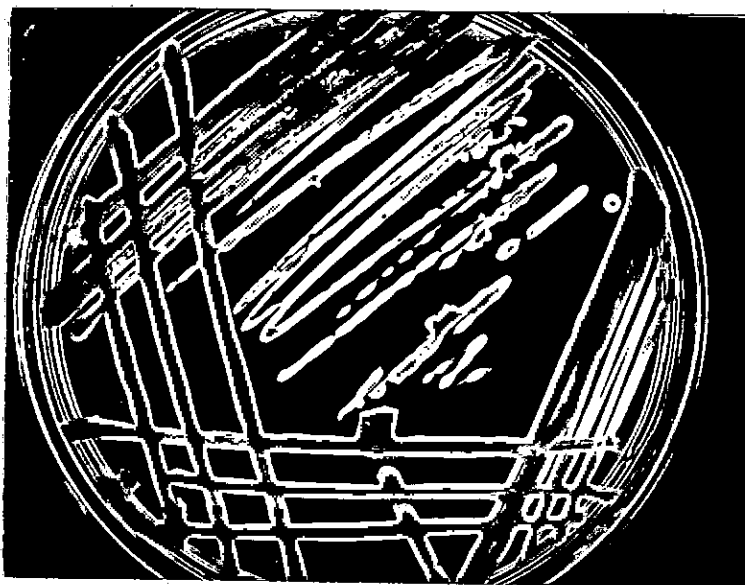
Plate 3. Chitinase production on agar plates by

Pseudomonas aeruginosa

(a) Bacterium on chitin glucose agar plate.

Heavy growth and clearing.

(b) Bacterium on glucose agar plate. Swarming

growth, but no clearing.

(c) Bacterium on chitin glucosamine hydrochloride

agar plate. Heavy growth and clearing.

(d) Bacterium on glucosamine hydrochloride agar

plate. No clearing.

a

b
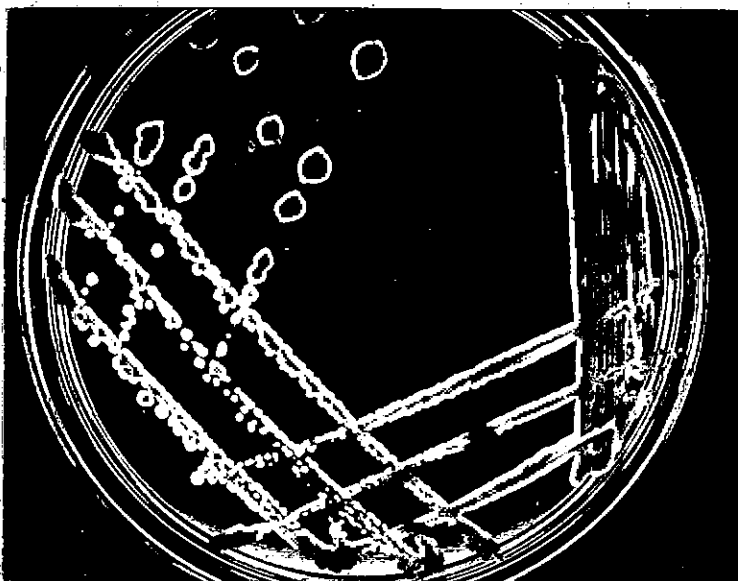
c

<u>Plate 4</u> <u>Chitinase production by B. cereus on agar</u>
<u>plates.</u>

(a) Bacterium on chitin glucosamine hydrochloride
     agar plate. Heavy growth and considerable
     clearing.

(b) Bacterium on glucosamine hydrochloride agar
     plate. Considerable growth but no clearing.

(c) Bacterium on glucose chitin agar plate. Slight
     growth and no clearing.

Fig. 3

<u>The effect of temperature on enzyme activity</u>

This is a plot of normalized results for the rate of
enzyme activity (V) against temperature. The values were
normalized at $45^{\circ}C$ and enzyme activity expressed as percentage
of that at $45^{\circ}C$. These values were:

(1)o————o   V = 0.020 μmol/min of 3, 4-DNP released.

Enzyme concentration was 75 μg

protein/ml of stock enzyme solution.

(2)●————●   V = 0.016 μmol/min of 3, 4-DNP released.

Enzyme concentration is 50 μg protein/ml

of stock enzyme solution.

V = enzyme activity expressed as 3, 4-DNP- released

(μmol/min).

The standard assay using 3, 4-DNP-TNAC was used. The
mixture was reacted at the different temperature and V
recorded for 10 minutes at $37^{\circ}C$ using SP 1800 at 400 nm.
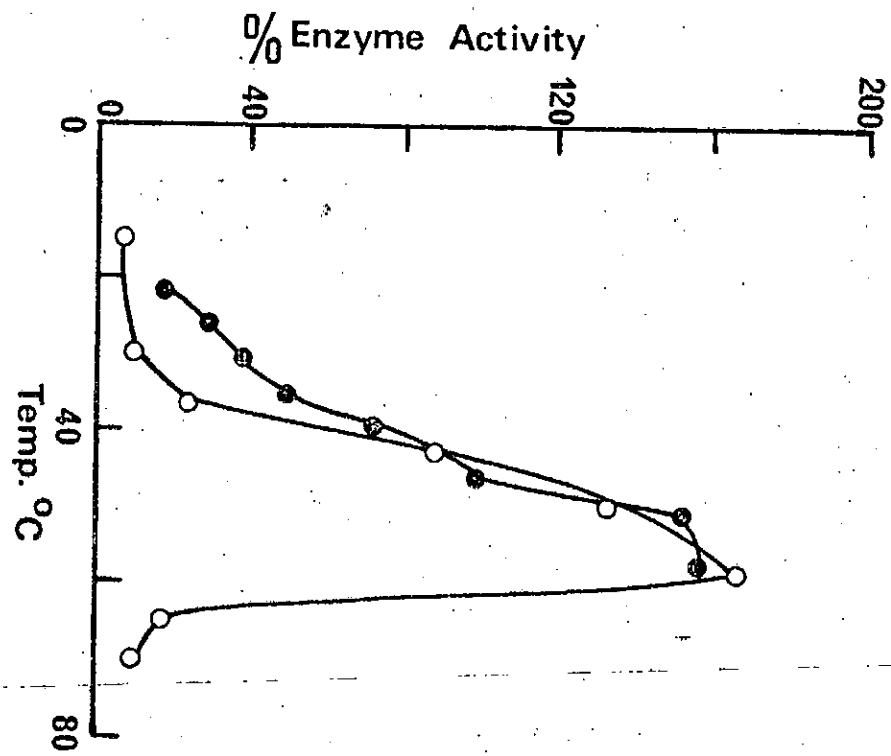
Fig. 14   The effect of preincubating enzyme before assay

Enzyme activity was expressed as percentage
of that at $40^{\circ}C$. Actual value at $40^{\circ}C$ was $V = 0.016$
$\mu mol/min$ of 3, 4-DNP released.

Enzyme solutions were heated in an electrical
heating block at the different temperatures for
15 minutes. The enzyme solutions were cooled
down to $37^{\circ}C$ and rate of activity measured using
the standard 3, 4-DNP-TNAC assay.
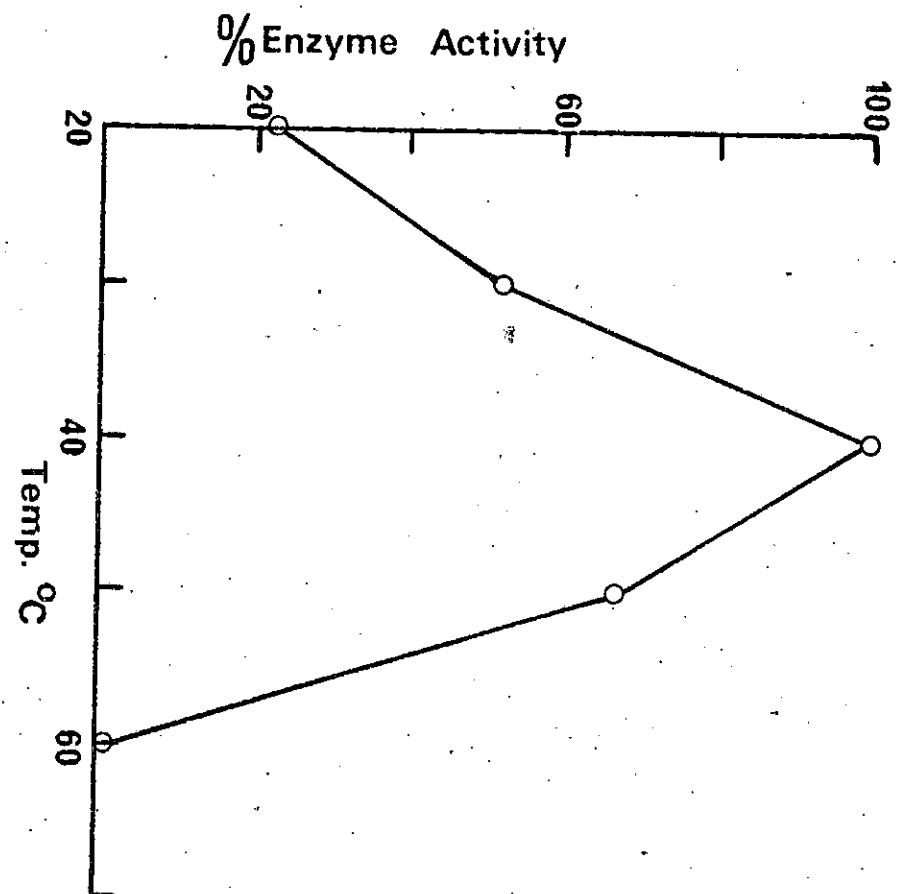
(b) Effect of pH on enzyme activity.

The effect of pH on enzyme activity is shown in figure 15. The assay results indicated a pH optimum of 5.5 - 6.0 for enzyme activity. Subsequent assays were run at pH 6.0. The optimum pH for activity obtained for enzyme using the 3, 4-DNP-TNAC assay was corrected using the pH curve for the compound 3, 4-DNP-TNAC. There was a complete absence of activity at pH 3.0.

(c) Effect of buffers on enzyme activity.

Table 31 shows that there was no inhibition of enzyme activity by the 2 biological buffers and McIlvaine buffer. The biological buffers could only be made up at pH 7.0 and above, which was not the optimum range of enzyme activity, therefore the McIlvaine buffer was used for all assays. At pH 6.0, McIlvaine buffer had the same value for V as the 2 biological buffers.

Decreasing the molarity of the buffer solution had no effect on enzyme activity (Table 32). The buffer concentration did not inhibit or stimulate the enzyme in this range.

Fig. 15   <u>Effect of pH on enzyme activity</u>

All values are a percentage of maximum

enzyme activity (V) achieved. The two values

corresponding are:

(a) 95 µg glucose/ml released at pH 5.5 using

the reducing sugar test . o————o

V was expressed as µg glucose/ml released.

(b) 0.018 µmol/min of 3, 4-DNP released at pH

6.0 using 3, 4-DNP-TNAC assay ⊕————●

V was expressed at µmol/min of 3, 4-DNP-

released.
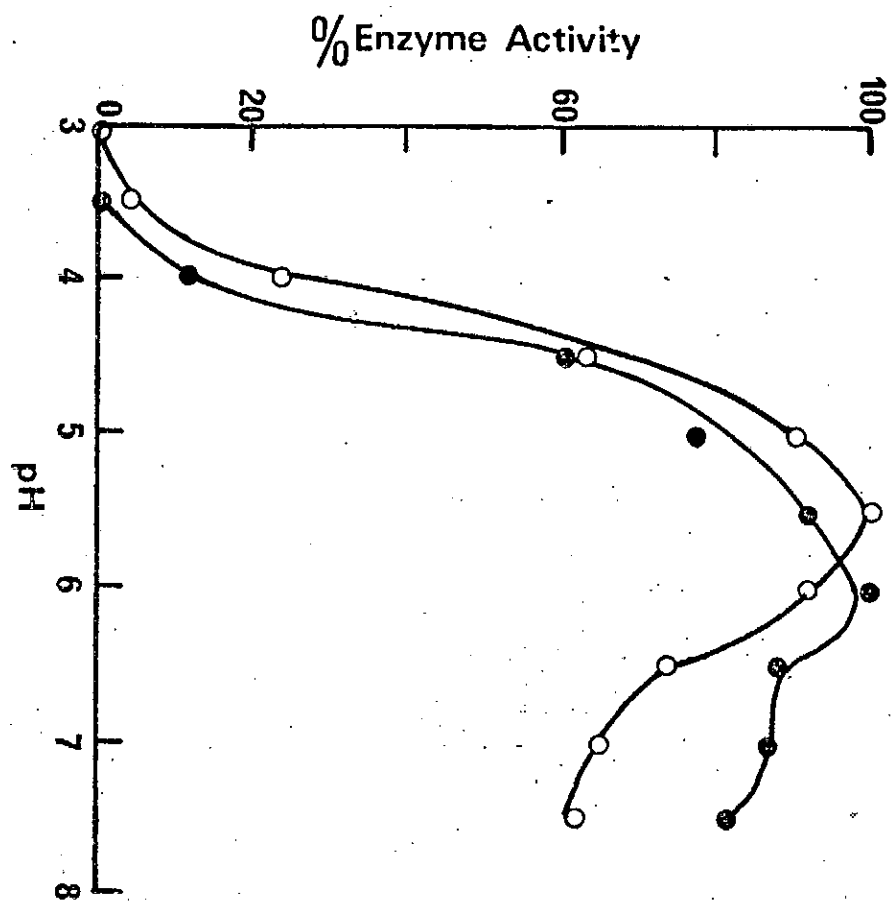
Table 31.

