FERMENTATION OF PRAWN SHELL WASTE AND APPLICATION OF ITS PRODUCT AS DIETARY INGREDIENT FOR THE INDIAN WHITE PRAWN, *PENAEUS INDICUS* (H. MILNE EDWARDS)

Thesis submitted to the
Cochin University of Science and Technology
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

BY

BEATRICE AMAR

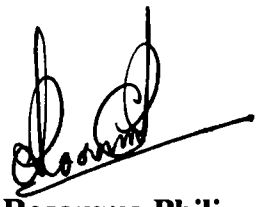
DIVISION OF MARINE BIOLOGY,
MICROBIOLOGY AND BIOCHEMISTRY
SCHOOL OF MARINE SCIENCES

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-682 016

2001

CERTIFICATE

This is to certify that the thesis entitled **"Fermentation of prawn shell waste and the application of its product as dietary ingredient for the Indian white prawn, *Penaeus indicus* (H. Milne Edwards)"** an authentic record of research work carried out by **Ms. Beatrice Amar** under my supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.



Dr. Rosamma Philip

(Supervising Teacher)

Dr. Rosamma Philip,
Senior Lecturer,
Dept. Marine Biology,Microbiology and Biochemistry,
School of Marine Sciences,
Cochin University of Science and Technology
Cochin – 682 016

Cochin – 16
August 2001

CONTENTS

Chapter 1

General Introduction

At present aquaculture is in focus as answer to the growing demand for food. As production in this industry gains momentum, protein emerges as the most important and expensive input component. Since the cost of production keeps steadily increasing with declining natural resources, there is compulsion to find a viable means to ease this problem. It is estimated that aquaculture feed accounts for 40-60 % of the operational cost. Naturally our attention should turn to cost effective and easily available feed ingredients.

The shellfish processing industry in India generates 8.5 million tons of shell waste per year (FAO, 1998). About 35-45% by weight of shrimp raw material is discarded as waste when processed into headless shell-on products. Peeling process, which involves the removal of the shell from the tail of prawn, increases the total waste production up to 40-45%. On a global basis, the shrimp processing industry produces over 700 000 million tons of waste shell. Although, part of this is used for chitin/chitosan preparation, feed manufacture and as manure, a major portion still remains unused. Environmental implication of traditional disposal methods of such waste, coupled with the strengthening of environmental regulations in many countries, has created an interest in alternative methods of disposal/utilization of this waste.

Fig. 1 Shrimp head waste in peeling shed

Crab shells contain only 12.9-23.5% protein materials based on the part of the shell used while shrimp wastes have 42% protein (Naczk and Shahidi, 1990). Ash content is 29.2%-36.8%. Content of crude chitin varies from 30% from crab backs to 40% for the other parts of the shell. Shell waste may serve as a potential source of raw materials for the production of much value added products including chitin/chitosan (Ashford *et al.*, 1977, Muzzarelli, 1977; Johnson and Peniston, 1982), carotenoid pigments (Lambersten and Braekkam, 1971; Chen and Meyers, 1983; Manu-Tawiah and Haard, 1987), and flavourants (Shahidi *et al.*, 1989; Pan, 1989). Many of these processes have concentrated on the production of chitin/chitosan. However in order to minimise the cost of their production, it would be beneficial to produce other value added products, such as pigments and flavourants simultaneously from the shell wastes.

Table. 1.1 Proximate composition of shell waste

Composition range for crustacean waste (% dry weight) (Hansen and Illanes, 1994)	
Solids	30-40
Chitin	14-30
Protein	15-40
$CaCO_3 + Ca_3(PO_4)_2$	35-53
Lipids	0-10

According to Brzeski (1987),crustacean waste contains approximately 10-60 per cent chitin on a dry weight basis, depending on the processing method. Chitin is the second most abundant natural polymer, after cellulose. Bracannot

(1811) was the first to describe "chitin". He called it "fungine". Chitin is a polymer of β (1-4) –N-acetyl-D glucosamine. Chitin occurs in three polymorphic forms, which differ in the arrangement of molecular chains within the crystal cell. α chitin is the tightly compacted , most crystalline polymorphic form where the chains are arranged in an anti-parallel fashion; β chitin is the form where the chains are parallel and γ -chitin is the form where the chains are "up" to every one "down" (Muzzarelli, 1977).

The profile for crustacean waste protein is excellent, the content of essential amino acids being significantly higher than the standards, with sulfur amino acids higher than in soy bean meal and lower than in fishmeal (Hansen and Illanes, 1994). According to the species, the protein may contain significant amounts of the important natural pigment astaxanthin. In fact crustacean waste is an interesting source of natural pigments, especially astaxanthin and astaxanthin ester. However, these pigments are highly unstable to temperature and chemicals, which makes extraction a very critical operation (Perceval, 1978; Simpson, 1978).

Minerals in shellfish waste usually consist of 90% or more calcium carbonate, the rest being calcium phosphate. The high amount of calcium is a problem for recovery and disposal. Fats are largely derived from viscera and vary from 0% in clean crab-leg shells to perhaps 5% or more in some shrimp and lobster species (Johnson and Peniston, 1978) and 10% or more in krill waste (Anderson *et al.* 1978)

Chitin in crustacean cuticle exists as a mucopolysaccharide i.e., seen bound to proteins (Lafon 1941; Rudall, 1963; Hunt, 1970; Hackman, 1976; Muzzarelli, 1977; Austin *et al.*, 1981; Brine, 1982). Horst (1989) examined the synthesis of chitin in the post larval stages of *Penaeus vannamei* . Analysis of the cuticle revealed 73% (by weight) amino acids and 27% N-acetyl glucosamine. Major amino acids in the cuticle are Asx (aspartic acid and asparagines), Glx (glutamic acid and glutamine), Thr, Ala, His and Leu. (Threonine, Alanine, Histidine and Leucine).

It is estimated that worldwide, annually there is over 39000 tons of chitin available from shellfish. This estimate does not include krill, which has a potential of 56000 tons annually. Approximately 75% of the total weight of these shellfish is considered as waste and from 20-58% of the dry weight of the waste is chitin depending upon the processing method (Kreag and Smith, 1973)

Table 1.2 Estimated global potentials for chitin production using waste from shrimp, crabs and krill landings.

Resource MT	Landings (1991) MT	Waste Potential MT	Waste Available MT	Dry Waste MT	Chitin content MT
Shrimp	2647,345 (40)*	1058,938	710,0001 (0.25)**	77,500 (0.25)**	44,375
Squid	1991,094 (20)*	398,219	99,531 (0.25)**	24,882 (0.05)***	1244
Crabs	1348,323 (70)*	943,826	482,744 (0.30)**	144,823 (0.20)***	28,964
Krill	232,700 (40)*	93,080	93,080 (0.25)**	23,270 (0.07)***	1629
Total	6219462	76212			

* Multiplication factor for calculating waste
** Multiplication factor for calculating dry waste
*** Multiplication factor for calculating chitin
Source : S. Subasinge (Chitin & Chitosan, Ed. Mat. B. Zakaria *et al.*, UKM, Malaysia ,1991)

Shellfish waste and utilization

The constituents of shell waste make them worthy of further processing and utilization. Shell waste is mainly subjected to chemical processing for chitin/chitosan production. Bioconversion processes are also taken up by various workers for the complete utilization of the waste.

Chemical processing

Chitin in shellfish waste is tightly associated with proteins, lipids, pigments and calcium deposits. Current procedures for preparing chitin from shellfish waste consist of two main steps i.e. $CaCO_3$ separation or demineralization with dilute acids or chelating agents (Tsugita, 1991), followed by protein separation or deproteinization with dilute alkali or proteolytic enzymes. Alternatively, the deproteinization process may precede the demineralization step (Sandford, 1989). Subsequent to the demineralization and deproteinization steps, the product may be decolorized with acetone and /or hydrogen peroxide.

Bioprocessing

Bioconversion of shell waste is probably the most cost effective and environment-friendly procedure for waste utilization. With the exception of the first stage, demineralization of crustacean shell with dilute acid, preferably with EDTA (ethylene diamino tetra-acetic acid), a process of total bioconversion, involving enzymes and micro-organisms was found feasible by Shimahara *et al.* (1984).

In order to obtain a less degraded chitin, some alternative methods for deproteinization stage have been proposed i.e., proteolytic enzymes such as tuna trypsin (Takeda and Abe, 1962), Rhozyme–62 (Bough *et al.*, 1978) and cod trypsin (Simpson and Haard, 1985) have been used to deproteinize crustacean shell. Despite promising results on a laboratory scale, large scale application of such methods is unlikely because of high price for the purified enzymes required (Cano-Lopez *et al.*, 1987). Much work has been carried out on the chitin bioconversion itself (Carroad and Tom, 1978; Revah–Moiseev and Carroad, 1981; Cosio *et al.*, 1982). On the basis of such research and renewed world wide interest in total utilisation of whole crustaceans including Antarctic krill, Arctic shrimp, Temperate shrimp and prawn (Healy *et al.*, 1992), it is now essential to adopt and optimize an approach to isolation and extraction of all those constituents of marine crustacean whole shell waste. To this end, Shimahara *et al.*, (1984) have proposed an interesting approach to crustacean shell bioconversion .The process is directed once again, at chitin production, by means of utilizing a proteolytic bacterial culture, *Pseudomonas maltophila* LC102, for one stage of the process. No effort is made to recover pigment and protein.

A process was conceptualised for the conversion of chitin waste into single cell protein suitable for animal feed or aquaculture feed (Carroad and Tom, 1978). Design concepts were drawn from the process development work concerning the bioconversion of cellulose to ethanol and single cell protein

(Wilke *et al.*, 1976). Chitin waste is used to induce a selected microorganism to secrete into solution an extracellular chitinase system in submerged culture. The enzyme is harvested by filtration and, in a third step, is combined with the bulk of the chitin waste for chitin hydrolysis. The chitin is enzymatically hydrolysed, principally to the monomer N-acetyl glucosamine and its dimer. The hydrolysate is filtered free of undigested solids and in a fourth step is fed to a product generation stage where, for example, the hydrolysate is fermented in submerged culture to single-cell protein by an appropriate microorganism. Berkeley (1978) has suggested the use of chitinolytic and chitosanolytic microorganisms as sources of enzyme for the modification of chitinous materials for commercial purposes.

Carroad and Tom (1978) have developed a process for bioconversion of shellfish chitin wastes into yeast single cell protein of value in animal or aquaculture feed formulations. Revah-Moiseev and Carroad, (1981) described the conversion of the enzymatic hydrolysate of shellfish waste chitin to single cell protein employing the yeast, *Pichia kudriavzenii*. Lactic acid fermentation of scampi (*M. rosenbergii*) for chitin recovery was described by Hall and de Silva (1992). Fermentation by lactic acid bacteria allowed enzymes to solubilise a protein-rich liquor from the insoluble chitin-rich fraction, and yet suppressed production of spoilage compounds by other bacteria (Hall and de Silva, 1992). Proteolytic strains like *Pseudomonas maltophila, Bacillus subtilis, Streptococcus faecium, Pediococcus pentosaseus* and *Aspergillus oryzae* were used for

microbial extraction of chitin from prawn shell waste. Over 80% deproteinization of the demineralised prawn shell waste was achieved by this bacterial consortia (Bustos and Healy, 1994).

Healy *et al.* (1992), have proposed two overall, continuous processes for not only chitin production but for the recovery of protein and pigment. Optional in the proposed process is a step to further depolymerize the chitin to monomer sugars (N-acetyl glucosamine) on which yeast can be grown for production of microbial biomass. Healy *et al.*, (1994) have proposed a whole continuous process for shellfish waste utilization which considers microbial demineralization-deproteinization of the shell waste with the recovery of protein and pigment as by-products, both with potential use as fish feed. Further de-polymerisation of chitin to N-acetyl glucosamine allows the production of this important amino sugar, which has itself some medical applications (Muzzarelli and Biagini, 1993) as well as its use as a substrate for single-cell protein production. Other stages of the chitin hydrolysis have been described in detail in many related articles (Healy *et al.*, 1994 a, b; Healy and Bustos, 1993; Bustos and Healy 1994 a, b). The utility of marine yeasts for the bioremediation of prawn shell wastes was investigated for SCP protein generation with high final protein content (Rhishipal and Philip, 1998).

Healy *et al.*, (1994) investigated the viability of lactic acid fermentation on demineralization of prawn shell waste for deproteinisation under both aerobic and anaerobic conditions. An integrated bioconversion scheme was proposed whereby protein, pigment (astaxanthin) and the main biopolymer constituent chitin, can be isolated for industrial or commercial use. All the above-mentioned alternatives have the disadvantage of concentrating on only one particular aspect of shellfish waste processing. In this context a process, which involves the total utilisation of the prawn shell waste generated, is highly warranted. The present work is aimed at the microbial transformation of the shell waste through fermentation in to a product with enhanced nutritional quality, which can be used as an aquaculture feed ingredient.

Against this background the present study was undertaken with the following objectives.

1. To isolate and identify potential chitinoclastic and proteolytic microbial strains that can play an effective role in the bioconversion of prawn shell waste.
2. To estimate the nutrient enhancement by way of fermentation of prawn shell waste.
3. To screen the fermented products generated by selecting potential strains in a bio-assay system as dietary feed ingredient by growth studies
4. To optimise the culture conditions for fermentation process ensuring maximal nutrient enrichment
5. To estimate the efficacy of the final enriched fermentation products as dietary feed ingredient for *Penaeus indicus* juveniles in culture system.

The results of the present study are presented in Six chapters. Isolation and characterisation of microflora associated with prawn shell waste transformation are presented in Chapter 2. The third chapter deals with the comparison between the solid state and submerged state fermentation of prawn shell waste by the biochemical evaluation of the fermentation products thus generated. Preliminary screening of the fermented product was carried out by evaluating their efficacy as feed ingredient for Indian White prawn *Penaeus indicus* and the results are presented in chapter 4. Characterisation of selected strains was done by studying their growth and enzyme production. The effect of various physicochemical parameters on fermentation was assessed to optimise the fermentation conditions and the results of these studies are presented in chapter 5. In Chapter 6, the application of the fermented products generated under optimised conditions were evaluated as dietary ingredient for *P. indicus* juveniles in culture systems.

This is followed by a summary, list of references cited and appendices

Chapter 2

Microflora associated with prawn shell waste transformation : Isolation and Characterisation

2.1 Introduction

2.1.1 Chitinoclastic bacteria in water and sediment

It has been estimated that several million tons of chitin are produced annually by the copepods, a subclass of planktonic crustacea in the sea. This as well as the chitin produced by other organisms in the sea, is probably utilized by biological agents because little accumulates in marine sediments, and moreover, if the chitin were not decomposed its accumulation would soon cause a serious drain on the marine carbon and nitrogen (Zobell and Rittenberg, 1938). There are few animals, which can attack chitin, and it is generally agreed that its degradation in nature is largely due to microbial action. Since Benecke (1905) reported the isolation of *Bacillus chitinovorus* from the polluted waters of Kiel harbour, there have appeared numerous reports of the isolation of microorganisms capable of decomposing chitin. The reports (Steiner, 1931; Johnson, 1932; Waksman *et al.*, 1933; Zobell and Rittenberg, 1938; Hock 1941 and Stanier 1947) on the occurrence of chitin decomposing microorganisms from marine sources, together with the report of Stuart (1936) on the occurrence of halophilic chitinovorous bacteria in the marine salt pans from various parts of

the world, reveal the world wide distribution of chitin decomposing microorganisms in the seas.

Boyer and Kator (1985) showed that particulate ^{14}C-labeled chitin incubated with York river water at 20 ^{0}C was mineralised to $^{14}CO_2$ at a rate of 21% per day, with less than 4% recoverable in the dissolved pool at any time. It has been assumed that chitin degrades rapidly in sediments because it does not accumulate to any significant extent. Two pathways are important in the mineralization of chitin in the sediments, one involving chitinases and N-acetylglucosamine and another involving chitin deacetylase and chitosanase.

There are a number of reports on the isolation of chitinoclastic microorganisms from different environments like fertilised garden soil, lake waters, sediments, plankton, exoskeleton of insects and crustaceans, intestines of both vertebrates and invertebrates, muds and sands (as reviewed by Benton, 1935; Veldkamp, 1955). It is well known that marine environment is very rich in chitinous material and is an excellent source of chitinoclastic bacteria. Veldkamp (1955) observed that acid sandy soils always harboured higher concentrations of chitinoclasts and actinomycetes formed the major group of such chitinoclastic population.

One of the most comprehensive studies on the chitin utilizers is by Seki and his associates. While working in Sakami Bay, Seki and Taga (1963) found that each water mass had a characteristic qualitative and quantitative distribution

of chitinoclastic bacteria. Maximum numbers of chitinoclasts were noticed in surface waters and they decreased with depth. They could also observe that the abundance of chitin utilisers varied with temperature. The qualitative variation of chitinoclastic bacteria in different water masses was attributed to different species of plankton in each layer suggesting a relationship between bacterial type and specific plankton.

The population of chitinoclasts is significantly higher in shallow waters of coastal zone than in open ocean. Chan (1970) reported higher concentrations of chitin digesters (slightly less than 10 % of total bacteria) in seawater and sediment from Puget Sound estuary. Sediments from deep subtidal, intertidal and freshwater areas harboured $2.0x10^4$ cells/g, $6.8x10^4$ cells/g and $3.7x10^3$ cells/g respectively. As in pelagic region highest concentrations of chitin utilisers, were at the surface of the sediments and the concentration decreased with depth. Surface waters contained an average of $2.5x10^2$ cells/ml while bottom waters contained fewer cells, $2x10^2$ cells/ml.

In the neritic waters of Aburastsubo inlet, Seki and Taga (1963) noticed that only 0.4% of the total hetetrotropic bacteria were chitinoclastic. However, they observed that a considerable number of chitinoclastic bacteria were attached to living copepods suggesting a relation between the chitinoclasts and planktonic crustaceans. An inverse relationship was also noticed between the percentage of chitinoclasts present and chemical oxygen demand (COD). The chitinoclastic

bacteria were classified into five species of *Beneckea. B. lipophaga* predominated in summer and *B. hyperoptica* appeared in winter and early spring. *B. indolthetica and B. chitinovora* were always found in association with the plankton or suspended matter throughout the year.

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

Poole and Warnes (1981) reported that majority of the chitinoclastic bacteria isolated from a freshwater habitat were Gram negative-*Pseudomonas, Chromobacterium, Flavobacterium, Moraxella and Serratia* spp. Gram-positive organisms showing chitin hydrolysis were predominantly *Actinomycetes.* Thomas (1982) has reported the isolation of *Vibrio, Aeromonas, Alcaligenes, Pseudomonas*, members of Enterobacteriaceae, *Bacillus* and *Micrococcus* associated with body surface of prawns. *Vibrio* was found to be the most common genus in sediment, water, prawns and fishes.

Chitinoclastic bacteria from the following genera have been previously reported. *Achromobacter, Aeromonas, Arthrobacter, Bacillus, Clostridium, Corynebacterium, Cytophaga, Enterobacter, Flavobacterium, Haloanaerobacter, Micrococcus, Pseudomonas, Serratia and Vibrio* (Cody *et al.*, 1990). Tsujibo *et al.* (1991) reported the characterisation of a chitin degrading marine bacterium belonging to the genus *Alteromonas*. Ashmin (1997) studied the microflora associated with prawn shell degrading systems. Coryneforms were found to be the predominant group associated with the transformation of prawn shell waste. Other groups of importance were *Bacillus, Vibrio, Staphylococcus, Pseudomonas, Micrococcus, Moraxella*, etc.

Chitinoclastic bacteria associated with aquatic animals

Chitinoclastic bacteria are closely associated with certain marine vertebrates and invertebrates. Reports also suggest a commensal or symbiotic relationship between these microorganisms and planktons. It appears that both external and internal regions of marine animals offer an excellent microenvironment for the growth and survival of chitinoclasts. Jones (1958) demonstrated that the surface of the radiolarian *Castanidin longispinum* contained at least 1000 fold increase in chitin digesters compared to the biomass of chitinoclasts in the surrounding seawater. Similarly higher concentrations of chitinoclasts were recorded in large copepod species when compared to the surrounding water. They also identified the chitinoclasts as *Beneckea* species and showed that they were indigenous to copepod and were able to reproduce within

the crustacean under certain conditions. Seki and Taga (1965) found significant numbers of chitinoclasts attached to living copepods within the water column. The observation of Lear (1961) also confirmed this, suggesting that the external surface of planktonic forms serve as the major area of microbial attachment. The adsorption of *Vibrio parahaemolyticus,* a chitinoclastic bacteria onto chitin and copepods was observed by Kaneko and Colwell (1975). Earlier they also observed a correlation between the population of zooplankton and concentration of *V. parahaemolyticus* in Chesapeake Bay.

Among the marine fishes examined, almost 90% of all the teleost stomachs and intestines contained chitinoclastic bacteria while the elasmobranchs had a much lower incidence of these bacteria (Chan, 1970). In addition to this, the existence of chitinoclasts in the digestive tract of whales has also been reported (Seki and Taga, 1965). In many cases, the chitinoclastic bacteria in the digestive tract of aquatic animals have been identified and species of *Vibrio* and its closely related species are found to be the major component of the chitinoclasts. Sera (1968) reported that chitin decomposing bacteria present in the digestive tract of black sea bream, *Acanthopagrus schlegeli*, were found to be *Vibrio* species. Sera and Ishida (1972) later reported the dominance of vibrio group as chitinoclasts in the digestive tracts of many marine fish. In the case of penaeid prawns the chitinoclasts of digestive tract were found to be members of *Pseudomonas, Vibrio and Beneckea* (Hood and Meyers, 1973, 1977).

The chitinase system which is responsible for the chitin degrading capacity in chitinoclasts is widely distributed among bacteria like *Serratia* (Monreal and Reese, 1969; Reid and Ogrydziak, 1981), *Chromatium* (Clarke and Tracey, 1956), *Klebsiella* (Jeuniaux, 1959), *Clostridium* (Clarke and Tracey, 1956).

Sugita *et al.* (1996) have added a new discovery of *Streptococcus* spp. with chitinoclastic activity from Pinnipedian gut flora. High chitinase activities were exhibited by *Bacillus* and Enterobacteriaceae isolates from Cape fur seal. Similarly, *Bacillus*, Coryneforms, Enterobacteriaceae and *Streptococcus* from the intestine of California sea lion produced high chitinase activities (Sugita *et al.*, 1996).

2.1.2 Proteolytic microflora associated with water, sediment and aquatic animals

Williams *et al.* (1952) recorded that the microflora associated with shrimps generally consists of *Achromobacter, Bacillus, Micrococcus* and *Pseudomonas*. The microbial spoilage of prawns is mainly due to psychrophilic *Pseudomonas* (Shewans, 1977). Most frequent amongst organisms involved in proteolytic activity in meat samples were *Pseudomonas, Vibrio, Moraxella, Bacillus, Micrococcus* and *Flavobacterium*. Pseudomonads alone formed 71.2% in *P. indicus* and they were most active in proteolysis leading to the spoilage of frozen fish and shellfish samples (Philip, 1987). Shaw and Shewan (1968) found that *Pseudomonas* spp. were by far the most active spoilers. Most of the

Moraxella spp. tested grew well producing a slight rise in pH 6.5 to 7.8. The association of *Pseudomonas* and *Bacillus* species to prawn flesh degradation have been recorded by Cobb and Vanderzant (1971).

In an attempt to understand the ecological implications of extracellular protein degradation promoted by estuarine bacteria, Sizemore *et al.* (1973) studied the distribution and activity of proteolytic bacteria in the sediments from the North inlet estuary near Georgetown, South Carolina. About 56% of the isolates obtained were proteolytic. The widespread distribution and occurrence of proteolytic forms especially in marine water and sediments have been studied by a number of workers (Murchelano and Brown, 1970; Reinheimer, 1972; Boeye *et al.*, 1975; Little *et al.*, 1979; Nitkowski *et al.*, 1977).

Chandrasekaran (1985) have reported that of 219 isolates from *P. indicus* belonging to the species of *Vibrio, Aeromonas, Pseudomonas, Alkaligenes, Acinetobacter*, *Micrococcus, Bacillus*, Coryneforms and Enterobacteriaceae, 63% were potential proteolytic forms.

Microbial bioconversion being the focus of study, isolation of hetertrophic bacteria with potential hydrolytic (chitinoclastic and proteolytic) property was carried out.

2.2 Materials and Methods

2.2.1 Collection of prawn shell waste

Prawn shell waste was collected from the peeling sheds in Cochin. This was dried in an oven at 80 ^{0}C overnight and stored in airtight containers for study.

2.2.2 Isolation of the microflora

Enrichment system

In situ

For obtaining a good collection of chitinoclastic and other groups of microflora associated with prawn shell transformation the following enrichment systems were set up.

Prawn shell waste dried and stored in the laboratory was mixed with sediment collected from Cochin backwater and put in perforated (upper half) bottles. These bottles were hung with weights in the calm regions of the backwater for the chemotactic attraction/enrichment of microflora associated with prawn shell degradation especially the chitinoclastic forms.

Ex situ

A sediment bed was prepared in a glass trough, overlaid with prawn shell waste and then flooded with 50% seawater. At 2 days interval fresh seawater collected from the backwater was added as inoculum for the enrichment of the chitinoclastic and other flora associated with the shell transformation.

Sampling and plating

Samples consisting of partially degraded shell and sediment were taken from both the enrichment systems at random intervals for a period of two weeks. The samples were then homogenised using a tissue homogeniser and serial dilutions were made up to 10^{-4}. These samples were used for plating.

Besides this direct plating, the samples were subjected to secondary enrichment by inoculation into 50% seawater containing 5% colloidal chitin. For this 1 ml of the 10^{-1} dilution of the sample was transferred to 9 ml 50% sea water with colloidal chitin. After seven days incubation at room temperature ($28\pm2^{\circ}C$) it was used as an inoculum for plating.

The conventional pour plate method was employed using the following media.

(1) Nutrient agar medium (modified)

Peptone	0.5g
Beef extract	0.3g
Dried prawn shell powder	2g
Agar	2g
pH	7
50% Sea water	100ml

(2) Colloidal Chitin Agar media (CCA) (Hsu and Lockwood, 1975)

*Colloidal chitin	4.0g
KH_2PO_4	0.3g
K_2HPO_4	0.7g
$MgSO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
$ZnSO_4.7H_2O$	0.001g
$MnCl_2.4H_2O$	0.001g
Peptone	0.1g
Yeast extract	0.1g
pH	7
Agar	20g
50% Sea Water	1000ml

*15g of purified chitin (obtained from the Central Institute of Fisheries Technology, Kochi, India) was taken in a beaker and 100 ml of 50% (v/v) H_2SO_4 was added with continuous stirring in an ice bath. After 60 min two litres of deionised distilled water was added into the dissolved chitin in order to precipitate the chitin. The suspension was kept overnight at 4^0C, the supernatant decanted out. The sedimented residue was resuspended in deionised distilled water, allowed to settle and decanted. This washing was repeated until the pH of the liquid became neutral. The colloidal chitin was collected by centrifugation at 5000 rpm for 10 min and stored at 4^0C. It was used as a substrate for estimation of chitinase activity.

(3) Sea Water Colloidal Chitin agar (SWCCA)

Colloidal chitin	4g
NaCl	1.5g
Agar	2g
50% Sea Water	100 ml
pH	7

(4) Actinomycete Isolation agar (Hi Media)

Sodium caseinate	0.2g
Asparagine	0.01g
Sodium propionate	0.4g
Magnesium sulphate	0.01g
Ferrous sulphate	0.0001g
Agar	1.5g
pH	8.1 ± 0.2
50% Sea Water	100 ml

(5) CSPY-ME Agar

Casein	0.3g
Maize starch	1g
Peptone	0.1g
Yeast extract	0.1g
Malt extract	1g
$K_2HPO_4.7H_2O$	0.15g
pH	7.5
Agar	2g
50% Sea Water	100 ml

Incubation was done at room temperature (28±2°C) for 3-5 days in the case of media 1 and 5, and for 2 weeks with media 2, 3 and 4. Random isolations of the colonies were made onto nutrient agar slants, purified and then stocked in soft nutrient agar vials overlaid with sterile liquid paraffin.

2.2.3 Identification

Based on gram staining, spore staining, morphological examination and biochemical characterization, all the cultures (220 strains) were identified up to generic level as per Bergey's Manual of Sytematic Bacteriology (Buchanan and Gibbons, 1974).

2.2.4 Testing for Chitinoclastic and Proteolytic Property

All the isolates were tested for their chitinoclastic and proteolytic potential.

2.2.4.1 Chitinoclastic Property

Chitin agar plates were prepared and after surface drying the isolates were spot inoculated. Incubation was done for 2 weeks at room temperature (28±2°C). Formation of clearing zone around the colony was recorded as positive. Diameter of the halo was also recorded.

Composition of chitin agar medium

Peptone	0.5g
Beef extract	0.3g
Colloidal chitin	5g
Agar	2g
50% Sea water	100 ml
pH	7

2.2.4.2 Proteolytic property

All the chitinoclastic strains were segregated based on the above test (2.2.4.1) and they were further screened for proteolytic property. Gelatin agar and prawn flesh agar were used for the study.

a. Gelatin agar [Frazier's gelatin agar medium (modified)]

Composition of the medium

Peptone	: 10 g
Beef extract	: 10 g
Gelatin	: 4 g
Agar	: 20 g
50% sea water	: 1000ml
pH	: 7.2

Gelatin agar plates were prepared, spot inoculation of the isolates were done and incubated at room temperature (28±2°C) for 3-5 days. Gelatinolytic activity was tested by flooding the plates with mercuric chloride solution ($HgCl_2$-20g, Conc. HCl-20ml, distilled water-100 ml) and observing the halo zone formation around the colonies.

b. Prawn flesh agar

Composition

Prawn flesh broth	100 ml
Agar	2g
pH	7

Preparation of flesh broth

Tissue homogenate of prawn was prepared by taking 100 g of flesh in a waring blender, added 200 ml distilled water with 0.5 % sodium chloride and homogenized for 5 min. This solution was made up to 1000ml (1:100w/v) and was centrifuged at 5000g for 30 min in a refrigerated centrifuge and the supernatant solution was taken and used as prawn flesh broth.

The isolates were spot inoculated on prawn flesh agar plates and incubated for 5 days at room temperature (28±2°C). Strains which developed a halo around the colonies when the plates were flooded with 20% solution of mercuric chloride indicated proteolytic property and the diameter of the halos were recorded.

2.3 Results

Microflora associated with prawn shell waste transformation

The percentage composition of microflora associated with prawn shell waste transformation is given in Table 2.1. *Bacillus* spp. (33%) were found to be the predominant group associated with the transformation of prawn shell waste. Other groups of importance were Coryneforms (17%), *Vibrio* (11%), *Streptococcus* (10.5%), *Pseudomonas* (7.2%) and *Acinetobacter* (4.5%).

Chitinoclastic property

Out of the various genera tested for chitinoclastic property, *Bacillus* was found to be the most prominent group consisting of 11.3% chitinoclastic forms.

Coryneforms consisted of 7% and the vibrios and *Streptococci* 6% each of the chitinoclastic forms (Table 2.2).

Nutrient agar (supplemented with prawn shell powder) was found to support the largest number (42%) of chitinoclastic forms isolated (Table 2.3). This was followed by Actinomycete isolation agar (32%), Colloidal chitin agar (13%) and Sea Water Colloidal Chitin Agar (7%).

Proteolytic property

Out of the 30 chitinoclastic strains tested for proteolysis, 25 were found to be positive on gelatin agar (Table 2.4). However, seven of these proteolytic forms were found negative on prawn flesh agar. Five strains were found to be negative on both gelatin and prawn flesh agar plates. These strains were considered non proteolytic forms.

Segregation of strains

Based on the chitinoclastic and proteolytic property of the strains, they were segregated into two.

(1) Chitinoclastic and Non Proteolytic forms

(2) Chitinoclastic and Proteolytic forms

These strains were used for the fermentation of prawn shell waste.

2.4 Discussion

The principal organic components of shell waste being protein and chitin, the associated microflora found would be mostly proteolytic/chitinolytic in nature. Hydrolysis of chitin into simpler components would make the shell waste easily available for the cultured organism when used as an aquaculture feed ingredient. Therefore prime importance was given to the isolation of chitinoclastic bacteria. Various media were used to serve the isolation of the target microflora. Nutrient agar supplemented with prawn shell powder was found to support the largest number of chitinoclastic forms. Prawn exoskeleton is rich in protein (approximately 40%) besides chitin which may constitute about 14% of the dry weight. Therefore a medium (Nutrient agar) rich in protein supplemented with prawn shell powder would be a good choice for the isolation of desirable microflora. Secondary enrichment using colloidal chitin broth supported the selective growth of chitinoclastic forms.

Occurrence of chitinoclastic bacteria are low in natural waters and the presence of chitinous material enhances their proliferation and increase in population. However proteolysis is very common with aquatic bacterial population and non proteolytic forms are rare. In this study the highly chitinoclastic forms could be segregated into proteolytic and non proteolytic forms. The potential of these two groups in the biotransformation of prawn shell waste is to be thoroughly examined. The hypothesis is that the non proteolytic forms may spare native protein while hydrolyzing chitin into oligomers and

monomers. Since protein is an easily utilizable substrate chitinoclastic cum proteolytic strains would be utilizing protein first and chitin at a later stage, a catabolite repression phenomenon. Simultaneous hydrolysis of protein and chitin also may take place giving a total transformation of the shell waste. Based on these hypotheses this work is planned and the isolation for the target flora has been done.

In the present study, *Bacillus* sp. was found to be the predominant group associated with trasnsformation of prawn shell waste. *Bacillus chitinovorous*, a chitinoclastic form was reported by Benecke in as early as 1905. Association of *Bacillus* sp. with prawn shell degradation has already been reported by Hock (1941) and Gray and Baxby (1968). *Bacillus* species have been found to possess potential chitinase activity by Chigaleichick *et al.* (1976), Tominaga (1977) and Cody *et al.* (1990). Coryneforms have also been reported to be chitinoclastic in nature (Cody *et al.*, 1990).

The chitinase activity of *Serratia marcescens* which belongs to the genus Enterobacteriaceae has been previously reported by Monreal and Reese (1969). Young and Carroad (1981) have reported the extracellular chitinase activity of *Serratia marcescens* QMB1466 in a study on its potential in treating chitin-containing solid waste of shellfish processing. Boer *et al.* (1999) reported a rapid degradation of chitin in soil samples by chitinoclastic Streptomycetes. Thomas (1982) has reported the isolation of *Vibrio, Aeromonas, Alcaligens,*

Pseudomonas, members of Enterobacteriaceae, *Bacillus* and *Micrococcus* associated with body surface of prawns. *Vibrio* was found to be the most common genus in sediment, water, prawns and fish samples collected. In a study by Ashmin (1997) on microflora associated with prawn shell degrading system, Coryneforms were found to be the predominant group. Other groups of importance were strains belonging to the genus *Bacillus, Vibrio, Staphylococcus, Pseudomonas, Micrococcus* and *Moraxella*. The various strains isolated in the present study are similar to that reported by Ashmin (1997). Isolates of Streptomyces spp. including actinomycetes were isolated from tropical soil by Gomez *et al.* (2000). They noted that around 60% of the 49 actinomycetes isolated were chitin degraders.

Almost all the chitinoclastic strains were found to be proteolytic except a few. Proteolytic potential of the isolates on gelatin agar and prawn flesh agar was found to be different. Seven of the gelatinolytic forms were found to be non proteolytic on prawn flesh agar plates. Perhaps the extent of degradation as observed in the gelatin and prawn flesh agar plates may not be at a qualitative (visible) detection level. Five strains were found to be non proteolytic on gelatin agar and prawn flesh agar. These isolates are considered to be potential since they are likely to spare native protein while hydrolyzing chitin, the complex component (polymer) of the shell.

Table.2.1 Generic composition of microflora associated with Prawn shell waste transformation

Genera	No. of isolates	Percentage composition
Bacillus	73	33
Coryneforms	39	17
Vibrio	25	11
Streptococcus	23	10.5
Pseudomonas	16	7.2
Acinetobacter	10	4.5
Aeromonas	7	3.2
Staphylococcus	6	2.7
Micrococcus	6	2.7
Serratia	6	2.7
Moraxella	5	2.3
Planococcus	3	1.4
Propionibacterium	1	0.5
Total	220	

Table 2.2 Percentage of chitinoclastic bacteria among the various bacterial genera isolated from prawn shell waste

Genera	No. of isolates	% of Chitinoclastic forms
Bacillus	73	11.3
Coryneforms	39	7
Vibrio	25	6
Streptococcus	23	6
Pseudomonas	16	4.5
Acinetobacter	10	3.6
Aeromonas	7	2.7
Staphylococcus	6	0.5
Micrococcus	6	0
Serratia	6	1.3
Moraxella	5	0.1
Planococcus	3	0.05
Propionibacterium	1	0
Total	220	

Table 2.3 Details of the media which supported the recovery of chitinoclastic bacteria from the enrichment system

Media	No. of Chitinoclastic Bacteria
Nutrient Agar + Prawn shell powder	42
Actinomycete Isolation Agar	32
Colloidal Chitin Agar	13
Casein Starch Peptone Yeast-Malt Extract Agar	6
Sea WaterColloidal Chitin Agar	7
Total	100

Table 2.4 Proteolytic potential of the selected chitinoclastic bacteria

Culture	Genera	Physiological grouping			
		Chitinoclastic	Proteolytic		
			Gelatin agar	Prawn flesh agar	
C4	*Streptococcus*	+	+	+	
C14	Coryneforms	+	+	+	
C15	*Bacillus*	+	+	+	
C18	Coryneforms	+	+	+	
C37	*Vibrio*	+	+	+	
C38	Coryneforms	+	+	+	
C48	Coryneforms	+	+	+	
C52	*Vibrio*	+	+	+	
C56	*Bacillus*	+	+	+	C
C63	*Streptococcus*	+	+	+	&
C84	*Pseudomonas*	+	+	-	P
C98	*Planococcus*	+	+	-	
C111	*Bacillus*	+	+	-	
C112	*Streptococcus*	+	+	-	
C113	*Acinetobacter*	+	+	-	
C123	Coryneforms	+	+	+	
C124	*Bacillus*	+	+	+	
C134	*Bacillus*	+	+	+	
C146	*Staphylococcus*	+	+	+	
C149	*Serratia*	+	+	+	
C153	Coryneforms	+	+	+	
C154	*Bacillus*	+	+	+	
C157	*Vibrio*	+	+	-	
C163	*Serratia*	+	+	+	
C217	*Vibrio*	+	+	-	
C29	*Vibrio*	+	-	-	
C33	*Pseudomonas*	+	-	-	C
C218	*Vibrio*	+	-	-	&
C219	*Vibrio*	+	-	-	NP
C220	*Bacillus*	+	-	-	

C&NP - Chitinoclastic and Non Proteolytic
C&P - Chitinoclastic and Proteolytic

Chapter 3

Fermentation of prawn shell waste: Comparison of Solid State and Submerged State Fermentation, and biochemical evaluation of product quality

3.1 Introduction

A successful fermentation is one in which a specific microflora has been encouraged to develop in a preferred direction by applying a system of physical, chemical, biochemical and environmental factors to prevent the growth of all undesirable microorganisms. Fermentation also implies transformation of organic substances into simpler compounds by the action of enzymes and microorganisms. It can generate new food components such as vitamins and essential amino acids, which are not present in the original food, thus improving its nutritive value. All fermented products have aroma and flavour characteristics that result directly from the fermenting organisms. In some instances, the vitamin content of the fermented product is increased along with an increased digestibility of the raw materials. The fermentation process reduces the toxicity of some foods, while others may become extremely toxic during fermentation. Fermentation is one of the oldest methods of preserving food and it continues to be one of the most important methods of preserving foods. Fermentation not only involves production of preservative or antibiotic ingredients, notably acids, CO_2 and alcohol, but also

results in chemical and physical changes that substantially alters the food and thus improves the flavour of food (Erichsen, 1983).