Effect of different buffers on enzyme activity

pH 7.0 0.1 M solution	V^+	*% Activity
1. TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid)	0.018	100
2. HEPES (N-2-hydroxyethyl piperazine N-2-ethane-sulphonic acid)	0.018	100
3. McIlvaine buffer	0.017	94.4

* Activity expressed as percentage of velocity with TES.

\+ $V = \mu mol/min$ of 3, 4-DNP liberated.

Table 32.

The effect of buffer concentration on
enzyme activity

Molar concentrations McIlvaine buffer pH 6.0	V^+	* % Activity
0.1	0.0163	93.14
0.01	0.0175	100.00
0.001	0.0173	98.85
0.0001	0.0170	97.14

* Activity expressed as percentage of velocity at 0.01 molar concentration

+ $V = \mu mol/min$ of 3, 4-DNP liberated.

12. Kinetic properties of chitinase from V. alginolyticus.

(a) Effect of enzyme concentration on enzyme activity.

There was a linear relationship between enzyme concentration and activity. Increase in enzyme concentration resulted in loss of linearity after 500 µg protein/ml concentration (Fig. 16) until increasing the enzyme concentration had no effect on the enzyme activity. The proportionality indicates that the assay was free of inhibiting substances which could cause a decline in activity as the concentration of enzyme was increased. The enzyme activity was linear for only 1 minute at an enzyme concentration of 980 µg protein/ml, 2.25 minutes at 490 µg protein/ml, and 10 minutes at 245 µg protein/ml and lesser concentrations.

(b) The effect of substrate concentration on enzyme activity.

The routine assay of the enzyme was based on hydrolysis of the synthetic substrate 3, 4-DNP-TNAC by the enzyme. Fig. 17 is a graphical determination of the Michaelis-Menten enzyme-substrate complex. At low substrate concentrations, the rate of release

Fig. 16   Effect of enzyme concentration on enzyme
activity

Enzyme activity (V) was expressed as
percentage of that at 980 μg protein/ml. The
value (V) at 980 μg protein/ml of enzyme solution
was 0.12 μmol/min of 3, 4-DNP-released. The
standard 3, 4-DNP-TNAC assay was used.
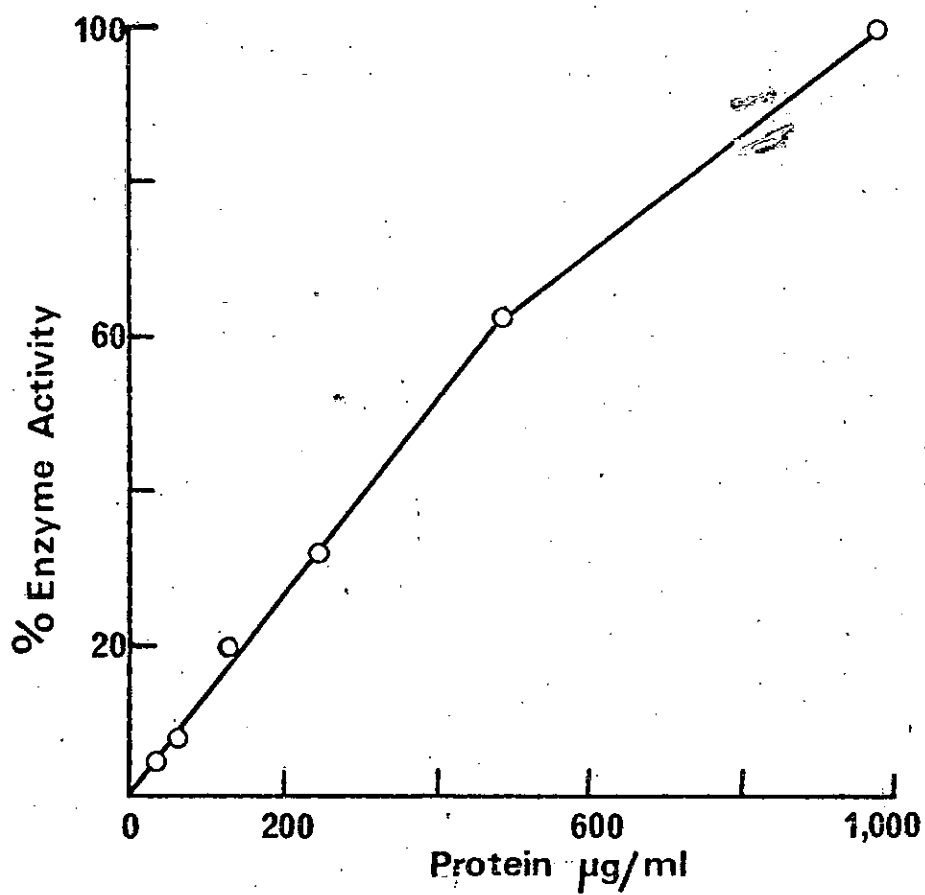
Fig. 17

Graphical determination of enzyme-substrate
complex using Michaelis-Menten plots

The 3, 4-DNP-TNAC was the substrate (S)
used.

V is the μmol/min of 3, 4-DNP liberated by
enzyme.
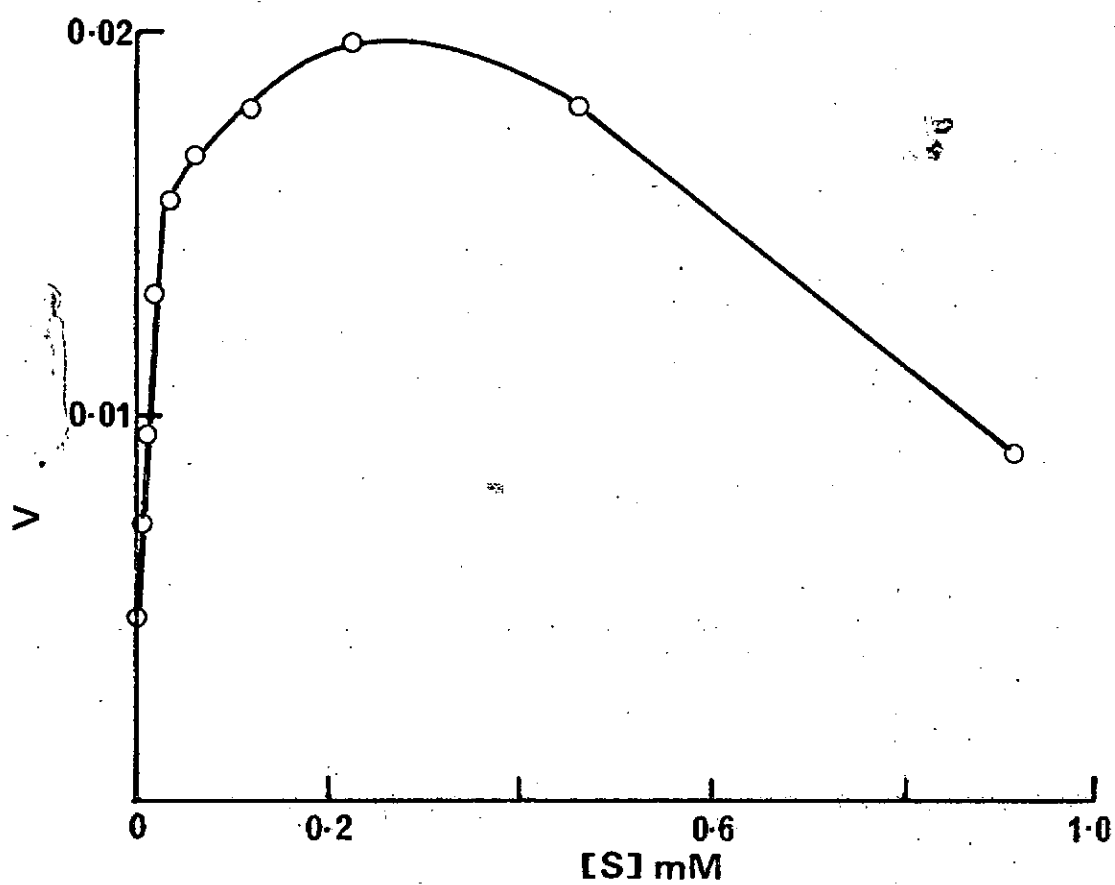
of 3, 4-DNP/min. by enzyme was constant. The

maximum velocity of reaction (V) occurred at a

substrate concentration of 0.227 mM, when the

enzyme was saturated with substrate.

The substrate concentration at half maximum

velocity of reaction (Km) and V values were

obtained from reciprocal plots (Lineaweaver-Burk).

The km values obtained for 2 experiments were

0.0071 mM and 0.0077 mM (Figs. 18 and 19).

Enzyme inhibition in the presence of excess

substrate was observed at a substrate concentration

of 0.45 mM and above (Fig. 19).

(c) Enzyme activity on other substrates.

The enzyme preparation was tested for other enzyme

activities associated with the preparation. This was

ability to hydrolyse p-Nitrophenyl-N-acetylglucosaminide

(substrate for N-acetylglucosaminidase) and Micrococcus

lysodeikticus cells (substrate for lysozyme).

The enzyme was incapable of hydrolysing either of these

substrates. A control experiment for hydrolysis of

M. lysodeikticus cells carried out simultaneously by

reacting lysozyme with M. lysodeikticus cells.

The rate of lysis of the cells by this lysozyme

Fig. 18   <u>Lineaweaver-Burk reciprocal plot showing</u>
<u>inhibition of chitinase by excess substrate</u>
<u>(3, 4-DNP-TNAC)</u>.

Data for this plot were obtained by varying

the substrate concentration under otherwise

standard assay conditions, using 3, 4-DNP-TNAC.
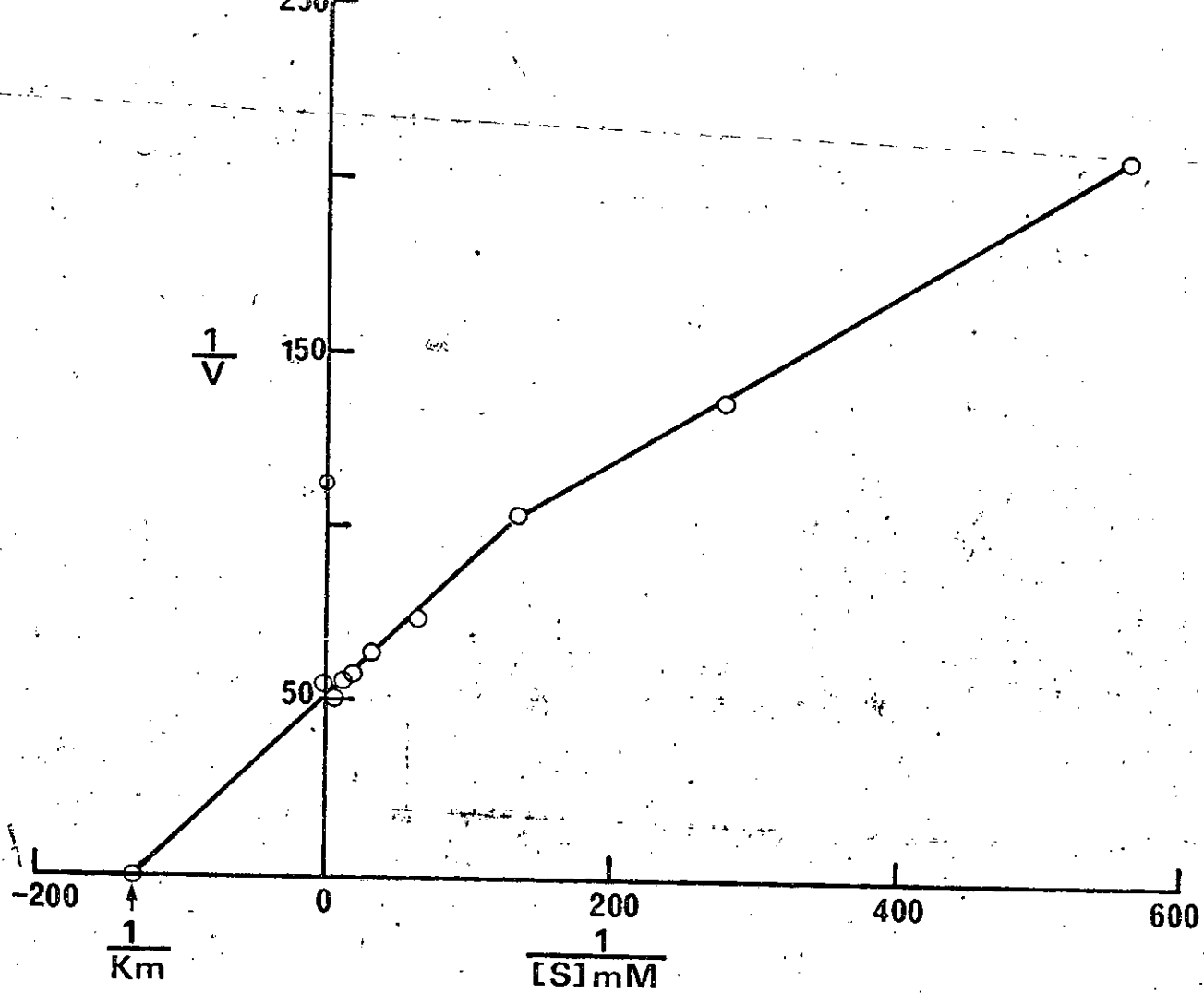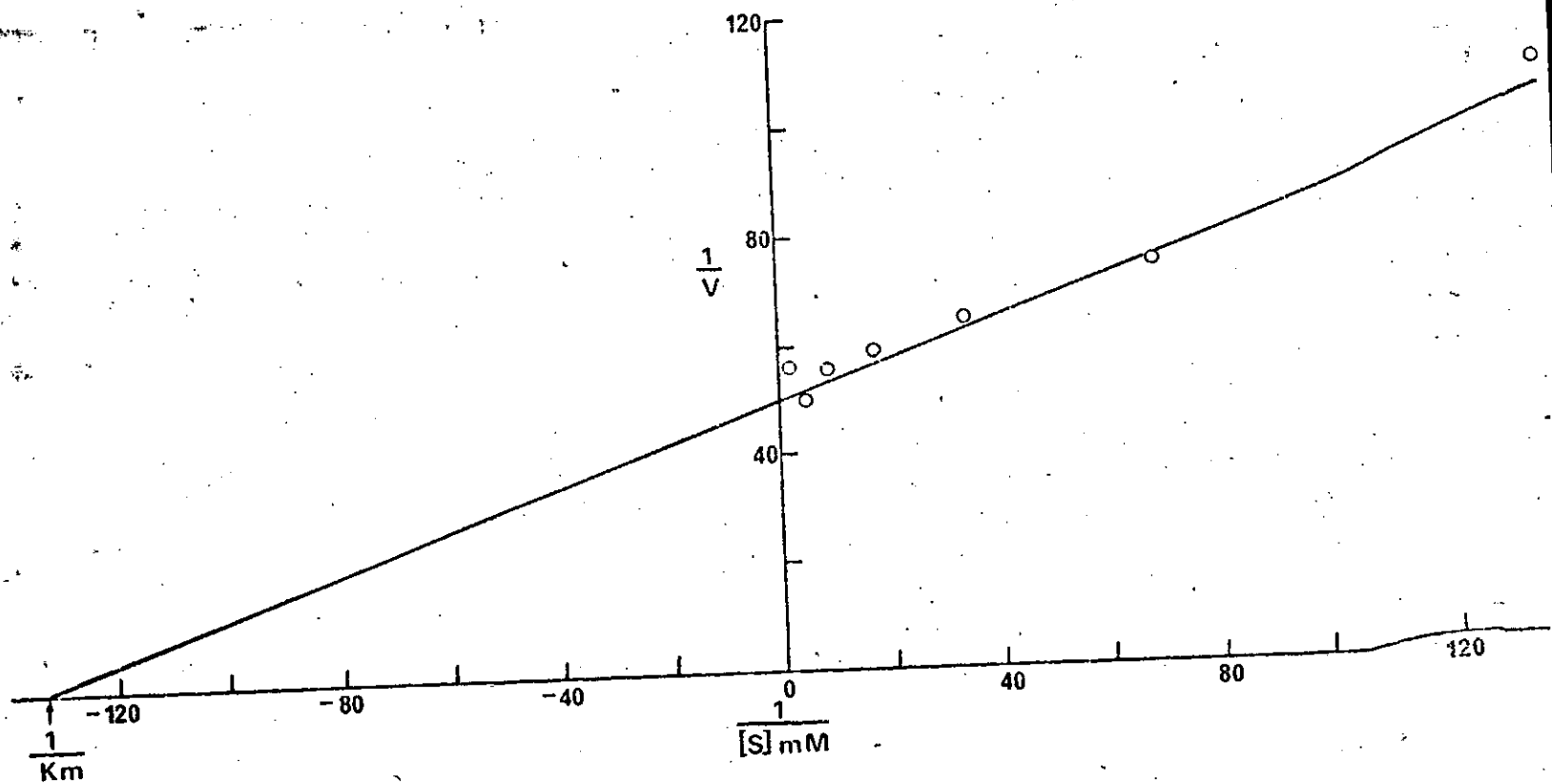
V in the μmol/min of 3, 4-DNP liberated by

enzyme.

Figure 19   <u>Lineaweaver-Burk reciprocal plot</u>

    Km values were obtained from this reciprocal
plot. This is an expanded graph of figure 22 to
show more clearly enzyme inhibition by excess
substrate.

    V is the μmol/min of 3, 4-DNP liberated by
enzyme.

was 24 units. One unit is equal to a decrease in absorbance of 0.001 per min. at 450 nm at $30^{o}C$ on a spectrophotometer. There was no change in absorbancy, when the enzyme preparation of V. alginolyticus was reacted with the lysozyme substrate. Lysozyme had an enzyme activity (V) of .006 μmol/min of 3, 4-DNP released, when tested on 3, 4-DNP-TNAC.

There was no detectable hydrolysis of p-Nitrophenyl-N-acetylglucosaminide by V. alginolyticus enzyme preparation after incubation for up to 40 minutes. N-acetylglucosaminidase when reacted with 3, 4-DNP-TNAC did not hydrolyse the substrate.

(d) Effect of various compounds on enzyme activity.

The enzymic hydrolysis of 3, 4-DNP-TNAC was not affected by most of the added compounds (Table 33). However, colloidal chitin and alpha chitin (Sigma) behaved as inhibitors.

(c) Effect of chitin on enzyme activity.

Colloidal chitin proved to be a very powerful inhibitor of the enzyme activity on 3, 4-DNP-TNAC. From plots of Vc/Vi (control velocity over velocities in presence of chitin) against colloidal chitin concentration (Fig. 20), the V (μmol/min of 3, 4-DNP-released)

Table 33.

Effect of various compounds on enzymic activity

Compounds	nmol/min 3, 4-DNP-released	% Activity
Control	8.0	100
N-acetyl-D-mannosamine	8.4	105.0
N-acetyl-D-glucosamine	8.2	102.5
N-acetyl-neuraminic A.	7.8	97.5
Agglutinin wheat germ	7.9	98.75
*Micrococcus lysodeikticus cells	8.2	102.5
*Chitin (Sigma)	7.3	91.25
*Colloidal chitin	3.9	48.75

1 mg of the various compounds were added to 0.5 ml enzyme preparation, 0.5 ml
of 0.1 M McIlvaine buffer pH 6.0, and 0.1 ml of 0.45 mM substrate (3, 4-DNP-TNAC).
After 10 minutes incubation at $37^{\circ}C$, the absorbance was read as for standard assay.

*Colour yield read after centrifugation.

Figure 20

(a) Effect of different concentrations of colloidal chitin on rate of hydrolysis of 3, 4-DNP-TNAC.

(b) Dixon plot (reciprocal of enzyme activity against inhibitor concentration) for the effect of colloidal chitin on enzyme activity. Two substrate (3, 4-DNP-TNAC) concentrations were used:

S_1 ●————●   0.045 mM

S_2 ○————○   0.0225 mM

The standard assay conditions were used, except that the substrate concentration was varied, and varying colloidal chitin concentrations were added.

After 10 minutes incubations at 37°C, the reaction was stopped by heating at 100°C. The tubes were centrifuged and colour production of supernatant read at 400 nm.

was greatly decreased, because the enzyme was adsorped by chitin, and the enzyme-chitin complex does not break down to yield the colour.

Figures 20b and 21 showed that the slopes varied at the 2 different substrate concentrations 0.045 mM and 0.0225 mM. As the slope was dependent upon the substrate concentration, this is consistent with competitive inhibition (Dawes, 1972).

The degree of inhibition was dependent on the relative concentrations of the substrate. From the plot of velocity (V) against substrate concentration (S) (Fig. 22), the V increased with increase in substrate concentration. The inhibition was lower at higher substrate concentration.

The plot of reciprocal enzyme velocity (1/V) against reciprocal substrate concentrations (1/S) (Fig. 23) illustrates the differences between the degrees of inhibition by colloidal chitin and alpha chitin. There was little inhibition by alpha chitin. The Km for 3, 4-DNP-TNAC in the absence of chitin was 0.0077 mM. A linear regression line was calculated using the formula built into a Sumlock Compucorp 340 Microstatistician Calculator. The regression coefficient (r) was 0.98 and the slope was 1.27.

Figure 21.

Effect of different concentrations of alpha
chitin (Sigma) on the rate of hydrolysis of
3, 4-DNP-TNAC by enzyme, at two substrate
concentrations.
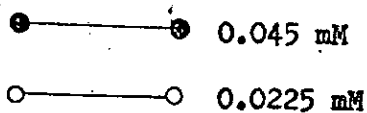
●————————● 0.045 mM

○————————○ 0.0225 mM

The standard assay conditions were used except
that the substrate concentration was varied, and
varying alpha chitin concentrations were added.

After 10 minutes incubation at $37^{\circ}C$, the
reaction was stopped by heating at $100^{\circ}C$. The
tubes were centrifuged and colour production of
supernatant read at 400 nm.

$$\frac{Vc}{VI}$$

∝ Chitin mg/ml
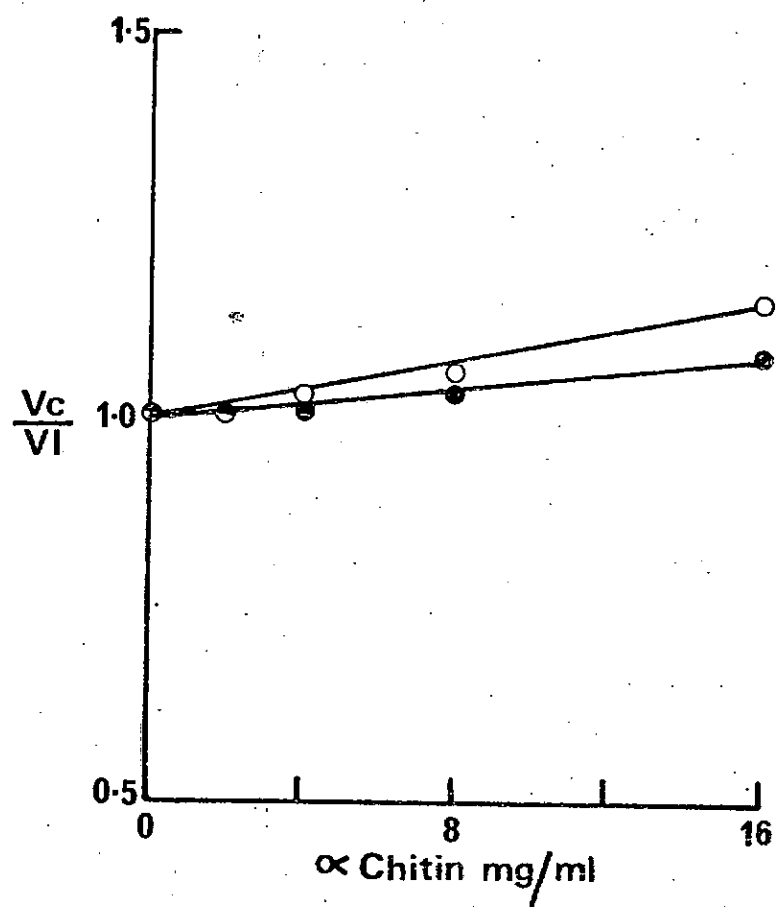
Figure 28. Effect of substrate concentration on
inhibition of enzyme activity by chitin

The standard assay conditions were used
except that the substrate concentration was
varied and that to one series of substrate was
added colloidal chitin   ⊕————⊕ and to another
alpha chitin   △————————△

both to a final
concentration of 4 mg/ml. A third series ○————○
had no addition.