3.1.1 Solid State Fermentation (SSF)

Solid-state fermentation (SSF) involves the growth of microbes on moist solid materials in the absence or near absence of free water (Mitchell, 1992). The moisture content could vary between 40 and 80 per cent (Channel and Moo-Young, 1980). This limited availability of water makes SSF quite different from submerged state fermentation (SmF). The major difference between SSF and SmF is that in the former the substrate is a moist solid, which is insoluble in water but not suspended in liquid (primarily water), whereas in the latter the substrate is a solid dissolved or submerged in liquid. The solid substrates act as a source of carbon, nitrogen and minerals as well as growth factors, and they have a capacity to absorb water, which meets the vital requirement for water by the microorganism. SSF simulates the fermentation reactions that occur in nature, which include wood rotting, composting and food spoilage by moulds. SSF processes can be conducted under controlled conditions, which are useful for producing valuable products like enzymes or secondary metabolites (Hesseltine, 1977; Bailey and Ollis, 1977; Ulmer *et al.*, 1981).

More recently, a number of modern applications of SSF technology have been developed including production of proteases, amylases, lipases and other enzymes, organic acids such as citric and lactic acids, flavour components and spores for use as inocula for biopesticides. In addition, SSF technology can be used to produce products such as composts and animal feeds from solid wastes such as wheat and rice bran, rice straw, peels and cores from tuber, and vegetable and fruit processing waste.

The advantage of SSF over submerged state fermentation is the high volumetric productivity, high product concentration and the simplicity of fermentation equipment and management obviating highly trained labour for operation (Tengerdy, 1998). The commercial applications of SSF can be divided into two types: (1) socio-economic applications such as composting of waste, ensiling of grass and upgrading of lignocellulosic products and (2) profit-economic applications such as production of enzymes, organic acids, and fermented foods (Mitchell and Lonsane, 1991).

The major groups of microorganisms used in fermentation are bacteria, actinomycetes, yeasts and fungi (Hesseltine, 1987). Solid substrate fermentation (SSF) may be used advantageously for enzyme production, especially in those agrobiotechnological applications where the crude fermented product may be used directly as enzyme source. Such applications are enzyme assisted ensiling, bioprocessing crops and crop residues, fiber

processing (eg. retting), enzyme enriched feed supplements, biopulping and directed composting for soil improvement, enhancing biopesticide action, post harvest residue decomposition, waste recycling and soil remediation.

Solid state (substrate) fermentation (SSF) has been known for centuries and used successfully for the preparation of Oriental foods. More recently, it has gained importance in the production of microbial enzymes due to several economic advantages over conventional SmF (Hesseltine, 1972). A thorough study of the literature showed that almost all the organisms used in solid state fermentation are of terrestrial origin except for a few reports on the use of marine bacteria (Renu, 1991; Nagendra and Chandrasekaran, 1995; Nagendra and Chandrasekaran, 1996; Shoby, 1996; Nagendra and Chandrasekaran, 1997). Marine microorganisms, which are salt tolerant and have, the potential to produce novel metabolites are highly suitable for use in SSF by virtue of their ability to adsorb onto solid particles (Chandrasekaran, 1994; Chandrasekaran, 1996). Their great potential to produce novel metabolites employing SSF remains untapped (Nagendra and Chandrasekaran, 1996).

Solid state fermentation (SSF) techniques are now considered suitable for both bacterial and fungal cultivation (Lonsane and Ramesh, 1990). Main applications include protein enrichment of the raw materials (biomass production), edible mushrooms, enzymes, organic acids, ethanol and special secondary metabolites like mycotoxins, antibiotics and flavours (Moo-Young

et al., 1983). Several type of food fermentation also belongs to the solid state cultivation family, as for example cheese manufacture and ripening or oriental fermented foods (Saono *et al.*, 1986).

3.1.2 Submerged State Fermentation (SmF)

Compared to solid state fermentation, very little information is available on submerged state fermentation. The advantages of SSF for protein enrichment and bioconversion of substrates over submerged state fermentation are very clear and significant. This has lead to SmF being utilized exclusively for enzyme production especially by fungal cultures. Ghildyal *et al.* (1984) have compared the economics of submerged and solid state fermentation for the production of amyloglycosidase. They have reported that the enzyme titre obtained by SSF is 10 times more than that obtained by SmF. The broth from SmF can be concentrated to the level obtained by SSF but proves to be cost intensive. Ohno *et al.* (1992) have described the production of an antifungal peptide antibiotic, Iturin by *Bacillus subtilis* NB 22 by SSF using soybean curd residue (okara). Aeration, temperature and moisture content were the controlling factors for the efficent production of Iturin. It was found that SSF was 6-8 times more efficient with respect to Iturin production than SmF on the basis of unit wet weight.

Senecal *et al.* (1992) have reported that SmF was favoured over SSF in biotransformation of corn stover when used as a carbon source. Yields of protein were around 83 mg in SmF and 30 mg in SSF. The most optimal yield of sugar was 972 mg total in liquid culture for 7 days when hammer-milled newsprint was used as the carbon source.

The nature and amounts of by-products formed during conversion of sugar beets to ethanol by *Zymomonas mobilis* in conventional submerged fermentation (SmF) and solid state fermentation (SSF) were investigated by Amin and Allah (1992). It was found that the bacterium produced fewer by-products in SSF than SmF, and that by-products profile was different. Stredansky *et al.* (1999) showed that bacterial exopolysaccharide production by *Rhizobium meliloti* and *R. frifolis* was better in solid substrate fermentation compared to submerged cultivation. The higher productivity in SSF might be attributed to a number of factors primarily higher oxygen availability as compared to liquid flask experiments.

Crestini *et al.* (1996) have described a method for the production and isolation of chitosan (polyglucosamine) by liquid and solid state fermentation from *Lentinus edodes*. The yield of isolated chitosan was 120mg/L of fermentation medium under SmF conditions and 6.18g/kg of fermentation medium under SSF conditions. The SSF methods which give up to 50 times more yield than other chitosan production methods from fungi, provides a new

flexible and easy procedure for production of low acetylation degree chitosan. Maximal values of chitosan yields were got 12 days after inoculation and concentration of dry cell biomass reached maximal value after 9 days of cultivation both under SSF and SmF.

3.1.3 Fermentation of bio-waste and nutritional enrichment

Recent advances in the area of SSF have led to the development of bioprocesses and their products. The last decade has witnessed an increase in interest in SSF for bioremediation and degradation of hazardous compounds, detoxification of agro-industrial residues, biotransformation of crops and crop residues for nutritional enrichment, pulping and production of value added products such as biologically active secondary metabolites, pesticides, surfactants, biofuel and aroma compounds (Pandey *et al.*, 2000).

Significant increase in the demand for livestock products in recent years in developing countries has required an increase in animal feed supply. Increasing interest has been paid by the researchers to the enrichment of protein of agricultural wastes and subproducts through solid state fermentation (SSF) (Aidoo *et al.*, 1982; Senez *et al.*, 1983; Gibbon *et al.*, 1984). A preferable mode for using the SSF technology is to integrate them in the existing agro-industrial complexes. The SSF technology has the advantage of direct utilisation of none or very few pre treated solid substrates under aerobic conditions to produce Microbial Biomass Products (MBP), which contain a

mixture of unused substrates, cell substances of the microorganisms and externalized metabolites.

Majority of the solid state fermentation studies are aimed at the bioconversion of agricultural wastes into forage or feed for livestock. The complex polysaccharides are hydrolyzed into simpler components supported by bacterial activity that enriches the fermented product by its metabolites. The fermented product characterized by its high protein content, enhanced digestibility and valuable nutrient profile can make an important component in feedstuffs for animals.

The use of microorganisms to convert carbohydrates, lignocelluloses and other industrial wastes into foodstuffs rich in protein is possible due to the following characteristics of microorganisms (Balagopalan, 1996).

a) Microorganisms have a very fast growth rate.

b) They can be easily modified genetically for growth on a particular substrate under particular culture conditions.

c) Their protein content is quite high varying from 35 to 60%.

d) They can be grown in slurry or on solids.

e) Their nutritional values are as good as those of other conventional foods rich in protein.

Table 3.1 Some typical SSF processes for the production of protein/animal feed

Sl. No.	Substrates	Microorganisms	Reference
1.	Canola meal	Aspergillus carbonarius	Alasheh & Duvnjak, 1995
2.	Carob pods	*Aspergillus niger*	Smail et al., 1995
3.	Apple pomace	*Candida utilis* *Kloeckera apiculata*	Rahmat et al., 1995
4.	Coffee pulp	*Penicillium verrucosum*	Roussos et al., 1994
5.	Sugarcane baggase	*Chaetomium cellulolyticum*	Bravo et al., 1994
6.	Sugar beet pulp and molasses	*Fusarium oxysporum* *Chaetomium cellulolyticum* *Trichoderma ressei* *Trichoderma viride*	Nigam, P. 1994
7.	Apple pomace	*Saccharomyces cerevisiae* *Candida utilis* *C. tropicalis* *T. viride* *A. niger*	Bhalla & Joshi, 1994
8.	Orange peels, Grape stalks	*Pleurotus ostreatus* *Agrocybe aegirata* *Armillarialla mellea*	Nicolini et al., 1993
9.	Cassava	*Rhizopus arrhizus*	Saegal et al., 1993
10.	Oil palm wastes	*P. sojor-caju*	Kume et al., 1993
11.	Cassava	*A. oryzae*	Zvauya & Muzondo, 1993
12.	Cotton stalks, Perlite	*P. ostreatus* *Ph. chrysosporium*	Kerem & Hadar, 1993
13.	Wheat straw	*P. ostreatus*	Tripathi & Yadav, 1992
14.	Grape pomace, Corn stover	*Chaetomium cellulolyticum* *+Candida utilis*	Girujie et al., 1992
15.	Rice straw, Maize stover	*Cyathus stercoreus* *Dichotomus squalens* *Ph. chrysosporium*	Karunandaa et al., 1992
16.	Sugarcane baggase	*Polyporus species*	Nigam, p. 1990
17.	Sugarbeet pulp	*T.reesei,* *Fusarium oxysporum*	Nigam & Vogel, 1990
18.	Sugarcane bagasse	Mixed cultures (*Polyporus, Pleurotus, Trichoderma)*	Nigam, P. 1989
19.	Corn cob	A. niger	Singh et al., 1989

Sl. No.	Substrates	Microorganisms	Reference
20.	Starchy raw materials	*A.oryzae, A .niger, A. awamori*	Czajkowska & Ilnicka, 1988
21.	Sugarbeet pulp	*T.viride*	Durand & Chereau, 1988
22.	Sugarbeet pulp	*T.reesei* *Fusarium oxysporum*	Nigam & Vogel, 1988
23.	Wheat straw	*Coprinus* species	Yadav, J.S. 1988
24.	Sugarbeet pulp	Thermophilic fungi	Grajek, W. 1988
25.	Cassava	Rhizopus oryzae	Daubrasee et al., 1987
26.	Saccharum munja residues	*Pleurotus* species	Gujral et al., 1987
27.	Straw	Candida utilis	Han, Y.W. 1987
28.	Cassava	*S. pulverulentum*	Smith et al., 1986
29.	Banana wastes	*A. niger*	Baldensperger et al., 1985
30.	Dried citrus peel	*A. niger*	Rodriguez et al., 1985
31.	Wheat straw	*T. reesei* *Ch. cellulolyticum* *C. utilis*	Abdullah et al., 1985
32.	Sugarcane bagasse	*Polysporus* species	Nigam & Prabhu, 1985
33.	Wheat straw	*T. reesei* *Endomycopsis fibuliger*	Laukevics et al., 1984
34.	Fodder beets	*S. cerevisiae*	Gibbon et al., 1984
35.	Pulpmill wastes	*Ch. cellulolyticum*	Tautorus & Chalmers, 1984
36.	Soyabean	Rhizopus oligosporus	Rathbun & Shuler, 1983
37.	Cassava	*T. reesei* + yeast	Opoku & Adoga,1980
38.	Starch substrates	Various cultures	Senez et al., 1980
39.	Alfalfa	A. terreus	Bajracharya & Mudgett, 1979
40.	Straw, corn stover	*Ch. cellulolyticum*	Moo-Young et al., 1979
41.	Rye-grass	*Cellulomonas, A. faecalis*	Yu et al., 1976
42.	Rye-grass	*T. reesei* *A. pullulans* *C. utilis*	Han & Anderson, 1975
43.	Newsprint	*Sporotrichum thermophilae*	Barnes et al., 1972

Using microbial consortia, primary and secondary fermentation can be combined to give products including single cell protein, mushrooms, enzymes and microbial cells. Bioconversion is an effective way of reprocessing waste material into useful value added products for the agricultural sector. Biotechnological potential of agro-industrial residues has been described by many workers but there is hardly any such reference available on seafood processing wastes.

Upgrading of paddy straw into protein rich animal feedstuff was accomplished by Kahlon and Dass (1987). Wheat straw, corn starch and manure fibres could be enriched with protein for animal feed by SSF using cellulolytic fungi (Tengerdy *et al.*, 1983). Similar works in bioconversion of lignocellulosic wastes for protein feedstuff preparation were carried out by a number of workers (Lynch, 1985; Hatakka and Pirhonene, 1985; Beg *et al.*, 1986; Milstein *et al.*, 1986; Viesturs *et al.*, 1987; Yadav, 1987).

Solid state fermentation of agricultural by-products with lignocellulose degrading fungi increases the digestibility and feed value of wheat straw due to partial hydrolysis of cellulose and hemicellulose and enrichment of straw with fungal biomass. Acid-hydrolysed and alkali-neutralised cellulose substrates (rice paddy straw, wheat straw, cotton stalks, fur stalks and peanut hulls) were enriched with *Beijerinekia mobilis*, a free living nitrogen fixing bacterium, by a solid state fermentation process. There is a twofold increase in

crude protein in paddy straw and peanut hulls, but only a marginal increase for other substrates. A very significant *in vitro* digestibility occurred with peanut hulls (Balasubramanya and Bhatawolekar, 1988). Rice and wheat straws treated with urea-ammonia and subjected to SSF with *Coprinus finetarius* showed the degradation of 50% cellulose, hemicellulose, lignin and 40% cell wall constituents within a one-week period. A good quality biomass for animal feedstuff was produced by Singh *et al.* (1989). SSF is reported to have tripled the protein content of the substrate. A novel ensiling process involving the use of a silage additive containing *Lactobacillus, Plantarum* and 0.5% molasses resulted in generation of fermentable sugars and 45% increase in digestibility. Similarly SSF of urea supplemented wheat straw with *Neurospora sitophila* resulted in protein enriched feed additive (Sondhi *et al.*, 1990).

Weichert *et al.*, (1991) studied the SSF of wheat straw, cotton waste, wheat bran and wine husks and its potential application as feedstuff. There was 3.4-fold enrichment in protein content by the growth of *Fusarium* spp.
The nutritional value and the free amino acid and vitamin content of rye fodder meal (13.5% protein and 2% ash) were enhanced by SSF with *Fusarium* (Klappach *et al.*, 1991). The content of free amino acids increased more than 10 fold in comparison to the non-fermented crude material. The lysine content was increased 14 fold and the methionine content was increased about 3 fold. The fermented rye was produced to substitute wheat and corn in feed mixtures (Klappach *et al.*, 1991).

Jacob (1991) reported the enrichment of wheat bran by *Rhodotorula gracilis* through solid-state fermentation. The upgradation of wheat bran a very rich agro-industrial waste material with lipid producing *Rhodotorula gracilis* led to the production of red carotenoids and a cyanocobalamin precursor. This significantly increased the nutritional value of the fermented bran, which was aimed for use as feedstuff for fowl. This fermentation gave an increase of 68.1% in the lipid content of the wheat bran (initial 3.6%). A maximum protein increment of 25% was noteworthy.

An SCP product containing 30.4% protein was obtained by bioconversion of cassava peel with high levels of methionine 3.3gm/100gm crude protein (Smith *et al.*, 1986). A feedstuff of improved digestibility (reduced lignin content) was produced after 8 days of bagasse fermentation by Nigam (1990). Cassava bagasse contains approximately 30-50% starch on a dry weight basis and due to its rich organic nature and low ash it could serve as an ideal substrate for microbial processes producing value added products (Pandey *et al.*, 2000).

Gonzalez-Blanco *et al.* (1990) carried out the protein enrichment of sugarcane by-products using solid state cultures of *Aspergillus terreus*. Bravo *et al.*, (1994) reported 80% protein enrichment by microbial fermentation of sugarcane bagasse.

Yang (1988) considered the production of feedstuff protein enrichment using amylolytic yeasts, moulds and *Streptomyces* spp. on starchy agricultural residues like sweet potato. The final product after 2-3 days fermentation contained 16-20% protein. *Aspergillus oryzae* on various starch containing raw materials resulted in the protein content in post-culture products increasing by 46-88%. The contents of 13 amino acids in post-culture products rose substantially (i.e. 34-63%) (Czajkowska and Olejniczak, 1989).

Gumbira-Said *et al.* (1992) have carried out solid state cultivation of sago starch with *R.oligosporus* UQM 145F followed by *in situ* drying of the microbial product with the aim of developing an animal feed. The product quality was assessed by analyzing the amino acids in the dried microbial product. The dried microbial protein-enriched sago beads contained 8-10% (w/w dry basis) protein determined by Folin-Lowry method and could be used as monogastric animal feed. Biochemical analysis showed that the protein produced was of superior quality.

Solid wastes from olive oil processing were delignified by *Phanerochaete chrysosporium* and the fermented material got was saccharified by *Trichoderma* sp. to provide substrate for yeast growth. Success was judged by the level of crude protein generated, and a combination of fermentation involving *Phanerochaete chrysosporium*, *T. reesei* and *Saccharomyces cerevisiae* increased the level of crude protein from 5.9% in

the raw pomace to 40.3% in the fermented material which was envisaged for use as fowl feed (Haddadin, *et al.*, 1999).

Elimination of antinutritional factors is another advantage of microbial transformations of waste residues whereby recycling of unacceptable products transforms them into nutritious products by reducing the antinutritional or antimetabolic factors (Murata *et al.*, 1967; van Veen and Steinkraus, 1970; Zamora and Veum, 1979; Liener, 1980; Wang and Hesseltine, 1981; Beuchat, 1983; Canella *et al.*, 1984). Enhancement of the nutritive value of oilseed cakes by bioprocessing to increase the bioavailability of nutrients, reduces or removes antinutritional factors, and the inclusion of appropriate additives could result in oilseed meals being incorporated at higher levels in fish feeds (Wee, 1991).

Kirk (1979) reported a significant decrease in phytate in wheat flour and whole-wheat bread by fermentation with yeast. Phytate could also be significantly decreased from soyabean tempeh (Sudarmadji and Markakis, 1977; Sutardi and Buckle, 1985) and oncom (Fardiaz and Markakis, 1981) by the process of fermentation with *Rhizopus oligosporus*.

Aspergillus ficuum grown on canola meal helped in reducing the phytic content to zero thereby improving its feed value (Nair and Duvnjak, 1991; Nair *et al.*, 1991). SSF with *Rhizopus* resulted in detoxification and protein enrichment of rapeseed meal. The antinutritional substances in the substrate

were considerably reduced. The protein intake of rats fed fermented meal diet was identical to that of rats fed the dehulled, defatted and heated meal diet. The combined effects of feeding trial showed that fermentation improved greatly the biological and nutritional value of the obtained products (Bau *et al.*, 1994). The antinutritional factor, phytic acid from raw sesame seed meal, could be reduced below detection limit by fermentation with lactic acid bacteria, *Lactobacillus acidophilus* (Mukhopadhay and Ray, 1999).

Solid state and submerged state fermentation differ basically in the amount of moisture content. This difference is one of the crucial factors that govern the processes that occur during fermentation. Moisture content being related to many factors can greatly influence the path of enrichment, leading to products of different quality. The objective of the study in this chapter is to find the optimal moisture content of the fermentation media and moisture preference of each strain selected that lead to the best nutrient enhancement.

3.2 Materials and Methods

3.2.1 Preparation of prawn shell waste for fermentation

Prawn shell waste collected from peeling sheds in Cochin was dried in an oven at 80^0C for 24 hrs. This dried prawn shell waste was then powdered and stored in polypropylene containers under refrigeration (4°C). This was used for carrying out the fermentation studies. Five grams each of this shell

Fig.3.1 Dried prawn shells

waste was transferred into conical flasks, plugged with cotton and then autoclaved at 121^0C for 15 minutes.

3.2.2 Strains used for fermentation

Two types of cultures were used for fermentation

1. Chitinoclastic and proteolytic strains –25 Nos.
2. Chitinoclastic and non proteolytic strains –5 Nos.

(See Table 3.1)

3.2.3 Inoculum preparation

The 30 selected strains were inoculated into nutrient agar slants, incubated for 24 hrs and harvested using sterile saline (0.85% NaCl). The optical density of the cell suspension was adjusted to 1 at 600 nm using a Hitachi 200-20 UV-Visible Spectrophotometer. 0.5 ml of this cell suspension was used as inoculum for 5 gm substrate.

3.2.4 Fermentation of the shell waste

0.5 ml each of the cell suspension (3.2.3) of 30 selected strains was inoculated into the sterile shell waste (3.2.1). Sterile 50% seawater was used for moistening the substrate.

Solid State Fermentation

After the addition of inoculum, the shell waste was mixed with 50% sea water in the ratio 1: 1.5 (w/v) i.e. shell waste : moistening medium. The flasks

in duplicate for each strain were incubated at room temperature (28 $\pm$ 20^{0}C) for two weeks.

Submerged State Fermentation

The procedure for SmF is same as that of SSF, but the moisture content was in the ratio 1:3 (w/v). Prawn shell waste powder maintained at SSF and SmF conditions without the addition of inoculum was used as the control.

3.2.5 Biochemical analysis of the fermented product

After incubation the products were oven dried at 80^{0}C for 24 hrs. The fermentation product was then stored in sealed polythene bags at 4^{0}C for biochemical analysis. The preference of each strain for solid state/submerged state fermentation was found out by analyzing the nutritional quality of the fermented product in terms of protein, lipid and carbohydrate content. Protein was estimated by Lowry's method (Lowry, 1951), lipid by phosphovanillin method following chloroform-methanol extraction of the sample (Folch *et al.*, 1957) and carbohydrate by phenol sulphuric acid method.

3.3 Results

Raw prawn shell waste (dried) contained 38% protein, 5.36% lipid and 1.86% carbohydrate. Fermentation resulted in substantial increase in the nutritive value of the product. The biochemical composition of the fermented products is given in Fig 3.1 and Table A.1. The maximum protein content obtained was 59.15% in FP111 (SmF), lipid 8.97% in FP124 (SmF) and carbohydrate 3.29% in FP163 (SSF). Eventhough there was not much

variation in protein enrichment between SSF and SmF, generally SmF was found to be better for most of the strains. Lipid enrichment was also found to be better in SmF compared to SSF. However the increase in carbohydrate was observed more in SSF. No significant difference could be observed between the performances of the two types of strains i.e. chitinoclastic/proteolytic and chitinoclastic/non proteolytic.

Solid State Fermentation

The protein content of the various fermentation products varied widely ranging from 29.15 to 58.24%. The highest protein enrichment in SSF was noted in FP111 (58.24%). Almost all the fermented products registered substantial increase in protein content compared to the control. FP 220 showed a distinctive increment in protein in the fermentation product recording 50.0% which is comparable to the best values recorded by the proteolytic forms.

Lipid content in the fermentation product varied from 2.51% to 7.27% with the maximum being observed in FP163 followed by FP48 (7.09%) indicating 35.6% and 32.3% increment respectively. The non proteolytic strains also showed varying performance with values ranging from 2.51 to 5.41%. (Table 3.3)

In the case of carbohydrate content, almost all fermented products showed comparatively higher values than the control (raw prawn shell

powder) (1.86%). The values ranged from 1.69 to 2.88% with the highest value for FP4 indicating 54.8% increase.

Submerged Fermentation

The highest protein content recorded in SmF is 59.15% by FP111 with an increase of 55.6%. The overall values ranged from 32.96% (FP33) to 59.15% (FP111). Lipid content ranged from 3.87% (FP217) to 8.97% (FP124). Some of the non proteolytic forms FP33 (6.39%), FP218 (7.58%) and FP220 (7.43%) showed substantial increase in lipid content. Total sugars in the fermentation product of SmF showed the highest value of 2.44% (FP63), which resulted in 31.2% increase. The values recorded for total sugars ranged from 1.32% (FP 218) to 2.44% (FP 63).

3.4 Discussion

Fermentation of the shell waste with various selected microbes recorded substantial increase in the nutritional quality. This might be due to the hydrolysis/transformation of the shell waste, formation of extracellular metabolites by microorganisms and biomass build up. The overall comparison of the data pertaining to SSF and SmF reveal a few distinct features.

3.4.1 Protein enrichment

The difference in protein enrichment attained by SSF and SmF was not very significant. A maximum value of 58.24% (FP111) protein content in the fermentation product attained by SSF shows protein enrichment by 53%. On

the other hand, in the case of submerged state fermentation, the maximum protein value recorded was by the same strain (C111) which was 59.15% (FP111) indicating an increment by 55.66%. A similar pattern of performance has been noted by Barrios-Gonzales *et al.* (1993).

The general protein enrichment by way of fermentation of the chitinous substrate by selected strains with varying proteolytic and chitinolytic potential can be attributed to the proliferation of bacteria in the chitinous substrate leading to protein enrichment due to biomass buildup. The highly varying results by the thirty selected strains in both fermentation types reflect the varying hydrolytic/transformation potential exhibited by these strains.

An overall comparison study reveals that the few strains that showed poor protein buildup in the fermentation product showed a corresponding increase in the levels of other nutrients like lipid and carbohydrate. This compensatory enhancement of other nutrients enhances the value of the fermented product thereby confirming the fact that fermentation is a very complex process with a wide variety of transformations. It was interesting to note that these strains showed lower value compared to the initial protein content in the shell waste. This could indicate the transformation of the initial protein into other nutrient forms by way of transformation.

Protein enrichment was given emphasis in this study as this was the target nutrient that formed the most important component in the feed

formulations. Protein enrichment of prawn shell waste has been previously reported by Rhishipal and Philip (1998) where marine yeasts have been employed for biotransformation of prawn shell waste to single cell protein. Similarly protein enrichment of cellulosic residues like rice husks fungi has shown promising results, and the digestibility of the product was notable (Bertolin *et al.*, 1996).

Czajkowska and Olejniczak (1989) have recorded an increase of 46-88% protein by *Aspergillus oryzae* on various starch containing raw materials. Singh *et al.*, (1990) have described the bioconversion of wheat straw by SSF that tripled the protein content of the substrate. Bravo *et al.* (1994) reported protein enrichment of 80% by microbial fermentation of sugarcane bagasse.

3.4.2 Lipid enrichment

Comparatively higher lipid enrichment in SmF by the various strains was noteworthy. An increase of 67.35% lipid could be recorded in FP124 whereas in SSF the lipid content was lower than that of the control. This shows the importance of moisture content in directing the metabolic processes of the microbial strains used. However culture No.C163 that showed maximum enrichment of lipid (35%) by SSF notably exhibited almost the same quantity in SmF (32%) also. Another important observation made with SmF is that out of the 5 non-proteolytic strains used in the fermentation study, 2 showed more than 37% increase in the lipid value whereas among proteolytic forms (25 numbers) only 4 strains registered a comparable increase

in lipid. Similar lipid enrichment has been noted by Jacob (1991) who has reported upgradation of wheat bran with lipid-producing *Rhodotorula gracilis* exhibiting an increase of 68.1% lipid in wheat bran.

3.4.3 Carbohydrate enrichment

Preference of SSF for increase in carbohydrate may be due to the increased activity of chitinase enzymes and release of more sugars in SSF. However, much work has to be done in this area for a better understanding of the finding. The maximum carbohydrate content was recorded in FP163 (SSF) with an increase of 76.88% followed by FP153 and FP111. Increase in carbohydrate content may be explained by the hydrolysis of complex polymers like chitin into simpler sugars and monomers, thereby enhancing digestibility and availability of such nutrients when used as a feed ingredient.

3.4.4 Performance of proteolytic and non proteolytic strains.

In this study, the performance of proteolytic forms were found to be better in terms of nutritional quality of the fermented products. Eventhough a better protein enrichment was expected with non-proteolytics due to the non utilization of the native protein, a notable increase in protein could not be witnessed. The hydrolysis of the shell waste protein is found to be supporting biomass buildup, elaboration of extracellular metabolites and therefore better enrichment of the substrate.

3.4.5 Moisture content: comparison of SSF/SmF

Moisture is the key element for regulating and optimizing fermentation process. Too much moisture compacts the substrate, prevents oxygen penetration and facilitates contamination by fast growing bacteria. Too little moisture inhibits growth, enzyme activity, accessibility to nutrients (Laukevics *et al.*, 1984) and reduces substrate swelling (Wijeyaratne *et al.*, 1979).

It was only recently, that it became apparent that the biochemical and physiological responses of many microorganisms in solid state culture differ greatly from those in SmF, leading to variations in productivity. This was supported by the findings that the higher performing strain in SmF seemed to have a lower activity in SSF. So it is imperative to study the preferences of the selected strains to obtain optimal performance (Balakrishnan and Pandey, 1996).

In this study preference for SSF/SmF varied with the strains and with the biochemical criteria analysed. More importance was given to protein enrichment since it was the major ingredient in the product and is of great importance when being utilized as a feed component for aquaculture applications. Majority preferred SmF when protein and lipid enrichment was considered. By comparing the protein enrichment in SSF and SmF, the preference of the culture for SSF/SmF was finalized for further experiments.

Shankaranand *et al.* (1992) have reported that some strains of *Aspergillus niger* and *Bacillus licheniformis*, which produce citric acid and α-

amylase respectively in submerged fermentation, did not produce these products when grown on wheat bran in a solid state fermentation system. Cultures that produced high titres of α -amylase in SmF were poor producers in SSF system and vice-versa. These discrepancies indicate the need for intensive screening to select potent cultures particularly suited to the system concerned since it is clear that the cultures that are good producers in SmF process cannot be relied upon to perform well in SSF.

It was interesting to note an increase in the moisture content in the solid state fermentation flasks during the course of fermentation. The water availability could change during the course of fermentation as a result of variations in the moisture content and also owing to the modifications of the water sorption properties of the fermenting matter (Gervais *et al.*, 1988; Oriol *et al.*, 1988). Three main sources of variation of the moisture content can be considered namely, evaporation caused by metabolic heat evolution, water consumption for polysaccharide hydrolysis and water produced by the carbohydrate and nutrient metabolism (Dorta *et al.*, 1994).

The present study revealed that moisture content influences the nutrient profile of the fermentation product generated. SSF was found favourable for maximal protein and carbohydrate enrichment by most of the strains. Lipid enhancement was clearly supported by submerged state fermentation by the majority of the strains tested.

Table 3.2. List of bacterial strains used in the fermentation study and the corresponding fermented products

S.No.	Culture	Genera	Enzyme production		Fermented product Code name
			Chitinase	Protease	
1	C4	*Streptococcus*	+	+	FP4
2	C14	Coryneforms	+	+	FP14
3	C15	*Bacillus*	+	+	FP15
4	C18	Coryneforms	+	+	FP18
5	C37	*Vibrio*	+	+	FP37
6	C38	Coryneforms	+	+	FP38
7	C48	Coryneforms	+	+	FP48
8	C52	*Vibrio*	+	+	FP52
9	C56	*Bacillus*	+	+	FP56
10	C63	*Streptococcus*	+	+	FP63
11	C84	*Pseudomonas*	+	+	FP84
12	C98	*Planococcus*	+	+	FP98
13	C111	*Bacillus*	+	+	F111
14	C112	*Streptococcus*	+	+	FP112
15	C113	*Acinetobacter*	+	+	FP113
16	C123	Coryneforms	+	+	FP123
17	C124	*Bacillus*	+	+	FP124
18	C134	*Bacillus*	+	+	FP134
19	C146	*Staphylococcus*	+	+	FP146
20	C149	*Serratia*	+	+	FP149
21	C153	Coryneforms	+	+	FP153
22	C154	*Bacillus*	+	+	FP154
23	C157	*Vibrio*	+	+	FP157
24	C163	*Serratia*	+	+	FP163
25	C217	*Vibrio*	+	+	FP217
26	C29	*Vibrio*	+	-	FP29
27	C33	*Pseudomonas*	+	-	FP33
28	C218	*Vibrio*	+	-	FP218
29	C219	*Vibrio*	+	-	FP219
30	C220	*Bacillus*	+	-	FP220

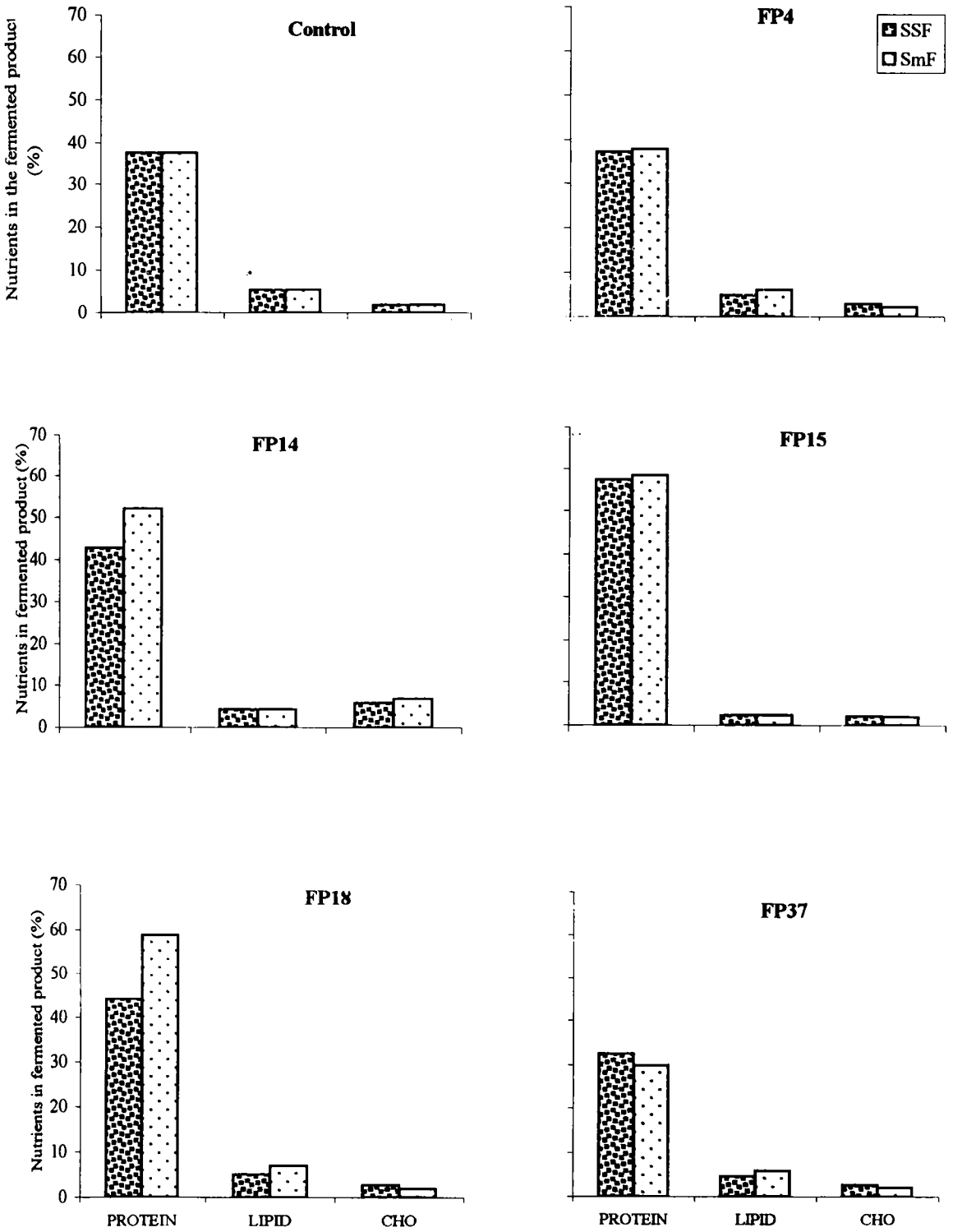
Table 3.3 Percentage increase in protein, lipid and carbohydrate in the fermented products generated by various strains

Fermented products	Percentage increase in the nutrients compared to the control (prawn shell waste)					
	Protein (%)		Lipid (%)		Carbohydrate (%)	
	SSF	SmF	SSF	SmF	SSF	SmF
FP4	*	*	*	12.50	54.84	12.90
FP14	36.89	12.58	*	*	17.74	16.67
FP15	50.97	53.55	*	25.56	5.91	2.15
FP18	28.68	15.37	*	29.66	47.85	2.15
FP29	*	31.53	*	*	31.72	39.78
FP33	20.26	*	*	19.22	50.54	17.20
FP37	*	*	*	9.14	41.94	9.68
FP38	14.66	9.79	*	33.96	26.88	13.44
FP48	50.97	19.79	32.28	*	20.97	0.54
FP52	23.63	15.61	*	29.85	17.74	16.67
FP56	28.34	15.68	*	2.43	12.90	9.14
FP63	20.84	5.13	*	0.93	40.32	31.18
FP84	6.58	53.47	*	24.07	37.63	*
FP98	*	38.45	23.32	41.23	12.37	*
F111	53.26	55.66	5.22	11.75	68.82	8.06
FP112	38.18	36.03	2.24	*	8.60	11.29
FP113	38.97	36.53	12.69	*	35.48	*
FP123	35.29	35.58	31.90	35.26	42.47	*
FP124	43.00	21.95	*	67.35	2.15	12.37
FP134	19.79	50.21	6.72	28.54	4.84	*
FP146	24.58	15.68	*	*	18.82	4.84
FP149	35.79	26.95	*	*	*	19.35
FP153	38.53	25.13	*	*	76.88	5.38
FP154	21.95	0.95	*	51.12	24.19	28.49
FP157	*	0.58	*	44.59	6.99	*
FP163	37.79	26.29	35.63	32.65	71.51	*
FP217	35.29	10.68	*	*	*	17.74
FP218	22.76	40.71	0.93	41.42	16.67	*
FP219	*	10.74	*	*	*	*
FP220	50.03	13.11	*	38.62	4.84	29.57