After 10 minutes incubation at $37^{\circ}C$ the
reactions were stopped by heating at $100^{\circ}C$ cooled,
centrifuged, and the supernatant read for colour
production at 400 nm.
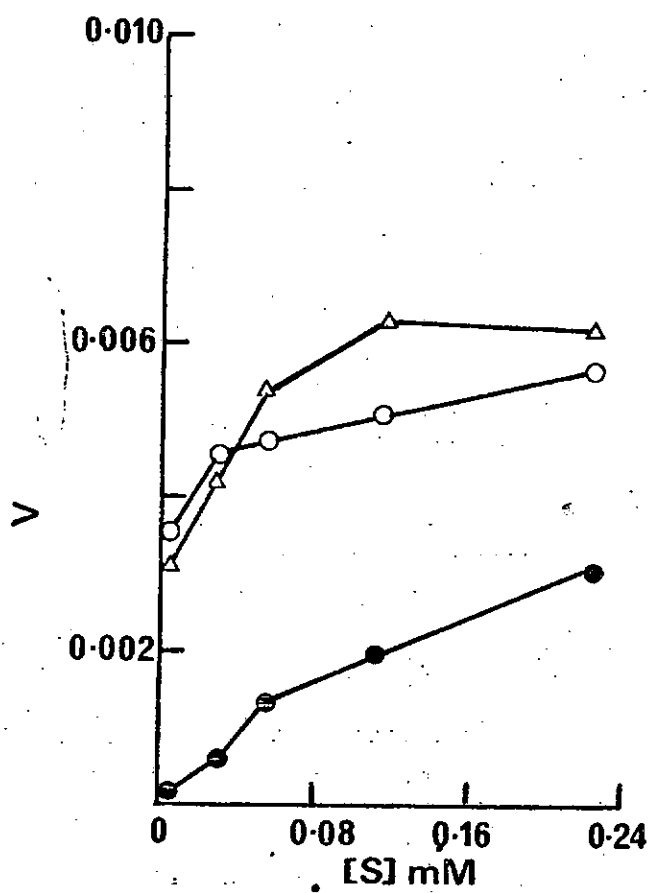
Figure 23.

Effect of chitin on hydrolysis of 3, 4-DNP-TNAC by V. alginolyticus chitinase

The standard assay conditions were used except that the substrate concentration was varied and that to one series of substrate dilutions was added colloidal chitin o————o and to another alpha chitin △————△ both to a final concentration of 4 mg/ml. A third series ⊕————⊕ had no additions.

After 10 minutes incubation at 37°C the reactions were stopped by heating at 100°C, cooled, centrifuged, and the supernatant read at 400 nm for colour production.

V is μmol/min of 3, 4-DNP liberated by enzyme.
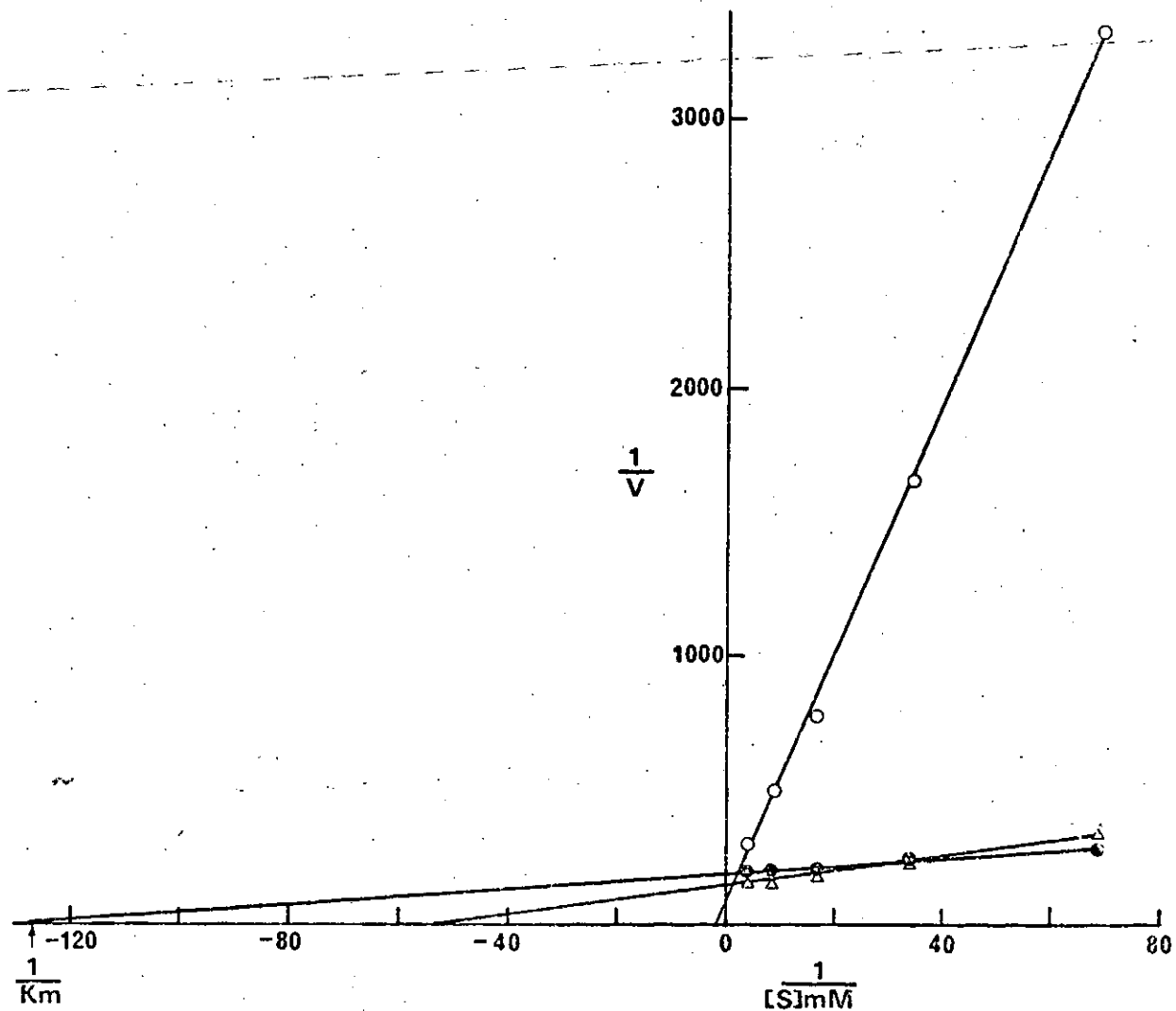
In the presence of colloidal chitin the Km was increased to 0.6 mM with a regression coefficient of 0.99 and a slope of 46.90. In the presence of alpha chitin the Km was 0.018 mM with a regression coefficient of 0.99 and a slope of 2.52.

The Dixon plot (1/V against I) gave a ki value of 72 mg for alpha chitin (Fig. 24). The ki value for colloidal chitin could not be determined from a Dixon plot, because there was the adsorption of enzyme to chitin.

A sample of 1 ml of enzyme was added to 4 mg colloidal chitin and immediately centrifuged for 2.5 minutes. The supernatant thus obtained was used as the enzyme solution and tested for chitinase activity using the 3, 4-DNP-TNAC standard assay. This gave an enzyme activity (V) of 0.002 μmol/min of 3, 4-DNP released. A control tube with 1 ml enzyme but no added chitin was treated the same way. The supernatant obtained from the control when assayed for chitinase activity had a V of 0.018 μmol/min of 3, 4-DNP released, which showed loss of activity from the supernatant obtained after reacting with colloidal chitin. This adsorption of enzyme by colloidal chitin made it impossible to obtain Ki values for colloidal chitin.
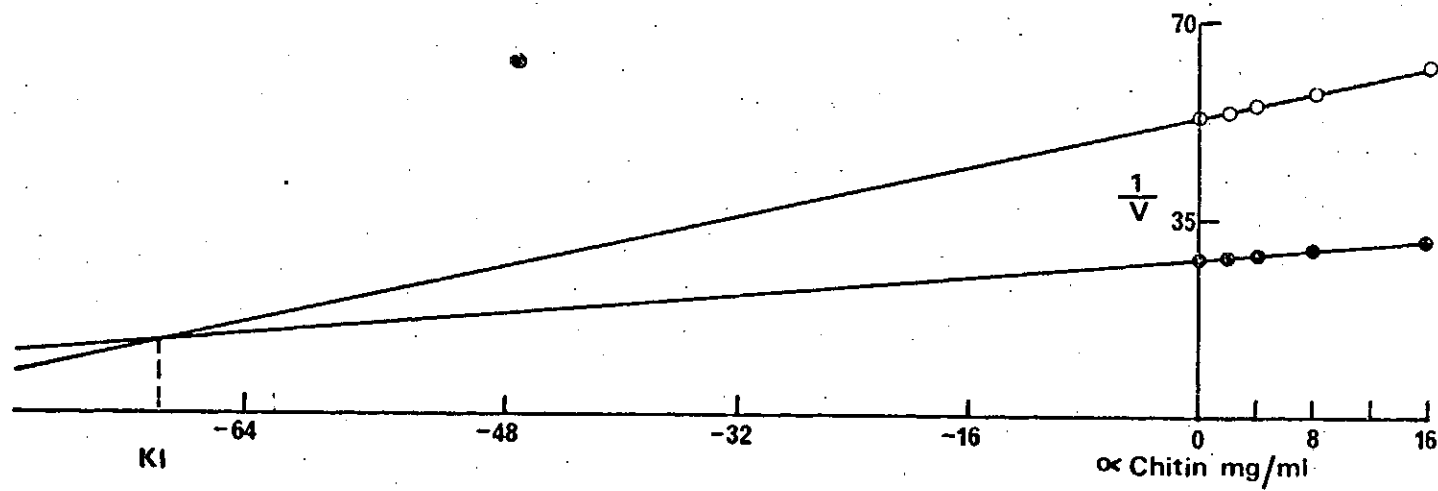
Figure 24.

Dixon plot (reciprocal of enzyme activity against inhibitor concentration) for the effect of alpha chitin on enzyme activity at 2 different substrate concentrations.

1. ⊖————————⊕   0.045 mM

2. ○————————○   0.0225 mM

The intersection of these 2 lines gave a value of K_i of 72 mg/ml.

V is the μmol/min of 3, 4-DNP liberated by enzyme.

13. **Chitinase production by V. alginolyticus in liquid medium.**

V. alginolyticus was grown in sea water medium
(peptone 0.5%) and yeast extract 0.1%) in the absence and
presence of chitin (1%). The protein content was higher
at zero hour because of the peptone in the culture. As
the peptone was used up by the bacteria as a carbon
source, the protein decreased in quantity.

The chitinase activity in culture media with chitin
reached a peak at the 6th day (Fig. 25). The enzyme
was extremely stable in the culture. Weak chitinase
activity was observed in cultures without chitin (Fig. 26).
Since there was a higher yield of enzyme activity
in the presence of chitin, chitinase production by
V. alginolyticus appears to be inducible to a greater
extent.

14. **Gut content of shrimps.**

Phase contrast microscopy of wet mounts of the gut
content of shrimps revealed spines, bristles, strips of
exoskeleton, and mouth parts of arthropods, sand, pieces
of silica material (Plate 5). Most of these structures
except sand probably contain chitin as a major component.
Some regions of the spines were closely packed with bacteria.

Chitin was recovered from the gut and gut contents
of shrimps.

Figure 25.

Liberation of chitinase by V. alginolyticus
in sea water chitin medium

V. alginolyticus was grown in sea water chitin
medium and incubated on a rotary shaker at $25^{o}C$ for
14 days: 25 ml samples were taken at zero time and
every 2 days and tested for chitinase activity using
3, 4-DNP-TNAC assay ⊕————⊕ , and reducing
sugar test △————△ , and protein of the
enzyme preparation was measured ○————○ .

Enzyme activity was expressed as percentage
of values at the 6th day of incubation. The values
were 0.030 μmol/min of 3, 4-DNP released using the
3, 4-DNP-TNAC assay and 93 μg glucose/ml released
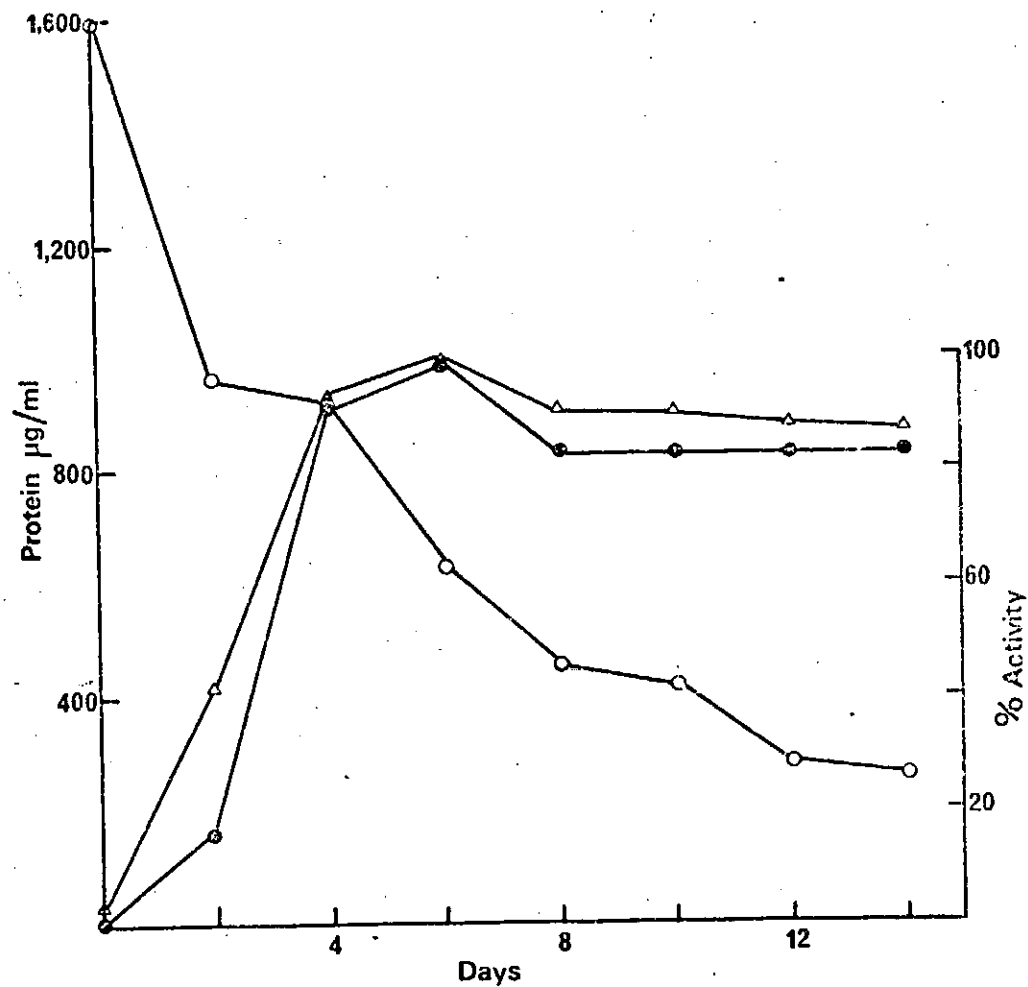using the reducing sugar test.

Figure 26?

<u>Liberation of chitinase by V. alginolyticus</u>
<u>in sea water medium</u>

V. <u>alginolyticus</u> was grown on sea water
medium and incubated on a rotary shaker at $25^{\circ}C$
for 14 days. 25 ml samples were taken at zero
time and every 2 days __ tested for chitinase
activity, using 3, 4-DNP-TNAC assay ⊕————⊕ ,
and reducing sugar test △————△   , and
protein in the enzyme preparation was measured

○————○ .

Enzyme activity was expressed as percentage
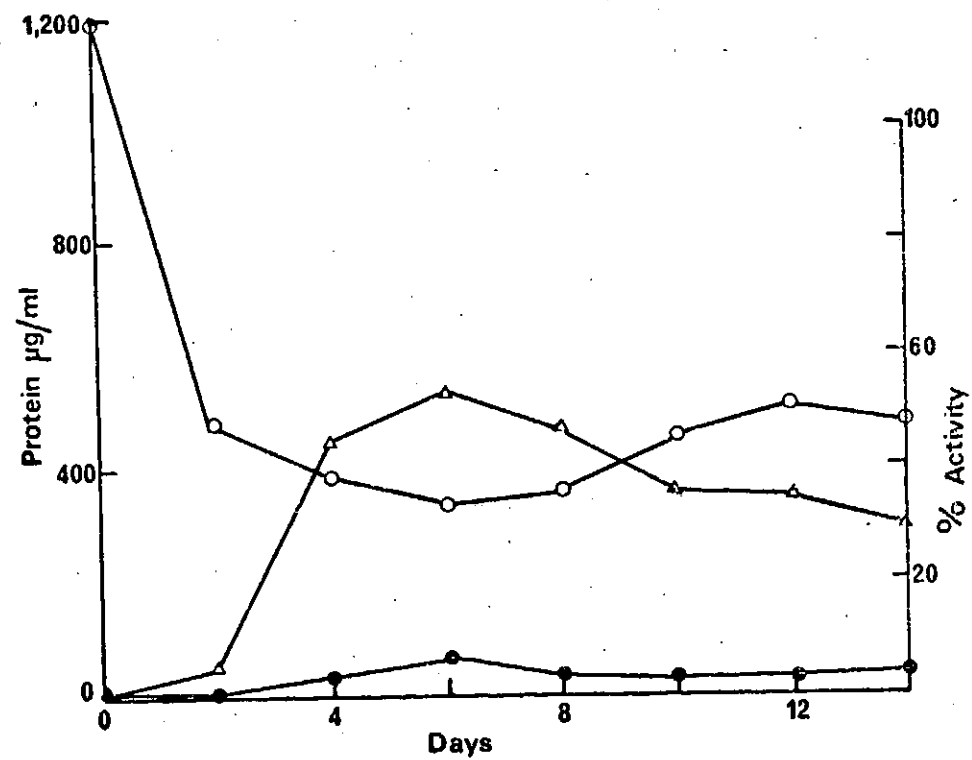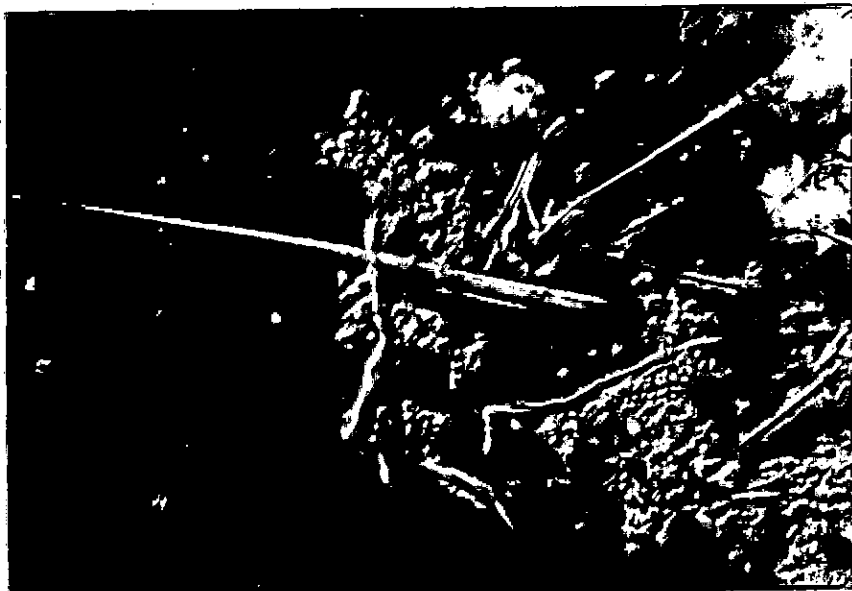of values at the 6th day of incubation in
figure 29.

Plate 5.

Gut contents of shrimp

(a)   and   (b)   Spines and bristles
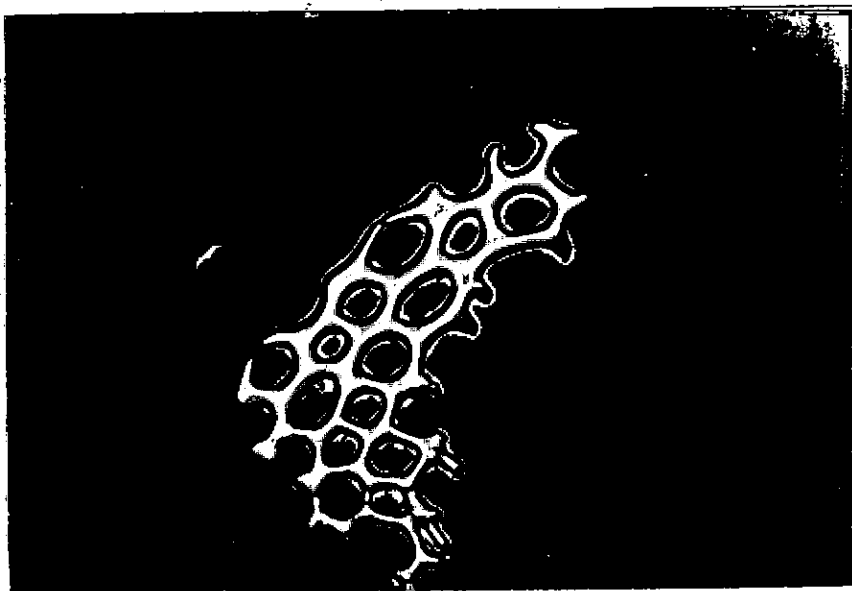
(c)   Silica material

a

b

c

15. Purification of chitinase.

    The culture supernatant of V. alginolyticus was precipitated with ammonium sulphate (80% saturation). A percentage of 90-96 of the total chitinase activity in the culture supernatant (Table 34) was recovered by this process. There was a two-fold increase in the specific activity. Between 7% and 13% of the total chitinase activity was lost by this procedure.

    Chitinase was separated from many other proteins on G-75 Sephadex. The effectiveness of the gel chromatography is illustrated by the elution profile (Fig. 27). Through this procedure, a further 11-fold increase in specific activity was achieved from the partially purified enzyme preparation (Table 35).

    The final preparation on G-200 Sephadex resulted in a 60-fold increase in specific activity compared with the culture supernatant (Table 35). Nearly 100% of the enzyme was eluted in a single peak on G-200 (Fig. 28). This did not correspond to the single protein peak.

Table 34.

Purification using ammonium sulphate (80% saturation)

Material	Volume assayed ml	Enzyme concentration μg protein/ml	Chitinase* activity	% Activity recovered	Specific+ activity
Culture supernatant (a)	0.5	1.270	0.032	100	0.050
(b)	0.5	1.310	0.040	100	0.061
(1) $(NH_4)_2SO_4$ (a) Precipitate	0.5	630	0.030	93.75	.095
(b)	0.5	650	0.035	87.5	.108
Supernatant (a) after precipitation with	0.5	340	.001	3.125	.006
$(NH_4)_2SO_4$ (b)	0.5	340	.001	2.5	.006

*   Chitinase activity expressed as μmol/min of 3, 4-DNP released

\+   Specific activity expressed as μmol 3, 4-DNP/min/mg protein

(1)   25 ml of culture supernatant was precipitated with $(NH_4)_2SO_4$ for 2 hours
       The precipitate was diluted to 4 ml with distilled water, and used as
       enzyme solution.

Figure 27.

Elution diagram of chitinase on sephadex
G-75 column

A 1 ml sample of enzyme preparation from
V. alginolyticus culture was applied to a column
of sephadex G-75 (35 cm x 0.9 cm). The column
was washed with 0.1 M McIlvaine buffer pH 6.0.
The size of the effluent fractions was 5 ml.
Samples were assayed for enzyme activity o———o
and protein was measured ●———● .