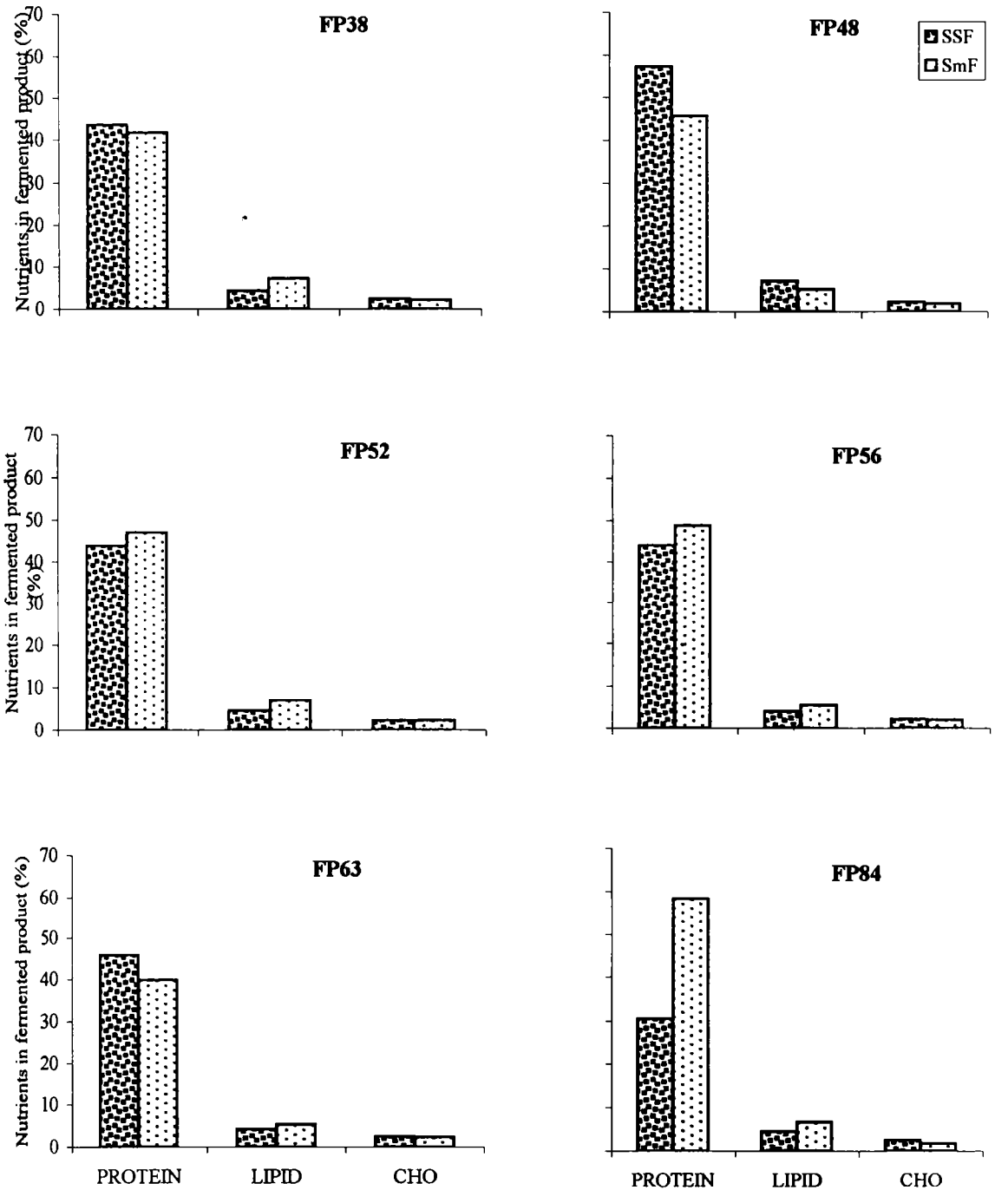
* value is lesser than that of control

Figure 3.1 (a&b) Protein, lipid and carbohydrate (CHO) content of the prawn shell waste fermentation products generated by the various strains (in % dry wt)

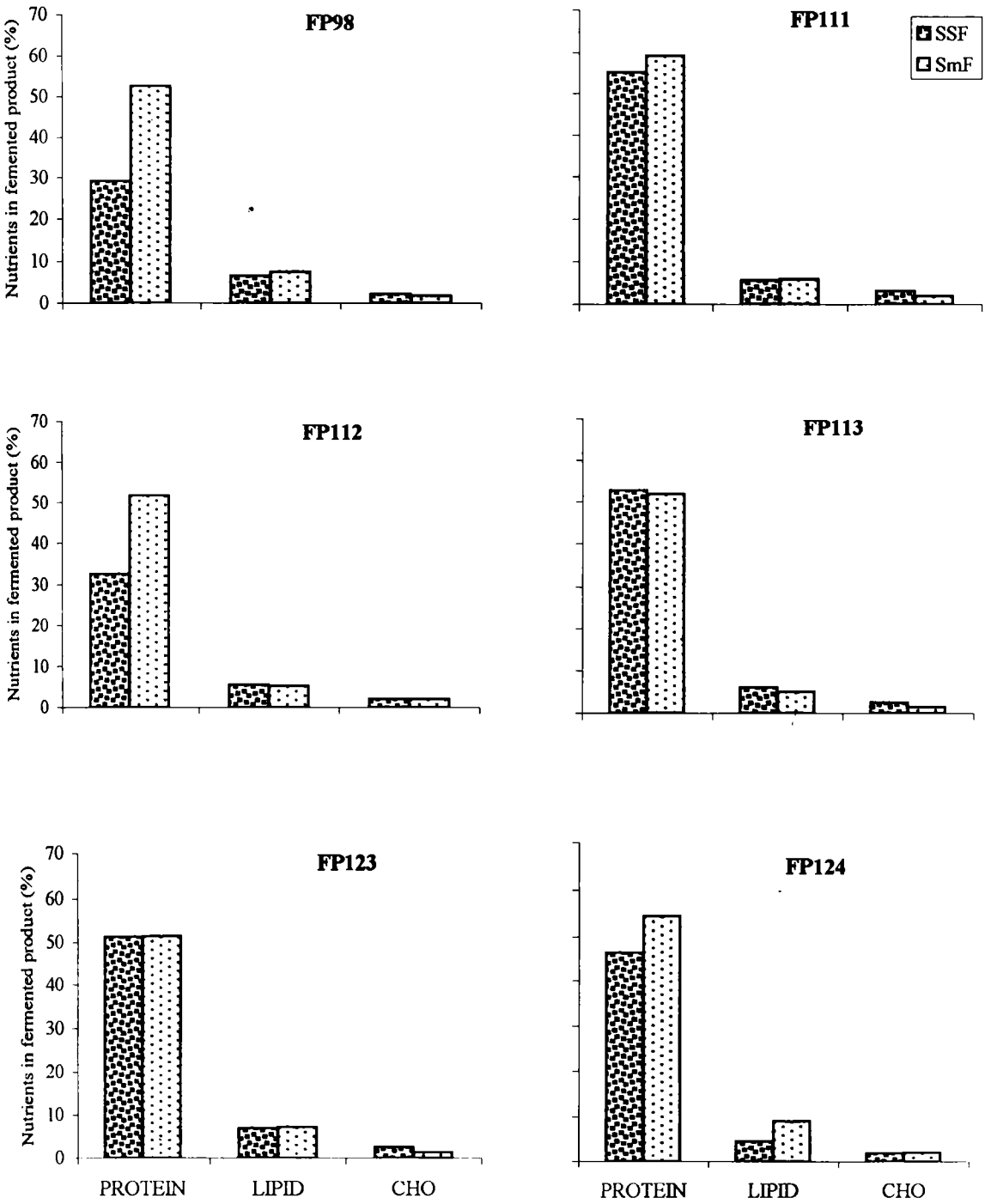
a. Chitinoclastic cum Proteolytic strains



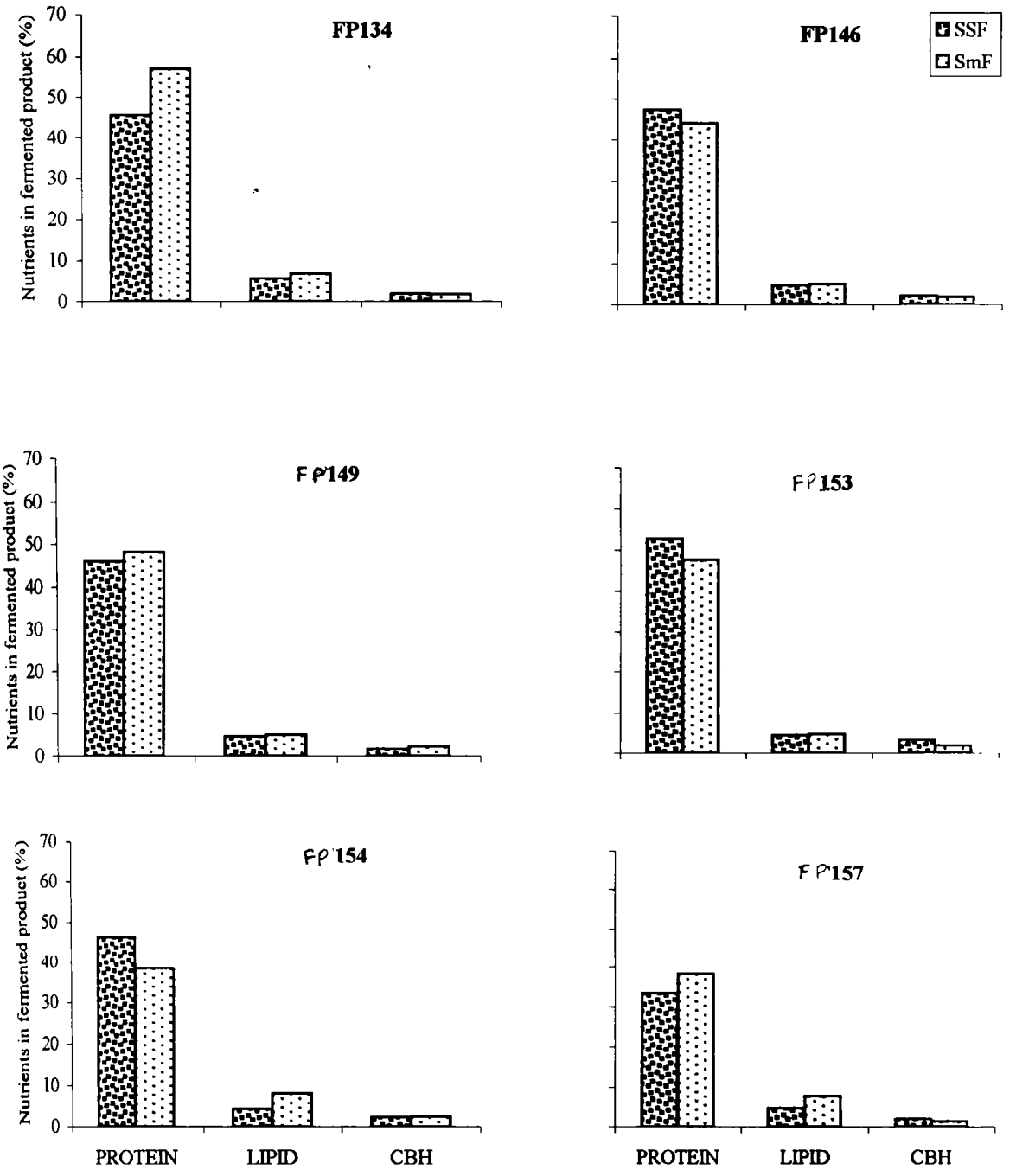
Chitinoclastic cum Proteolytic strains



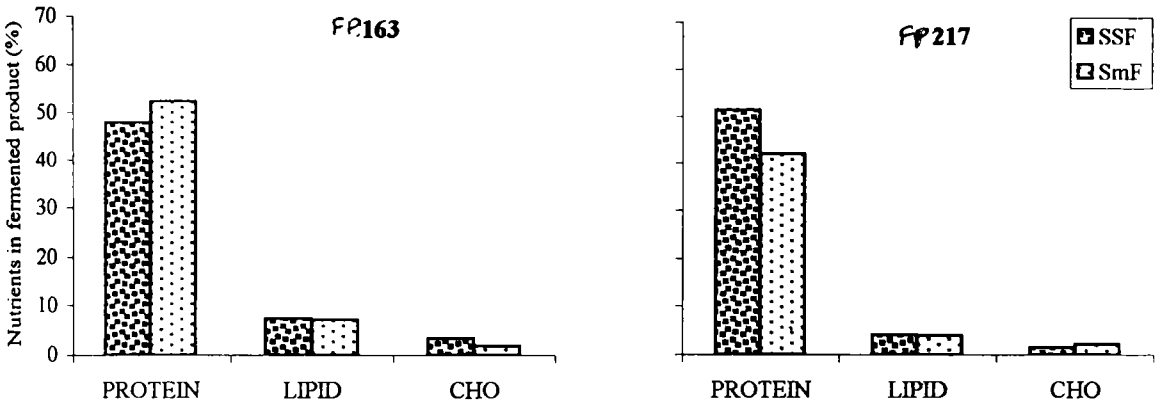
Chitinoclastic cum Proteolytic strains



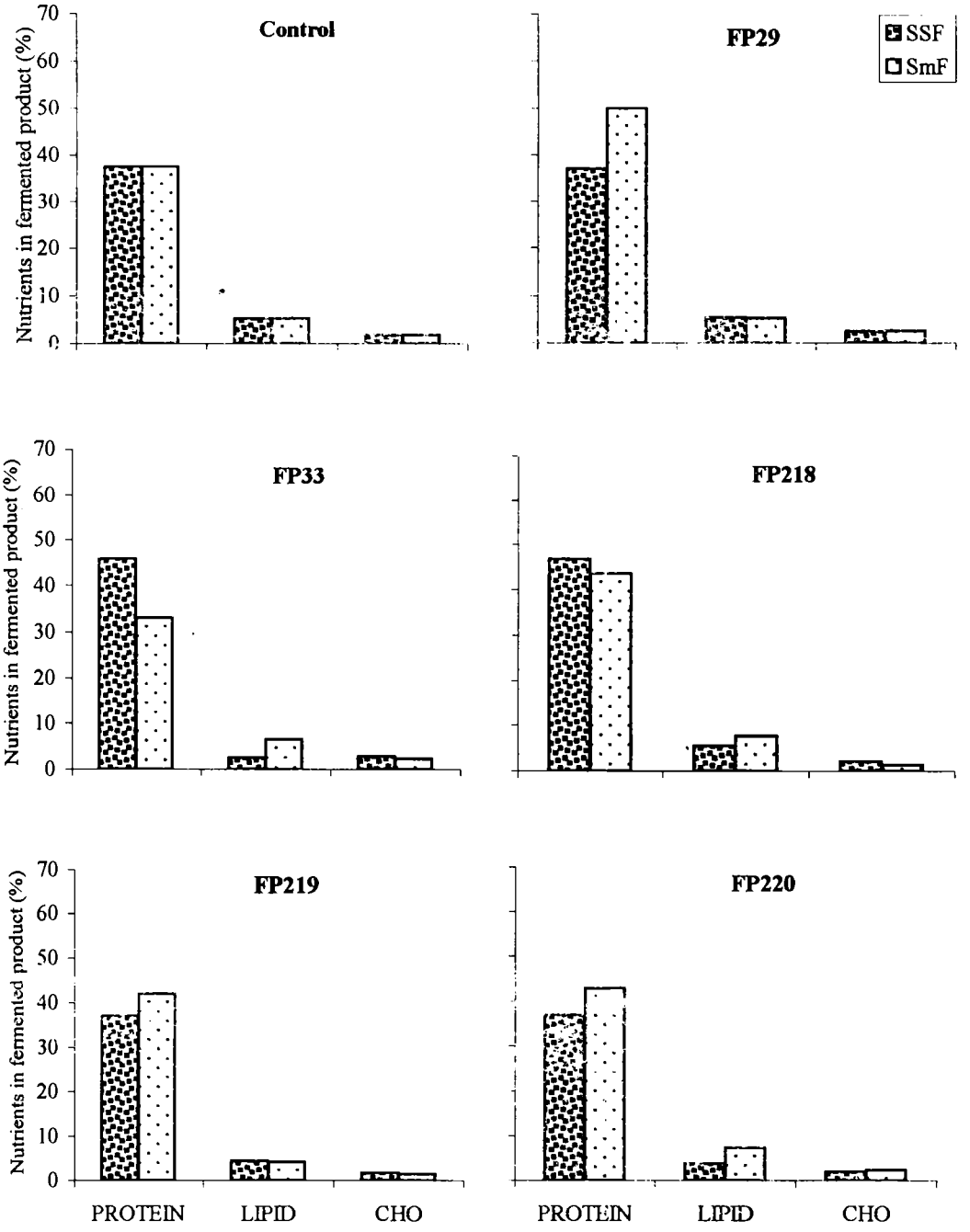
Chitinoclastic cum Proteolytic strains



Chitinoclastic cum Proteolytic strains



b. Chitinoclastic cum Non Proteolytic strains



Chapter 4

Screening of the shell waste fermentation product for its efficacy as a dietary ingredient for the Indian White Prawn, *Penaeus indicus*

4.1 Introduction

During the past few decades, there has been an increasing interest to use alternative plant and animal protein as low cost substitutes of fishmeal (Moore and Stanley, 1982; Kohler and Kruegger, 1986; Koshio *et al.*, 1992; Tidwell *et al.*, 1992, 1993a &b, 1994). Shrimp meal has been a well-known ingredient, which is both cheap and nutritious. Investigations on alternative protein sources in practical diet for shrimps have proved the suitability of prawn meal as a major protein source. The utilization of shrimp wastes in shrimp diets has been well studied (Venkitaramaiah *et al.*, 1978; Ali, 1982; Ali and Mohammed, 1985; Akiyama *et al.*, 1989; Penaflorida, 1989; Lim and Dominy, 1990). The potential of shrimp processing waste as flavour components in livestock feed formulations, sources of carotenoids for pigmentation purposes and as a source of chitin, protein, fatty acids and minerals was examined by Meyers (1986). Research in USA and Equador into the uses of shrimp wastes from the processing plants for shrimp meal, shrimp head meal and shrimp silage has resulted in the identification of their potential importance as proteinaceous source of food in animal feeds (Brzeski, 1987). Sudaryano *et al.*, (1996) have shown that fish processing waste products (eg. scallop waste, sardine, shrimp

head and lobster waste) can be used as alternative protein sources in diets for *Penaeus monodon* with high digestibility values.

Its flavour seems to be an attractant in shrimp diets (Pascaul and Destajo, 1978) and the shell (chitin) of the shrimp waste is found to have good growth promoting effect in *Penaeus indicus* (Vaitheswaran and Ali, 1986). Content and digestibility of essential amino acids are crucial to the biological value of the protein. Essential amino acid index (EAAI) used to evaluate protein quality of feeds gave a result in the range of 0.94-0.96 for shrimp waste based diet indicating its suitability as an alternative protein source in shrimp diets (Penaflorida, 1989). Balazs and Ross (1976) have reported the differential feed intake by the freshwater prawn due to the presence of shrimp meal which may act as a feeding stimulant and the results showed that prawns fed with shrimp head meal augmented diet grew significantly larger. Final mean biomass and food utilization efficiency in the experimental group were about twice that of the control (Sick *et al.*, 1972; Balazs *et al.*, 1973; Forster and Beard, 1973; Sandifer and Joseph, 1976). Chitin in shrimp meal is believed to have a growth promoting effect and a minimum level of 0.5% chitin in shrimp feeds was recommended (Clarke *et al.*, 1993). Dietary glucosamine, was found to be a growth promoting factor in shrimp (Kitabayashi *et al.*, 1971; Kanazawa *et al.*, 1985). This growth effect indicates the shrimp's ability to absorb and utilize dietary sources of glucosamine but equal amounts of chitin do not produce the same growth promoting response (Kitabayashi *et al.*, 1971). Kono

et al., (1987) studied the effect of feeding chitin, chitosan and cellulose supplemented diets to fishes like red sea bream, Japanese eel and yellow tail. Chitinases, chitosanase and cellulase activity in the stomach of these fishes which were fed with these separate specific diets were noted. The levels of chitinase activity in the stomach of these fishes were in proportion to the rate of growth when fed with chitin supplemented diet. The growth rate of all these fishes fed with 10% chitin recorded highest value indicating diet superiority in terms of growth and feed efficiency (Kono *et al.* 1987). A weight gain in broiler chicken fed on chitin diet was significantly higher than that in the birds fed on commercial diet only. There was an increase of 5% feed intake by these birds showing better appetite (Nair *et al.*, 1987). It is commonly observed that shrimp consume cast exuviae following ecdysis. Several studies have reported measurable levels of chitin degrading enzymes in the alimentary tract of various crustacea (Chandramohan and Thomas, 1984; Lynn, 1990; Spindler-Barth *et al.*, 1990). These observations suggest that shrimps may be able to utilize dietary chitin. Apparent chitin digestibility was investigated in three species of penaeid shrimps, *Penaeus vannamei, P.setiferus* and *P.dourarum* and it was found that chitin was digested and the extent of digestion varies among species (Clarke *et al.*, 1993). The effect of dietary chitin on the growth, survival and chitinase levels in the digestive gland of juvenile *Penaeus monodon* was well studied (Fox, 1993; Lan and Pan, 1993; Das *et al.*, 1995; Sudaryono *et al.*, 1996).

crustaceans in particular *P.japonicus* (Kanazawa *et al.*, 1977a, b; Kanazawa *et al.*, 1978, 1979; Guary *et al.*, 1976), *Penaeus duorarum* (Sick and Andrews, 1973) and *Macrobrachium rosenbergii* (Sandifer and Joseph, 1976). Feedstuffs such as shrimp meal, fish meal and fish oil, etc., are commonly used in feeds for shrimps to meet this end as they are very rich in polyunsaturated fatty acids (Bautista and Subosa, 1997). Several workers have studied the inclusion of lipids in diet and emphasized their role in growth and survival of prawns. Increased survival and growth were observed when prawns were fed n-3 highly unsaturated fatty acid (HUFA)-enriched diets (Kanazawa *et al.*, 1978, 1979; Martin, 1980; Read, 1981; Sorgeloos and Leger, 1992; Rees *et al.*, 1994; D'Abramo and Sheen, 1993). High levels of dietary HUFA have increased survival, growth and resistance to salinity stress in penaeid post larval stages (Sorgeloos and Leger, 1992).

Bioconversion of chitin to single cell protein as animal feed is an interesting alternative of shellfish utilization (Carroad and Tom, 1978, Cosio *et al.*, 1982). Carroad and Tom (1978) studied the bioconversion of chitinous waste to single cell protein as animal feed. Chitin is degraded by chitinases produced by *Serratia marcescens* QMB1466.

The use of yeast and bacterial single cell protein as feed ingredient has gained importance with the understanding of its nutrient value. Bacterial cell walls contain peptidoglycan rich in N-acetylglucosamine and N-acetylmuramic acid (White *et al.*, 1979). The essential amino acid index of almost all bacteria

was noted to be in the range 91 to 94. Being above the value 90, it is of good quality for use as aquaculture feed ingredient (Penaflorida, 1989). Bacteria like *Pseudomonas* and *Methylophilus* spp. have been investigated for use as single cell protein in aquafeeds. They have approximately 73% crude protein and 5.7% lipid and 2.7% NFE by weight (Kant, 1996). Brown *et al*, (1996) have analysed the composition of a few strains of marine bacteria including *Methylophilus methylophilus*, *Aeromonas* sp., *Derxia* sp., *Pseudomonas* sp., to assess their nutritional value for bivalve aquaculture. Protein was the major constituent of the bacteria (25-49% dry weight), with lipid a minor component (2.5-9.0%). Carbohydrate in bacteria ranged from 2.5-11%. Ash occurred in high levels 29-40%. Glucose was the major polysaccharide in bacteria (12-73% of total sugars). Nucleic acids ranged from 3.3 to 8.4% dry weight. Polyunsaturated fatty acids 20:5n-3 and 22:6n-3 were absent in the bacteria. High levels of good quality protein however indicate their potential to provide important nutrients in a mixed diet.

The process of fermentation is therefore a feasible alternative that envisages the bioconversion of shell waste into a partially hydrolysed product with enriched microbial biomass. In the present study an attempt was made to evaluate the efficacy of fermented prawn shell waste by various bacterial strains as potential feed ingredient for Indian white prawn, *P. indicus*.

4.2 Materials and methods

4.2.1 Selection of strains

Based on the nutritional quality studies (chapter 3), 20 fermented products were selected and the corresponding bacterial strains were set apart for further study (Table 4.1).

4.2.2 Preparation of prawn shell powder

Prawn shell waste collected from peeling sheds in Cochin was oven dried at 80°C overnight. The dried shells were then powdered, sieved through 1 mm sieve and used for the study. 100 gm each of this shell powder was transferred to 1000 ml conical flasks, plugged with cotton and sterilized in an autoclave at 121°C for 15 minutes.

4.2.3 Inoculation preparation

The selected 20 bacterial strains were streaked onto nutrient agar slants, harvested with sterile physiological saline (0.9% NaCl solution) after 16-24 hrs. The culture harvest was mixed with a vortex mixer and the optical density of the cell suspension solution was adjusted to 0.5 at 600 nm in a Hitachi 200-20 UV-Visible spectrophotometer.

4.2.4 Fermentation.

5 ml each of the inoculum was added to 100 gm of the shell substrate in conical flasks and subjected to solid state and submerged state fermentation by adding sterile 50% seawater based on the preference of the strains. Moisture

levels of SSF and SmF were 1:1.5 and 1: 4 (w/v shell : 50% water). Fermentation was carried out at 28±2°C for a period of 14 days with regular mixing of the substrate with a sterile glass rod once per day. After 14 days the fermentation product was dried at 60°C for 24 hrs and used as an ingredient for feed preparation.

4.2.5 Proximate composition of the fermented products

Biochemical composition of the fermentation products was analysed to assess their nutritional quality. Protein was estimated by Lowry's method (Lowry, 1951), lipid by phosphovanillin method following chloroform-methanol extraction of the sample (Folch *et al.*, 1957) and total sugars by Anthrone method (Roe, 1955), N-acetylglucosamine was estimated as per Reissig *et al.*, (1955) and free amino acids according to Yemm and Cocking (1955).

4.2.6 Experimental feed preparation

Powdered ingredients as given in Table 4.2 were mixed well into a dough with 100 ml water. This was steamed for 10 min in an autoclave and pelletized using a laboratory model hand pelletizer having a 1mm die. Pellets were dried in an oven at 50°C for 18 hrs. The pellets were broken into pieces of 4-5 mm size. 20 different feeds were prepared incorporating the 20 fermented products plus the control diet with raw prawn shell powder. Water stability of feed was checked by immersing pellets in seawater for 18 hrs and examining

stability by visual observation. Feeds were stored in air tight polythene bags at -20^{0}C in a freezer.

4.2.7 Proximate composition of the experimental diets.

Protein content of the experimental diets was determined by microkjeldhal method (Barnes, 1959) and lipid by chloroform- methanol extraction (Folch *et al.*, 1957). Ash was determined by incineration at 550°C in a muffle furnace for 5 hrs and moisture content by drying in an oven at 80°C to constant weight. Fibre content was determined by acid and alkali treatment following AOAC (1990). The nitrogen free extract (NFE) was computed by difference (Crompton and Harris, 1969).

4.2.8 Feeding experiment

Experimental animals

Post larvae (PL-21) of Indian white prawn, (*Penaeus indicus* H.Milne Edwards) of the size range 30-50 mg were brought to the laboratory from a commercial prawn hatchery in Kannamali, Kochi.

Rearing facility

Fibre reinforced rectangular plastic (FRP) tanks of 30 L capacity were used for the study (Fig 4.1). Water quality was monitored daily and was maintained as per Table 4.3. On alternate days after removing the faeces and unconsumed feed, 50% of water was exchanged from all the experimental tanks. Aeration was provided from a 1h.p. compressor through air stones. Physiochemical parameters like salinity, nitrogen and dissolved oxygen of the

rearing water were estimated daily by following standard procedures (APHA, 1995).

Design of experiment

The post larvae of *P. indicus* were maintained on prepared control diet for a period of one week. The larvae were then stocked into 30 L rectangular fibreglass tanks containing 20 L seawater with 25 individuals per tank and reared on the experimental diets for 21 days. Feeding trials were conducted using triplicate tanks for each treatment.

Feeding schedule

Twenty-one different feeds were given to the prawns including one control diet. Prawns were fed twice daily at 10 a.m and 5 p.m. at the rate of 10-15% the body weight per day. Pre weighed experimental diets were placed in petri dishes in the tank. Faecal matter was removed by siphoning twice daily. Uneaten feed was also collected twice daily by siphoning and was washed gently with distilled water to remove salt and filtered through a pre weighed filter paper and dried to constant weight in an electric oven at 80^0C for 24 hrs.

Measurements

The initial wet body weight of all the prawns in each rearing tank was recorded. They were weighed on a precision balance after they were blotted free of water by tissue paper. The mean weight of all the prawns in a tank was calculated (mean $\pm$ 0.01mg). After 21 days, final weights of all the prawns were measured and mean weight was found. Parameters including individual

4.2.9 Challenge experiment

After termination of the feeding experiment (21 days) all treatment groups including the control, were maintained under the same rearing conditions. A challenge with white spot virus (WSSV) was performed through oral administration. For this, prawns were fed with white spot virus infected prawn flesh (*P.indicus* adult) in the morning (after a starvation period of 12 h) and evening *ad libitum* for one day ensuring availability of infected meat to all the prawns in the tank and then maintained on the corresponding experimental diets for the following days. All the rearing conditions were also maintained as earlier.

Survival rate was recorded every day for a period of seven days by which time almost complete mortality was recorded in some treatment groups (F29, F52, F123, F124, etc.). Mortality by WSSV infection was confirmed by checking the characteristic cuticular white spots on the carapace and other shell parts of the infected animal.

4.3 Results

4.3.1 Proximate composition of the fermented products

The proximate composition of the fermented products are given in Table 4.4. Protein was maximum in FP124 (47.2%) followed by FP29 (46.2%) and FP84 (45.9%). Lipid content varied from 3.2 to 10.7%. Lipid was highest in FP111 and FP134 (10.7% and 10.6% respectively). In the case of N-acetyl glucosamine, the monomeric form of chitin except for FP15 (0.045%) and

was recorded for feed F134 (53.02 mg) followed by F124 (33.81 mg). Food conversion ratio (FCR) showed significant variation ranging from 1.7 to 6.47 (Fig. 4.4), the best FCR obtained being with *P. indicus* fed F134 (1.7) followed by F124 (2.21). SGR was maximum with feed F153 (12.9%) followed by F134 (9.9%) (Fig. 4.5). Similar to FCR, GGE was also found highest for feed F134 (58.9%) followed by F124 (45.16%) (Fig. 4.6).

Relative growth rate (RGR) was lowest (0.035) for control feed and highest for F153 (0.08) followed by F134 (0.071) (Fig. 4.7). Consumption per unit weight per day (CUD) ranged from 0.11g to 0.23g. It was found to be lowest for the control feed (Fig. 4.8). Protein efficiency ratio (PER) varied from 1.4 to 5.2 for the various feeds (Fig. 4.9). The highest PER for feed F134 (5.2) was found to be significantly different from the next highest value of 3.6 by feed F153.

The percentage survival of the post larvae after 21 days of feeding on experimental diets is depicted in Fig. 4.10. The shrimp fed with control feed showed 80% survival. The percentage survival for treatment diets varied from 41% to 100%. The survival was best with feed F218 (100%) followed by F157, F15, F33, F48 and F52, all showing above 90% survival. The percentage survival of the post larvae fed on experimental diets after challenge with white spot virus (WSSV) is given in Fig. 4.11. Post challenge survival after 7 days

was best with F111 followed by F 48. Very low survival was recorded for shrimps fed with diet F163 (i.e. below 10%).

Duncan's multiple range test showed that the performance of the feeds in terms of bio-growth parameters differed significantly and it is presented in the supporting data for the fig. 4.3 to 4.9. Feeds F134 and F124 gave the best performance. (Table 4.7)

The correlation between the bio-growth parameters and proximate composition of the various fermented products incorporated in the feeds is given in Table 4.6. Correlation of bio-growth parameters with that of the proximate composition of the fermented products did not show a significant positive correlation with any of the factors except for the free fatty acids, which showed a positive correlation ($p<0.1$) with SGR and RGR. Survival percentage before and after challenge with white spot virus had a positive correlation ($p<0.1$) with the lipid content in the fermented products.

4.4 Discussion

Considerable increase in nutrient content was noted in terms of protein, lipid and total sugars in most of the fermented products. Proliferation of bacteria in the chitinous substrate might have contributed to the enrichment of protein due to biomass buildup. The chitinoclastic and proteolytic strains performed better in terms of product enrichment as evidenced by the biochemical analysis of the fermented product. Except for F29 (46.2%) which

is a product by a chitinoclastic non proteolytic strain, almost all other high protein enrichment values have been observed for products by chitinoclastic cum proteolytic forms like FP124 (47.2%), FP48 (45.9%), FP15 (44.9%) and FP163 (44.8%). Protein enrichment has been already reported for agricultural residues through fermentation for use as feedstock (Tengerdy *et al.*, 1983; Lynch, 1985; Hatakka and Pirhonene, 1985; Beg *et al.*, 1986; Milstein *et al.*, 1986; Viestrus *et al.*, 1987; Yadav, 1987; Kahlon and Das, 1987). Hydrolysis of chitin in prawn shell and to a certain extent the protein hydrolysis would have enhanced the nutritional quality in addition to improvement in texture and odour of the product. Microbial protein is believed to contribute significantly to the protein content of the fermentation product. A similar enrichment of protein and lipid was noted in fermented sesame seed meal by Mukhopadhyay and Ray (1999).

Increase in total sugars can be attributed to the hydrolysis of the complex substrate (chitin) leading to increased levels of soluble oligosaccharides thereby enhancing digestibility. Very low value of NAG obtained by fermentation may indicate the rapid uptake of monomeric NAG for bacterial metabolism as soon as it is produced by the bacterial hydrolysis of the substrate. Free amino acid enrichment in the fermented product helps in assimilability of the product. Increase in free amino acids (10 fold) and vitamin content of rye fodder by fermentation has been reported by Penoloza *et al.* (1985), Czajkowska and Olejniczak (1989) and Klappach *et al.* (1991). In this

study only very few products showed an increase in FAA, may be due to the utilization of FAA by the microorganisms themselves for their metabolism.

The protein content of the feeds ranged from 41.6% (F52) to 57.4% (F124). This range was found acceptable for optimum growth in penaeid prawns as shown by various earlier feeding experiments. Dietary protein has been reported as the most essential nutrient for the growth of prawns (Andrews *et al.*, 1972; Balazs *et al.*, 1973; Forster and Beard, 1973; Venkataramiah *et al.*, 1975; Alava and Lim, 1983). Penaeid shrimps require 35 to 40% protein, 8-10% fat rich in PUFA and 35% carbohydrate in their diet. Vitamins, minerals, fish oil, highly unsaturated fatty acids, phospholipids and cholesterol are essential additives to the basic diet (Ali, 1989) for optimal growth in shrimp. The protein quality of a feed ingredient depends on several variables, digestibility and content of essential amino acids, which are also crucial to the biological value of the protein.

The protein requirement of juvenile *Penaeus monodon* has been repeatedly studied using different sizes of animals and types of protein (Lee, 1971; Lin *et al.*, 1982; Alava and Lim, 1983; Bautista, 1986; Millamena *et al.*, 1986; Shiau *et al.*, 1991; Shiau and Chou, 1991). In general, the optimal level of protein is about 40% in the diet on the basis of weight gain and feed conversion. Juvenile or adult penaeids have been shown to attain optimum growth on diets containing 22-60% protein (Hanson and Goodwin, 1977). The presence of protein rich in lysine and methionine (Miranda and Horowitz,

1978) together with n-3 fatty acids (Kanazawa *et al.*, 1979; Menesveta *et al.*, 1983; Sandifer and Joseph, 1976; Sick and Andrews, 1973) in prawn shell waste was found to enhance growth in shrimps. The quality of shrimp meal protein in terms of amino acid content was found to be close to that of prawn muscle (Lim *et al.*, 1979; Deshimaru *et al.*, 1985; Penaflorida, 1989).

In the present study, a part of the protein in the fermented product maybe contributed by microbial protein. As the strains selected were highly proteolytic in nature, luxuriant bacterial growth was observed during the fermentation of the substrate. The nutritional value of the microorganisms used in aquaculture depend on their digestibility and assimilation characteristics and the target animal. Very few reports are available on the effects of feeding bacteria directly to prawns.

Moriarity (1977) has reported that penaeid prawns being omnivorous also eat some microorganisms including bacteria. Bacteria were found to constitute less than 2% of the organic matter in the gut content in the adults of all species tested, but in many juveniles of *Penaeus merguensis*, bacteria were more important constituting up to 14% of organic matter (Moriarity and Barclay, 1980). Yeast was examined as a replacement for fishmeal in rainbow trout diets by Dabrowski *et al.* (1980). Yeasts and bacteria have been evaluated as food for bivalve aquaculture. Protein is the major constituent of both yeast and bacteria (25 to 49% dry weight). In this study, bacteria belonging to the genera *Bacillus, Vibrio*, *Pseudomonas*, *Serratia* and Coryneforms were used

for the fermentation of prawn shell waste. Product generated with *Bacillus* sp. gave the best performance in terms of various growth parameters.

In the present study, lipid was found to be highest (13.9%) in (F111) followed by F134. Lipid enhancement could be by bacterial production. Yongmanitchai and Ward (1989) have reported marine bacteria that produced EPA. Microorganisms contain a diverse range of fatty acid composition and are rich sources of useful unsaturated fatty acids like PUFA (Brown *et al.*, 1996). Other studies have shown that some bacteria spp. contains 20:5n-3 (Yazawa *et al.* 1988). A positive correlation eventhough not significant could be obtained between the percentage survival and the lipid concentration in the various diet. A detailed analysis of the fatty acid profile might reveal the presence and quantum of specific fatty acid components responsible for this enhanced survival. Recommended lipid levels for commercial shrimp feeds range from 6% to 7.5% and a maximum level of 10% was suggested by Akiyama and Dominy (1989). Among the lipid compounds in the diets of shrimps, polyunsaturated fatty acids, phospholipids and sterols have received the most attention in crustacean lipid nutrition. Sheen and Chen (1993) found that growth of *P. monodon* fed iso-nitrogenous diets supplemented with 8, 10 and 12 % lipid was significantly higher than those with lower lipid content. Fatty acids are reported to promote growth in penaeids (Lee *et al.*, 1967; Castell *et al.*, 1972; Shewbart and Mies, 1973; Sick and Andrews, 1973; Watanabe *et al.*, 1974; Guary *et al.*, 1976). Millamena *et al.* (1988) noted greater survival and

growth in *Penaeus monodon* larvae that were fed lipid enriched *Artemia* nauplii. Similarly enhanced larval survival and development on diets rich in EPA and DHA are reported by Levin and Sulkin (1984) for a brachyuran crab (*Euypanopeus depressus*) until the megalopa stage and in other zoeal stages (Citarasu *et al.* 1998). Prawn fed shrimp head oil augmented diet grew significantly larger (Sandifer and Joseph, 1976). Similarly final mean biomass and efficiency of food utilization in the experimental groups were about twice than that in the control (Sick *et al.*, 1972; Balazs *et al.*, 1973; Forster and Beard, 1973).

Various studies with *Penaeus japonicus* have demonstrated that dietary phospholipids enhance growth and survival of larvae (Teshima *et al.*, 1982; Kanazawa *et al.*, 1985; Camara *et al.*, 1997) and growth and stress resistance in postlarval/juvenile stages (Sandifer and Joseph, 1976; Levin and Sulkin, 1984; Kanazawa *et al.*, 1979a, b; Teshima *et al.*, 1986 a,b; Camara *et al.*, 1997; Kontara *et al.*, 1997). Watanabe *et al.* (1994, 1995) have reported that yellowtail fed diets with alternative protein sources replacing fishmeal had lower levels of plasma lipid components with increased susceptibility to infectious disease. This correlation between plasma lipid level to resistance and immunity has been further shown by Maita *et al.* (1998).

Deshimaru and Yone (1978) and Abdel-Rehman *et al.* (1979) have shown that the type of the dietary carbohydrate in purified diets adversely or

positively affects growth and survival of *P. japonicus*. The ash content is found to be higher than that in the control feed. This can be due to the change in the overall composition of the fermented product from that of the raw prawn shell. More work has to be done in this direction for the elucidation of this increase.

The overall results showed superior growth performance by feeds F134 and F124. The performance of feed F111 supporting good survival during post challenge with WSSV was noteworthy. Strain C163 (*Serratia* sp.) used in this study is a red pigmented bacterium. It would thus be a good source of carotenoids for prawns for better growth, survival and colouration. The importance of carotenoids in survival and growth have been emphasized by a number of workers. Sandifer and Joseph (1976) reported that shrimp heads (*Penaeus setiferus*) were a good source of fatty acids and pigments for use in prepared feeds for *Macrobrachium rosenbergii.* The use of shrimp head oil in crustacean feed led to significantly greater pigmentation (Joseph and Williams, 1978). Since crustaceans are incapable of synthesizing carotenoids, these pigments have to be supplied as dietary ingredients (Estermann, 1994). The pigment can be stored directly and utilized more efficiently in the body (Petit *et al.*, 1991; Negree-Sadarguez *et al.*, 1993). Apart from good larval pigmentation, higher larval survival was obtained for *Penaeus indicus* when fed on lipid enriched pigmented nematodes (Kumlu *et al.*, 1998).