The void volume was 6.75 ml. Enzyme activity
was expressed as the percentage of the value at
fraction 5. The actual value at fraction 5 is
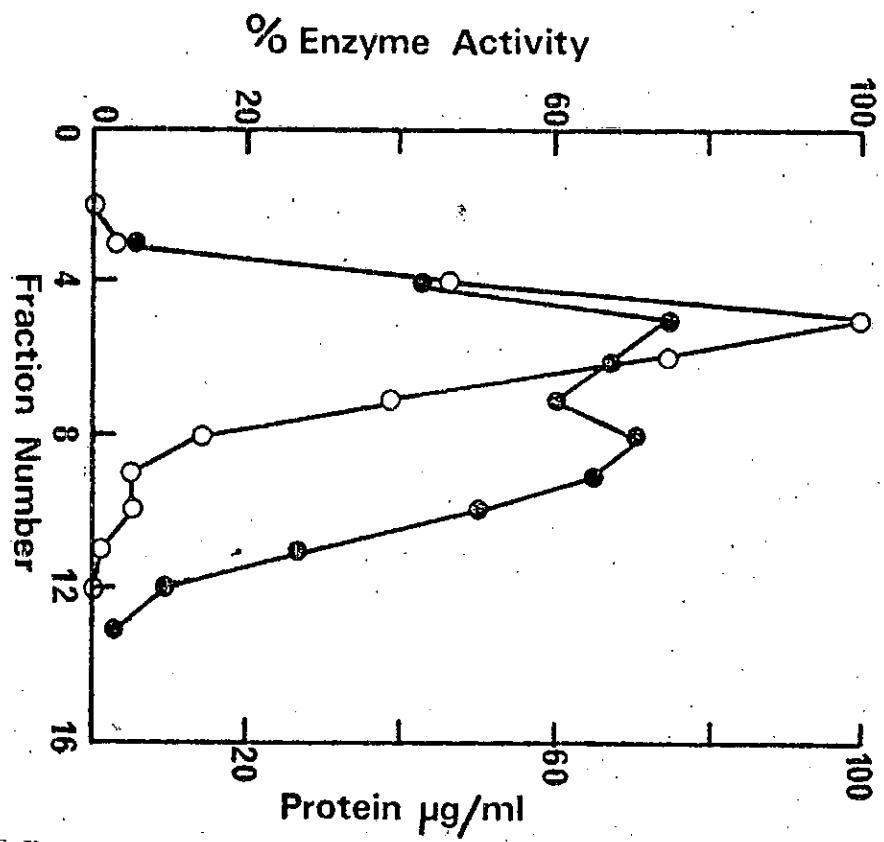0.056 μmol/min of 3, 4-DNP released.

Table 35.

Purification of chitinase

	Vol ml assayed	Protein $\mu g/ml$	Chitinase* activity	Specific[+] activity	Purification
Supernatant	0.5	1,270	.032	0.050	1
(1) $(NH_4)_2So_4$ (80% Saturation)	0.5	123	.020	.13	2.6
G-75	0.5	76	.056	1.5	30
G-200	0.5	2	.003	3.0	60

* Chitinase activity expressed as $\mu mol/min$ of 3, 4-DNP released

+ Specific activity expressed as $\mu ml/3,4\text{-DNP}/min/mg$ protein

(1) 1/20th dilution of stock enzyme solution used, with a protein concentration of 123 $\mu g/ml$.

Figure 28. <u>Elution diagram of chitinase on a sephadex</u>
<u>G-200 column</u>

The fractions 4-6 from G-75 fractions were
pooled. These were concentrated and dialysed
against 0.1 M McIlvaine buffer pH 6.0, and 0.5
ml of this applied to a Sephadex G-200 column
(60 cm x 1.5 cm) equilibrated in the same buffer.
The size of the effluent fractions was 3.7 ml.
The fractions were assayed for enzyme activity

O————————O , and protein   ●————————●

was measured.

Enzyme activity was expressed as percentage
of the value at fraction 13. The actual value
was 0.003 μmol/min of 3, 4-DNP released.

The void volume was between 40 ml and 120 ml.
Fraction 1 on the diagram is equivalent to 40.7 ml
of the void volume.
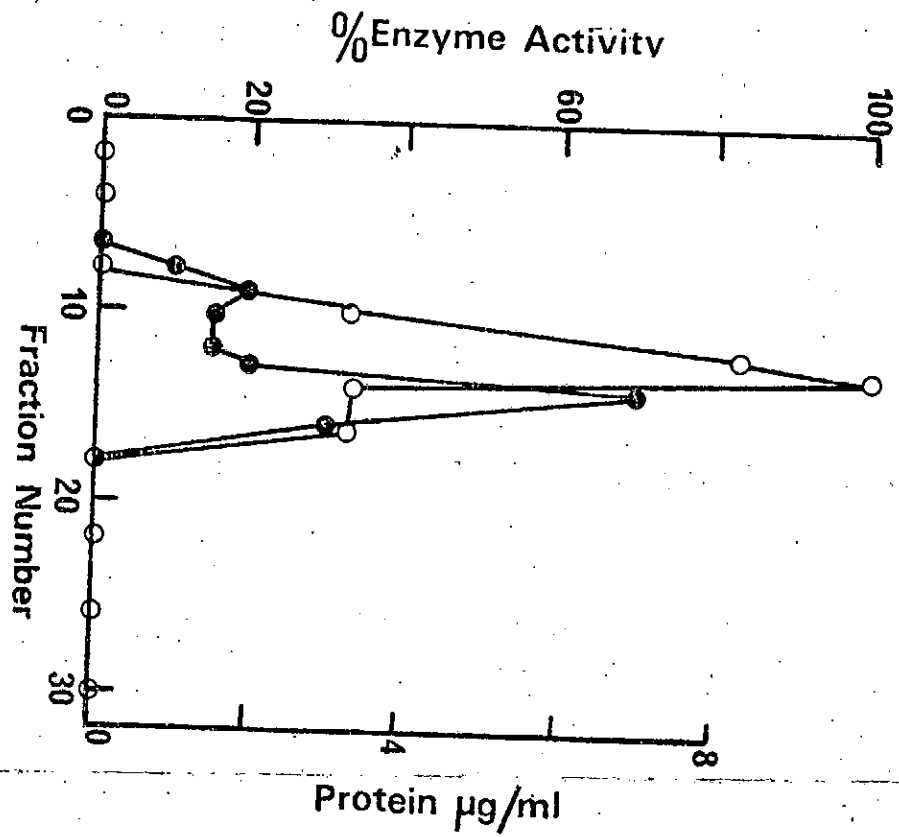
DISCUSSION

The flora of sea water and shrimps.

The occurrence of various bacterial genera in marine environment has been recognised by many microbiologists but there has often been considerable difficulty in defining the difference between marine and non-marine micro-organisms as they frequently have the same morphology, and many of them, the same physiological characteristics (Wood, 1967). Salt tolerance has been the only acceptable characteristic used to delineate marine organisms from non-marine. Marine micro-organisms are regarded as those that can tolerate and multiply in solutions of about 35 parts per thousand salinity. Wood (1967) applied the criteria of Baird-Parker (1965) for classing cocci especially micrococci, and those of Schewan, Hobbs and Hodgkiss (1960a, b) for non-sporing Gram-negative rods to classify into families and genera the bacteria found in the marine environment. He listed some 20 families and several genera of bacteria. All the bacterial genera isolated from sea water and from freshly caught shrimps, in the present study are included in Wood (1967) list.

In the present study, several bacterial genera were isolated from the sea water (table 4) and from freshly caught shrimps (table 3) from the sea. The bacterial flora on the freshly caught shrimps are certainly inhabitants of the waters from which they were caught.

The monthly fluctuations observed in the numbers of bacteria in the sea water (and therefore also on shrimps caught from such waters) (see figs 10 and 11) have been ascribed by some investigators to either water temperature (Zobell and Feltham, 1934) or plankton outbursts (Lloyd, 1930; Zobell and McEwan, 1935). It must be mentioned however, that in the present study the fluctuations in bacterial load of sea water and the shrimps cannot be entirely attributed to changes in water temperature because water temperature changes in the tropics are minimal, the higher temperature occurring mainly in January to March and not in October and September, the two months in which the highest number of bacteria were isolated from sea water and shrimps respectively.

Longhurst (1962) however observed upwelling in the West African coastal waters in the months of August and September. Since no data on plankton outburst were obtained in the present study and no data on plankton outbursts of Nigerian waters is available yet, it is not possible to relate the fluctuations in bacterial populations to this factor.

Green (1949) found that bacteria $\log_{10}$ counts of freshly caught shrimps in the Gulf of Mexico ranged between $\log_{10}$ 3.2 and $\log_{10}$ 5.2/gm shrimp tissue. Similarly Magar and Shaikmahmud (1956) obtained counts ranging from $\log_{10}$ 4.0 to $\log_{10}$ 6.1/gm shrimp tissue

for Bombay prawns. Harrisson and Lee (1969) worked on freshly caught Pacific shrimps and obtained counts of $\log_{10}$ 6.1 to $\log_{10}$ 6.4/gm shrimp tissue. The figures obtained for Nigerian shrimps in the present study were considerably higher, ranging from $\log_{10}$ 3.1 to $\log_{10}$ 8.7/gm shrimp tissue at 20°C. This may imply that Nigerian waters are very rich in organic nutrients which support large bacterial populations. The higher loads on Nigerian shrimps and sea water appear to confirm Kriss'finding (1971) of greater numbers of bacteria in water from hotter areas than in colder regions.

The high level of contamination and variation in the bacteria loads of shrimps arriving at the processing plants is of great importance to the processor, for the attainment of high bacteriological standards of the finished product depends on the level of contamination of raw materials and the level of hygiene maintained during processing.

Campbell and Williams (1952) reported that freshly caught shrimps may have bacterial counts as high as $\log_{10}$ 8.7/gm shrimp tissue at 20°C, and $\log_{10}$ 6.0/gm shrimp tissue at 37°C.

The various bacterial genera isolated from freshly-caught shrimps (prawns) by various workers are tabulated in table 36 for the ease of comparison. The composition of bacterial flora of Nigerian shrimps (Table 36) is similar to that of Thai shrimps (Cann, 1973) in which coryneforms formed over 50% of the flora on shrimps. Williams, Campbell and Rees (1952b) while working on microflora of Gulf shrimps, Magar and Shaikmahmud (1956) on Bombay prawns observed that Achromobacter,

Bacillus, Micrococcus and Pseudomonas were the main groups present in freshly caught shrimps.

The microflora of Pacific shrimps consisted of 46.8% Acinetobacter/Moraxella, 21.4%, Flavobacterium; 9.9%, Pseudomonas; and 4.4% Bacillus. Cann (1973) reported that the microflora of tropical shrimps consisted of coryneforms and micrococci, forming about 51-91% of the bacterial load; Achromobacter/Pseudomonas, 2-15%; Flavobacterium/Cytophaga, 0 - 20%; and others were 6-14% (Table 36).

The differences in the types of microflora of shrimps from temperate waters and those from tropical waters has been attributed by Shewan (1961) to climatic effects on the marine environment which influences the microflora of the fish. This worker stated that a greater percentage of mesophiles (Bacillus spp. coryneforms and micrococci) and fewer psychrophiles, such as Pseudomonas, Achromobacter, and Flavobacterium species, occur in the warmer waters off India, the East coast of South Africa, Australia and the Adriatic. In addition to climatic affects, other factors which may influence the types of microflora on marine shrimps include species of shrimps, type of fishing gear, fishing grounds, fishing methods, age of the samples, degree of washing and icing on the boat, duration and temperature of storage prior to processing (Shewan, 1944).

Table 36.