The most commonly used carotenoids in commercial shrimp growout diets are canthaxanthin and astaxanthin (Bird and Savage, 1990; Johnson and

Ann, 1991), but studies with crustaceans have shown that astaxanthin is the most effective pigment (Yamada *et al.*, 1990) found richly in prawn shell waste. Recent studies on the pigmentation of *Penaeus japonicus* showed that dietary carotenoids may improve survival and growth (Chein and Jeng, 1992; Negree-Sadarguez*et al.*, 1993). Similar results were obtained by Yamada *et al.*, (1990) who demonstrated a significantly better survival of shrimps fed on an astaxanthin-supplemented diet. Negree-Sadarguez *et al.*, (1993) also reported a positive correlation between survival rate and pigment concentration in prawn tissue and suggested that pigments could play an important role in improving the survival of penaeid prawns. Yamada *et al.*, (1990) found no significant effect of various pigments on weight gain in *P. japonicus* juveniles. Similar conclusions were also made by Chein and Jeng (1992) and Negree-Sadarguez *et al.*, (1993) who showed the effect of different pigment sources and their various levels on the pigmentation, survival and growth of *Penaeus indicus*. The amount of carotenoids in the fermented product could have had an important role in the survival of prawns in this experiment.

Growth performance coupled with high post challenge survival could not be obtained for any of the strains. Feeds which showed best growth performance (F134 and F124) exhibited an average post challenge survival. F111 on the other hand exhibited an average performance for all other bio-growth parameters while exhibiting a significant post challenge survival. Growth performance of some of the experimental feeds was superior when

compared to earlier studies with raw prawn shell waste feed (Balazs and Ross, 1976; Menesveta *et al.*, 1983). An FCR of 2.8 was recorded for shrimp meal diet by Jayalakshmy and Natarajan (1994). In the present study, better FCR could be obtained with F134 (1.7) and F124 (2.21). According to Forster (1970) only an FCR of 2-3 could be anticipated in prawns due to loss incurred during moulting, though the order of acceptance of the feeds varies in different species (Goswami and Goswami, 1982).

In conclusion a positive correlation could not be obtained between protein, NAG, free amino acids, etc with that of the growth parameters. A detailed analysis of amino acid profile as well as other factors in the fermented product may give a better picture of the fermented product as a potential shrimp feed supplement with enhanced properties. Correlation of bio-growth parameters with that of the proximate composition of the fermented product did not show a significant positive correlation with any of the factors except for free fatty acids, which showed a positive correlation ($p<0.1$) with SGR and RGR. The survival percentage after challenge with WSSV had significant correlation with the lipid content. This observation indicate the importance of lipids as a component of the diet and necessitate a detailed analysis of the lipid profile of various fermented products to find out the specific fraction responsible for boosting growth as well as immune system leading to better survival.

Table 4.1 List of bacterial strains used for the generation of shell waste fermented products for testing its efficacy as a dietary ingredient for *Penaeus indicus*

Culture No.	Genera
C14	Coryneforms
C15	*Bacillus*
C18	Coryneforms
C29	*Vibrio*
C33	*Pseudomonas*
C48	Coryneforms
C52	*Vibrio*
C84	*Pseudomonas*
C111	*Bacillus*
C123	Coryneforms
C124	*Bacillus*
C134	*Bacillus*
C149	*Serratia*
C153	Coryneforms
C154	*Bacillus*
C157	*Vibrio*
C163	*Serratia*
C218	*Vibrio*
C219	*Vibrio*
C220	*Bacillus*

Table 4.2 Composition of experimental diets

Ingredients	Control diet (F1) (gm/100gm)	Experimental diet (gm/100 gm)
Prawn shell powder	25	-
Fermented prawn shell[a]	-	25
Fish meal	28	28
Groundnut oil cake[b]	8	8
Soyabean meal[c]	15	15
Maida[d]	10	10
Rice bran[e]	10	10
Vitamin and mineral mix[f]	2	2
Agar	2	2
Water	100 ml	100 ml

[a] 20 Different fermented products were prepared which were incorporated in the 20 experimental diets prepared.

[b] Prepared by grinding the cake remaining after oil extraction from ground nuts

[c] Prepared by grinding the flakes remaining after oil extraction from soyabean

[d] Refined wheat flour

[e] Finely ground Rice bran

[f] Vitamin and mineral mix (mg/g vitamin and mineral mix)

Thiamine	0.61 mg
Riboflavin	0.48mg
Panthothenic acid	2.42mg
Pyridoxine	0.72mg
Cyanocobalamine	0.02mg
Biotin	0.02mg
Retinol	0.13mg
Menaptone	0.24mg
Folic acid	0.13mg
Niacin	2.42mg
∝ tocopherol	2.42mg
Banox	0.30mg
Cholecalciferol	0.06mg
Ascorbic acid	6.05mg
K_2HPO_4	4.68mg
$Ca_8(PO)_4$	6.36mg
$MgSO_4.7H_2O$	7.12mg
$NaH_2PO4.2H_2O$	1.84mg

Table 4.3 Rearing conditions and Water quality parameters of the experimental system

Rearing and feeding conditions	
Initial body weight (average)	: 40.33 ± 10.2 mg
Number of prawn stocked	: 25 prawns per tank
Tank capacity	: 30L
Feeding level	: 10-15% body weight
Feeding frequency	: Twice daily
Feeding period	: 21 days
Water Quality Parameters	
Water temperature	: 28 - 30°C
pH	: 7 - 7.5
Salinity (ppt)	: 26 - 28 ppt
NH_3 (mg/l)	: 0.01 -0.02 mg/L
NO_3 (mg/l)	: n.d
NO_2 (mg/l)	: < 0.01 mg/L
Dissolved O_2 (mg/l)	: 7 - 8 mg/L

n.d. = not detectable

Table 4.4 Proximate composition of the various fermented products (% on dry weight basis)

Fermented Product	Protein	Lipid	NAG*	Total sugars	Free amino acids
Control	32.5	5.1	0.043	2.5	1.5
FP14	23.9	9.6	0.030	2.2	0.7
FP15	44.9	5.0	0.045	2.8	0.9
FP18	35.5	7.0	0.030	3.8	0.7
FP29	46.2	3.6	0.029	2.7	1.4
FP33	25.8	3.2	0.029	4.5	1.7
FP48	27.8	7.2	0.026	4.6	0.5
FP52	23.8	7.5	0.029	2.8	1.4
FP84	45.9	2.8	0.025	2.5	1.3
FP111	37.8	10.7	0.024	4.2	2.2
FP123	44.8	4.8	0.025	2.2	0.8
FP124	47.2	8.2	0.012	3.0	1.0
FP134	29.0	10.6	0.027	4.3	1.6
FP149	33.9	5.2	0.024	4.1	1.3
FP153	26.2	4.9	0.026	2.3	1.6
FP154	39.0	5.2	0.037	2.6	1.0
FP157	27.1	8.7	0.014	2.7	0.8
FP163	44.8	8.1	0.039	4.4	1.4
FP218	32.2	7.3	0.044	3.7	1.0
FP219	30.6	3.5	0.023	2.3	1.8
FP220	36.5	7.3	0.032	4.3	2.6

**NAG - N-acetyl glucosamine*

Table 4.5 Proximate composition of the experimental feeds (%)

Feeds	Protein (%)	Lipid (%)	Fibre (%)	Ash (%)	Moisture (%)	NFE* (%)
Control	47.47	8.3	4.18	10.15	3.50	26.4
F14	41.71	10.6	3.42	12.72	2.93	28.6
F15	55.7	5.6	3.78	12.14	4.88	17.9
F18	49.45	8.4	5.38	12.98	3.87	19.9
F29	56.55	5.1	4.76	12.90	3.70	17.0
F33	42.98	4.8	4.02	12.70	6.61	28.9
F48	44.28	9.4	4.25	12.70	5.83	23.5
F52	41.64	10.6	2.84	12.50	5.96	26.5
F84	56.37	4.1	4.09	10.60	3.04	21.8
F111	50.97	13.9	4.81	12.76	5.03	12.5
F123	42.31	6.7	4.19	12.13	5.38	29.3
F124	57.24	10.8	5.08	12.30	5.31	9.3
F134	45.1	13.6	4.48	12.80	5.52	18.5
F149	48.35	7.0	4.61	13.16	5.91	21.0
F153	43.22	6.9	4.08	13.34	4.46	28.0
F154	51.79	7.5	3.25	12.31	5.18	20.0
F157	43.85	11.9	4.81	13.10	4.30	22.0
F163	55.63	10.5	6.77	12.35	5.39	9.4
F218	47.22	9.1	4.05	12.35	2.98	24.3
F219	46.21	4.2	4.77	12.56	3.98	28.3
F220	44.04	10.1	2.99	12.62	4.12	26.1

**NFE - nitrogen free extract*

Table 4.6 Correlation between the biogrowth parameters and biochemical components of the various fermented products. (r value)

Bio-growth parameters	Proteins	Carbohydrates	Lipids	NAG	Total Sugars	Free fatty acids
PRODUCTION	0.01	-0.107	0.248	-0.298	0.153	0.122
FCR	0.1156	0.201	-0.091	0.204	0.005	-0.093
SGR	-0.375	0.074	0.109	-0.288	-0.027	0.364*
RGR	-0.285	0.155	0.233	-0.275	0.064	0.393*
GGE	0.069	-0.271	0.113	-0.243	0.080	0.08
C/UD	-0.274	0.417*	0.126	0.007	-0.007	0.208
SURVIVAL (Pre Challenge)	-0.300	0.012	0.421**	-0.072	0.265	-0.268
SURVIVAL (Post Challenge)	-0.183	0.026	0.385**	-0.237	0.188	-0.094

*P<0.1

Table 4.7 Relative position of various feeds with respect to their performance in terms of bio-growth parameters and percentage survival in *P.indicus* postlarvae maintained on experimental diets

Para-meter	PRO	FCR	SGR	GGE	RGR	PER	Survival 1	Survival 2
Experimental Feeds	**F134**	**F134**	F153	**F134**	F153	**F134**	F218	F111
	F124	**F124**	**F134**	**F124**	**F134**	F153	F157	F48
	F15	F157	F219	F157	F52	F157	F15	F157
	F163	F219	F220	F219	F220	F123	F48	**F134**
	F149	F84	F52	F84	F219	F220	**F124**	F15
	F153	F153	**F124**	F153	**F124**	**F124**	F33	F18
	F157	F218	*F163*	F218	*F163*	F218	F111	F153
	F111	F15	F149	*F163*	F149	F149	F123	F33
	F84	F149	F18	F15	F218	F219	**F134**	F14
	F123	F220	F218	F149	F14	F52	Control	F84
	F154	F123	F14	F220	F111	F14	F18	F219
	F33	Control	F111	F123	F157	Control	F14	F154
	F220	F33	F15	Control	F18	*F163*	F52	Control
	F18	F111	F157	F33	F123	F84	F29	F29
	F218	F14	F123	F111	F15	F33	F149	**F124**
	F48	F18	F33	F14	F33	F15	F153	F149
	F219	F48	F84	F18	F154	F48	*F163*	F218
	F14	F154	F48	F48	F84	F18	F220	F123
	Control	F52	F154	F154	F48	F111	F154	F52
	F29	F29	F29	F52	F29	F154	F84	F220
	F52	*F163*	Control	F29	Control	F29	F219	*F163*

Survival 1: - Survival after 21 days feeding experiment (pre challenge)
Survival 2: - 7 days post challenge survival with WSSV

Fig.4.1 Culture facility used for the screening of feeds incorporated with fermented products employing *Penaeus indicus* post larvae

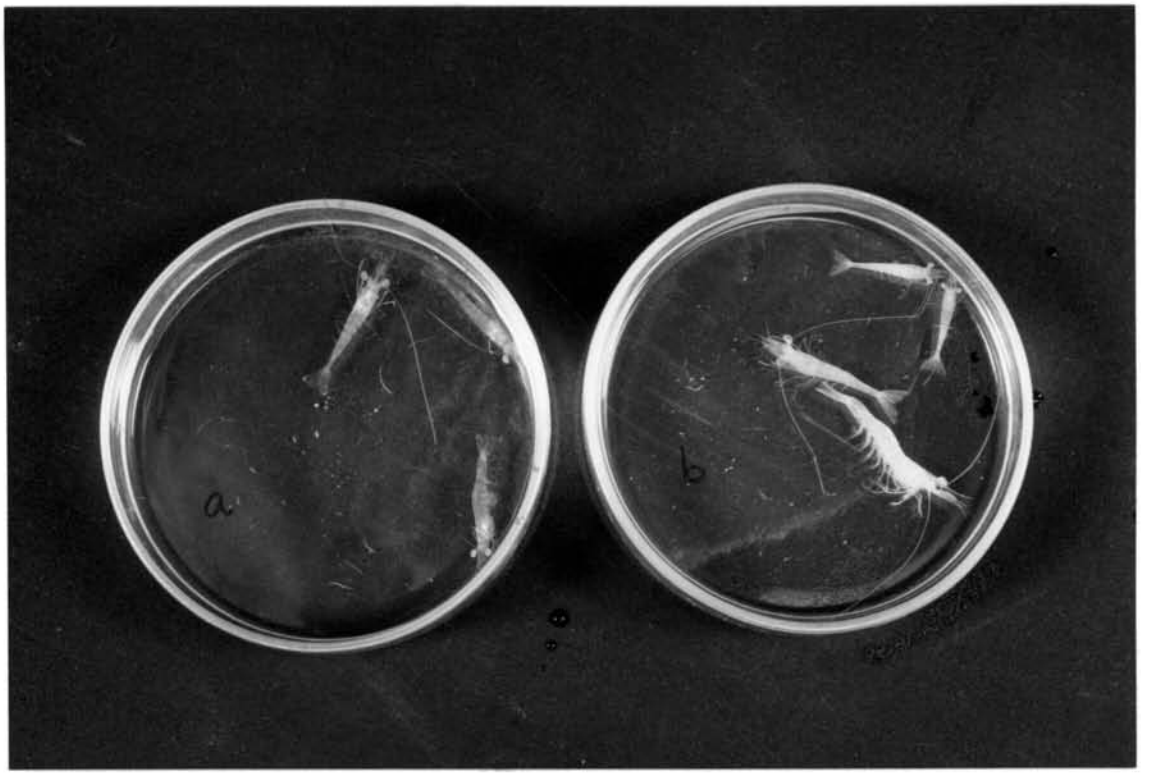
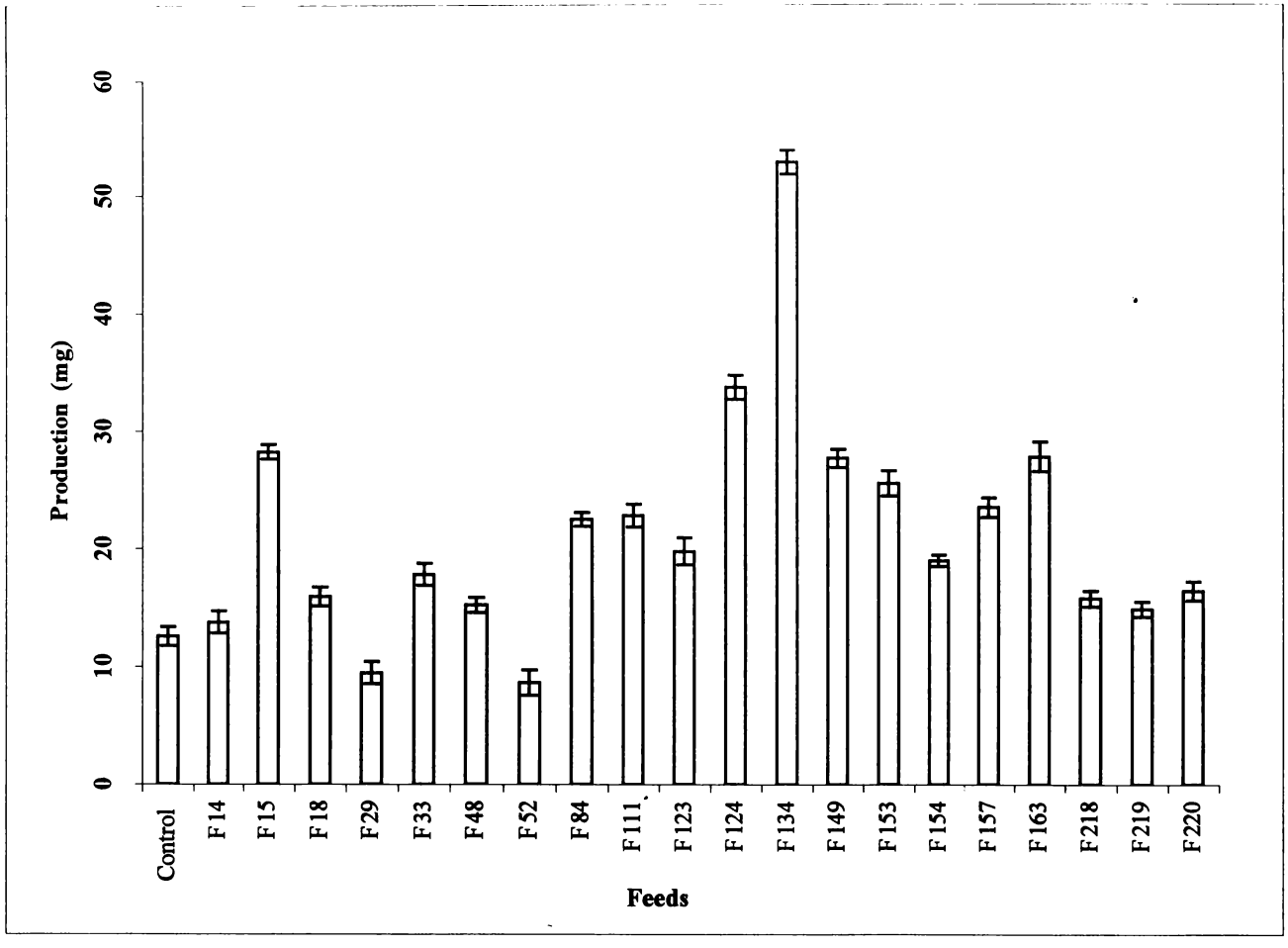


Fig.4.2 Post larvae of *Penaeus indicus* after challenge with white spot virus

a Uninfected prawns

b. Infected prawns

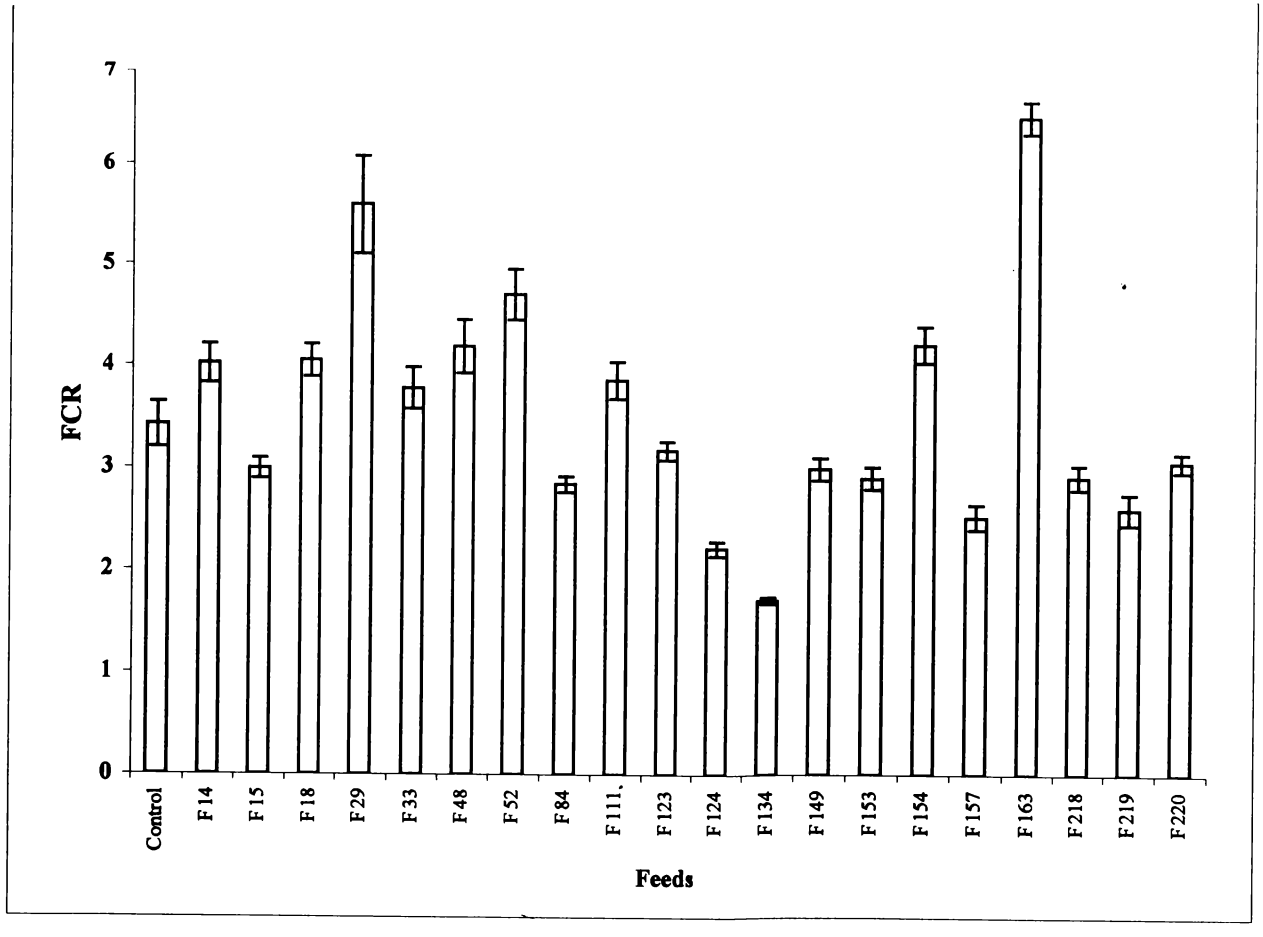


Diets	*Production (mg)
Control	12.5 ^{b} ± 0.8
F14	13.76 ^{b} ± 0.8
F15	28.32 ^{h} ±0.9
F18	15.96 ^{c} ± 0.6
F29	9.47 ^{a} ± 0.8
F33	17.83 ^{cd} ± 0.9
F48	15.24 ^{bc} ± 0.9
F52	8.64 ^{a} ± 0.7
F84	22.54 ^{ef} ± 0.7
F111	22.86 ^{fg} ± 1.1
F123	19.83 ^{de} ± 0.6
F124	33.81 ^{i} ± 1.0
F134	53.02 ^{j}± 1.2
F149	27.78 ^{h} ± 1.0
F153	25.66 ^{g} ± 1.0
F154	19.03 ^{de} ± 0.8
F157	23.6 ^{fg} ± 1.1
F163	27.93 ^{h} ± 0.5
F218	15.83 ^{c} ± 0.8
F219	14.93 ^{bc} ± 1.3
F220	16.46 ^{c} ± 0.7

*mean ± s.d

* Values with the same superscript does not vary significantly.

Fig. 4.3 Weight gain (Production) of *P. indicus* post larvae fed with various experimental diets.

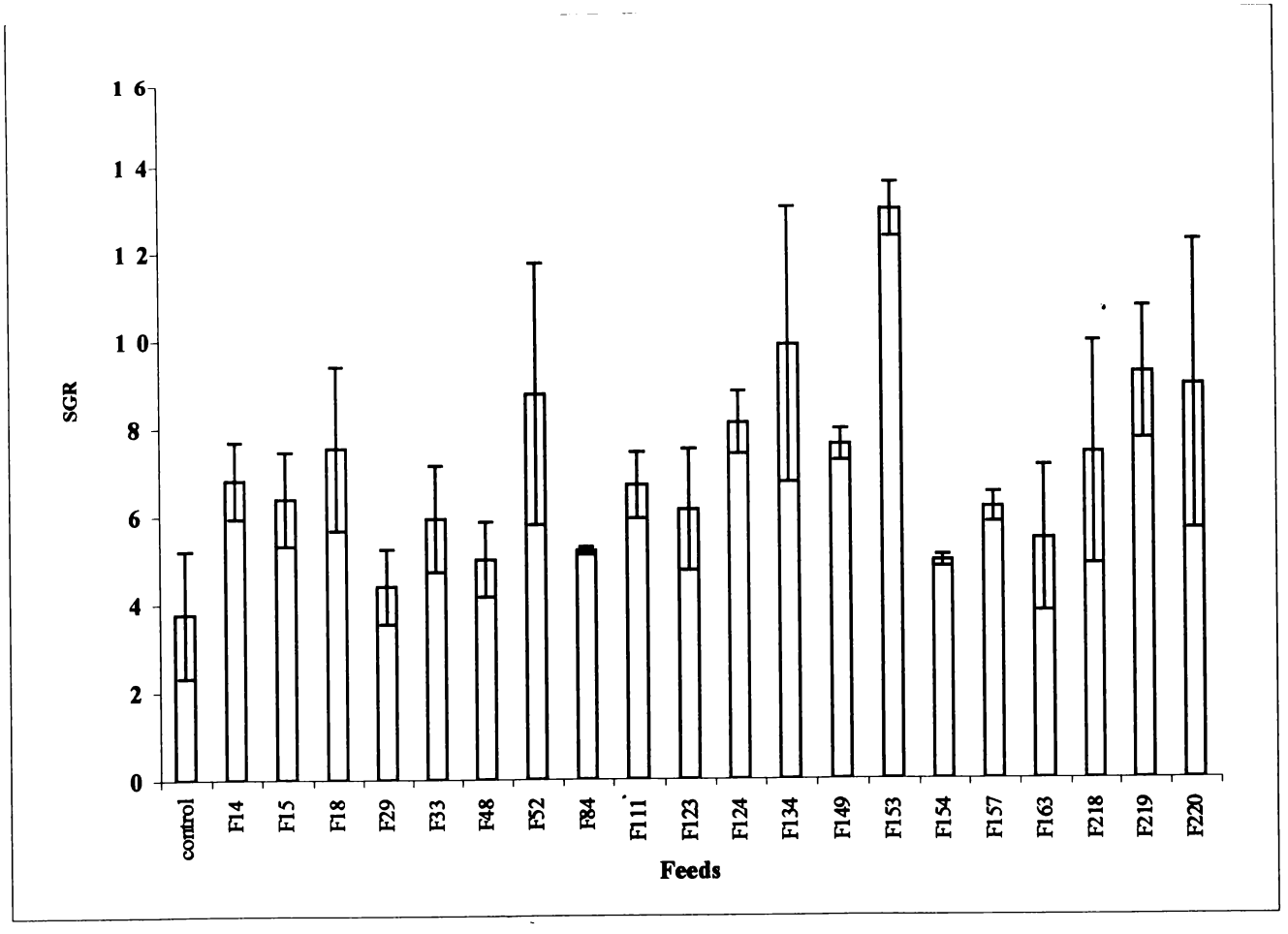


Diets	*FCR
Control	$3.42^{e} \pm 0.2$
F14	$4.02^{fg} \pm 0.2$
F15	$2.99^{d} \pm 0.0$
F18	$4.05^{g} \pm 0.2$
F29	$5.59^{i} \pm 0.5$
F33	$3.78^{fg} \pm 0.2$
F48	$4.19^{g} \pm 0.3$
F52	$4.70^{h} \pm 0.3$
F84	$2.84^{cd} \pm 0.1$
F111	$3.86^{fg} \pm 0.2$
F123	$3.17^{de} \pm 0.1$
F124	$2.21^{bc} \pm 0.01$
F134	$1.70^{a} \pm 0.03$
F149	$2.99^{d} \pm 0.1$
F153	$2.90^{d} \pm 0.1$
F154	$4.21^{g} \pm 0.2$
F157	$2.52^{bc} \pm 0.1$
F163	$6.47^{j} \pm 0.1$
F218	$2.91^{d} \pm 0.1$
F219	$2.60^{cd} \pm 0.2$
F220	$3.06^{d} \pm 0.01$

*mean $\pm$ s.d

* Values with the same superscript does not vary significantly.

Fig. 4.4 Food Conversion Ratio (FCR) of *P. indicus* post larvae fed on various experimental diets.

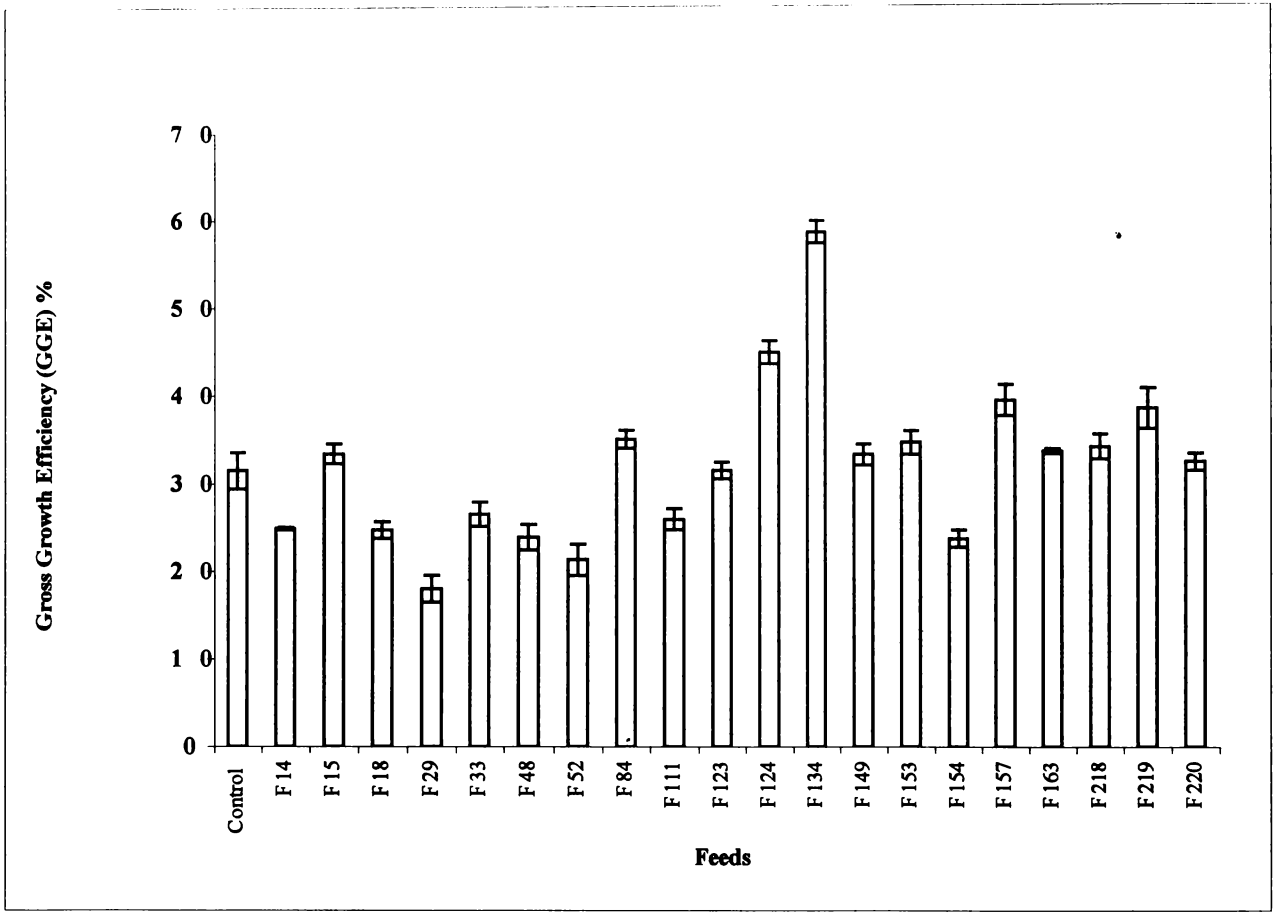


Diets	*SGR
Control	3.76 [a] ± 1.4
F14	6.80 [bc] ± 0.9
F15	6.37 [bc] ± 1.1
F18	7.53 [cd] ± 0.3
F29	4.39 [b] ± 0.9
F33	5.93 [bc] ± 1.2
F48	5.00 [bc] ± 0.9
F52	8.76 [d] ± 3.0
F84	5.20 [bc] ± 0.1
F111	6.68 [bc] ± 0.8
F123	6.13 [cd] ± 0.7
F124	8.10 [cd] ± 0.7
F134	9.88 [d] ± 3.1
F149	7.6 [cd] ± 0.4
F153	12.96 [d] ± 0.6
F154	4.95 [bc] ± 0.1
F157	6.17 [bc] ± 0.3
F163	7.66 [cd] ±1.7
F218	7.41 [bcd] ±2.5
F219	9.23 [d] ±1.5
F220	8.95 [d] ± 3.3

*mean ± s.d

* Values with the same superscript does not vary significantly.

Fig. 4.5 Specific Growth Rate (SGR) of *P. indicus* post larvae fed on various experimental diets.

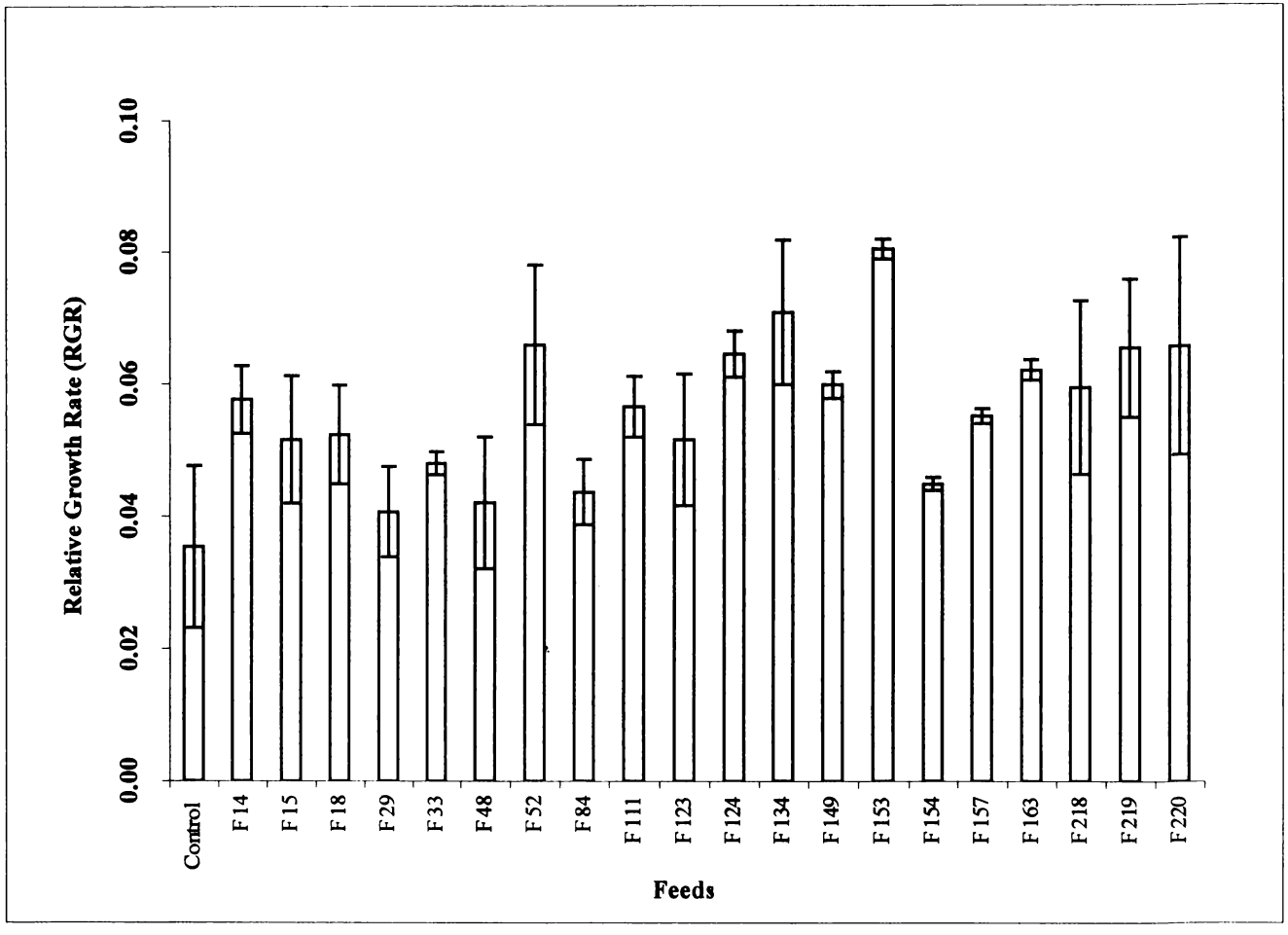


Diets	*GGE
Control	$31.14^{e} \pm 2.1$
F14	$24.87^{cd} \pm 1.2$
F15	$33.43^{ef} \pm 1.1$
F18	$24.72^{cd} \pm 0.9$
F29	$18^{a} \pm 1.5$
F33	$26.52^{d} \pm 1.4$
F48	$23.94^{c} \pm 1.5$
F52	$21.33^{b} \pm 1.8$
F84	$35.17^{f} \pm 1.0$
F111	$25.99^{cd} \pm 1.2$
F123	$31.59^{e} \pm 0.9$
F124	$45.16^{h} \pm 1.3$
F134	$58.95^{i} \pm 1.3$
F149	$33.84^{f} \pm 1.4$
F153	$34.84^{f} \pm 1.4$
F154	$23.78^{cd} \pm 0.9$
F157	$39.69^{g} \pm 0.9$
F163	$33.86^{ef} \pm 0.3$
F218	$34.38^{f} \pm 1.5$
F219	$38.8^{g} \pm 2.3$
F220	$32.65^{ef} \pm 1.0$

*mean ± s.d

* Values with the same superscript does not vary significantly.

Fig. 4.6 Gross Growth Efficiency (GGE) of *P. indicus* post larvae fed on various experimental diets.

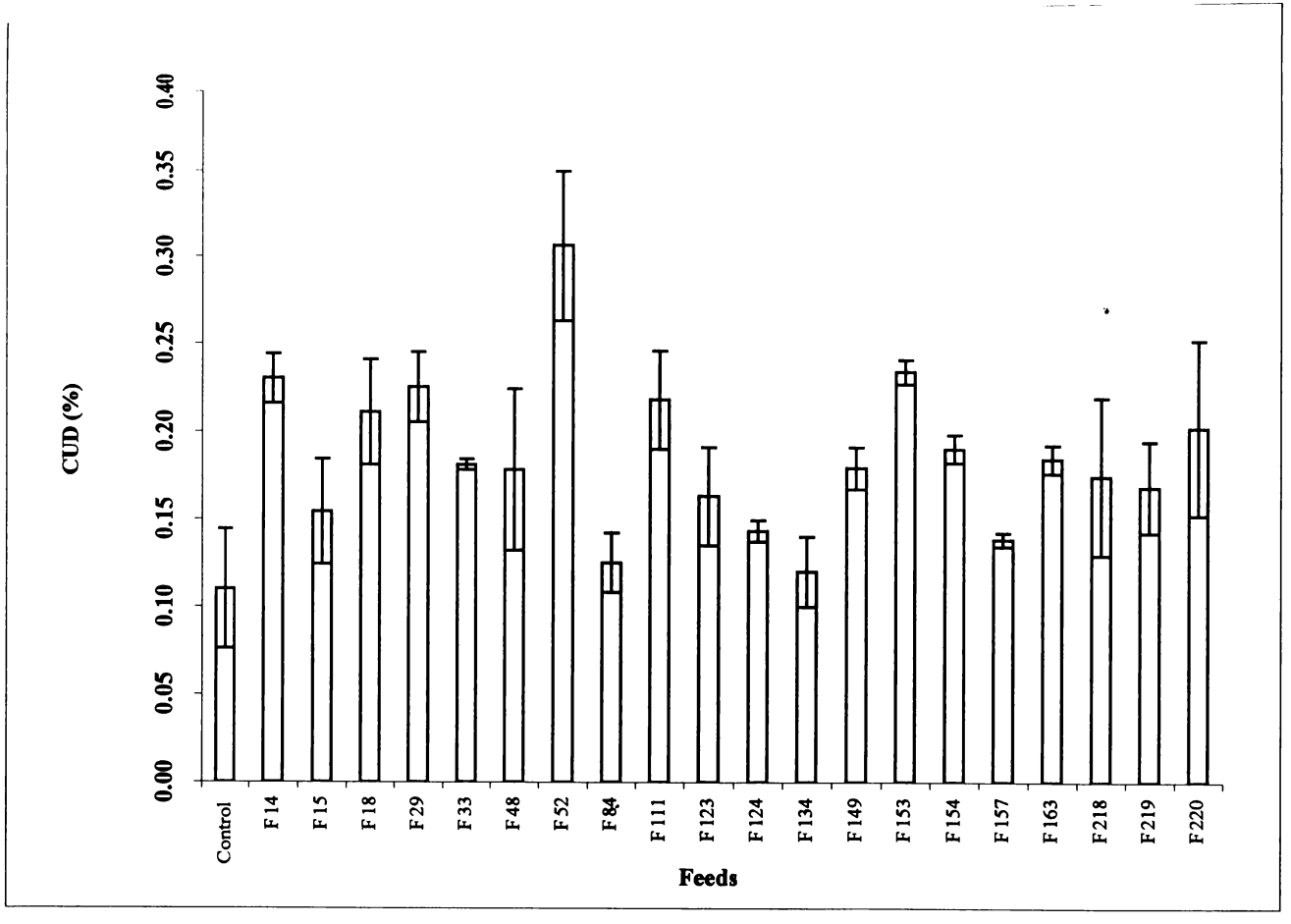


Diets	RGR
Control	0.035 [a] ± 0.012
F14	0.057 [bcde] ± 0.015
F15	0.51 [bcd] ± 0.009
F18	0.52 [bc] ± 0.007
F29	0.040[a] ± 0.007
F33	0.048 [ad] ± 0.002
F48	0.042 [a] ± 0.010
F52	0.066[bc] ± 0.012
F84	0.043 [ad] ± 0.005
F111	0.056 [bcde] ± 0.005
F123	0.051 [bcd] ± 0.01
F124	0.064 [be] ± 0.004
F134	0.071 [e] ± 0.011
F149	0.06 [bce] ± 0.002
F153	0.08 [f] ± 0.001
F154	0.045 [ad] ± 0.001
F157	0.055 [bcde] ± 0.001
F163	0.062bce ± 0.002
F218	0.059 [bcde] ± 0.013
F219	0.065 [be] ± 0.011
F220	0.066 [b] ± 0.0165

*mean ± s.d

* Values with the same superscript does not vary significantly.

Fig. 4.7 Relative Growth Rate (RGR) of *P. indicus* post larvae fed on various experimental diets.

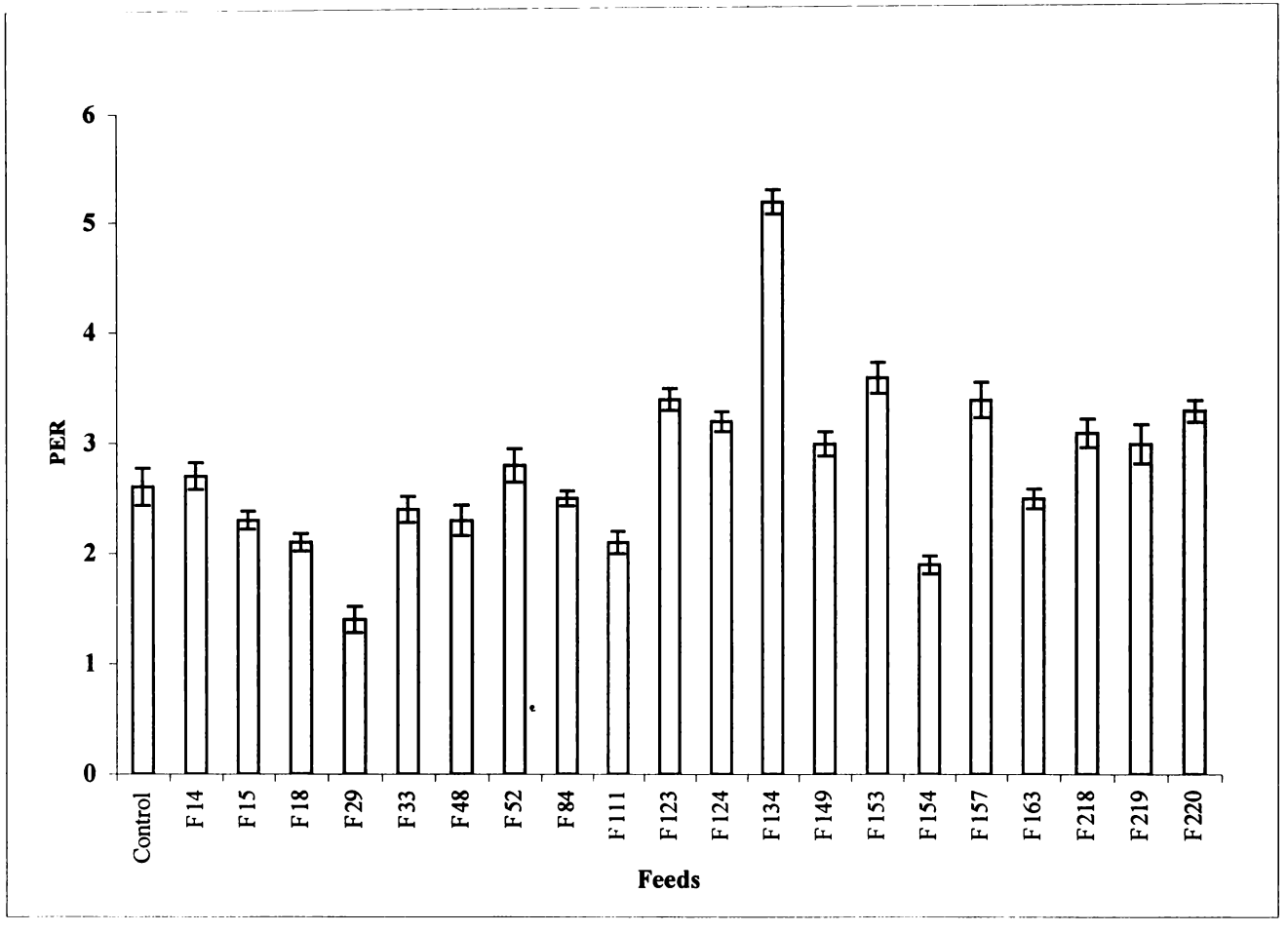


Diets	*CUD
Control	$0.11^{a} \pm 003$
F14	$0.23^{ef} \pm 0.01$
F15	$0.15^{bc} \pm 0.03$
F18	$0.21^{def} \pm 0.02$
F29	$0.23^{def} \pm 0.22$
F33	$0.18^{cd} \pm 0.05$
F48	$0.18^{cd} \pm 0.05$
F52	$0.31^{g} \pm 0.04$
F84	$0.13^{b} \pm 0.02$
F111	$0.22^{def} \pm 0.03$
F123	$0.16^{bc} \pm 0.03$
F124	$0.14^{b} \pm 0.006$
F134	$0.12^{b} \pm 0.02$
F149	$0.18^{cde} \pm 0.01$
F153	$0.23^{f} \pm 0.001$
F154	$0.19^{cdef} \pm 0.01$
F157	$0.14^{b} \pm 0.004$
F163	$0.18^{cdef} \pm 0.01$
F218	$0.17^{cd} \pm 0.05$
F219	$0.17^{cb} \pm 0.03$
F220	$0.2^{cdef} \pm 0.05$

*mean $\pm$ s.d

* Values with the same superscript does not vary significantly.

Fig. 4.8 Consumption per Unit weight per Day (CUD) of *P. indicus* post larvae fed on various experimental diets.



Diets	*PER
Control	$2.6^{ef} \pm 0.17$
F14	$2.7^{fg} \pm 0.12$
F15	$2.3^{cd} \pm 0.08$
F18	$2.1^{c} \pm 0.08$
F29	$1.4^{d} \pm 0.12$
F33	$2.4^{de} \pm 0.12$
F48	$2.3^{cd} \pm 0.14$
F52	$2.8^{gh} \pm 0.15$
F84	$2.5^{ef} \pm 0.07$
F111	$2.1^{c} \pm 0.10$
F123	$3.4^{kl} \pm 0.1$
F124	$3.2^{jkl} \pm 0.90$
F134	$5.2^{n} \pm 0.11$
F149	$3^{hi} \pm 0.11$
F153	$3.6^{m} \pm 0.14$
F154	$1.9^{b} \pm 0.08$
F157	$3.4^{l} \pm 0.16$
F163	$2.5^{def} \pm 0.09$
F218	$3.1^{ijk} \pm 0.13$
F219	$3^{hij} \pm 0.18$
F220	$3.3^{kl} \pm 0.1$

*mean $\pm$ s.d

* Values with the same superscript does not vary significantly.

Fig. 4.9 Protein Efficiency Ratio (PER) of *P. indicus* post larvae fed on various experimental diets.

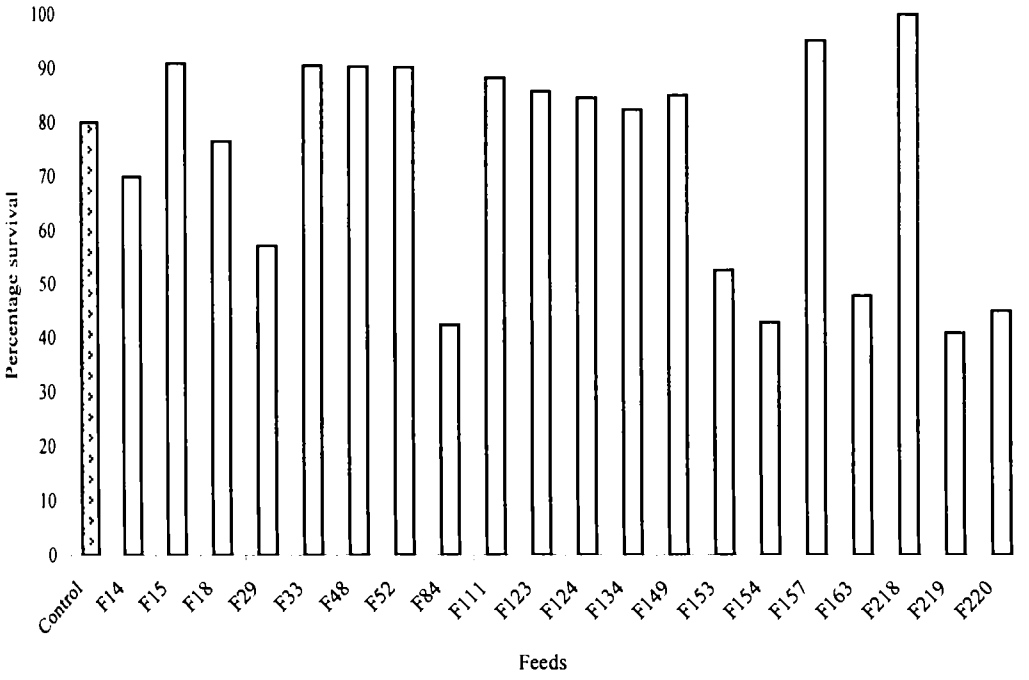


Fig.4.10. Survival of the post larvae after 21 days feeding experiment with various experimental diets

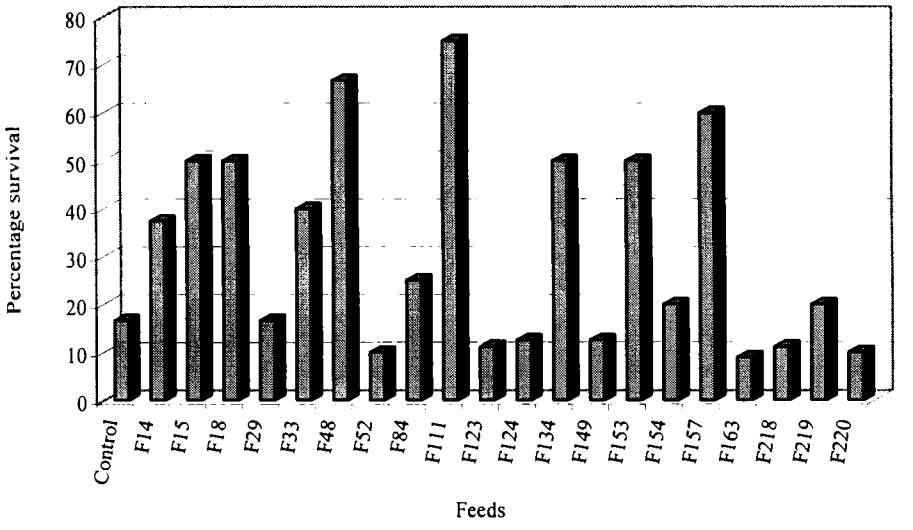


Fig. 4.11. Percentage survival of post larvae fed on experimental feed after challenge with WSSV

Chapter 5

Characterisation of growth and enzyme production by the selected strains and optimisation of the fermentation conditions

5.1 Introduction

The growth of aquatic microorganisms is influenced by various physico-chemical factors. Incubation temperature, pH values and salt concentrations may influence their metabolism and reproduction. The synthesis of enzymes and in consequence the ability to breakdown substrates may be either promoted or inhibited by the prevailing physical and chemical conditions (Reinheimer, 1980).

Several studies were earlier conducted to assess the effects of various physico-chemical parameters on enzyme activity and growth of various microorganisms during fermentation with a view to characterize the selected strains of microorganism to improve the quality and quantity of the end product. An optimization study is essential to determine the best combination of suitable culture conditions for maximal production of extracellular enzymes.

5.1.1 Proteases

Makino *et al.*, (1981, 1983) conducted studies on proteases from marine bacteria, with special reference to the extracellular proteases of marine

Pseudomonas sp. was observed by Duong *et al.* (1981). The enzyme had maximum activity at 18% NaCl concentration and optimal pH 8.0. Kobayashi *et al.* (1985) purified and studied the properties of alkaline proteinases by *Pseudomonas maltophila.* The optimum pH and temperature were 10.5 and 55°C respectively and calcium ions were found to activate the enzyme activity.

Culture conditions to promote highest levels of production of extracellular protease by *Bacillus brevis* was studied by Wight *et al.* (1988). They found that an inoculum size of 5% resulted in consistently good growth and protease production (more than 3g/litre) in *Bacillus brevis.* It was noted that the pH of the medium at 24 hr was always 8.5 which was unfavourable for the process regardless of the extent of protease production, glucose consumption, etc. This suggested that pH had reached an unfavorable level. An initial pH of 6.5 was found to favour maximal enzyme production. There was an unconditional rise of the pH of the medium to a final value of 8.5, which limited the protein production. Providing à lower pH at the outset may allow the cells to produce additional protein/ enzyme before the final pH of 8.5 is reached. External regulation of the pH during course of fermentation should therefore also enhance protease production (Wight *et al.,* 1988). There have been extensive studies on the characterisation of alkaline proteases from *Bacillus* spp. such as *Bacillus subtilis* (Tsuru *et al.,* 1967), *B. thuringiensis* (Kunitate., 1989). *B. thuringiensis* var. *kurstaki* HD-255 was found to produce

an extracellular thermostable protease after the end of the vegetative growth phase. The enzymes showed maximal proteolytic activity at 70 °C and pH 8.5-9.0. Protease activity was detected after 4-5 hr of fermentation at the end of the logarithmic growth phase and reached maximum level 14-15 hr after the start of the fermentation and the pH of the culture after 15 hr was about 8.4.

Yang *et al.* (2000) reported the production and purification of protease from a *Bacillus subtilis* that can deproteinise crustacean wastes. The optimized conditions for protease production was found when the culture was shaken at 30 °C for 3 days in 100 ml of medium (phosphate buffer adjusted to pH 6.0) containing 70% shrimp and crab shell powder (SCSP), 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.0% arabinose, 1.5% $NaNO_3$ and 1.5% $CaCl_2$. Under such conditions, the protease of *B. subtilis* attained highest activity of 20.2 U/ml. The protease was most active at pH 8.0 and 50 °C with casein as substrate.

In commercial practice, medium composition is optimized continuously with time so that the balance between the various components is maintained, thus maximizing productivity of the microorganism and minimizing the amount of utilized components at the end of the fermentation. The balance of optimal activity is highly dependent on the interaction between the particular strain of the microorganism, the type of medium/substrate components, the fermentation equipment and the process conditions. Fermentation time is also of importance towards achieving the

highest possible enzyme concentrate and also to minimize the total cost of the production process while the quality of the product is kept high (Aunstrup, *et al* 1979).

Parameters for the production of a thermostable alkaline protease by solid state fermentation from a new *Pseudomonas* sp. B45 have been optimized by Chakraborty and Srinivasan (1993). Similarly, *Pseudomonas* sp. B45, a soil isolate, was grown on wheat bran (WB) supplemented with salt solution for the production of thermostable alkaline protease. High enzyme titre were obtained when the WB moisture content was 60-80%, with an optimum at 74-78%. The enzyme titre was highest with 74% initial moisture content after 96 hrs of growth at 37 °C. Growth was faster at 74.0 and 78.5% moisture level (96 hrs) than at 70.4 or 81.0% (120 hrs). The enzyme was stable in the pH range 7.5-9.5. Enzyme titres were stable in the pH range 7.5-9.5. Enzyme titres were similar at 30 and 37 °C, but decreased at higher temperature. Dissacharides and complex sugars like fructose and sucrose upto 2g/10g WB or 1% starch, stimulated enzyme production. Proteinaeceous nitrogen sources did not enhance production. Takiguchi *et al.* (1985) reported that *Vibrio anguillarum* E-383a, isolated from seawater accumulated a large amount of N,N'-diacetyl chitobiase $(GlcNAc)_2$ in a medium containing colloidal chitin (Takiguchi *et al.,* 1985).

5.1.2 Chitinase

Chitinolytic properties of *Bacillus pabuli* K1, isolated from mouldy bran was studied by Fraendbarg and Schnurer, (1994). Optimum conditions for chitinase production were 30 °C and initial pH 8.0. Chitinase production was induced when it was grown on colloidal chitin. Ulhoa and Peberdy (1992) reported extracellular chitinase production by *Trichoderma harzianum.* The pH optimum for the enzyme was 4.0-4.5, and maximum activity was obtained at 40 °C. It was also noted that chitin addition (6g/litre) at the end of the fermentation reactivated the growth but did not bring about further chitinase accumulation. *Cellulomonas flavigena* NTOU1 could grow well and show higher chitinase activity in modified salts-pectin broth additionally containing 1% (w/v) shrimp shell powder and 0.5% yeast extract. The optimum pH and temperature were 10.0 and 50 °C respectively (Chen *et al.,* 1997). Bhushan and Hoondal (1998) reported the purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. It produced extracellular chitinase constitutively but the presence of chitin (1%) as a carbon source in the production medium enhanced the chitinase yield by 40%. The optimum production of chitinase was 76 U/ml of culture supernatant obtained after 72 hrs at 50 °C, pH 8.5 under stationary conditions. Impact of process parameters on chitinase production by an alkalophilic marine *Beauveria bassiana* in solid state fermentation was reported by Suresh and Chandrasekaran (1999). The production of chitinase on wheat bran was

enhanced under optimized conditions. Optimal NaCl and chitin concentration for chitinase production was dependent on the type of moistening media used (aged sea water or 2NaOH). Enzyme yields were higher using a mycelial inoculum than with a conidial inoculum and when phosphate and yeast extract were added to the medium. Under optimized conditions, enzyme yield was 246.6U/g initial dry substrate. The optimization of the parameters for the extracellular chitinase production by *Trichoderma harzianum* was described by Felse and Panda (1999). A suitable combination of parameters, pH aeration rate and agitation rate, increased an initial value of 0.109 U of enzyme activity under unoptimized conditions to a final value of 0.384 U after optimization. A controlled pH of 4.9, an aeration rate of 1.51 per min, and an agitation rate of 224 rpm was found to be the best combination.

Barboza-Corona *et al.,* (1999) detected different chitinase activities by *Bacillus thuringiensis* in a culture medium supplemented with chitin as the major carbon source. A proteo-chitinolytic strain of *Bacillus thuringiensis* able to grow in shrimp waste media was reported by Rojaz-Avelizapa *et al* (1999). The importance of process optimization in fermentation having been emphasized by similar works cited in the review of literature, it was imperative to understand the optimal conditions for maximal growth and enzyme activity. The present study is an attempt to characterize the process conditions favourable for the selected strains by assessing the growth and

enzyme activities in nutrient broth. The derived optimal conditions specific to each strain was applied to the optimization of the fermentation process.

5.2 Materials and methods

5.2.1 Characterisation of the four selected strains in terms of growth, protease production and chitinase production in Nutrient broth.

5.2.1.1 Selection of bacterial isolates.

Four isolates were selected based on their performance in terms of nutrient enrichment of prawn shell waste by fermentation and the efficacy of their fermentation products as feed ingredients in prawn feed formulations. the four isolates selected were: (Fig 5.1 and 5.2)

1. *Bacillus* sp. (C111)
2. *Bacillus* sp. (C124)
3. *Bacillus* sp. (C134)
4. *Serratia* sp. (C163)

Serratia sp. C163 was selected due to its carotenoid content and to test its efficacy as a dietary component in prawns.

5.2.1.2 Media used

Nutrient broth with the following composition was used as basal medium for the estimation of growth and enzyme production of these strains under varying culture conditions.

Nutrient Broth

Peptone	: 0.5g
Beef extract	: 0.3g
Colloidal chitin	: 0.5g
NaCl	: 1.5g
pH	: 7
Distilled waster	: 100 ml

5.2.1.3 Preparation of inoculum

The bacterial isolates were streaked onto nutrient agar slants and after incubating for 18-20 hrs at room temperature (28±2°C), the cells were harvested using sterile physiological saline (0.9% NaCl in distilled water). The optical density of the cell suspension was adjusted to 1 O.D. (approximately $4x10^8$ cells per ml) concentration and this cell suspension was used as inoculum for experiments wherever necessary. 1 ml was inoculated to 100 ml medium.

5.2.1.4 Measurement of growth

The bacterial growth was measured by measuring the optical density at 600 nm in a Hitachi Model 200-20 UV-Visible Spectrophotometer.

5.2.1.5 Estimation of growth and enzyme production.

Each media prepared was inoculated with the four bacterial inoculum prepared and incubated at room temperature (28±2°C) for 4 days and the growth and enzyme production were assessed.

5.2.1.5.1 Preparation of the enzyme

The cell suspension was centrifuged at 10,000 x g for 15 min and the cell free supernatant fluid (as crude enzyme) were assayed for protease and chitinase activity.

5.2.1.5.2 Protease enzyme assay

Protease production was measured in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Protease activity was assayed by a modification of the casein digestion method of Kunitz (1946). 1% casein (Hammersten) in 0.1M phosphate buffer (pH 7.6) was used as the substrate. To 2 ml of the casein substrate, 1 ml of the culture supernatant (crude enzyme) was added and incubated at 35 °C for 20 min. The reaction mixture was stopped with 3 ml of 5% Trichloroacetic acid (TCA) solution. The precipitated protein was filtered through Whatman No.1 filter paper. Optical density was measured at 280 nm in a Hitachi 200-20 UV-Visible Spectrophotometer. The tests were run in duplicate and the average values were taken. A unit of proteolytic activity was defined as the enzyme quantity which liberated 1μg of tyrosine per ml of reaction mixture per minute under standard conditions.

5.2.1.5.3 Chitinase enzyme assay

The method employed was essentially that of Hood and Meyers (1977). Bacterial chitinase activity was measured by the release of N-acetyl glucosamine from re-precipitated chitin.

The enzyme assay mixture consisted of colloidal chitin (5mg/ml)-1.0 ml, 1.0 ml of Mineral Sea Water Salts solution and I ml of enzyme. 0.2 ml of toluene was also added as a bacteriostatic agent. The tubes containing the enzyme assay mixture were incubated at 37 °C in a water bath for 2 hrs. Control solution which had all the reagents except the crude enzyme, were maintained likewise. After incubation, the contents were centrifuged (10,000 x g) and the amount of N-acetyl glucosamine released into the solution was estimated as given in below. The tests were run in duplicates and the average values were taken. Chitinase activity was expressed as μg of N-acetyl glucosamine liberated in 1 ml of the incubation mixture under assay conditions.

Preparation of Minimal Sea Water Salts (MSWS) solution.

This solution contained most of the major ions of seawater and it was prepared according to Goodrich and Morita (1977).

The composition was as follows:

NaCl	: 24g
$MgSO_4.7H_2O$	: 7.0g
$MgCl_2.6H_2O$	: 5.3g
KCl	: 0.7 g
Distilled water	: 1000 ml
pH	: 7.5

Estimation of N-acetyl D-glucosamine (NAG)

The N-acetyl-D-glucosamine assay developed by Morgan and Elsen (1934) and modified by Reissig *et al.*. (1955) was followed in the present study. 0.5 ml of the sample was mixed with 0.1 ml of potassium tetraborate (0.8M) and heated in boiling water bath for 3 min. After this, it was cooled under tap and 3.0 ml of DMAB reagent was added. The mixture was incubated at 37 °C in a water bath for 20 min. The intensity of the colour developed was read at 585 nm in a spectrophometer. Standard curve was prepared with analytical grade NAG in a similar way and the quantity of NAG in the sample was estimated using this curve.

Preparation of DMAB reagent

10.0g of analytical grade p-Dimethyl amino benzaldehyde (DMAB) was dissolved in 100 ml of glacial acetic acid containing 12.5% v/v conc. HCl. This stock solution was diluted with 9 volume of glacial acetic acid before use.

5.2.1.6 Effect of pH on growth, protease and chitinase enzyme production

Nutrient broth of different pH (5,6,7,8 and 9) were prepared using various buffers as given below:

Buffer	pH
Sodium acetate-acetic acid	5.0
Tris-Maleic acid	6.0-7.0
Tris-HCl buffer	8.0
$NaHCO_3$-Na_2CO_3	9.0

Inoculation was made and incubated at room temperature (28±2°C) for 4 days.

5.2.1.7 Effect of Sodium chloride concentration on growth, protease and chitinase enzyme production.

Nutrient broth with different concentration of sodium chloride (0, 1, 1.5, 2, 3 and 3.5 %) were prepared. Inoculation was made and they were incubated at room temperature (28±2 °C) for 4 days. Growth and enzyme production were estimated as before.

5.2.1.8 Effect of different concentrations of colloidal chitin on growth, protease and chitinase enzyme production.

Nutrient broth with four different concentrations (0.25, 0.5, 0.75 and 1%) of colloidal chitin were prepared. Inoculation was made and kept for incubation at room temperature (28±2 °C) for 4 days. Growth, protease and chitinase activity was assessed as mentioned earlier.

5.2.1.9 Effect of Calcium carbonate concentration on growth, protease and chitinase enzyme production.

Nutrient broth with different concentrations of $CaCO_3$ (0.2, 0.4, 0.6, 0.8 and 1%) were prepared. The inoculation was made and incubated at room temperature (28±2 °C) for 4 days. Growth, protease and chitinase activity was assessed as described before.

5.2.2 Optimization of fermentation conditions

5.2.2.1 Preparation of prawn shell powder

Prawn shell waste was collected form peeling sheds in Cochin. They were oven dried at 80 °C overnight. The dried shells were then powdered and stored in tightly sealed packets and kept under refrigeration (4 °C).

5.2.2.2Preparation of fermentation substrate

Five grams of dried prawn shell powder was weighed and put in to 250 ml Erlenmeyer flask, plugged with cotton and sterilized by autoclaving at 121°C for 15 min.

5.2.3 Inoculum preparation and inoculation

Inoculcum preparation was done as in 5.2.1.3. One ml of the cell suspension was added to 5 gm shell substrate.

5.2.3.1 Optimization of process parameters

The protocol adopted for the optimization of process parameters influencing prawn shell fermentation was to evaluate the effect of a individual parameter and to incorporate it at the standardized level. In all the parameters optimized, initial substrate and inoculum preparation was carried out as described in 5.3.1.2.

The standard parameters used for the present study were:

Incubation temperature - 28±2 °C

moistening media - 50% sea water

Moistening amount - 1: 1.5 (substrate : moistening media)

Inoculum - 1 ml of cell suspension (10^8 cells / ml) into 5 gm substrate

While doing the optimization for each parameter, a range of conditions for that particular parameter was employed keeping others as given above. After incubation time, the fermentation products were dried in an oven at 80°C for 24 hrs and stored in a dessicator for further analysis. These products were analysed for protein, lipid and total sugars.

Initial moisture content

Five ratios of substrate to moistening media were selected for study. 50% of sterilized seawater was used as the moistening media. The ratios selected were 1: 1.5, 1:1.75, 1:2, 1:3 and 1:4. One ml of the cell suspension of all the selected strain was inoculated into the fermentation substrate. Incubation was done at 28±2 °C for a period of 10 days. After incubation, the fermentation products were dried at 80 °C for 24 hrs and stored in a dessicator for further analysis.

Sodium chloride concentration the substrate

Distilled water with different NaCl concentrations (0, 0.5, 1, 1.5, 2%) were prepared and it was used as the moistening media. Incubation was done at room temperature 28±2 °C for 10 days and the products were dried at 80°C for 24 hrs and stored for further analysis.

Incubation time

Six sets of fermentation substrate were prepared for each strain employed for fermentation. After inoculation, samples were taken initially and at intervals of 5 days for a period of 25 days. Samples were dried at 80°C for 24 hrs and stored for further analysis.

Incubation temperature

Fermentation media were inoculated and incubation was done at different temperature (25, 30, 35, 40, 45 and 50 °C) for 10 days. After this, the fermentation products were dried at 80 °C for 24 hrs and stored for further analysis.

Initial pH

The initial pH of the substrate was adjusted to various levels (4,5,6,7,8,9) by adding either 1N HCl or IN NaOH to the moistening media before inoculation. The pH was checked using pH indicator paper (MERCK). After incubation for 10 days at room temperature 28±2 °C the samples were dried at 80 °C in an oven and stored for further analysis.

Optimum inoculum concentration

Cell suspension was prepared as in 5.2.1.3 and added in different amounts (0.5, 1, 1.5, 2, 2.5 and 3 ml) to the fermentation substrate and the incubation was done for 10 days room temperature 28±2 °C. One ml of this cell suspension contained 10^8 cells/ml. Then the products were dried at 80 °C for 24 hrs and stored for analysis.

5.3 Results

5.3.1 Characterisation of strains using nutrient broth

Effect of pH on growth and enzyme production

The effect of different pH on growth and enzyme production are presented in Fig. 5.3. Generally pH 7.0 was found to be favourable for growth and chitinase production by the various strains. Maximum chitinase activity (23.2 mg NAG/unit enzyme) was achieved with *Serratia* sp. C163 at pH 7.0. However protease production was found to be maximum at pH 8.0. The highest protease activity of 1.14 mg tyrosine/unit enzyme was at pH 8.0 for strain C124 (*Bacillus* sp.). Growth was nearly completely inhibited at pH 5.0 and 9.0.

Effect of NaCl concentration on growth and enzyme production

Generally, 1.5 to 2 % NaCl concentration was found to be optimal for growth and enzyme production. Growth was maximum at 1.5% NaCl for strains C163 (*Serratia* sp.), C111 and C124 (*Bacillus* spp.) recording 0.98, 0.74 and 0.97 for growth respectively. Protease production of C111 and C124 was also enhanced at 1.5% NaCl (with 6.77 and 8.3 mg tyrosine/unit enzyme respectively). 2% NaCl was preferred by C163 for protease (0.63 mg tyrosine/unit enzyme) and chitinase (2.64 mg NAG/unit enzyme) production and by *Bacillus* sp. C134 for protease (6.29 mg tyrosine/unit enzyme) production (Fig. 5.4).

Effect of colloidal chitin on growth and enzyme production

Growth and protease production was found to be at maximal level with 0.5% colloidal chitin (Fig. 5.5). Maximum chitinase enzyme production of 12.5 mg tyrosine/ unit enzyme for *Serratia* sp. C163 was at 1% colloidal chitin. However, 1% colloidal chitin favoured maximum chitinase production. There was not much difference in growth with different chitin concentration in the case of strains C111 and C134 (*Bacillus* spp). However, C124 and C163 showed considerable decrease in growth at 1.5% colloidal chitin concentration.

Effect of calcium carbonate on growth and enzyme production

Presence of 0.8% $CaCO_3$ in the medium was found to be most favourable for growth and chitinase production for strain C111 (*Bacillus* sp.) and C124 (*Bacillus* sp.) whereas (*Bacillus* sp.) C134 preferred a concentration of 0.6% $CaCO_3$. Protease production was found to be best at lower concentrations (0.2 - 0.4%) of $CaCO_3$ for C111 and C124 (Fig. 5.6). *Bacillus* sp. C124 registered maximal values in both protease 6.4 mg tyrosine / unit enzyme and chitinase (5mg NAG / unit enzyme) production.

5.3.2 Optimization of fermentation conditions

Nutritional enrichment of the substrate (shell waste) evaluated by estimating protein, lipid and total sugars in the fermented product. Protein was estimated by Lowry's method (Lowry, 1951), lipid by phosphovanillin method following chloroform-methanol extraction of the sample (Folch *et al.*,

1957) and total sugars by Anthrone method (Roe, 1955). 50% sea water (17ppt) was used as the moistening media. The optimum for parameters like moisture, pH, temperature of incubation, inoculum size and sodium chloride concentration varied with the different nutrients (protein,. lipid and carbohydrate) tested in the fermented products and among the strains. Since protein is the major constituent of the fermented product, priority was given to protein enrichment of the substrate during fermentation and the optimum was finalized based on the protein content.

Substrate : moistening media

The optimal moisture content was 1:1.75 ratio for strains *Bacillus* sp. C111 and C134 (49.5 and 47% protein respectively) whereas for *Bacillus* sp. C124 and *Serratia* sp. C163 (46.7% and 47.7% protein respectively), the optimum was at 1:2 ratio (Fig5.7). The lipid content was maximum at 1:4 ratio for all the strains. It was interesting to note that the lipid content did not vary much with the variation in the moisture content of the fermentation substrate. Total sugars were found to be maximum at 1:3 ratio for *Bacillus* spp. C124 and C134. However, *Bacillus* sp. C111 and *Serratia* sp. C163 registered maximum total sugars in the fermented product at 1:2 ratio.

Sodium chloride concentration

Moistening medium (distilled water) with 1.5 - 2% NaCl was found to be optimal for protein enrichment with the various strains (Fig.5.8). The best protein enrichment was in FP111 and FP134 with 45% protein in each.

There was not much variation in the lipid and total sugar content with the different NaCl concentration of the moistening medium.

Incubation time

Protein content in the fermentation product was found to increase gradually and steadily over the period of incubation up to 20 days and after which there was no considerable increase (Fig.5.9). Maximum protein enrichment of 48.4% among the four strains was attained in FP163 on the 20^{th} day.

Incubation temperature

Maximum protein enrichment could be observed at 35 °C for all the four strains studied (Fig.5.10). Maximum protein enrichment of 47% among all the four strains was observed in fermented product FP124 at 35 °C. Lipid content was significantly high in all the fermented products at 35 °C compared to the other incubation temperatures and reduced considerably at 40, 45 and 50 °C. Total sugar content remained almost the same in all the fermentation products irrespective of the incubation temperature.

pH

An initial pH of 6.0 was found to be optimal for protein enrichment with all the strains (Fig.5.11). Maximum protein enhancement (67.7%) was in FP163 at initial pH 6.0. However, only a very low reduction in protein content of the fermented product could be observed when the initial pH was set at a higher level of 8.5. Lipid content also did not show much variation with the

varying intial pH. However, pH 8.0 and pH 7.0 was found favorable for *Bacillus* spp. C111 and C124 respectively while an intial pH of 6.0 was found favourable for *Bacillus* sp.C134 and *Serratia* sp. C163. The amount of total sugars remained the same in the fermented products irrespective of the initial pH of the fermentation substrate.

Inoculum size

An inoculum size of $2x10^8$ cells/5 gm of substrate was found to be best for protein enrichment in the fermented product (Fig.5.12). Up to an inoculum size of $5x10^8$ cells, the protein enrichment was more or less at the same. However, a drastic reduction was observed with $6x10^8$ cells. Lipid and total sugar level remained almost the same without many alterations irrespective of the inoculum size.

5. 4 Discussion

Characterisation of strains using nutrient broth

The strains used for the study were isolated from a brackish water environment and all of them were found to prefer a NaCl concentration of 1.5 to 2% for growth and enzyme production. In this study the protease production was found to be maximum at pH 8.0 and this observation was in consonance with that of Epremyan *et al.* (1981) who reported the maximum protease activity of *Bacillus thuringiensis* at pH 8.5. Alkaline protease with optima pH 8.0-10.0 was reported earlier by Lecadet *et al.* (1977).

The results obtained in the present study suggest that colloidal chitin is inducing chitinase production as evidenced by the enhanced production at 1% colloidal chitin concentration by almost all the strains. Simultaneous production of both the enzymes could be observed in this study. A catabolite repression phenomenon was not witnessed i.e., presence of an early utilizable susbstrate (protein) inhibiting the enzymatic system for a less easily utilizable susbstrate (chitin). For the initial screening of these cultures for chitinolytic activity, nutrient agar supplemented with colloidal chitin was used. This was with the intention that the chitinolytic strains should be able to produce chitinase enzyme in the presence of a proteinaceous substrate. In this study the shell waste employed is highly proteinaceous and therefore the chitinolytic activity should happen along with the proteolytic process. A succession of proteolytic activity followed by chitinolytic process may not happen due to product inhibition.

About 33% of the shell waste contain minerals especially $CaCO_3$ (Rojaz-Avelizapa *et al.*, 1999). Therefore the influence of $CaCO_3$ concentration on the growth and production of chitinase/protease was tested. Growth as well as chitinase production was found to be very much influenced by $CaCO_3$ concentration, with the peak being observed at 0.8% level in the media. However protease production was found to be adversely affected by $CaCO_3$ showing higher production at lowest concentration of $CaCO_3$. This

shows that decalcification process can result in enhanced protease production in a shell waste transformation system.

With shell waste as substrate, it is difficult to assess the influence of all these parameters (NaCl, pH, colloidal chitin, and $CaCO_3$) on growth and enzyme production. Therefore a well-defined medium such as nutrient broth was used for the characterisation of the selected cultures.

Optimization of fermentation conditions

Fermentation process is predominantly influenced by pH, aeration rate, temperature, incubation time and inoculum size of the selected microorganisms. Optimization of these parameters was essential to achieve maximum enhancement of the nutritional quality of the fermented products. A moisture level of 1:1.75 to 1: 2 indicate the preference of the strains for solid state condition for fermentation by the various strains in this study. Earlier reports also suggest the preference of solid-state condition for shell waste transformation by *Serratia marcescens* (Tom and Carroad, 1981). The initial moisture content significantly affected hydrolytic enzyme production since the moisture content of the substrate is a critical factor that determines microbial growth and product yield in SSF (Nishio *et al.,* 1979; Ramesh and Lonsane, 1990). Moisture is reported to cause substrate swelling and thereby facilitates better utilization of the substrate by microrganisms (Kim *et al.,* 1985). Higher moisture levels were found to inhibit product enrichment in terms of protein content by all the strains. At lower and higher initial moisture

levels, the metabolic activities of the culture and product synthesis are variously affected. Higher moisture levels are reported to cause decreased porosity, loss of particle structure, development of stickiness, reduction in gas volume and decreased gas exchange (Lonsane *et al.,* 1985).