Percentage composition of bacterial flora of freshly caught prawns in various parts of the world (for comparison only)

Fishing area	Coryneforms	Micrococcus	Staphylococcus	Flavo/Cyto	Achromobacter	Streptococcus	Pseudomonas	Aeromonas	Enterobacteriaceae	Bacillus	Streptomycetes	Others	Reference	Incubation Temperature °C.
Nigerian coast	46.6	16.5	14.0	2.6	5.9	–	2.7	4.0	3.5	4.2			Aribisala, 1976	20
Lagoon, Ilubirin Village	14.5	4.54	10.0	8.1	6.3	–	0.9	6.3	37.2	11.8	–	–	Aribisala, 1976	37
Gulf of Thailand	55.0	24.0		–	5.0	–	1.0	1.0	3.0	–	9.0		Cann, 1974	20
Pacific shrimps	2.7	7.1	–	21.4	46.8	–	9.9	–	–	4.4		7.7	Herrison and Lee 1969	20
Off shore Chilan Sri-Lanka	18.0	15.0		17.0	12.0		18.0	5.0	4.0	1.0		10.0	De Silva, 1976 (Unpublished)	20
Lagoon – Mulatiu (Sri-Lanka)	23.3	20.0		20.0	7.6	1.4	3.2	1.0	12.1	2.88	7.9		De Silva, 1976 (Unpublished)	20
Gulf coast	–	15.0	–	–	27.3	0.8	14.3	–	14.7	20.8		2.0	Williams Campbell and Rees, 1952b	20

There are few descriptions in literature of non-pigmented
coryneforms isolated from soil and water, and no reports of such
bacteria have been found in the literature dealing with marine and
fish bacteriology. It is therefore likely that many of the
non-pigmented strains, 230 from water and 211 from shrimps, will be
found on further examination to be new species. Many of the yellow
and orange strains were similar to isolates from fish described by
previous workers, particularly Wood (1950). The coryneforms were
biochemically heterogenous although all presented the typical
coryneform morphology. Since complete description of saprophytic
coryneforms are rare, attempts to relate the present strain to
previously described strains isolated from soil, water and marine
sources, met with little success. These attempts were further
complicated by the lack of correlation between the results of
biochemical tests used in classifying the strains. Cann (1973)
found that the number of coliforms on Thai shrimps ranged between
180 to 1,240/100 gm shrimp tissue but he did not isolate any
pathogenic bacteria. The range of coliform count on Nigerian shrimps
in this study was found to be between 1,600 to 1,800/100 gm shrimp.
No Salmonella sp, Vibrio cholera, or Vibrio paraheamolyticus were
isolated. The high coliform count does not mean that the shrimps
could constitute a health hazard as long as food poisoning bacteria
are not present in addition to the coliforms.

A succession pattern was established for the microflora involved in spoilage of shrimps held at chilled temperature. The pattern was different from observed microflora of shrimps held at room temperature. The results obtained for the succession pattern of bacteria on shrimps at chilled temperature were similar to those previously recorded by Cann (1971) for the microflora of Thai shrimps, the outstanding feature being the predominance of Pseudomonas. The important feature of the result of experiments on microbial spoilage at chilled temperature is the dominance of the Pseudomonas group among the spoilage bacteria flora. Since icing is an important aspect of processing of shrimps in Nigeria, this observation is very important.

Because of higher ambient temperatures, the bacterial flora of shrimps held at room temperature consists of Aeromonas species which are mesophiles and which were not very active at chilled temperatures.

Investigations have shown that faecal streptococci are present on various heat treated foods, e.g.; pasteurized milk and powdered eggs, (Solowey and Watson, 1950; White and Shewan, 1944). Faecal streptococci are mildly heat-resistant organisms. This might have accounted for the high 'MPN' values for faecal streptococci in smoked shrimps. Larkin, Litsky and Fuller (1955) have shown that faecal streptococci can be eliminated from vegetables by boiling for 1 minute at $88^{\circ}C$.

The preliminary culturing experiments of chitinolytic bacteria indicate that chitin without supplementary factors was not a suitable medium for isolating the bacteria. There was appreciable delay in growth of colonies and appearances of clearing. The addition of 0.1% yeast extract and 0.5% peptone to chitin agar supported the growth of bacteria well, and there was a quicker response to chitinase production, by the cultures in this medium.

Constituents of the exoskeleton of crustaceans are chiefly chitin and calcium carbonate. Seki (1965) described calcified and uncalcified layers in the exoskeleton of crustaceans, the latter of which is favourable for the decomposition of chitin after the death of the custaceans. Both of the layers contain chitin which amounts to about 60-80% of the dry weight of the organic fraction of the exoskeleton. Waterman (1960) reported the exoskeleton of lobster (Homarus) as having water soluble protein and protein bound firmly to the chitin, which contain serine, alanine, threonine, tyrosine, tryptophan and other amino acids. This will probably be similar to the exoskeleton of shrimps. The exoskeleton of these crustaceans contains favourable amino acids for growth of micro-organisms. The growth and chitinolytic activities of bacteria isolated from the crustaceans must be stimulated by these nutrients.

The literature on microbial degradation of chitin consists of reports of the isolation of chitinolytic bacteria from a variety of marine sources, sand, mud, intestine of frogs, snipe, trout, decaying crayfish, etc. These chitinolytic bacteria are distibuted over a number of known genera of bacteria, some of which are Streptomyces, Bacillus, Vibrio, Aeromonas, Pseudomonas, Cytophaga, Flavobacterium, Achromobacter, Micrococcus, Clostridium.

During this study, the chitinolytic flora of the gut and the surface of the shrimps are similar. _Vibrio alginolyticus_, _Pseudomonas_ spp and _Bacillus cereus_ were isolated from both the surface and gut of shrimps. These are representatives of well known genera, although these species have not been previously reported as chitinolytic from any marine source, including shrimps. Members of these two genera which have been reported as chitinolytic from marine sources are _Vibrio gerris_, _V. labrakos_, and _V. orphus_ from digestive tract of _Lateolabrax japonicus_ (Okutani, 1966).

This is the first report of chitinolytic bacteria from shrimps. _V. alginolyticus_ often form the majority of the _Vibrio_ population in marine samples (Donovan, Lee and Furniss, 1976). So far, this bacterium has not been associated with gastroenteritis in man. They are often isolated from localised tissue infections as well as infections of the eye and ear (Baumann, Baumann and Reichert, 1972). Variable flagellar organisation is frequently encountered among _V. alginolyticus_ strains (Baumann, Baumann and Reichert (op.cit.). They are often peritrichously flagellated when grown on solid media and polarly flagellated in liquid media as observed for the strains isolated in this study. Scheffers, De Boer and Looyaard, (1976) put forward an ecological hypothesis that the peritrichous flagella may be aids in holding bacteria to a solid substrate. It may be appropriate to suggest that the _V. alginolyticus_

use their numerous flagella produced on the chitin agar plate
to establish close contact with the solid media.

Bacillus cereus is common in sediments of polluted areas
and marine areas (Bonde, 1976). It has also been isolated in
several countries from a wide variety of foods, where it has been
associated with food poison (Mossel, Koopman and Jongerius, 1967;
Kim and Goepfert, 1971; Gilbert, Stringer and Peace, 1974).
Gilbert, Stringer and Peace (op.cit) reported B. cereus as causing
food poison in boiled and fried rice, especially in Chinese
restaurants. The isolation of B. cereus from shrimps is important
from the point of view of health hazards because they are heat
resistant, and thorough cooking may not destroy the spores.

The predominance of Pseudomonas on shrimps during spoilage
in ice has been reported by the author from the previous work
on spoilage of shrimps during chilling in ice. Cann (1971)
also reported a predominance of Pseudomonas during spoilage of
Thai shrimps in ice. The presence of Pseudomonas sp. on shrimp
is of significance to the fishing industry from the point of
view of shrimp preservation.

Micro-organisms which utilise substances of high molecular
weight generally do so by liberating into the medium enzymes
which break down the substrate into smaller permeable units.
All the species of chitinolytic bacteria isolated from shrimps
liberated chitinase extracellularly.

All chitinolytic species isolated are aerobes, although
V. alginolyticus is a facultative anaerobe. This does not mean
that there are no anaerobic chitinolytic bacteria on shrimps as
the isolation techniques used were designed to obtain only aerobes.

Chitinolytic bacteria have been isolated from the gut of
shrimps. Some research workers have also isolated a vast amount
of chitinolytic bacteria from the digestive tract of fish and
other marine animals (Benton, 1935; Zobell and Rittenberg, 1937;
Hock, 1937; Seki and Taga, 1963; Okutani, 1966). The gut wall and
the gut contents were found to contain an appreciable amount
of chitin. This raises a question as to the functional aspect
of these bacteria in the gut. Some investigators suggested
that these chitinolytic bacteria from the digestive tract of fish
should not be neglected as symbionts (Zobell, 1938; Okutani, 1966).
Seki and Taga (1963) suggested that the possibility of symbiosis
by the chitinolytic bacteria in aiding the marine animals for the
digestion of chitin might be negligible from the point of view
of the importance of mechanical aid provided for digesting food
and the small amount of end products released during chitin
degradation.

These shrimps are bottom dwellers. A vast amount of
chitinolytic bacteria has been reported from marine mud (Benton, 1935;

Zobell and Rittenberg, 1938; Hock, 1940; Campbell and Williams, 1951). From the sand and other gut contents of the shrimps, they probably feed on organic matter from the mud. They may likely ingest these chitinolytic bacteria with their food.

Another group of workers have reported the presence of lysozyme with chitinase activity from the digestive tract of plaice (Fletcher and White, 1973) and chitinase from the gastric mucosa of fish (Okutani, 1966), and the tissue, plasma and lymph of fishes (Fange, Lundbhad and Lind, 1976), Jeuniaux (1971)pointed out that the distribution of chitinases and chitobioses in bacteria suggested that the synthesis of both chitinolytic enzymes was a property attributed to the primitive unicellular organisms. From the results of the investigation of Okutani (1966) and Fange, Lundbhad and Lind (op.cit.) the origin of the chitinase in the organs seems to be secreted from the pancreas and gastric mucosa of these fishes. The possibility of the chitinase being of bacterial origin in the digestive tract and diffusing through the gut wall into the blood stream and thereby transported to some organs of the body should not be overlooked.

Chitinases are specific for linear polymers of N-acetyl-D-glucosamine, but do not split diacetyl-chitobiose. They hydrolyse chitin to diacetyl-chitobiose (EC 32.1.14). Other substrates

that have been described for chitinases apart from chitin (colloidal chitin) include carboxymethyl chitin (Hultin, 1955), glycol-chitin (Otakara, 1961; Senju and Okimasu, 1950), and chitin sulphates (Hackman, 1954).

The common methods of measuring chitinase activity using the following substrates, colloidal chitin, and chitin powder, are estimating the amounts of reducing sugar liberated and N-acetylamino sugar colorimetrically. Viscosimetric determinations have been used for chitinase activity using glycol-chitin and carboxymethyl chitin. Turbidimetric method (Jeuniaux, 1958) has also been used to measure the turbidity variation of a colloidal chitin suspension during chitinolysis.

Two assays were used in this study; reducing sugar assay using colloidal chitin and alpha chitin as the substrates, and a new colorimetric assay using 3, 4-dinitrophenyl-tetra-N-acetyl-chitotetraoside (3, 4-DNP-TNAC) as the substrate. This substrate was synthesised by Ballardie and Capon (1972) as a lysozyme substrate and they reported the cleavage of NAG-NAG bonds occurring at about the same rate as release of 3, 4-dinitrophenyl (3, 4-DNP). Otaki and Kimura (1975) have used this 3, 4-DNP-TNAC assay for determining quantitatively the activity of human milk lysozyme, and found it to be less sensitive than the turbidimetric procedure using the commercial substrate Micrococcus lysodeikticus cells.

It is most likely that the present investigation is the first on chitinase activity colorimetrically determined using the 3, 4-DNP-TNAC assay. Using a partially purified enzyme preparation from V. alginolyticus culture, the specificity of the assay was determined. The results of the pH optimum of enzymic activity in 3, 4-DNP-TNAC was compared with the pH optimum obtained for the enzyme using the reducing sugar assay. The pH optimum of the enzyme preparation using the two tests fall between 5.5 and 6.0, which is similar to the optimum pH reported for other bacterial chitinases (Berger and Reynolds, 1958; Jeuniaux, 1963; Okutani, 1966).

The enzyme did not exhibit any muranidase or lysozyme activity using M. lysodeikticus cells as the substrate; also, there was no competitive inhibition exhibited by M. lysodeikticus on cells during enzyme activity with 3, 4-DNP-TNAC.

There was no release of p-nitrophenyl from p-nitrophenyl-N-acetylglucosaminide by the enzyme, showing that the enzyme preparation has no N-acetylglucosaminidase activity. N-acetylglucosaminidase when reacted with 3, 4-DNP-TNAC could not hydrolyse the substrate.

Inhibition experiments in which structural analogous of N-acetylglucosamine were used showed that none of them has an

inhibitory effect on the degradation of 3, 4-DNP-TNAC by V.alginolyticus enzyme. Chitin and colloidal chitin showed a competitive inhibition to the enzyme activity on 3, 4-DNP-TNAC. Colloidal chitin has a stronger inhibitory effect, and there was also an adsorption of the enzyme to chitin when reacted with colloidal chitin, showing there was a chitin-enzyme complex.