An intial pH of 6.0 was found to be very much favourable for nutritional enhancement of the substrate. All the strains employed for the study were proteolytic and hydrolysis of protein in the shell waste may lead to elevation of pH. Thereby a substrate with a lower initial pH can support or accommodate more alkaline products without adversely affecting the metabolism (i.e., enzyme production and hydrolytic activities) of the microorganism by drastically changing the pH of the medium to inhibitory levels. A pH of 5.5 was found to be optimal for the SSF of sugar cane baggase for animal feed production (Nigam, 1990). Sugars formed as a result of chitinolytic process might be utilized by the microorganisms themselves. This may be the reason for the presence of more or less constant amount of total sugars in the fermented product.

All the four strains (3 *Bacillus* spp. and *Serratia* sp.) used in the study showed 35°C as their optimal temperature for growth and production in shell waste substrate. All of them are mesophilic strains isolated from the brackish water system and the elevated temperature might have inhibited the growth and enzyme production leading to a lower nutritional profile of the fermented

product. Philip (1987) has reported an optimum of 35°C for *Vibrio* spp. isolated from brackish water environment for protease production. The optimum NaCl concentration of the moistening media (1.5-2%) indicate the brackish water origin of the various strains. The effect of the various NaCl concentration on the nutritional quality of fermented product is not much as evidenced from the graph in Fig. 5.8.

The effect of inoculum in solid state fermentation is very much important. Inoculum size of $2x10^8$ to $5x10^8$ cells was optimum and the lower and higher inoculum resulted in low initial nutritional values compared to the others. This decrease seen with larger inocula may be due to the fact that the cultures had already crossed the stationary phase (after 10 days incubation) and had entered the death phase resulting in hydrolysis of microbial protein and other components of the product resulting in lower inoculum the culture may be still in log phase registering a lower protein content due to lower levels of microbial biomass.

Fermentation of the shell waste has taken large incubation time unlike other SSF studies of enzyme production. In this study, a biotransformation of the substrate is envisaged rather than enzyme production. Enzyme production usually takes place during the later logarithmic phase or early stationary phase (Keil-Dlouha *et al.,* 1976; Reid *et al.,* 1980; Makino *et al.,* 1983; Philip, 1987). Such studies therefore take only 3-4 days for accomplishing the task of

maximum enzyme production. Here the substrate is a crude material and chitin constitute around 27% impregnated with $CaCO_3$ and other minerals (Rojaz-Avelizapa *et al.,* 1999). Chitin is a recalcitrant material and it is not an easily available form in the shell waste. Protein, which constitutes about 40% of the shell waste is easily hydrolyzed. However, the chitin hydrolysis takes place at a lower rate demanding more time for the transformation of the waste into simpler components enriching the nutritional quality.

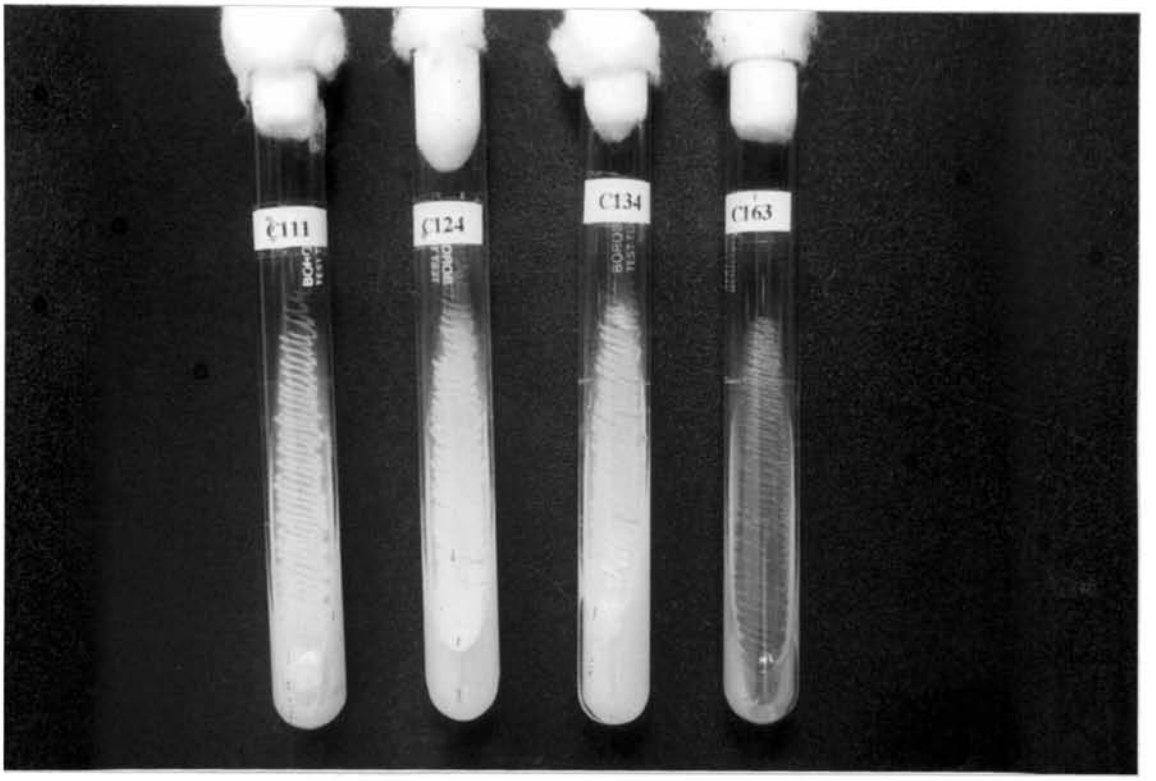
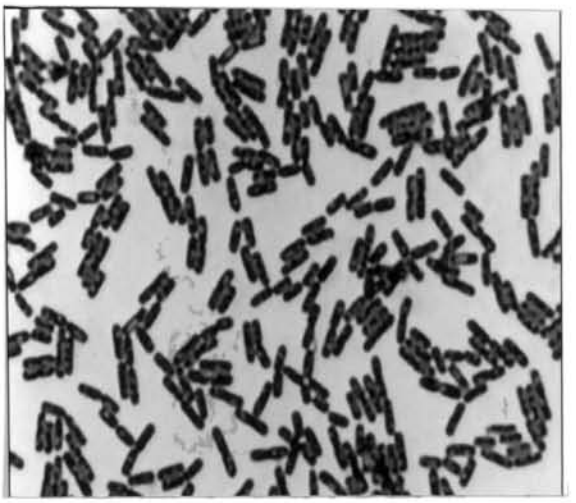
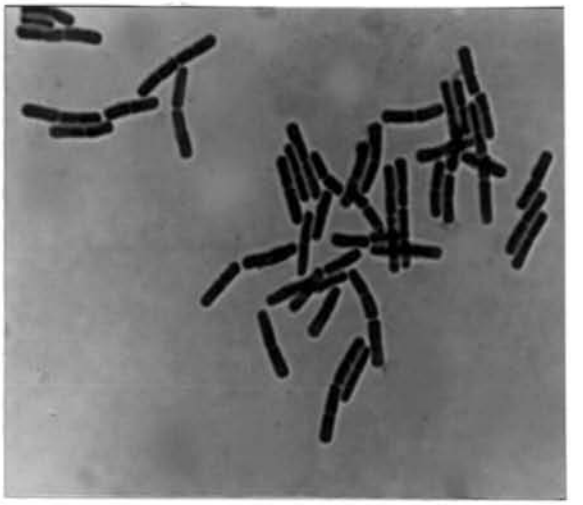


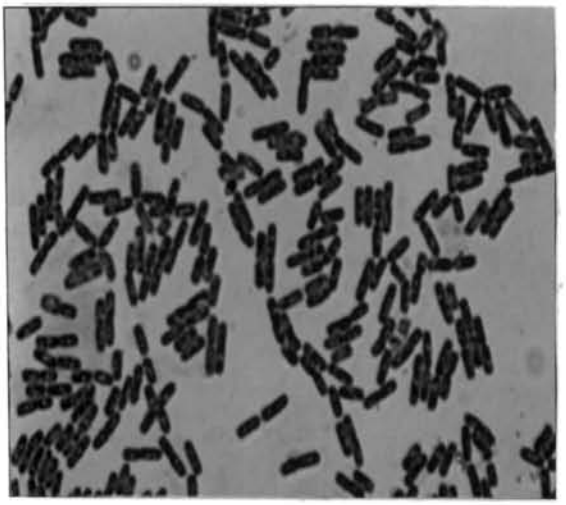
Fig.5.1 Slant culture of the selected bacterial strains in nutrient agar medium



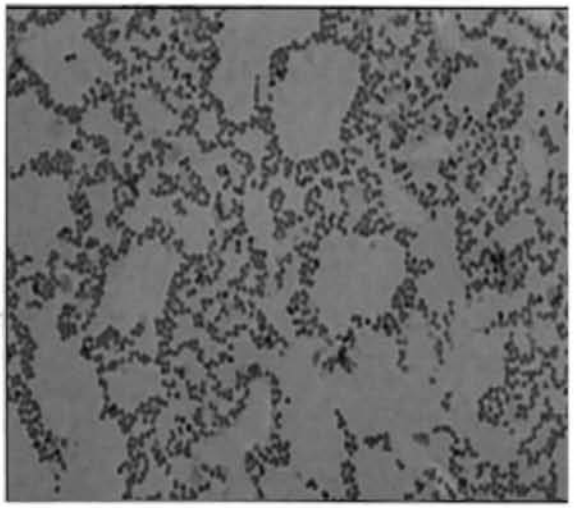
a. **C111** (*Bacillus* sp.)



b. **C124** (*Bacillus* sp.)



c. **C134** (*Bacillus* sp.)



d. **C163** (*Serratia* sp.)

Fig. 5.2 Gram stained preparation of selected bacterial strains

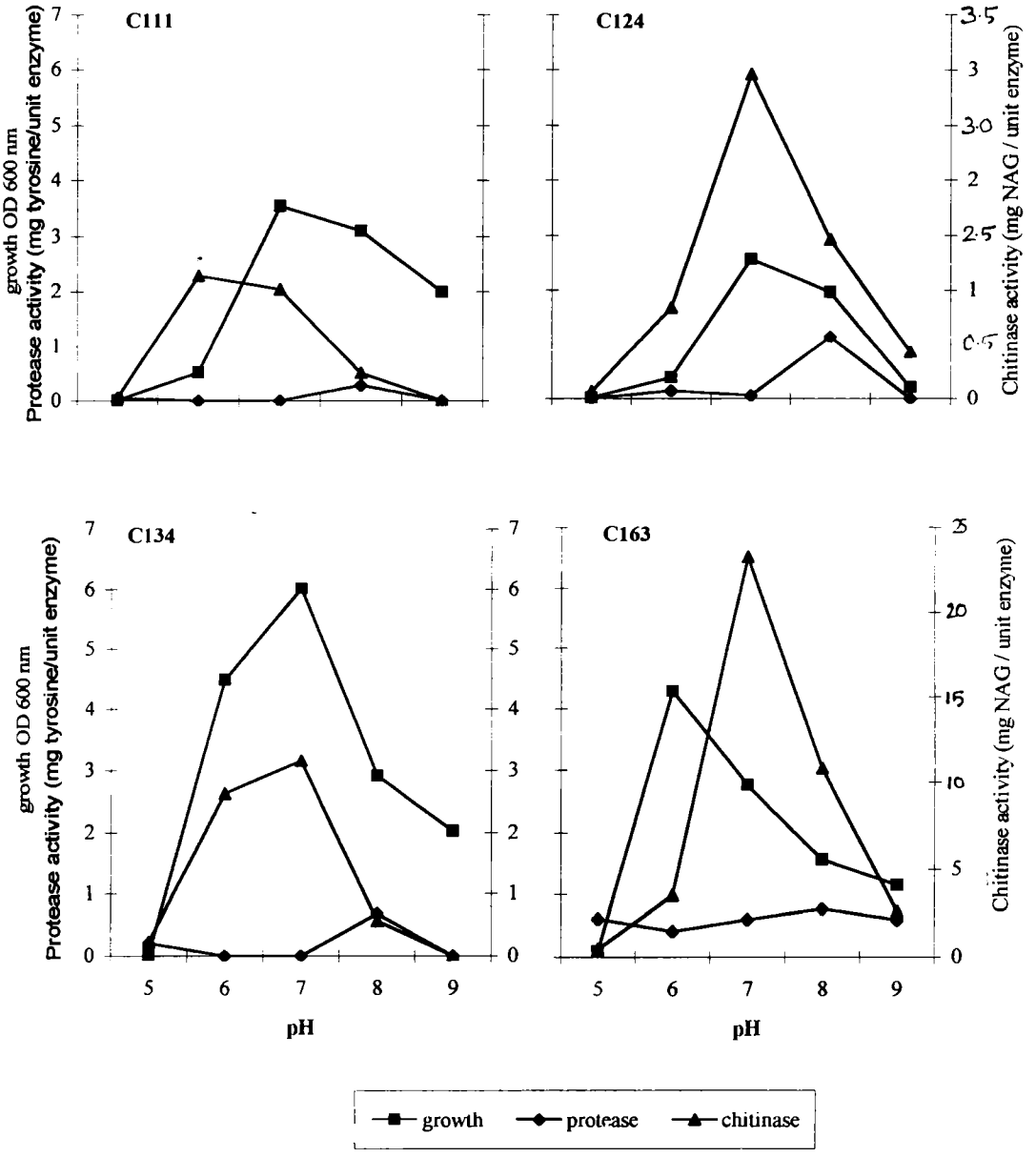


Fig. 5.3 Effect of pH on growth and enzyme (protease and chitinase) production

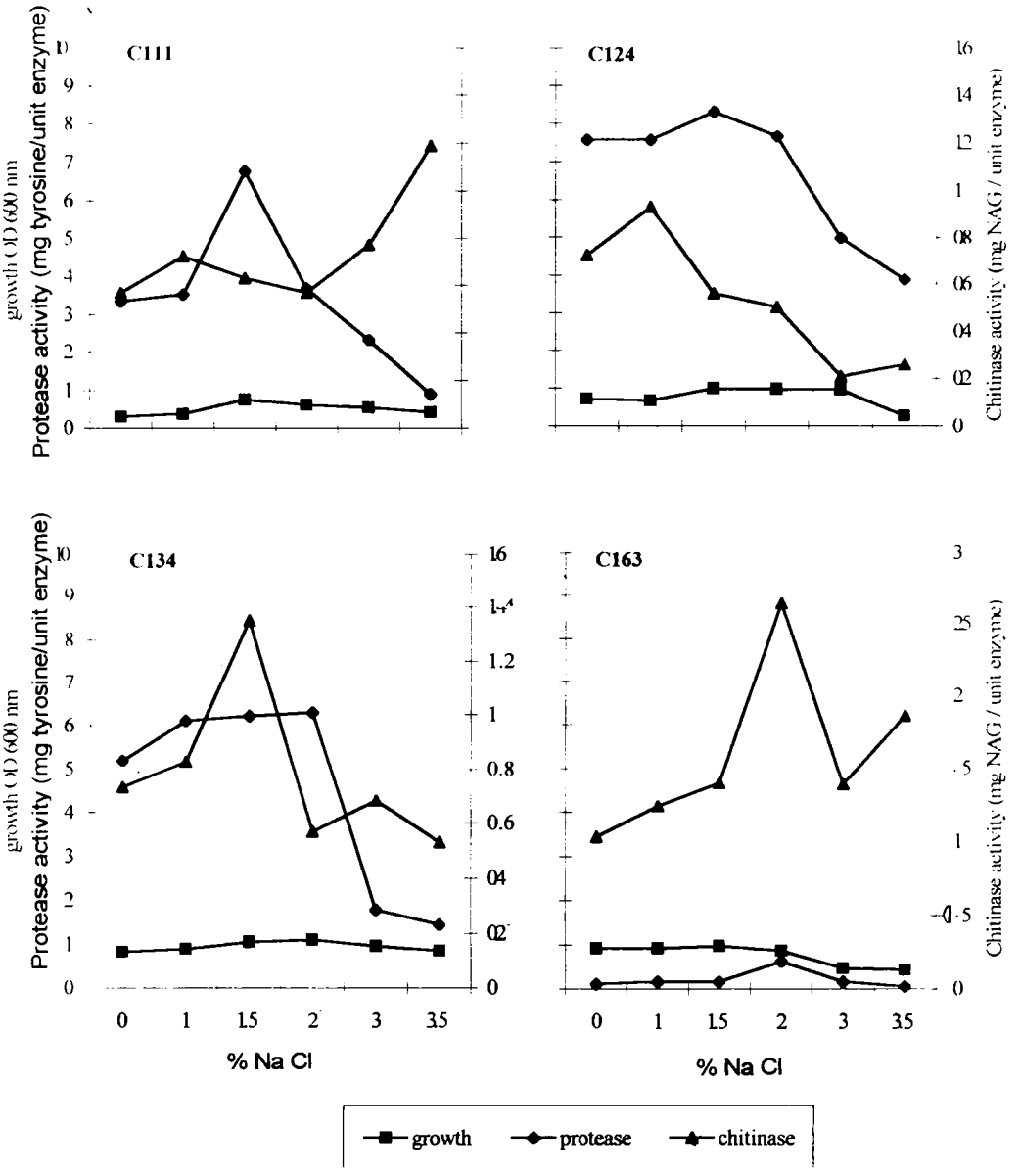


Fig. 5.4 Effect of NaCl on growth and enzyme (protease and chitinase) production

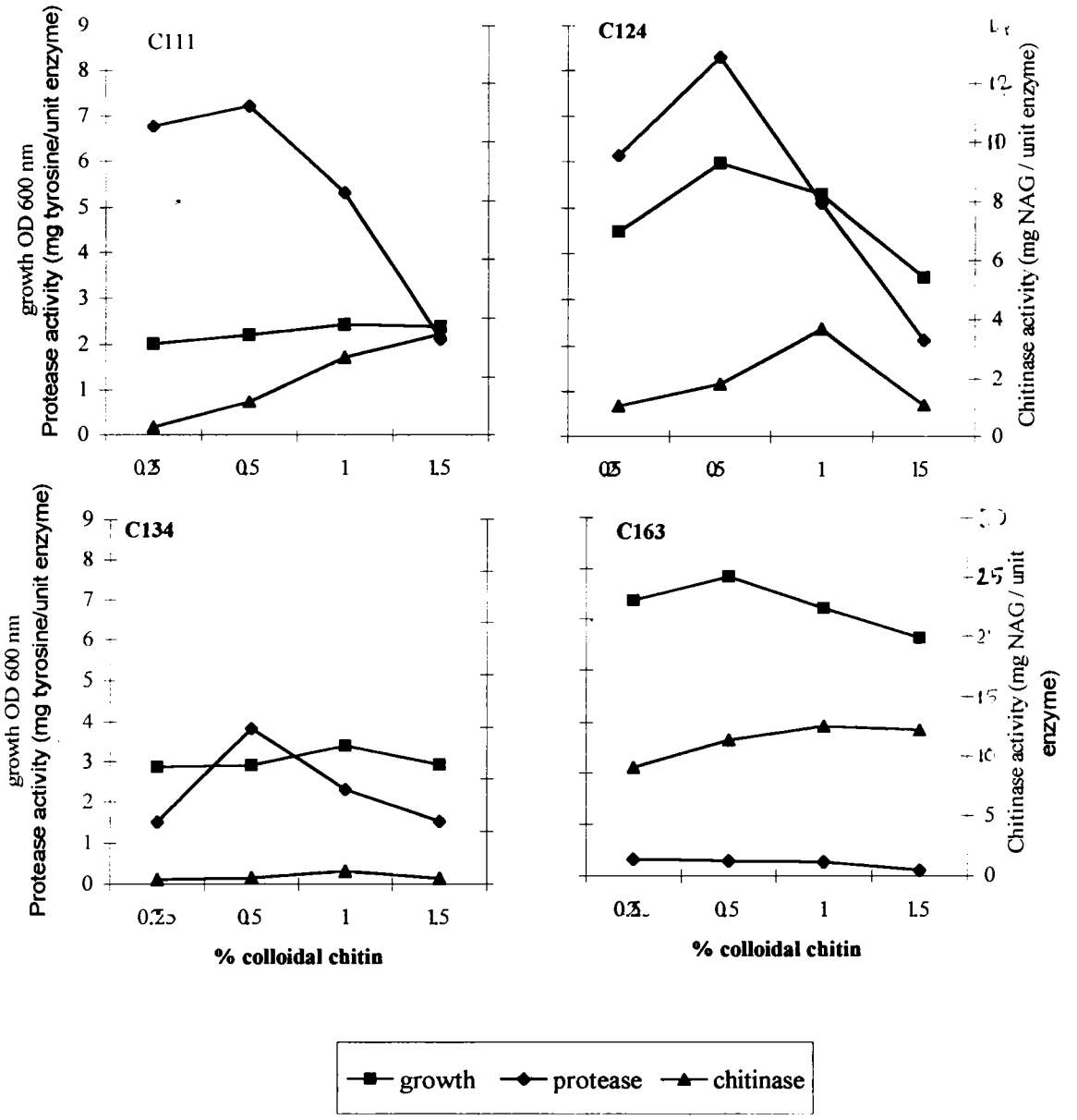


Fig. 5.5 Effect of colloidal chitin on growth and enzyme (protease and chitinase) production

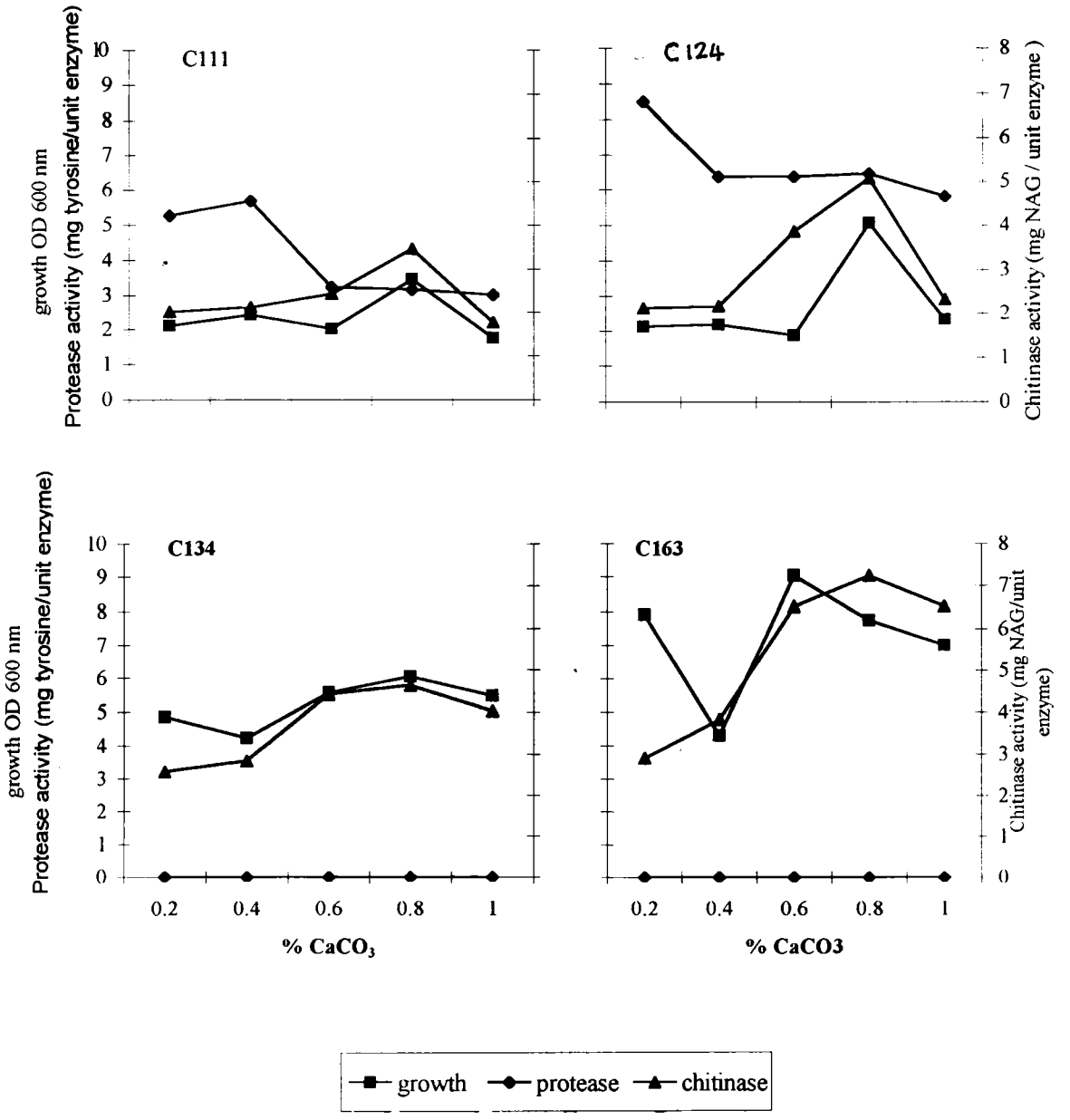


Fig.5.6 Effect of calcium carbonate on growth and enzyme (protease and chitinase) production

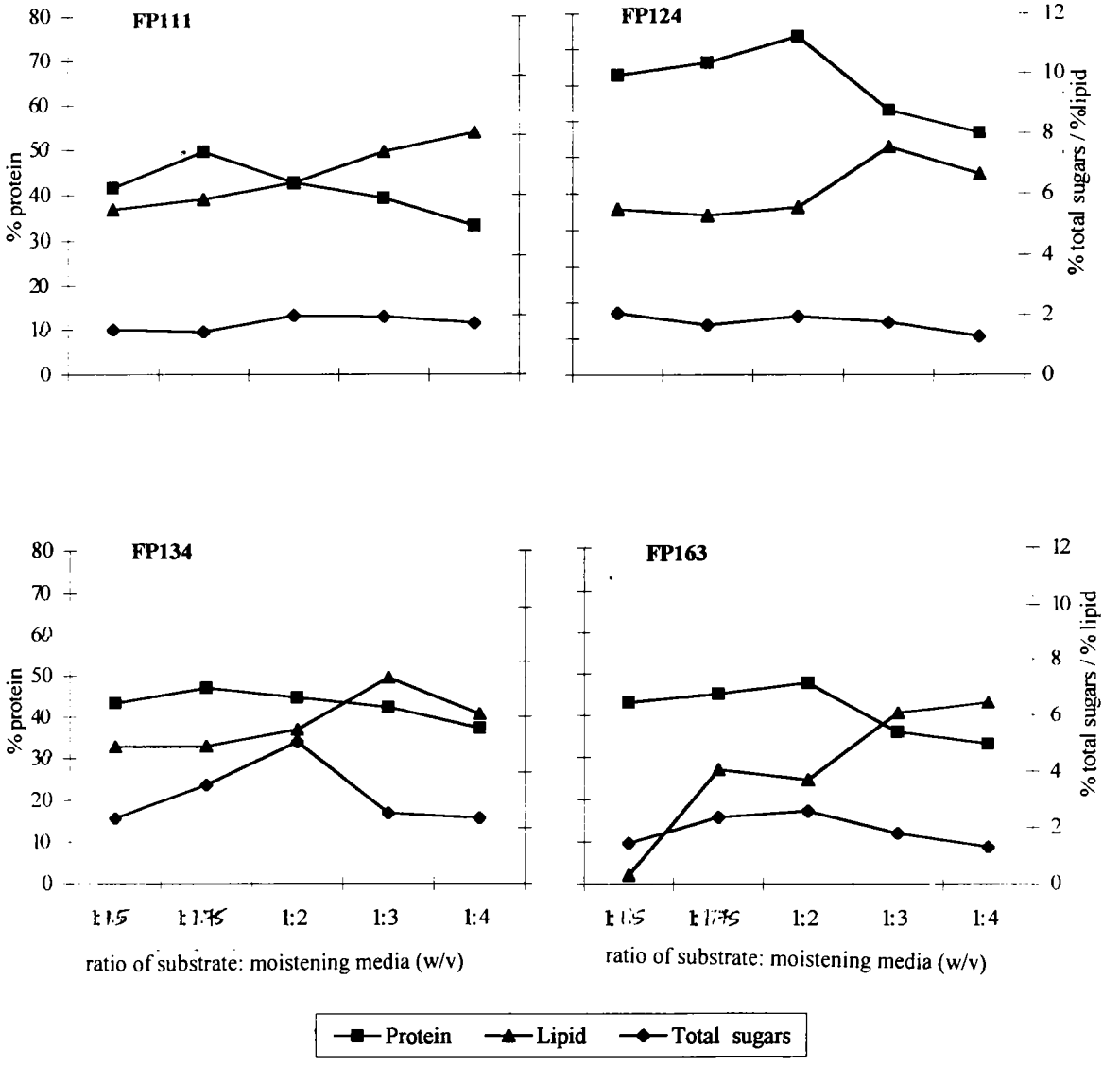


Fig. 5.7 Effect of substrate : moistening media ratio on the nutritional enrichment of the fermentation product by the various strains

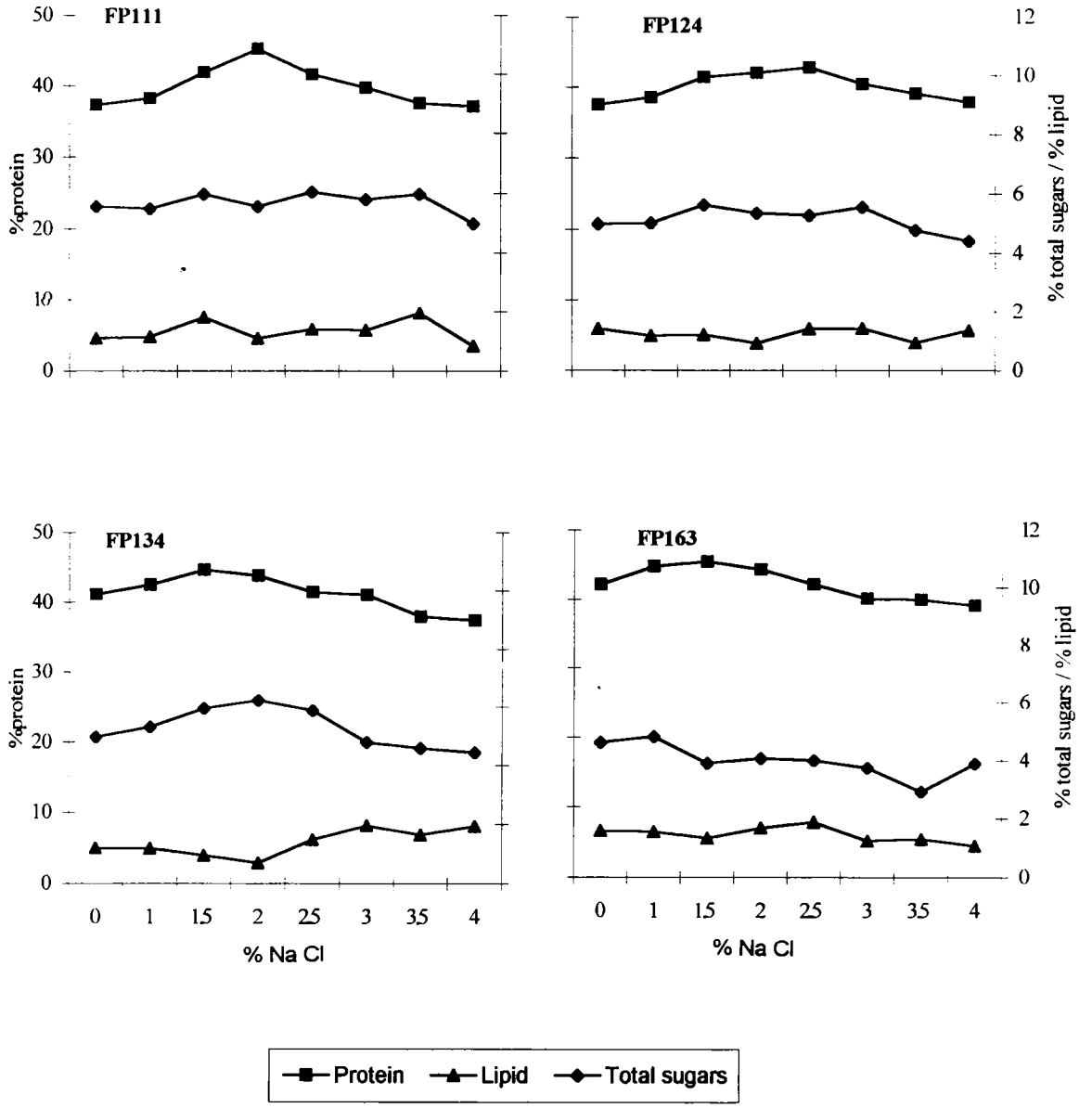


Fig. 5.8 Effect of sodium chloride concentration on the nutritional enrichment of the fermentation product by the various strains

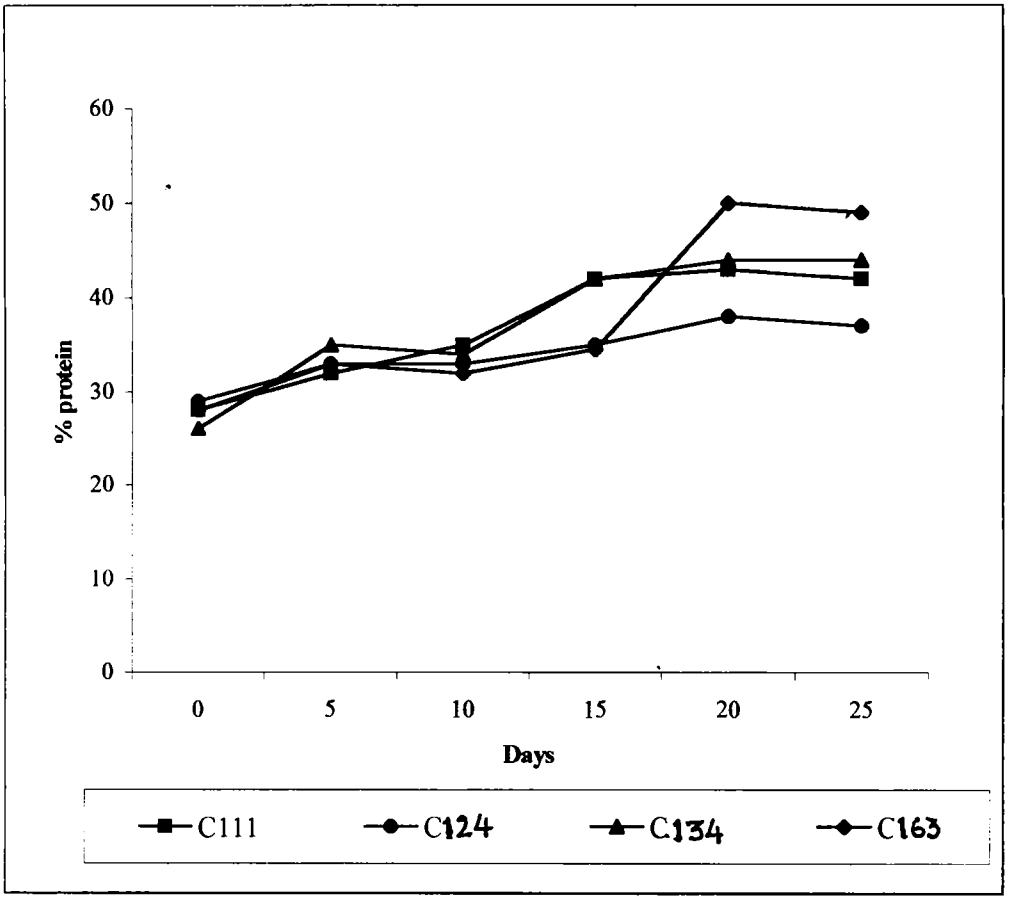


Fig. 5.9 Effect of incubation time on the protein enrichment of the fermented product

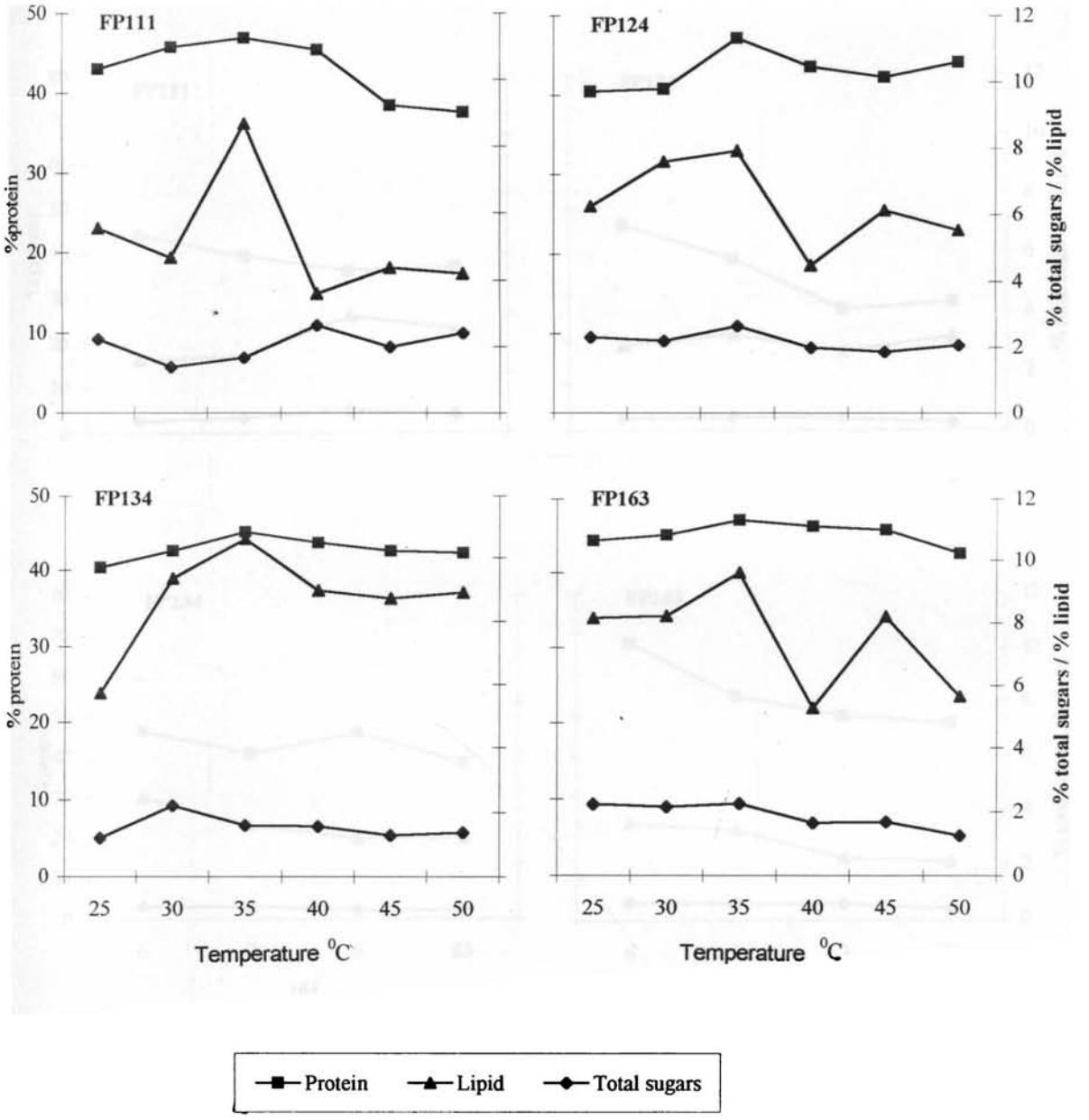


Fig.5.10 Effect of temperature of incubation on the nutritional enrichment of the of the fermentation product by the various strains

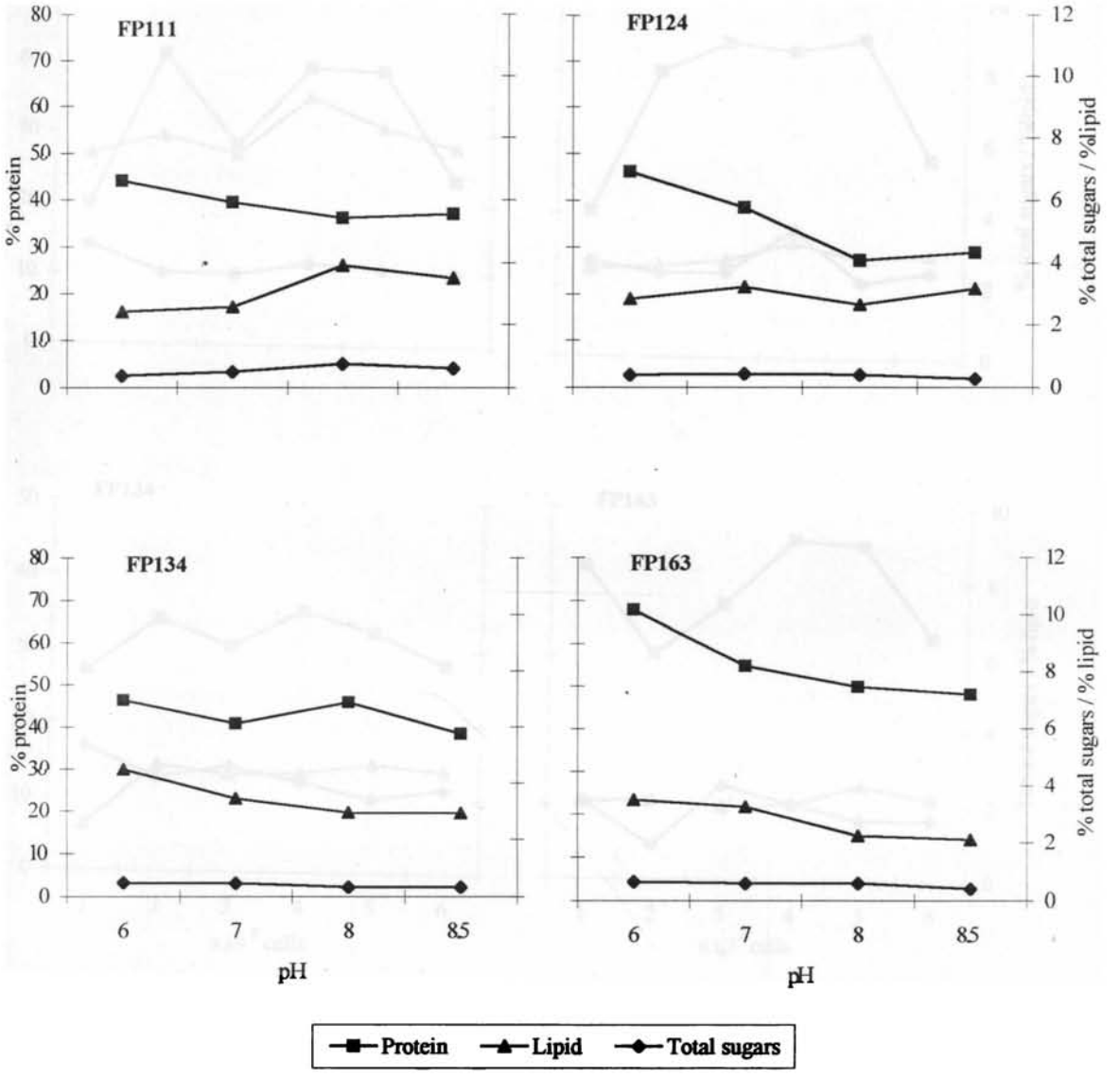


Fig.5.11 Effect of pH on the nutritional enrichment of the fermentation product by the various strains

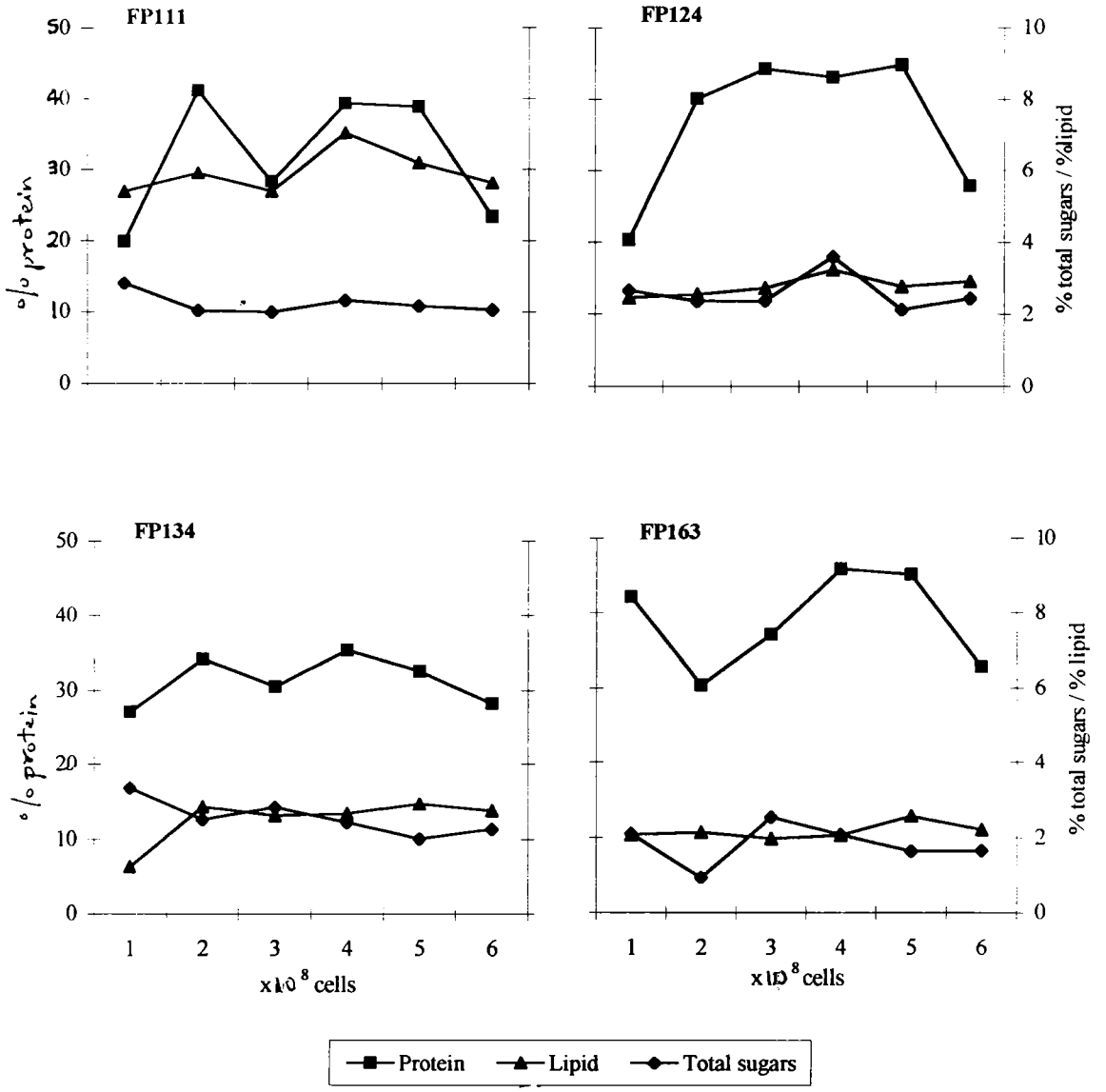


Fig.5.12 Effect of inoculum size on the nutritional enrichment of the fermentation product by the various strains

Chapter 6

Application of shell waste fermented product as a dietary ingredient for *Penaeus indicus* juveniles in culture systems and testing its efficacy as an immunostimulant

6.1 Introduction

Culture of penaeid shrimp species has increased dramatically during the last decade. Estimates from global production in 1995 are in the order of 800 thousand metric tons, with some forecasts suggesting that commercial cultivation of shrimp may grow to as much as 1.6 million tones by the turn of the century (Newman, 1996). Aquaculture is a fast growing industry in the world and a major threat to this industry is the frequent outbreak of diseases especially viral diseases. White spot syndrome virus (WSSV) is one of the worst shrimp viral diseases and it affects most of the commercially cultured shrimp species globally. Diseased shrimps show white spots on the carapace and reddish discolouration of the body. In the lightly infected specimens, the prevalence of WSSV is particularly high in the gills, periopod, hemolymph, followed in order of decreasing prevalence by the stomach, eyestalk, maxilliped, heart, integument, reproductive organs, midgut, abdominal muscle, nervous tissue and hepatopancreas (Huang and Song, 1998).

The prevention and control of diseases are now considered priorities for shrimp aquaculture in the vast majority of the shrimp producing countries. The maintenance and development of this industry are at stake as shrimp

aquaculture faces increasingly significant ecological and pathological problems on a global scale.

The use of antibiotics and other chemotherapeutics has several shortcomings including the risk of generating resistant pathogens, the problems of drug residues in the treated animal, and the impacts of environmental pollution. The need for alternate methods for regulating the number of pathogenic bacteria and also detrimental effects of viruses in aquaculture, have led researchers to turn to different methods of treatment such as probiotics and immunostimulants. Probiotics aim at improving the intestinal microbial balance of the host animal with the objective of having beneficial microorganisms dominate the harmful bacteria that cause disease (Ewing and Haresign, 1989).

Immunostimulants on the other hand aim at enhancing the non-specific defense mechanisms in animals. A number of different biological and synthetic compounds have been found to enhance the non-specific defense system in animals, including shrimp (Song and Sung, 1990; Sung *et al.*, 1991). They have been shown to increase the barrier of infection against a series of pathogens simultaneously, both specific and opportunistic ones (Raa *et al.*, 1992). The use of immunostimulants may increase the resistance level sufficiently to abolish infections by opportunistic pathogens and therefore lead to improved performance, enhanced growth and reduced mortality throughout the production period.

Shrimp immunology is a key element in establishing strategies for disease control in shrimp culture (Bachere *et al.*, 1998). Shrimps have been shown to possess a primitive immune system that relies mainly on prophenoloxidase system, phagocytosis, encapsulation, agglutination and the lysis activity of the hemocytes (Smith and Soderhall, 1986). Central to any active cellular or humoral response to microbial or parasitic invasion is the initial recognition of foreignness by the host. Crustaceans accomplish this through a complex cascade of serine proteases and other factors in the hemocytes that are specifically triggered by foreign molecules. This is known as the prophenoloxidase system or proPO system, and is confined to the semigranular cells of the hemolymph (Smith and Soderhall, 1986). ProPO is activated in a stepwise process by microbial cell wall components such as β-1,3-glucans of fungi, or the lipopolysaccharides (LPS) and peptidoglycans of gram negative and gram positive bacteria, respectively (Soderhall, 1982; Ashida *et al.*, 1982; Soderhall and Hall, 1984; Soderhall and Smith, 1986; Saul and Sugumaran, 1987; Duvic and Soderhall, 1990). The proPO cascade serves as the receptor for non-self signals, released from the surface of microorganisms or parasites (Soderhall, 1982) and terminates in the conversion of proenzyme to activate phenoloxidase, which is needed to synthesize bactericidal melanin.

In crustaceans, glucans have been shown to activate prophenoloxidase in the hemolymph (Unestam and Soderhall, 1977; Soderhall *et al.*, 1990;

1994; Baracco *et al.*, 1991; Scholz *et al.*, 1999) and thereby increased survival (Sung *et al.*, 1994; Supamattaya and Pongmaneerat, 1998). β-1,3-glucan significantly enhances the resistance of post-larval juvenile and adult shrimp to WSSV infections (Su *et al.*, 1995; Liao *et al.*, 1996).

Most evidences indicate that shrimp pathogens are not the major cause of mortality and should be classified as opportunistic infections. It is believed that various types of stress reduce the resistance of the shrimp to diseases, making them susceptible to less virulent pathogens. Increasing their resistance to a specific pathogen does not protect them from other pathogens during their growth period. Therefore, increasing the non-specific immunity of shrimp to provide them with broad-spectrum defensive ability should effectively protect shrimp against infections from pathogens. (Table 6.1)

The immunostimulatory effects of glucan (Unestam and Soderhall, 1977; Robertsen *et al.*, 1990; Aakre *et al.*, 1994; Sung *et al.*, 1994; Sung and Song, 1996), laminarin (Dalmo *et al.*, 1994), chitin (Nishimura *et al.*, 1985; Sakai *et al.*, 1992, Kawakami *et al.*, 1998), and chitosan (Anderson and Siwicki, 1994; Andersen *et al.*, 1995) have been reported earlier. Bacterial derivatives like muramyl peptide (Olivier *et al.*, 1985; Kodama *et al.*, 1993), low molecular weight peptides (Bogwald *et al.*, 1995; Gildberg *et al.*, 1996; Kitao and Yoshida, 1986) and Freund's (complete) Adjuvant (Patterson and Fryer, 1974; Olivier *et al.*, 1985,1986) have also been proved to confer immunostimulation.

There are several evidences to suggest that bacterial cell walls have the ability to enhance the non specific resistance of fish and shrimp against bacterial infections. In another experiment using peptidoglycans, shrimps fed with peptidoglycan incorporated diet showed hemocytic activity, disease resistance and higher tolerance to variations in dissolved oxygen, salinity and stress (Booyratnapalin, 1990). The oral administration of peptidoglycans from *Bifidobacterium thermophilium* protected juvenile rainbow trout challenged with *Vibrio anguillarum* (Matsuo and Miazano, 1993). Peptidoglycan (PG) derived form the cell walls of the Gram-positive bacteria, *Brevibacterium lactofermentum* was continuously fed to black tiger shrimp (*Penaeus monodon*) larvae (aged 15 days) for 8 weeks. Shrimp fed with PG-supplemented feed showed better survival and higher disease resistance against yellow-head baculovirus (YHBV) than those fed a normal diet.

The prophylactic efficiency of oral administration of peptidoglycan (PG) was noted by Itami *et al.* (1998). Administration of *Bifidobacterium thermophilum*-derived PG to kuruma shrimp through the diet for 7 consecutive days was then alternated with 7 days without PG throughout a 95-day period. Treated shrimp showed an increased disease resistance when they were challenged through a water-borne infection of *Vibrio penaeicida* and white spot syndrome baculovirus respectively.

Lipopolysacharides (LPS), the outer cell envelope of the gram-negative bacteria is also known for its immmunostimualtory properties in activating both macrophages and lymphocytes (Burrel, 1990). LPS is less toxic to the fish and shrimp and has been shown to be active as an immunostimulatory complex that increases disease resistance in fish (Jorgensen, 1994) and shrimp (Song and Sung, 1990). Bacterin (LPS) from *Aeromonas stenohalis* was effective as an immunostimulant in mouse (Isogai *et al.*, 1989) and showed anti-tumour effects (Chang *et al.*, 1996). Sakai *et al.* (1995) reported the leucocyte activation, including phagocytosis and increased superoxide anion production in rainbow trout induced by *Clostridium butyricum* bacteria. The horseshoe crab proPO system which is composed of a cascade of serine proteases and coagulogen; is located in the secretory granules of the hemocytes and is released and activated by LPS (Levin, 1985).

Chitin is a polysaccharide forming the principle component of crustacean and insect exoskeletons and the cell walls of certain fungi. Sakai *et al.* (1992) reported that rainbow trout, *Oncorhyncus mykiss* injected with chitin showed stimulated macrophage activities and an increased resistance to *V. anguillarum* infection. Chitin is also reported to have shown adjuvant effects in mice and guinea pigs (Nishimura *et al.*, 1985). Several microbial constituents containing N-acetyl glucosamine residues were shown to display immunopotentiating activity. Suzuki *et al.* (1986) reported that both chitin and

chitosan are capable of displaying significant host defence effect in animals challenged with tumour cells and pathogenic microbes similar to those observed in many plant polysaccharides and hexosamine containing microbial constituents. Yellowtail injected with chitin alone also showed increased protection against *P. piscida* challenge which continued until 45 days after the treatment (Kawakami *et al.*, 1998).

Another disease management strategy currently under exploration is nutritional modification. Profound changes in the immune response are some of the earliest manifestations of malnutrition (Mac Farlane and Path, 1977). Improvements in the health status of aquatic organism can certainly be achieved by balancing the diets with regard to nutritional factors, in particular lipids and antioxidative vitamins, which is primarily an input of substrates and co-factors in a complex metabolic system. This is unlike immune-stimulants, which interact directly with the cells of the immune system and make them more active. Nevertheless, some nutritional factors are so intimately interwoven with the biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors beyond the concentration range sufficient to avoid deficiency symptoms of below a certain concentration range (Raa, 2000). This is in the focus of what has been designated nutritional immunology which has been studied in fishes also (Blazer, 1992; Waagbo, 1994).

Fermentation is a process that involves substrate biotransformation by microbial activity leading to enhanced nutritive value of the product. Recently fermented products have been recognized that conferred immuno-potentiating property. The oral administration of fermented chicken egg EF203 to rainbow trout stimulated the activity of leucocytes such as phagocytosis and chemiluminescence and increased protection against streptococcus infection (Yoshida *et al.*, 1993). Sakai *et al.* (1995) have described the immunostimulsatory effects of EF203 in rainbow trout vaccinated with *Renibacterium salmoninarum* bacterin. The vaccinated fish treated with EF203 showed higher phagocytic activities as compared to the vaccinated fish without EF203 treatment or the unvaccinated fish. Increased survival was also noted in EF203 treated fish compared to the other groups following a *R. salmoninarum* challenge.

However, there is a growing understanding of the importance of lymphoid tissues along the digestive tract relative to the immune functions and disease resistance of the whole body (Tristam and Orga, 1994), including fish and shrimp. Moreover, experimental data are beginning to accumulate which show a positive effect on growth and disease resistance in conjunction with immunostimulants in the feed. Yeast and chitosan have been reported to affect non-specific immunity and protection against furunculosis in rainbow trout when mixed into feed (Siwicki *et al.*, 1994). These results show that immunomodulants provide protection when administered the oral route. This

is promising news for aquaculture and aquafeed industry. However, there are still much uncertainities related to the dosage, time of administration, formulation of the feed, etc.

The growth promoting nature of the biotransformed prawn shell waste was demonstrated in the post larvae of *P. indicus* in the previous chapters. In the light of the scientific information gathered, an attempt was made in this chapter to study the efficacy of shell waste fermented products (prepared under optimal conditions) as a dietary ingredient for supporting growth as well as increased protection against microbial infection in *P. indicus* juveniles.

6.2. Materials and Methods.

6.2.1 Preparation of prawn shell fermentation product

Prawn shell powder was prepared following the procedure mentioned in chapter 4 section 4.2.2. Hundred grams each of this shell powder was transferred to 1000 ml conical flasks (6 numbers), plugged with cotton and sterilized at 121°C for 15 minutes. Four strains that gave the best yield in terms of nutritional enrichment under optimized fermentation conditions were selected for this study. They were inoculated separately into four conical flasks and all the strains together as a consortium into another flask. An uninoculated flask kept under similar conditions was the control. Inoculation and incubation were done under optimized conditions finalized by characterization and optimization studies described in chapter 5. Optimum

conditions finalized for the fermentation of prawn shell waste by the selected strains are given below.

Parameters	C111	C124	C134	C163
Moisture[a]	1: 1.75	1: 2	1: 1.75	1: 2
pH	6	6	6	6
Temperature	35°C	30°C	35°C	35°C
Inoculum size[b]	$2x10^8$ cells	$3x10^8$ cells	$2x10^8$ cells	$4x10^8$ cells
Incubation time	20 days	20 days	20 days	20 days

[a] substrate: moistening media (w/v)
[b] inoculum for 5 gm fermentation substrate

After incubation at room temperature (28±2 °C) for 20 days (Fig. 6.1), the fermented products were dried at 60 °C for 48 hrs and stored at 4 °C till used for nutrient analysis and feed preparation.

6.2.2 Preparation of experimental feeds

Experimental feed preparation was done as described in 4.2.6. Six different feeds (Fig.6.2) were prepared incorporating the five different fermented products and raw prawn shell waste (control). These feeds were dried at 50 °C overnight and stored at –20 °C in a freezer.

6.2.3 Proximate composition of fermented products and experimental diets.

Protein in the fermented product was estimated as per Lowry *et al.* (1951), lipid by phospho-vanillin method (Folch *et al.*, 1959) and total sugars by Anthrone method (Roe, 1955). Protein in the experimental diet was estimated by micro-kjeldhal method (Barnes *et al.*, 1959). Ash was determined by incineration at 550°C in a muffle furnace for 5 hrs and

moisture content by drying in an oven at 80°C to constant weight. Fibre content was determined by acid and alkali treatment following AOAC (1995). The nitrogen free extract (NFE) was computed by difference (Crompton and Harris, 1969).

6.2.4 Feeding experiment

Juveniles of Indian white prawn (*Penaeus indicus*) were brought from a farm in Maradu, Cochin, acclimatized to laboratory conditions and maintained on the control diet for a period of one week. They were distributed in fibre glass tanks in triplicates (Fig. 6.3). Feeding experiment was carried out for 28 days. The initial wet body weight of all the prawns in a tank was taken and the average was calculated and represented as initial body weight. Physico-chemical parameters of the rearing water were monitored daily (Table 6.3). Salinity, NH_3N, NO_2-N, NO_3-N and dissolved O_2 were estimated as per APHA (1995). The experiment was terminated on the 28th day and final weight and survival was noted. The final wet body weight of all the prawns in a tank was taken, average was calculated and represented as the final body weight. Statistical analysis of bio-growth parameters were performed using ANOVA. Differences between means were performed by Duncan's multiple range test (SPSS 7.5 package for Windows).

6.2.5 Histology

Histological analysis of hepatopancreas of the experimental animals was carried out to examine whether the fermented product incorporated feeds do have any toxic effect on the animals. For this just after the completion of

the feeding experiment one animal each was removed from all the six different experimental treatments and the hepatopancreas was dissected out and the histological analysis was done as given below.

Fixation and staining of hepatopancreas sections

The hepatopancreas tissue were fixed in Davidson's fixative solution. They were transferred to 70% alcohol for post fixation treatment. The tissues were then transferred to two changes of 90% alcohol and two changes of 100% alcohol for one hour each. They were then transferred to a 1:1 solution of absolute alcohol and methyl benzoate for 30 min until the tissues became transparent after which they were transferred to benzene for 15 min and then xylene saturated with paraffin wax for 6 hrs. The tissues were then infiltrated with two changes of paraffin wax at 58-60°C in a hot air oven for one hour each. The tissues were embedded in paraffin wax at 60-62°C. The blocks with the embedded tissue were sectioned using a microtome at 7.5μ thickness, heat fixed onto albumin coated glass micro-slides, deparaffinized in xylene, hydrated by passing through a descending series (absolute, 90%, 70%, 50% and 30%) of alcohol-distilled water solution. The sections were then stained with Haemotoxylin (Mayers) and Eosin (Scott's) stain and then subjected to an ascending series (70%, 90%, 95% and absolute) of alcohol-distilled water solution cleared in xylene and mounted in DPX.

The sections were viewed and photographed under a light microscope with 20x and 40x lens magnifications.

6.2.6 Challenge of the experimental animals with white spot virus

After the termination of the feeding experiment (28 days) all the treatment groups including the control were maintained under the same rearing conditions mentioned earlier. Challenge with white spot virus (WSSV) was performed through oral administration of the white spot virus (SEMBV) via diet (flesh of infected prawns). The animals were starved for 12 hrs before the challenge to ensure the feeding of the infected prawn flesh (*Penaeus indicus* adult). 24 hrs after challenge, they were maintained on the test feeds and survival was noted everyday till the 10^{th} day post challenge.

6.2.7. Hematological parameters

Hemolymph was taken from *Penaeus indicus* at different times during the feeding and challenge experiment for immunoassays i.e. at the beginning of the feeding experiment, just before challenge (0 hr), 10 hr post challenge and 3 days post challenge.

Hemolymph was drawn from 3 animals maintained on a particular test diet (n = 3) and the various assays were made.

Preparation of the anticoagulant buffer solution

An anticoagulant solution (0.025M Sucrose, 0.01M trisodium citrate in 0.01M Tris HCl) was prepared; pH was adjusted to 7.6 and stored at 4^0C. (Song and Hsieh, 1994)

Hemolymph collection

Hemolymph was drawn from one animal taken from each treatment. Using sterile distilled water, the region near the rostrum was washed. The spot for hemolymph withdrawal was wiped with sterile cotton swabs. Using specially

designed glass capillary tubes (rinsed thoroughly with anticoagulant) hemolymph was withdrawn from the pericardial cavity of the prawn and transferred to an eppendorf rinsed with anticoagulant and maintained at 4°C till further analysis.

The following parameters were then analysed.

1. Total hemocyte count
2. Phenol oxidase assay
3. Nitroblue tetrazolium (NBT) assay
4. Alkaline phosphatase assay

1. Total hemocyte count

An aliquot of fresh hemolymph soon after withdrawal was placed on Neubauer hemocytometer for enumeration of total count of circulating prawn hemocytes. The result was expressed as number of cells per ml hemolymph.

2. Phenoloxidase activity

Phenoloxidase activity was estimated with a spectrophotometer set at 420 nm using L-dihydroxy phenylalanine (L-DOPA, Sigma) as substrate. 100 µl haemolymph was taken in an eppendorf and 100 µl of 1% Sodium dodecyl sulphate (SDS) solution was added. This was added to 2 ml of substrate (0.01 M L-DOPA in 0.05M Tris-HCl, pH7) solution. SDS was used to activate the pro-enzyme (prophenoloxidase). The absorbance at 420 nm was recorded every 30 sec, for a period of 3 min in a Hitachi 200-20 UV-Visible spectrophotometer. One unit of enzyme activity is defined as an increase in absorbance/min/mg protein (Soderhall and Unestam, 1979).

Hemolymph protein

To 100 µl hemolymph 1.9 ml alcohol was added and centrifuged. The supernatant was discarded and the residual pellet consisting of precipitated hemolymph protein was dissolved in 1ml of 1N NaOH and used for protein estimation by Lowry's method (1951).

3. Intracellular superoxide anion (NBT) assay

This test allows to indirectly assess the intracellular superoxide anion ($O^{-2)}$ levels. Nitroblue tetrazolium (NBT) is reduced by O^{-2} produced by phagocytes during the respiratory burst giving a bluish colour.

100µl of haemolymph was added to 100µl 0.05M Tris-HCl buffer containing 2% NaCl taken in an eppendorf tube. 100µl NBT (2mg/ml NBT in Tris-HCl buffer, pH 7.6) solution was added to the samples and incubated at room temperature for 1hr. After incubation, the samples were centrifuged at 7000 rpm for 10 min. The supernatant was carefully tipped out and the residue was washed twice with phosphate buffered salt solution (PBS). After the final wash and centrifugation, the supernatant was discarded and 100% methanol was added to the residue and incubated for 10 min. The supernatant was again discarded after centrifugation and the tubes were dried in a vacuum dessicator for 1 hr. After this, the eppendorf tube was rinsed with 50% methanol 3 to 4 times to fix the residue. This residue was then allowed to solubilize with 60µl KOH. To this 70µl DMSO was added and mixed well. 2 ml of distilled water was added to this coloured solution to make it up to a readable volume. The optical density was read at 620 nm in a Hitachi 200

UV-Visible spectrophotometer against a blank. The blank was prepared using all reagents in the same volume with equal volume of distilled water instead of hemolymph. The optical density was expressed per mg hemocyte protein.

Hemocyte protein

100µl of hemolymph was centrifuged at 2000 rpm in a refrigerated centrifuge (Remi) and washed with phosphate buffered salt solution twice and the supernatant was decanted 0.1 ml of 1N NaOH was added to the residual pellet to dissolve it and this was used for protein estimation by Lowry's method (1951).

4. Alkaline phosphatase assay

Alkaline phosphatase catalyse the hydrolytic cleavage of phosphoric acid esters and their pH optima lie in the alkaline pH range of 9.0. The procedure was carried out according to Gonzales *et al.*, (1994).

100µl of hemolymph solution was added to 2 ml of 4-nitrophenyl phosphate substrate solution (0.5% in Glycine-NaOH buffer (pH 9.0)). The mixture was incubated at 37^0C for 30 min. At the end of the incubation period the enzyme reaction was terminated by adding 2.9 ml 0.1N NaOH. The yellow coloured solution was read against a blank at 405 nm. The blank is prepared by incubating a mixture of 2.9 ml 0.1N NaOH and 2 ml of substrate solution to which finally 100µl of hemolymph was added.

6.3 Results

6.3.1 Proximate composition of fermented product generated under optimum conditions.

Protein content was highest (51.0%) in fermented product produced by the consortia. The highest lipid (10.08%) and carbohydrates (5.4%) were observed in FPO124 produced by *Bacillus* sp. C124 under optimum conditions (Table 6.4). Considerable increase in the protein and lipid content could be observed in the various fermented products compared to the control. Fermented product generated under optimal conditions by the various strains.

Sl. No.	Culture	Fermented product generated under optimal conditions	Feed prepared
1	*Bacillus* sp. C111	FPO 111	F111
2	*Bacillus* sp. C24	FPO124	F124
3	*Bacillus* sp. C134	FPO134	F134
4	Serratiasp. C163	FPO163	F163
5	Consortia	FPOCs	F Cs

6.3.2 Proximate composition of experimental feed

The proximate composition of experimental feed prepared is tabulated in Table 6.5. Protein content varied in different feeds with the maximum in feed FCs (59.2%) followed by feed F111 (58.8%), F134 (57.8%) and feed F124 (52.3%). Lowest protein content (44.3%) was recorded in diet F163. Lipid was highest in F124 (9.9%) followed by F111 (9%) and F134 (9%). Ash content was slightly higher for all the fermented products compared to

the control but there was no considerable variation between the experimental feeds. Fibre content was more or less the same for all the feeds ranging from 4.3 to 5.4%. There was considerable variation in NFE of the experimental feed. Maximum (35.6%) was recorded in the control feed followed by feed F163 (28.6%). Feed FCs recorded the lowest NFE (11.8%).

6.3.3 Bio-growth parameters

The data collected from the feeding experiment were analysed and the bio-growth parameters like production, food conversion ratio (FCR), specific growth rate (SGR), relative growth rate (RGR), gross growth efficiency (GGE), consumption per unit weight per day (CUD) and protein efficiency ratio (PER) were determined and are presented in Fig. 6.5 to 6.11. An overall analysis of the bio-growth parameters showed that the performance of feed F111 and F134 was the best when compared to the other feeds. Production, FCR, GGE, etc. of the shrimps fed feeds F111 and F134 were found to be significantly different from the other four feeds. F124 is next in the order of performance and the growth parameters were found to be significantly different from those of the best two feeds (F111 and F134) and the other feeds (F163, FCS) including control feed. Performance of feeds F163 and FCS are found to be poor since no significant increase in the growth parameters could be observed when compared to the control.

The highest production was recorded in prawns fed with feed F111 (1.4gm) followed by F134 (1.25gm) and the lowest (0.48gm) was recorded in FCs. Increase in weight exhibited by feeds F111 and F134 was not

significantly different from each other but was significantly different from all the other feeds including the control feed. Food conversion ratio was found to be the best with feeds F111 and F134 (2.35 and 2.76 respectively) when compared to F124 (4.15) as given in Fig. 6.6. FCR obtained by feeds F163 and FCs (6.58 and 6.78 respectively) were found to be comparable to that of the control feed (7.7) and were significantly different from the values of F111 and F134. Specific growth rate (SGR), relative growth rate (RGR) and gross growth efficiency (GGE) are parameters which express the different facets of growth efficiency and a similar trend of best performance exhibited by diet F111 followed by F134 was observed for all these parameters recorded. Specific growth rate (Fig.6.7) among the dietary treatments varied significantly and the highest specific growth rate was with diet F111 (1.39). The SGR of the other diets F124, F134 (0.87 and 1.18) varied significantly from F163 (0.53) and FCs (0.55).

Relative growth rate ranged from 0.40 to 1.38 (Fig. 6.8). The highest value was recorded for F111 (1.38), which was significantly different, from all other feeds statistically. The RGR was significantly higher in F111 (1.38) followed by F134 (1.17) and F124 (0.87).

The highest GGE value was for feed F111 (43.04) followed by F134 (36.63) and F124 (24.67) (Fig. 6.9). The GGE of feeds F111 and F134 was significantly different from the rest of the feeds. In the case of consumption

per unit weight per day (CUD), no significant difference could be obtained between the various feeds including control (Fig.6.10).

PER was highest with F111 (1.84) followed by F134 (1.51). There exists no statistically significant difference in PER values among the dietary treatments (Fig. 6.11).

Duncan's multiple range analysis of the various growth parameters effected by the different feeds showed that the performance of feed F134 and F111 was significantly different from other feeds. Feed F124 also vary significantly from the control feed and the other two F163 and FCs.

6.3.4. Histological examination of hepatopancreas

Histological analysis of the hepatopancreas did not show any structural or functional abnormalities with feeds F111, F124, F134, F163 and control feed. However, feed FCs showed disruption of the basal membrane and damage of the tubules indicating possible presence of toxic components in the fermented product.

Fig. 6.12 a&b show the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed the control feed. This has been taken as the reference for the comparative studies to evaluate the toxic effects of the experimental diets. The mature B cells are seen in the tubules which are compactly arranged. The healthy tubules are with their form intact and possess a characteristic stellate luminal space.

Fig. 6.13 a&b show the CS of hepatopancreas tubules of the midgut-gland of shrimps fed feed F111. The B-cells are more in number and larger suggesting an active and healthy condition of digestive processes.

Fig. 6.14 a&b show the CS of hepatopancreas tubules of the midgut-gland of shrimps fed feed F124. The normal healthy structure comparable to that of the control is observed and no degenerative changes are noticeable in the tubules. B cells are distinctly visible signifying a healthy digestive function.

Fig. 6.15 a&b show the CS of hepatopancreas tubules of the midgut-gland of shrimps fed feed F134. There is a significant reduction in the number and size of the B cells in each tubule. There also appears to be a marginal shrinkage in all tubules.

Fig. 6.16 a&b show the CS of hepatopancreas tubules of shrimps fed feed F163. The details observable include active B cells which are comparable to those observed in those fed on the control feed. A compression of the lumen space is noticeable.

Fig. 6.17 a&b show the CS of hepatopancreas tubules of shrimps fed feed FCs. There is a clear and distinct variation observed in this treatment group. The tubules are not completely obliterated or damaged. A slight disruption of the basal membrane is observable.

6.3. 5. Post challenge survival

Post challenge survival was found to be significantly high with *P. indicus* maintained on feed F134 compared to the other feeds (Fig.6.24). 3rd day post challenge data was almost similar for feeds F124, F163 and FCs. However on the 7th day the survival percentage of *P. indicus* reduced considerably for all the feeds. 10th day post challenge survival was found to be significantly high (75%) for feed F134 and the RPS for all other feeds were almost at the same level on that day (Fig.6.25). Death by WSSV infection was confirmed by the presence of white spots on the carapace of the infected prawns (Fig. 6.23).

6.3.6 Hematological parameters.

Feed F134 exhibited significant immunostimualtory property being evidenced by better post challenge survival and hematological profile supporting an immune boost up. The other feeds F111, F124, F163 and FCs did not show a significant increase in the performance compared to the control feed.

Hemocyte count

Post challenge hemocyte count was also found to be maximal for prawns fed with feed F134 and a gradual increase was found over the post challenge period. *P. indicus* fed on feeds F111 and FCs also showed considerable increase in hemocyte count on the 3rd day (Fig. 6.18).

Phenol oxidase activity

Generally phenol oxidase activity was found to be high for *P. indicus* fed on feed F124 and F134 (Fig.6.19). The PO activity was considerably high at 10 hr post challenge for these two treatment groups. Considerable reduction in the PO activity could be noticed on the 3rd day post challenge for all the treatment groups.

NBT activity

Superoxide anion formation was estimated and is presented in Fig.6.20. *P. indicus* maintained on feed F134 showed the best activity followed by feed F163. Unlike phenol oxidase an elevated NBT activity could be observed on 3rd day post challenge with feed F134.

Alkaline phosphatase activity

Alkaline phosphatase activity of hemolymph expressed in terms of hemolymph protein was found to be high for *P. indicus* fed on F134 and maximum activity was found on 3rd day post challenge (Fig. 6.21). A gradual elevation of the alkaline phosphatase activity could be observed for this treatment group which was not observed with other feeds. A maximum at 10 hr PC could be observed for feeds F111, F163, FCs and control feed.

Feedwise comparison of hematological parameters

Fig.6.22 shows the hematological parameters supported by various diets at different time intervals post challenge with WSSV. A 10th hr peak could be observed for various hematological parameters in the case *P. indicus*

fed on of feeds F111, F163 and FCs whereas F134 supported a 3rd day hike in the various immunological parameters.

Duncan's multiple range analysis of the hematological parameters at different time intervals showed that significant variation exists between the performance at different time periods (post challenge WSSV) with the peak being at 10 hr for most of the feed except feed F134 which exhibited a third day peak (See Appendix 2).

6.4 Discussion

Optimal conditions employed for developing shell waste fermented product used in the final feeding experiment have favoured the nutrient quality of the product especially that of protein and total sugars with lipid content maintained at the maximum attained previously under unoptimized conditions.

6.4.1 Growth parameters

P. indicus fed on feeds F111 and F134 gave the best performance consistently in all the bio-growth parameters recorded. This showed difference in the profile of bio-growth parameter values recorded in chapter four, with feeds incorporating unoptimized fermented products. All these results strongly emphasize the favourability of optimal conditions to bring out a better enriched product whose efficiency is correspondingly reflected in the growth performance of the experimental animals. This also confirms the fact

that enhanced content of nutrients like protein, lipid and carbohydrate plays a significant role in weight gain and overall growth efficiency.

The superior growth performance obtained with the feeds may be attributed to several factors like the quantity of nutrient present, presence of hydrolysed fermentation products of chitin, which is a known growth promotor and other vitamins and minerals, microbial biomass, etc. The good quality of protein contributed both by shell waste hydrolysis and microbial biomass has been noted earlier (Sick and Andrews, 1973; Sandifer and Joseph, 1976; Lim *et al.*, 1979; Kanazawa *et al.*, 1979a; Menesveta *et al.*, 1983; Deshimaru *et al.*, 1985 ; Penaflorida, 1989).

Growth and survival are the most authentic parameters used to test the effectiveness of a diet. Variable external factors like water quality, temperature and disease might affect the results because these tests have to be conducted for several weeks (Storch, 1984). The survival of prawns fed fermented products showed better performance than the control feed. The optimization of the fermentation process could have contributed to the enhancement of nutrients, which are specific or necessary for supporting resistance in general. Bacterial components could also have supported growth to a certain extent. Three of the strains used in the present study for fermentation leading to fermentation product FP111, FP124 and FP134 belong to *Bacillus* sp..

6.4.2 Histology of hepatopancreas

Histological examination of the hepatopancreas of experimental animals from each treatment group was done at the end of the feeding experiment to assess the toxic effects of the diet if any. It is well known that a close correlation exists between the nutritional state and the ultrastructure of the liver or midgut-gland cells (Storch, 1984; Storch *et al.*, 1984). The effect of different diets on midgut-gland cells of crustacea was reported in decapods (Storch and Anger, 1983,), isopods (Storch, 1984) and amphipods (Storch and Burkhardt, 1984,). The principal functions of the midgut-gland are secretion of digestive enzymes and absorption of nutrients (Dall and Moriarity, 1983).

Basically four cell types can be recognized, which are widely known as the E-(embryonic or undifferentiated), F-(fibrillar), R-(resorptive or absorptive) and B-(secretory cells). Ultrastructural and histological studies indicate that each cell type possesses its own specific role in the functioning of the hepatopancreas. The F-cells, which synthesize digestive enzymes, become transformed into secretory B-cells. These are the largest of the cell types and characteristically contain a single enormous vacuole, which may occupy 80-90% of the total cell volume (Gibson and Barker, 1979). B cells are generally indicative of enhanced rate of digestion or elimination of waste materials (Gibson and Barker, 1979; Hopkin and Nott, 1980).