V. alginolyticus chitinase has a pH optimum around 5.5 for colloidal chitin and 6.0 for 3, 4-DNP-TNAC. Jeuniaux (1963) found out that most chitinases from bacteria have an optimum pH of about 5.0 when the substrate is chitin. The pH optimum of 5.5-6.0 is in agreement with many other bacterial chitinases previously studied: Pseudomonas hydrophila, pH 5.0 (Clarke and Tracey, 1956), Streptomyces griseus pH 6.3 (Berger and Reynolds, 1958), Aeromonas chitinophthora, pH 5.5-6.0 and Vibrio gerris pH 7.0 (Okutani, 1966), Serratia marcescens pH 6.4 (Monreal and Reese, 1969), Cytophaga johnsonie, pH 6.3-6.5 (Sundarraj and Bhat, 1972), Arthrobacter sp. pH 4.9 (Morrissey, Duggan and Koths, 1976), Aeromonas liquefasciens pH 6.5 (Chigaleichik and Piriera, 1976).

For V. alginolyticus chitinase, the activity was rapidly inhibited below pH 3.5. In contrast, gastric chitinases of non-bacterial origin from fish have a pH optimum of between 3.0 and 4.0 (Okutani, 1966; Fange, Lundbhad and Lind, 1976).

The optimum temperature for $\underline{V}$. alginolyticus chitinases was about $55^{\circ}C$. The optimum temperature for most bacterial chitinase is $40^{\circ}C$ (Jeuniaux, 1963; Okutani, 1966; Sundarraj and Bhat, 1972; Chigaleichik and Piriera, 1976). Monreal and Reese (1969) reported an optimum temperature of $50^{\circ}C$ for Serratia marcescens chitinase, also chitinase from Arthrobacter sp. had an optimum temperature of $50^{\circ}C$ (Morrisey, Duggan and Koths, 1976). Although $\underline{V}$. alginolyticus chitinase had a high optimum temperature, it was rapidly inactivated by preincubating enzyme for 15 minutes before assay at $50^{\circ}C$ and above. The heat stability of the enzyme was much greater at about $40^{\circ}C$.

The chitinase from the gastric mucosa of fish had an optimum temperature of between $50^{\circ}C-60^{\circ}C$. The enzyme was found to be stable at $60^{\circ}C$ for 30 minutes, (Okutani, 1966).

The Km value was very low, 007-007 mM with V max $0.020\,\mu$ mol/min of 3, 4-DNP released. Inhibition of enzyme activity by excess of substrate was observed. Chitin and colloidal chitin both showed competitive inhibition of enzymic activity on 3, 4-DNP-TNAC. The Ki value (72 mg) for alpha chitin was very high. The Ki value of colloidal chitin could not be obtained because the enzyme was adsorped by the colloidal chitin.

Using 3, 4-DNP-TNAC as a substrate for chitinase activity allowed the detection of weak chitinase activity in enzyme

preparations. Also, the procedure is shorter, more convenient, and more sensitive than all the known colorimetric methods for determining chitinase activity. Also, it does not need prolonged incubation like reducing sugar to detect end products of chitinase activity. When the result is positive, there seems to be no likelihood of its being false positive, although the substrate has been found to be degraded by lysozyme. Lysozyme activity could be separated from chitinase activity by testing the enzyme preparation on M. lysodeikticus cells to see whether it would be positive for lysozyme. Another way of doing this is by incubating enzyme preparation with both 3, 4-DNP-TNAC and M. lysodeikticus cells to see whether there would be any inhibition of enzyme activity on 3, 4-DNP-TNAC by the lysozyme substrate.

From Otaki and Kimura's report (1975) and this work, 3, 4-DNP-TNAC is specific to only lysozyme and chitinase.

The end product of V. alginolyticus chitinase activity on 3, 4-DNP-TNAC is production of diacetyl chitobiase. As reported by Ballardie and Capon (1972), the amount of colour produced from 3, 4-DNP-TNAC by chitinase is equivalent to the rate of hydrolysis of the NAG-4 bonds. The cleavage of bonds in this reaction as illustrated in figure 2 of this report may take place in four ways, the amount of colour released depending on the type of cleavage of NAG-4 bonds.

Chitinases may be produced by bacteria in the absence of chitin, and this is referred to as constitutive enzyme production (Clarke and Tracey, 1956; Jeuniaux, 1958). An induced (adaptive) chitinase in the presence of chitin has been reported by Reynolds (1954) for $\underline{S}$. $\underline{griseus}$ and Morrissey, Duggan and Koths (1976) for $\underline{Arthrobacter}$ sp. Jeuniaux (1963) suggested that addition of chitin to culture media greatly enhances enzyme production in both cases.

Chtinase production by $\underline{V}$. $\underline{alginolyticus}$ was to a greater extent induced and only to a very small extent constitutive. The presence of very weak chitinase activity in the growth medium without chitin would suggest that $\underline{V}$. $\underline{alginolyticus}$ chitinase may be constitutive but need the addition of chitin in the culture media to enhance enzyme production. There was over 90% more chitinase activity from the culture media with chitin.

More chitinase activity was detected by the reducing sugar test in the culture medium without chitin. This may be explained from two points of view. There may be two different enzymes involved in the chitinase system, only one may be degrading the 3, 4-DNP-TNAC substrate, while the two might have degraded chitin to release reducing sugars in the reducing sugar test.

Also, the reducing sugar liberated may not be linear with time, the amount released may tail off with time, which shows a time course effect. The first explanation could be checked by using Sephadex column.

The peak chitinase activity was reached at 6 days'
incubation.

An observation made by the author on the growth of
V. alginolyticus in liquid medium shows that the exponential
phase was between the 4th and 5th day and that chitinase was
probably liberated during this period, and peak chitinase
activity reached on the 6th day.

Ammonium sulphate precipitation has been used in this
study as an important part of the purification scheme for enzyme
preparation from V. alginolyticus culture. Eighty per cent
saturation was used and the recovery of chitinase activity was
good. About 90% of chitinase activity was recovered using this
method, and about 2-fold increase in specific activity was
obtained. The chitinase preparation was further purified and
separated from many other proteins on G-75 Sephadex and an 11-fold
increase in specific activity was obtained compared with the
partially purified chitinase (80% ammonium sulphate saturation).

A further purification on G-200 Sephadex gave a 60-fold
increase in specific activity compared with the culture supernatant.

Several workers have used ammonium sulphate precipitation
of chitinases as an important part of their purification scheme.
Berger and Reynolds (1958), using the culture supernatant for
S. griseus, obtained the greatest amount of chitinase with a

salt saturation of 70%. Monreal and Reese (1969) obtained a high yield of chitinase activity with a 40-80% ammonium sulphate precipitation of culture supernatant of S. marcescens. Skujins, Pukite and Mclaren, (1970) obtained the greatest amount of chitinase with a salt saturation of 70% and a pH of 7.0 working with S. griseus chitinase. Merrissey, Duggan and Koths (1976) obtained a three-fold increase in specific activity using 30-90% saturation. They also separated the Arthrobacter sp. chitinase they worked with from other proteins on a G-75 Sephadex and obtained a 12-fold increase in specific activity compared with the culture supernatant.

From this study the maximum contamination of freshly caught and smoked shrimps originate from:

(a) Contamination of the raw material at the fishing ground.

(b) Use of polluted water in washing the shrimps.

(c) Inavailability of ice to the local fisherman.

(d) Use of unclean equipment, including shrimp boxes during handling.

(e) Insanitary and unhygienic conditions in processing factories.

(f) Lack of personal hygiene among shrimp handlers.

(g) Underprocessing and exposure of processed products.

It would be in the interest of the shrimping industry that regulations are implemented to control the contaminants. To meet existing standard for most fishery products significant improvements are required. Recommended improvements are:

1. Supply of icing facilities to the fishing villages for preservation of shrimps after catch. Adequate icing and chilling below $4^{o}C$ immediately after catch must must be encouraged since _Aeromonas_ (spoilage organism at room temperature) would not grow well at low temperature.

2. Abolishing the use of raw river or harbour water for washing of shrimps. In the alternative, use of chlorinated water should be encouraged. Large proportions of bacteria isolated from smoked shrimps were Gram-positive cocci, which most likely had originated from human hands. Since there's a delay of between 3 - 4 hours between catching and processing, the use of chlorinated clean water for washing the shrimps would remove most bacterial contaminants on the surface of the shrimps.

3. Hot smoking or drying of shrimps at $80^{o}C$ for a minimum of 48 hours.

4. Packing of smoked or dried shrimps in clean polythene bags sealed with hot flame to avoid post processing contamination.

5. Health education of local processors on dangers of underprocessing and unhygienic handling of already processed products.

If the above recommendations are adopted, microbial contamination of shrimps would be greatly reduced, and their shelf-life thus lengthened.

SUMMARY

The bacterial counts obtained from Nigerian shrimps, Penaeus duorarum, on incubation at $20^{\circ}C$ ranged between $\log_{10} 3.1$ and $\log_{10} 8.7$/gm of shrimp tissue, while at $37^{\circ}C$ counts ranged between $\log_{10} 3.1$ and $\log_{10} 6.1$/gm of shrimp tissue. For sea water incubated at $20^{\circ}C$, the count of bacterial populations ranged between $\log_{10} 3.2$ and $\log_{10} 7.6$/ml; at $37^{\circ}C$, the range was between $\log_{10} 3.3$ and $\log_{10} 8.4$/ml.

The bacteria present on freshly caught shrimps and in the sea water from where the shrimps were caught were predominantly coryneforms. Fewer numbers of micrococci, staphylococci, Bacillus spp, Aeromonas spp, Acinetobacter spp and some members of the Enterobacteriaceae were isolated.

When shrimps were stored at temperatures of $6^{\circ}C - 8^{\circ}C$ (refrigeration temperature), Pseudomonas spp, increased in population and they became the most predomonant bacterial flora, irrespective of the initial bacterial flora of shrimps. Aeromonas spp were the predominant group causing spoilage in shrimps stored at room temperature ($22^{\circ}C$ to $25^{\circ}C$).

The most frequently isolated bacteria from smoked shrimps were Gram-positive cocci. Coryneforms, Bacillus spp, Pseudomonas spp, and Acinetobacter spp, were also isolated in large numbers.

Shrimps purchased at Adeniji Adele road and Ilubirin market in Lagos were rich in coliforms including Escherichia coli.

The percentage composition of the bacterial types of shrimps throughout the freezing period of about 21 days was similar to that on the freshly caught shrimps, with Gram-positive bacteria e.g. coryneforms, micrococci and streptococci predominating.

Chitinolytic bacteria isolated from sea water were Vibrio alginolyticus, Bacillus cereus, Flavobacterium spp, and Aeromonas spp. The chitinolytic bacteria isolated from the gut of freshly caught or frozen shrimps were V. alginolyticus, Pseudomonas spp, and Bacillus cereus.

The extracellular enzyme preparation from V. alginolyticus had chitinase activity, as detected by the chromogenic assay and by release of reducing sugars from chitin.

The optimum pH for V. alginolyticus chitinase activity was between 5.5 - 6.0. The temperature optimum for enzyme activity was about $56^{\circ}C$, with 10% of maximum activity at about $20^{\circ}C$ and $65^{\circ}C$. Preincubating the enzyme solution for 15 minutes before assay at the different pre-incubating temperatures showed that enzyme activity was stable at $40^{\circ}C$, but stability decreased at higher temperatures and the enzyme was inactivated at $60^{\circ}C$.

There was a linear relationship between initial rate of enzyme activity and protein concentration between 30 and 500 ug protein ml^{-1}. Plots of enzyme reaction velocity (V) against substrate concentration (S) showed maximum activity at about 0.23 mM. There was inhibition of activity by substrate concentration greater

than this value, so that at 0.9 mM the velocity was 45% of the maximum value. The apparent Km values obtained by linear extrapolation of points between 6 and 111 µM for different experiments were about 7.5 µM.

The hydrolysis of the substrate was inhibited by the addition of chitin. There was no inhibition by Micrococcus lysodeikticus cells, N - acetylglucosamine, N acetylmannosamine, N - acetyl-neuraminic acid, or wheat germ agglutinin when added individually to the standard assay. The enzyme preparation showed no detectable hydrolysing activity on M. lysodeikticus, dried cells or P- nitrophenol N-acetyl-glucosaminide.

Chitinase activity in culture media with chitin reached a peak at the sixth day, with 0.030µ mol 3, 4-DNP released min^{-1} ml^{-1}. Only very weak chitinase activity was observed in cultures without chitin. Thus chitinase production by V. alginolyticus appears to be largely inducible.

About 90 - 96% of the chitinase activity was recovered from the culture filterate by precipitation with ammonium sulphite (80% saturation) and about a two-fold increase in specific activity was obtained.

Chitinase purified on G-75 sephadex resulted in an eleven-fold increase specific activity. A final preparation on G-200 Sephadex resulted in a sixty-fold increase in specific activity compared with the culture supernatant.

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