The histological examination of the hepatopancreas was carried out in an attempt to evaluate the toxic effects, if any, in the fermented product

incorporated in the experimental feed fed to the prawn. The cross section of hepatopancreas tubules of the midgut-gland of shrimps fed the control feed has been taken as the reference for the comparative studies. Analysis of midgut-gland status was done assuming that a comparable state of digestion was maintained. The mature B cells are seen in the tubules, which are compactly arranged. The healthy tubules are with their form intact and possess a characteristic stellate luminal space. The B cells in the CS of hepatopancreas tubules of the midgut-gland of shrimps fed feed F111 are more in number and larger suggesting an active and healthy condition of digestive processes. This picture is comparable to that of the control feed and may even suggest better activity by the larger size and number of B cell vacuoles. The normal healthy structure comparable to that of the control is observed and no degenerative changes are noticeable in the tubules of hepatopancreas of shrimps fed on feed F124. The CS of hepatopancreas tubules of the midgut-gland of shrimps fed feed F134 depict a significant reduction in the number and size of the B cells in each tubule. There also appears to be a marginal shrinkage in all tubules. No explanation can be found for this at this point of study. The slight structural deformities could be suggestive of changes that develop after prolonged feeding of a particular feed type. In some cases, the B cells' secretion involves the total liberation of the cell (i.e., holocrine discharge of digestive enzymes), followed by a phase where post-secretory B cells must either ultimately degenerate and/or be

voided from the epithelium or become reconstituted (Gibson and Barker, 1979).

The details observable in the CS of hepatopancreas tubules of the midgut-gland of shrimps fed feed F124, include active B cells which are comparable to those observed in those fed on the control feed. The compression of the lumen space could indicate reduced feed intake.

In the CS of hepatopancreas tubules of shrimps fed feed FCs, there is a clear and distinct variation observable in this treatment group. The tubules are not completely obliterated or damaged. A slight disruption of the basal membrane could suggest the loss of membrane stability. A similar pattern was observed in the midgut-gland cells of *Penaeus monodon* fed diets deficient in vitamins (Reddy *et al.*, 1999). The nature and level of carbohydrates in the diet can have an effect on the histological structure of digestive glands of *P. monodon* (Pascaul *et al.*, 1983).

The histological analysis shows that the fermented product incorporated feeds (except FCs) do not contain any toxic components and they can very well be used as an ingredient in aquaculture feed.

6.4.3 Hematological parameters

Phenoloxidase activity was maximum for shrimp fed with feed F124 followed by F134, both registering the peak activity at 10 hr post challenge. Almost all 0 hr (before challenge) values for all feeds were comparable to the initial value which reflect the capability of experimental feeds to maintain the

immunity at the base level. The 10 hr post challenge results by the various feed vary with elevated responses exhibited by feeds F124 and F134 respectively. But feeds F111, F163 and FCs showed a significant dip at 10 hr PC that may signify different levels of activity triggered by different factors available. The quantity and type of available immunostimulant and many other factors could have a part in these varying responses. The dosage level of available immunostimulatory component has an important role in supporting disease resistance as shown by Cardenas and Dankert (1997) in crayfish.

An interesting feature observed during the measurement of phenoloxidase activity of the hemolymph in the present study was the darkening of the hemolymph just after it drawn from the shrimp fed with feed F163. An intensification of the dark colour was observed with time. However, the phenoloxidase activity by feed F163 was on the lower side when compared to the other feeds. This darkening of hemolymph could be due to the triggering of proPO enzyme activity and melanin formation. More studies must be pursued to unravel this phenomenon. However, the available literature suggests that in higher concentrations, the immunostimulant itself can trigger the phenoloxidase activity and lead to exhaustion, which may increase disease susceptibility, and priming of macrophages to lower doses of inducers may increase resistance.

The hemocytes constitute the first line of defense against invaders and are crucial in the defense reactions of crustaceans and it is essential to study

the hemocyte parameters. Hemocyte functions such as phagocytosis, chemotaxis, lysosomal enzyme activities as well as hemocyte production in the hemopoietic tissue have been studied by many workers (Supamattaya, 1998: Vargas-Alborez *et al.*, 1998).

Enhanced phenoloxidase activity was observed in hemocytes of *Penaeus monodon* given an oral administration of *Vibrio* bacterin and yeast glucan (Devaraja *et al.*, 1998a). The effect of different concentrations of LPS in the red swamp crayfish, *Procambarus clarkii* in the activation of proPO system has been studied by Cardenas and Dankert (1997).

When pathogens are engulfed by hemocytes a series of microbicidal substances are generated. These include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxide ions (OH^-), singlet oxygen (O_2^1); myeloperoxidase (MPO)-catalysed hypochlorite and digestive enzymes within cytoplasmic granules (Segal, 1989). They can either inhibit microbial activities or completely digest the microorganism. Since O_2^- is the first product released during respiratory burst, O_2^- concentration has been accepted as an accurate parameter to quantify the intensity of a respiratory burst (Secombes, 1990).

The results of the present study showed that NBT activity in prawn hemolymph was highest for feeds F134, F163 and FCs. While feed F134 supported a gradual increase with each sampling time period, with a very high activity by the 3rd day PC. Feeds F124, F163 and FCs showed increased

activity on the 10 hr post challenge. The response of F111 was almost similar in pattern to that of the control. The increasing trend in NBT activity supported by feed F134 showed that the activity confers extended immunoprotection. Those feeds with lower level 3rd day PC response after a hike at 10 hr PC, suggest the differential effect of various feeds as immunostimulants. Similar NBT reduction assays have been reported for immune potential determination in prawns and fishes (Santarenes *et al.*, 1997; Campoz-Perez *et al.*, 1997; Devaraja *et al.*, 1998b).

The potential for microbicidal activity through release of degradative enzymes during phagocytosis is another important method of cellular immune defence in invertebrates (Cheng, 1975; Coles and Pipe, 1994; Pipe, 1990; Lopez *et al.*, 1997). The alkaline phosphatase activity in the hemolymph collected from experimental animals was measured to understand its activity during WSSV challenge. Results of the present study showed that alkaline phosphatase activity could be used as one of the reliable assays for determining the immune potential of the animal.

Increased total numbers of circulating hemocytes occur by stimulation or migration of cells from tissues rather than by blood cell proliferation. This may or may not result in altered relative proportions of hemocyte types depending on the distribution within the tissues of blood cell types in a particular individual or a species (Pipe, 1990). The maximum number of hemocytes was recorded in *P. indicus* fed feed F134 especially on 3rd day PC.

FCs and F111 also showed a gradually increasing hemocyte count post challenge. The significant increase in hemocyte cell count with time by feed F134 may have contributed to its overall superior performance in terms of phenoloxidase activity, NBT activity and alkaline phosphatase activity. This supports the fact that the hemocytes in circulation are directly responsible for immunoresponses measured. A similar correlation can be drawn on F111 and F163, which showed comparatively poorer performance and comparable to the control feed. Similar changes in the number and character of circulating hemocytes have been recorded in mussels during exposure to WSSV (Pipe *et al*, 1992). The migration of hemocytes treated with *Vibrio* bacterin increased compared to non-treated controls (Itami *et al.*, 1989).

The control feed has shown a slight increase in immune response after stimulation though it wasn't strong enough to resist the disease as evidenced by the low survival rates recorded in the control feed. There was considerable increment in the NBT activity especially at the 10 hr PC. F111 showed a favourable response, which peaked at 10 hr. PC that was comparable to the 10 hr. PC response of the control feed in its pattern. Shrimps fed with feed F124 helped maintain the alkaline phosphatase activity on a higher side at 0 hr. when compared to the other feeds. However, after the challenge with WSSV, alkaline phosphatase activity of feed F124 was inferior to other feeds. It has fared poorer than the control feed. F134 has put forth a commendable performance as evidenced by its performance in stimulating all

immunological responses noted. A detailed analysis of the performance of F134 showed its consistent ranking as the best. Another interesting observation for feed F134 was its gradual increase in immunological response, which could indicate higher readings beyond the 3rd day PC. This sustained rise in defence activity is confirmed by the excellent survival rates registered by F134 (Fig. 6.24) suggesting a longer duration of immunoprotection. F163 has responded positively at the 10 hr PC in terms of NBT activity and alkaline phosphatase activity. The very insignificant phenoloxidase activity at the 10 hr PC may be due to the melanization observed by blackening of the hemolymph immediately after it was taken from the animal, an exhaustion of the phenol oxidase system before an estimation could be made. Feed FCs has elicited a response comparable to that of the control feed both in its pattern and quantity of each response observed.

As seen in all the feeds, it is confirmed that the maximum response was observed within 10 hours of the challenge or stimulation. The wide gap in time between 10 hr and 3rd day post challenge have obliterated the actual peak in activity of F134 but responses for the other feeds showed that the defence reactions are definitively on a higher side within 10 hr post challenge.

Feed F134 showed a remarkable resilience with 80% survival on 3rd day PC and it remained consistent at 75% on the 7th day and the 10th day PC showing a prolonged effect of protection. It has exhibited an equally good

performance in the immunoassays. Almost all other feeds reflected a diminishing resistance with time registering a gradual increase in mortality. The whole picture showed a general trend in all feeds tested as being capable of supporting greater survival compared to control feed apart from growth recorded earlier. Feed F134 proved its dual capacity to support growth and survival remarkably well taking up the second best position in all the bio-growth parameters recorded. On the other hand F111, which showed superior growth enhancing properties, failed to exhibit comparable performance in the face of the challenge registering poor survival, compared to the other feeds. Feed F134 holds promise as a valuable addition to the aquaculture industry by its cost effectiveness, growth enhancing and immunostimulating properties. There seems to be a strong correlation between growth promotion and immunoprotection conferred by the presence of some immunostimulants in fermented prawn shell waste. Such a correlation is evident in shrimps fed with F134 from the results obtained. Similarly, Laramore (1992) reported an increase of 17 percent in total yield in post larvae *Penaeus vannamei* exposed to formalin-killed suspension of *Vibrio*. Another similar experiment recorded 40% increase in yield in treated animals (Laramore, 1992).

Treatment of lipopolysaccharide (LPS) can have significant benefit on the survival, growth and disease resistance of shrimps (Itami *et al.*, 1992, 1994). Newman (1996) studied the effects of LPS of gram-negative bacteria on penaeids. The effect of LPS as immunostimulant for shrimp was further

reported by Rukyani *et al.* (1999). Takahashi *et al.*, (1999) reported the enhancement of disease resistance in kuruma shrimp, *P. japonicus*, against white spot syndrome after oral administration of a lipopolysaccharide extracted form *Pantoea agglomerans*. Hemocyte phenoloxidase activities of LPS fed shrimps were significantly higher.

There are two types of bacterial components of interest in the present experiment that are believed to confer immunostimulant properties. They are the peptidoglycan of gram-positive bacteria in feeds F111, F124 and F134 and lipopolysaccharide characteristic of gram-negative bacteria in F163. Feed F163 is a product of a red-pigmented Enterobacteriaceae strain, which could additionally contain the red pigment carotenoid.

The factors that could have contributed to the efficacy of feeds as immunostimulant are LPS, carotenoid, low oligomeric hydrolysed chitin from prawn shell, an optimal balance of nutrients and vitamins as products of microbial fermentation and the high lipid content rich in polyunsaturated fatty acids. High levels of n-3 polyunsaturated fatty acids have been proposed to increase disease resistance in fish (Salte *et al.*, 1998) and it has been demonstrated that the cell membranes of salmon feeding on such diets became stronger and more resistant to lysis (Erdal *et al.*,1991) because the fluidity of the membrane is a function of fatty acid composition of the membrane lipids, which are affected by dietary lipids (Waagbo, 1994).

Feed F134, F124 and F111 are of the gram-positive *Bacillus* sp. and the immunoprotection exhibited by the corresponding feed may be due to the peptidoglycan factor present. All the feeds tested have responded well compared to the control feed. F111, which gave superior performance in the growth studies, failed to show an equally good immunoprotective capacity. However, in the first feeding experiment, maximum survival could be observed with this feed. One of the factors that may explain its poor performance in this experiment may be the dosage related variance of immunostimulation. Usually immunostimulation does not show a linear relation between dose and effect. But a maximum effect at intermediate dose and no effect and even toxicity at high dose (Gialdroni-Grassi and Grassi, 1985) is noted. This has been established in fish in vivo (Olivier *et al.*, 1985; Yano *et al.*, 1989, 1991; Robertsen *et al.*, 1990; Andersen, 1992) and in vitro (Siwicki *et al.*, 1990; Robertsen, 1994). These authors observed an increment in the mortality and suppression of the nonspecific immune mechanisms at high dose of immunostimulants such as M-glucan (Robertsen *et al.*, 1990), levamisole (Andersen, 1992) and Freund's adjuvant (Olivier *et al.*, 1985). According to Andersen (1992), too little of immunostimulant will result in no protection where as too much may cause immunosuppression. Overdose of immunostimulant by feeding or bio accumulating sometimes will induce an adverse effect in shrimp that may be accounted for by overproduction of reactive oxygen intermediates (ROIs) or non-specific host injury.

Since prawns do not have specific memory immunity as in fishes, their non-specific immune system requires the regular use or at least intermittent use of immunostimulant. The results obtained for the duration of protection are similar to that of Sung and Song (1996) who traced the tissue location of heat killed *Vibrio vulnificus* antigen delivered by immersion to tiger shrimp *P. monodon.* At selected time intervals (5 min, 3, 6 and 12 hours and 1, 3, 7 and 14 days) following infection, they traced the mode and path of uptake in hemolymph and tissues. By day 14, the antigen was completely cleared undetectable. Survival/protection against infection may be attributed to an increase in resistance to disease due to an unknown nutrient/nutrients provided by the fermented product.

The phenomenon of immunoprotection exhibited by fermented prawn shell products incorporated experimental feeds reveal an interesting aspect which warrants further study. A detailed analysis of the fermented prawn shell product is necessary to understand the components in the nutrient profile that may contribute to this effect.

Table 6.1 Literature review of immuno-stimulants applied in shrimp

Source		Chemical structure	Host	Effect of Penaeid shrimp									References
				Phagocytic ctivity	Prod. of α -microbial substances	α -vibriosis	α -viral disease	Duration of protection (day)	Growth	Survival	FCR	Tolerance to stress	
Fungus	*Saccharomyces cerevisiae*	β-1, 3-1, 6-glucan	*P. monodon*		+	+	+	18	±	+	-	+	Sung et al.1994
	Schizophyllum commune	β-1, 3-glucan	*P. monodon*			+		120					Song et al. 1997
Bacterium	*Brevibacterium lactofermentum*	Peptidoglycan	*P. monodon*	+		+	+	20					Liao et al. 1996
	Bifidobacterium thermophilum	Peptidoglycan	*P. japonicus*	+		+	+	20	+	+	+	+	Boonyaratpalin et al. 1995
	Heat-killed *Vibrio alginolyticus*		*P. stylirostris*			+		95					Itami et al.1998
	Formalin-killed *Vibrio sp.*		*P. japonicus*			+		120					Lewis et al. 1982
	Vibrio alginolyticus		*P. monodon*			+		50					Teunissen et al. 1998
Dietary componen t	Astaxanthin and ascorbic acid – polyphosphate HUFA		*P. monodon*										Merchie et al. 1998
			P. kerathurus										Mourente and Rofriguez, 1997

Table 6.2 Percentage usage of feed ingredients in diets containing five different optimized fermented prawn shell product

Ingredients	Control diet (%) (F1) gm	Experimental diet (%) (F2-F6) gm
Prawn shell powder	25	--
Fermented prawn shell[a]	_	25
Fish meal	28	28
Groundnut oil cake	8	8
Soyabean meal	15	15
Refined wheat flour	10	10
Rice bran	10	10
Vitamin and mineral mix[b]	2	2
Agar	2	2
Water	100 ml	100 ml

[a] 5 different fermented products were generated using pawn shell waste under optimized fermentation condition with the four bacterial strains and a consortium of all these bacterial strains.

[b] Vitamin and mineral mix (mg/g vitamin and mineral mix)

Thiamine	0.61 mg
Riboflavin	0.48mg
Panthothenic acid	2.42mg
Pyridoxine	0.72mg
Cyanocobalamine	0.02mg
Biotin	0.02mg
Retinol	0.13mg
Menaptone	0.24mg
Folic acid	0.13mg
Niacin	2.42mg
∝ tocopherol	2.42mg
Banox	0.30mg
Cholecalciferol	0.06mg
Ascorbic acid	6.05mg
K_2HPO_4	4.68mg
$Ca_8(PO)_4$	6.36mg
$MgSO_4.7H_2O$	7.12mg
$NaH_2PO4.2H_2O$	1.84mg

Table 6.3 Rearing conditions and Water quality parameters of the growth experiment

Rearing and feeding conditions

Initial body weight	: 3.05 ± 0.33 g
Number of prawn Stocked	: 10 prawns/per tank
Water volume	: 30L
Feeding level	: 10-15 % body weight
Feeding frequency	: Twice daily
Feeding period	: 28 days

Water Quality parameters

Water temperature	: 27-30°C
pH	: 7-7.5
Salinity (ppt)	: 24 -25 ppt
NH_3 (mg/l)	: 0.01-0.02 mgL^{-1}
NO_3 (mg/l)	: n.d
NO_2 (mg/l)	: < 0.01 mgL^{-1}
Dissolved O_2 (mg/l)	: 7-8 mgL^{-1}

n.d = not detectable

Table 6.4 Proximate composition of nutrients in the optimized fermented product (% dry weight)

Fermented product	Protein	Lipid	Total sugars
Control	33.7	4.9	2.1
FPO111	50.1	8.2	4.3
FPO124	42.7	10.1	5.4
FPO134	50.0	8.8	2.6
FPO163	41.1	6.2	3.0
FPOCs	51.0	7.2	3.2

Table. 6.5 Proximate composition of feed

Feed	Crude Protein (%)	Crude lipid (%)	Ash (%)	Crude fiber (%)	Moisture (%)	NFE (%)[1]
Control	41.2	6.8	10.7	4.3	1.45	35.6
F111	58.8	9.0	12.8	4.7	2.40	12.3
F124	52.3	9.9	12.6	5.2	2.42	17.5
F134	57.8	9.0	12.9	4.9	2.18	13.2
F163	44.3	7.0	12.6	5.4	2.16	28.6
FCs	59.2	8.0	12.6	5.3	3.11	11.8

[1]By difference

NFE = nitrogen free extract

Fig 6.1 Fermentation of prawn shell waste being carried out in Erlenmeyer flasks under laboratory conditions

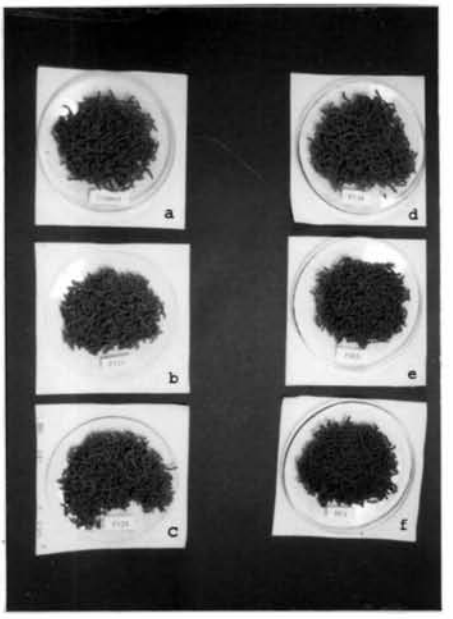
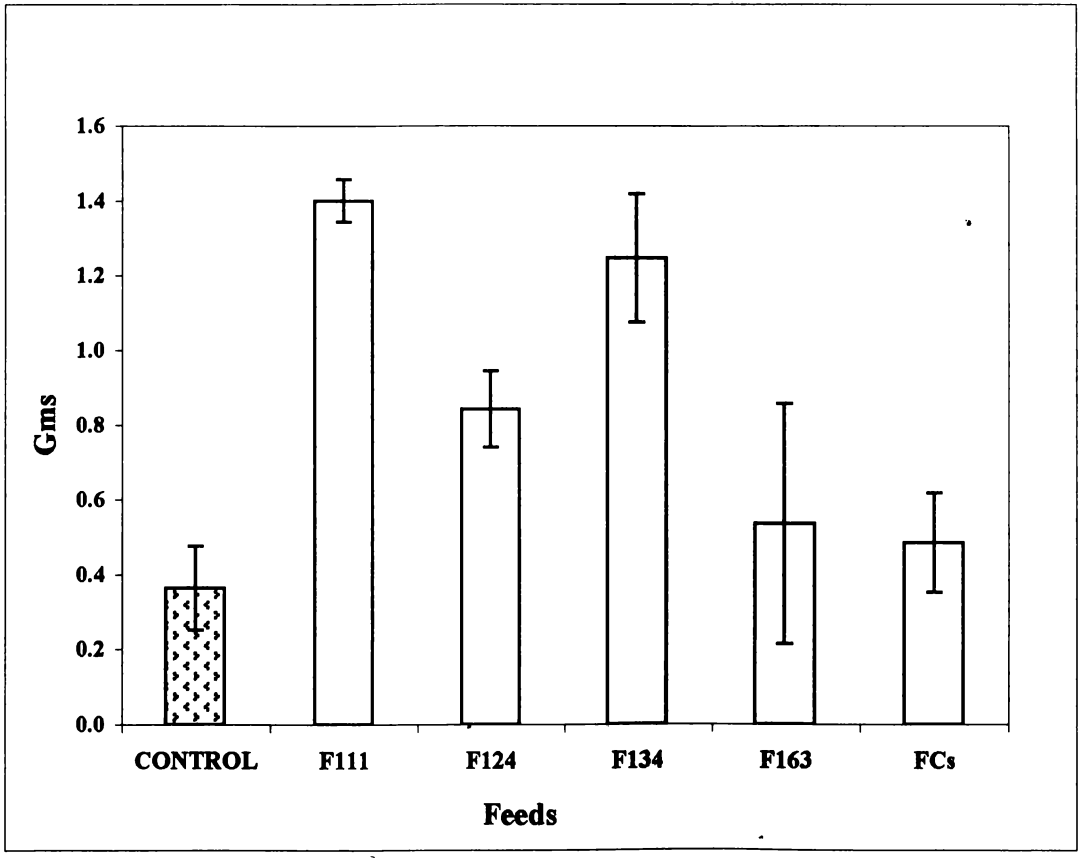


Fig 6.2 Experimental diets prepared by incorporating the fermented products generated by the various strains (a-f)

a. Control
b. F111
c. F124
d. F134
e. F163
f. FCs (consortium)

Fig. 6 3 Experimental setup for testing the efficacy of selected feeds in *Penaeus indicus* juveniles

Fig.6.4 Close-up view of single experimental tank.

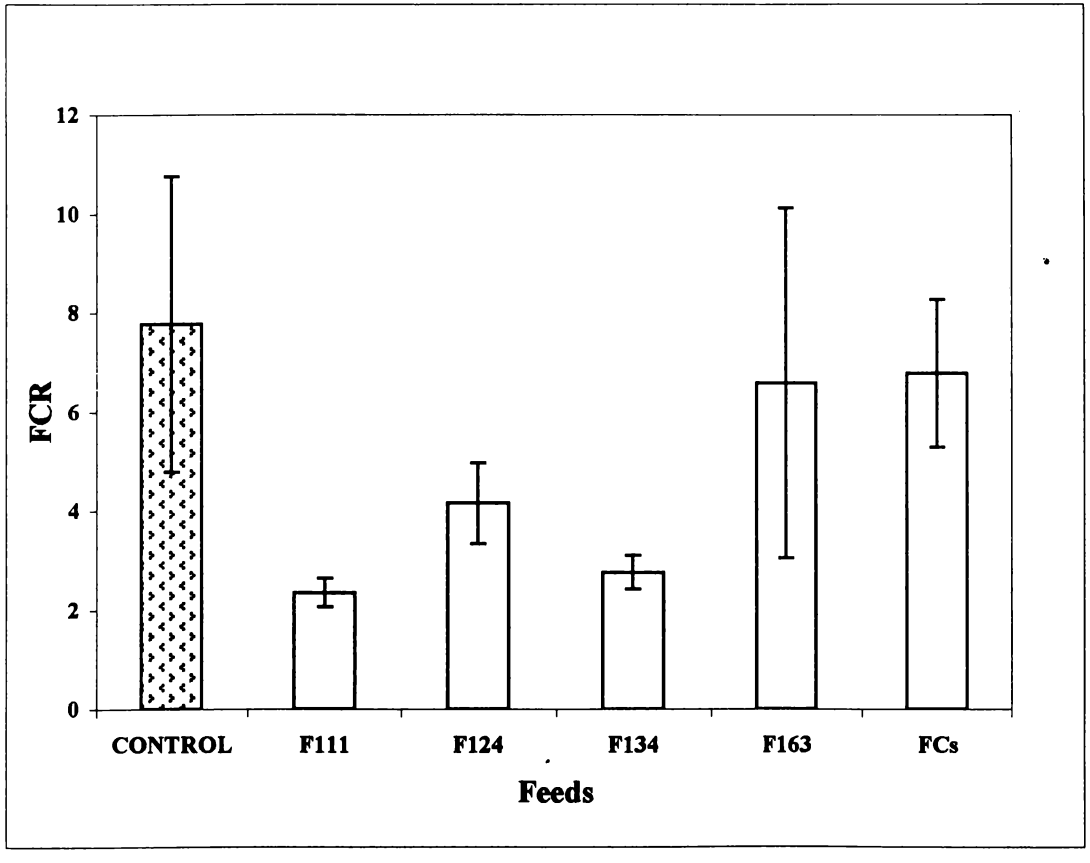


Feed	*PRO
CONTROL	0.36 ± 0.11^{a}
FC111	1.4 ± 0.06^{c}
F124	0.84 ± 0.10^{b}
F134	1.25 ± 0.17^{c}
F163	0.54 ± 0.32^{ab}
FCs	0.48 ± 0.13^{a}

*(mean + s.d.)

* Values with the same superscript does not vary significantly.

Fig. 6.5. Weight gain (production) obtained in *P. indicus* juveniles when fed with various experimental diets

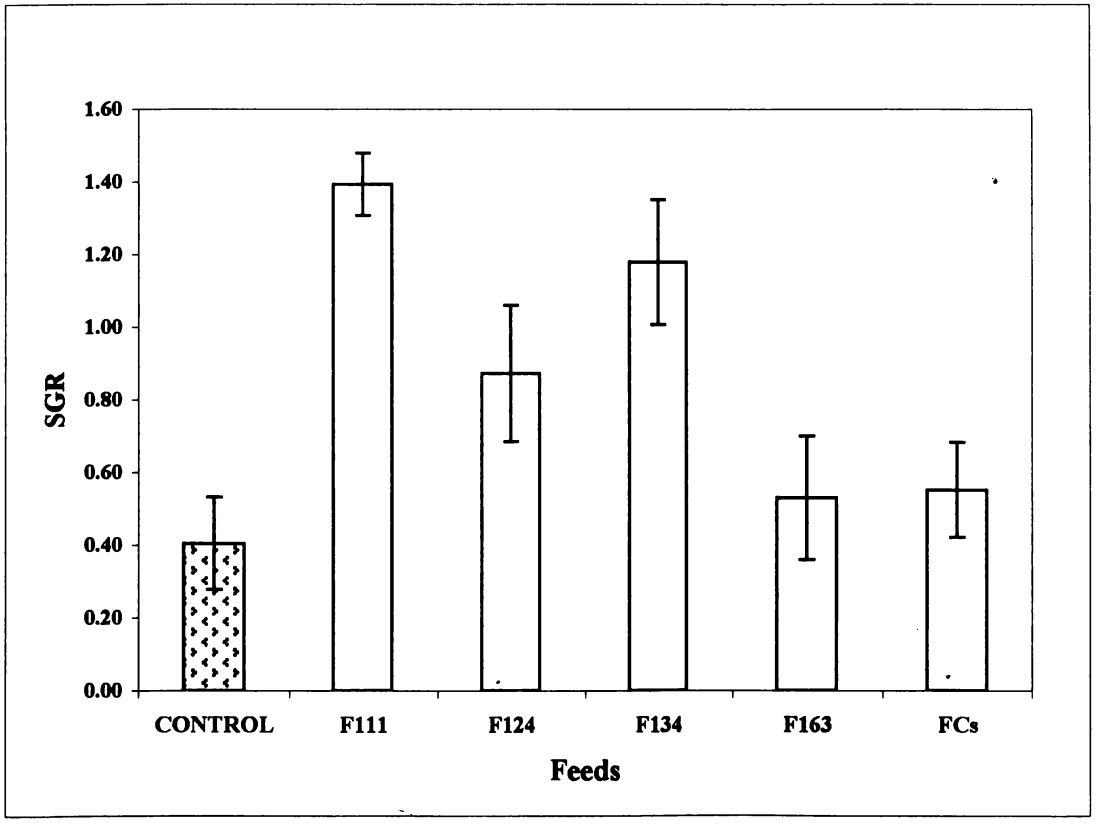


Feed	*FCR
CONTROL	7.7 ± 2.98^{b}
FC111	2.35 ± 0.28^{a}
F124	4.15 ± 0.81^{ab}
F134	2.76 ± 0.34^{a}
F163	6.58 ± 3.53^{b}
FCs	6.78 ± 1.49^{b}

*(mean ± s.d.)

* Values with the same superscript does not vary significantly.

Fig. 6.6. Food Conversion Ratio obtained in *P. indicus* juveniles when fed with various experimental diets

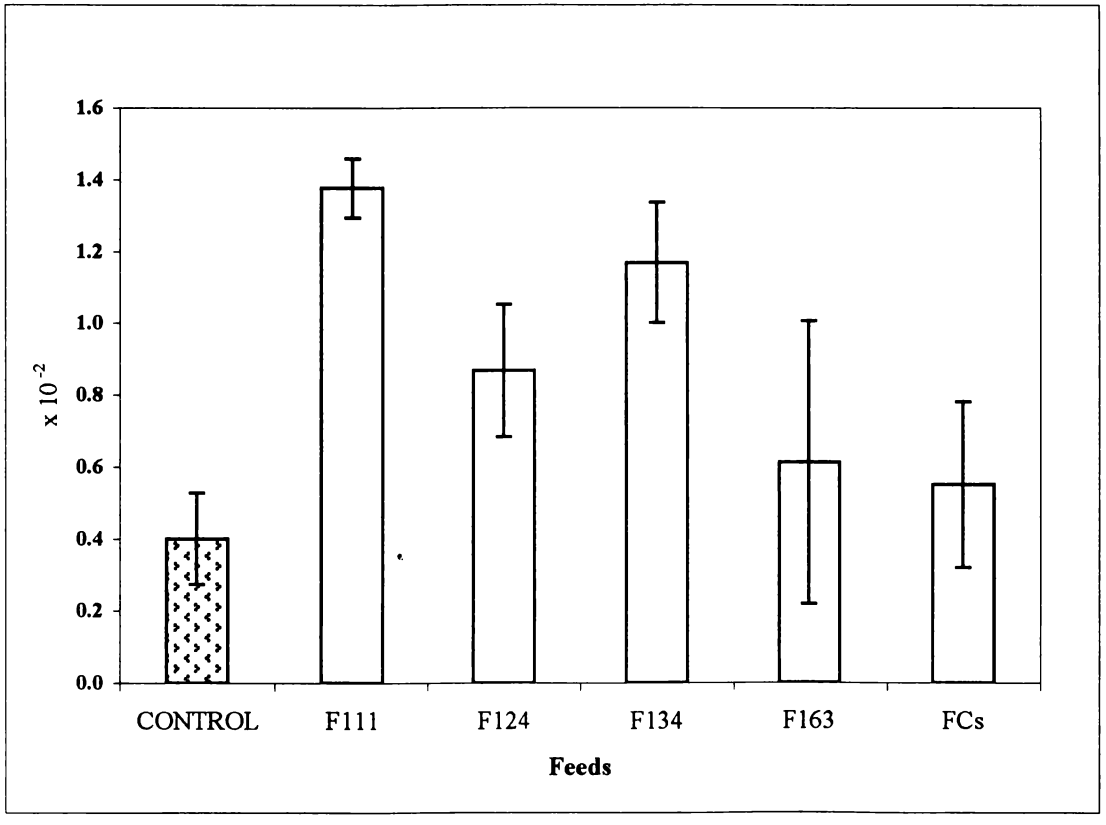


Feed	*SGR
CONTROL	0.40 ± 0.13^{a}
FC111	1.39 ± 0.09^{c}
F124	0.87 ± 0.19^{ab}
F134	1.18 ± 0.17^{bc}
F163	0.53 ± 0.17^{a}
FCs	0.55 ± 0.13^{a}

*(mean $\pm$ s.d.)

* Values with the same superscript does not vary significantly.

Fig. 6.7 Specific Growth Rate Ratio obtained in *P. indicus* juveniles when fed with various experimental diets

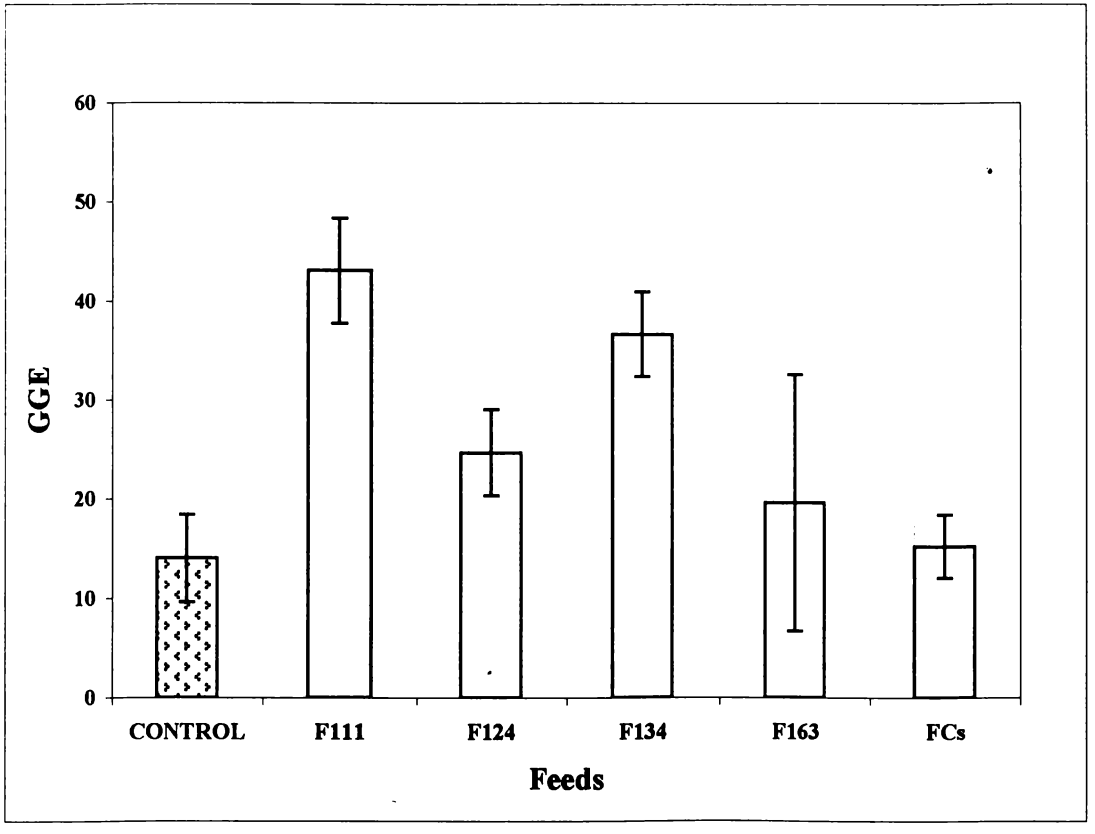


Feed	*RGR
CONTROL	0.40 ± 0.13^{a}
FC111	0.0138 ± 0.08^{d}
F124	0.0087 ± 0.18^{bc}
F134	0.0117 ± 0.17^{cd}
F163	0.0061 ± 0.39^{ab}
FCs	0.0055 ± 0.23^{ab}

*(mean ± s.d.)

* Values with the same superscript does not vary significantly.

Fig. 6.8 Relative Growth Rate (RGR) obtained in *P. indicus* juveniles when fed with various experimental diets

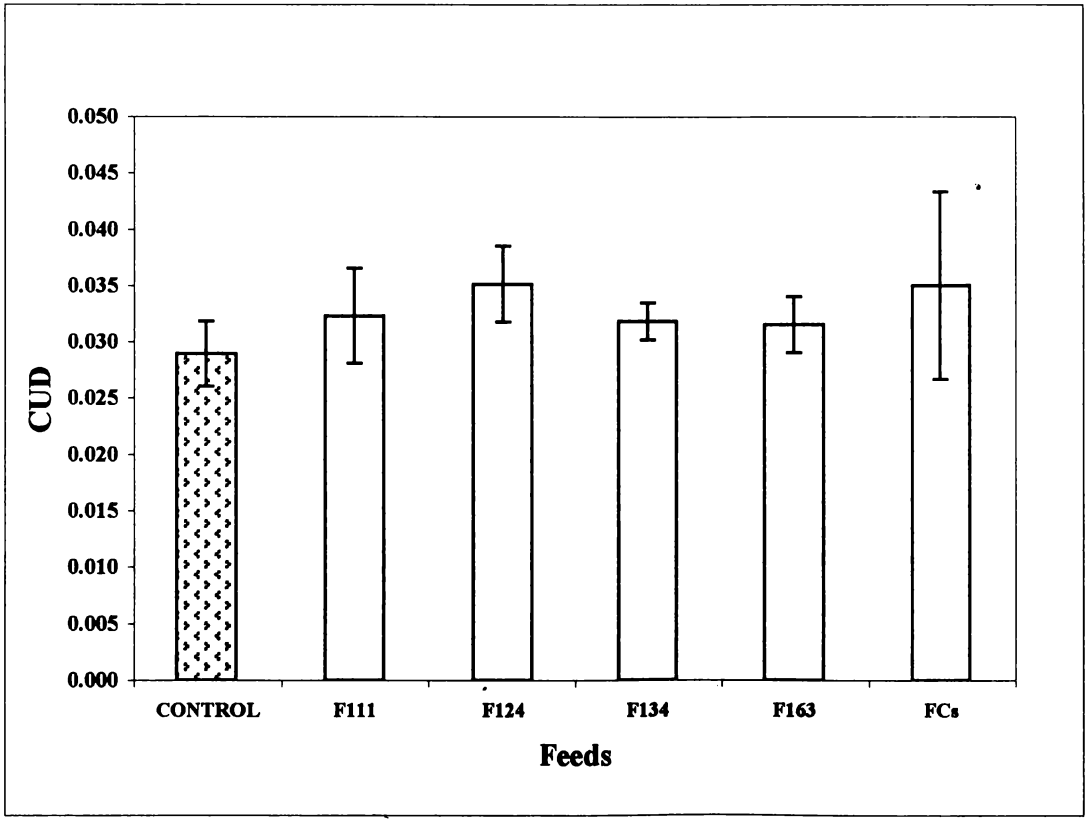


Feed	*GGE
CONTROL	14 ± 4.42^{a}
FC111	43.04 ± 5.33^{b}
F124	24.67 ± 4.36^{a}
F134	36.63 ± 4.28^{b}
F163	19.65 ± 12.95^{a}
FCs	15.22 ± 3.17^{a}

*(mean ± s.d.)

* Values with the same superscript does not vary significantly.

Fig. 6.9. Gross Growth Efficiency obtained in *P. indicus* juveniles when fed with various experimental diets



Feed	*CUD
CONTROL	0.029±0.003[a]
FC111	0.032±0.004[a]
F124	0.035±0.003[a]
F134	0.032±0.002[a]
F163	0.032±0.003[a]
FCs	0.035±0.008[a]

*(mean ± s.d.)

* Values with the same superscript does not vary significantly.

Fig. 6.10 Consumption per Unit weight per Day obtained in P. indicus *indicus* juveniles when fed with various experimental diets

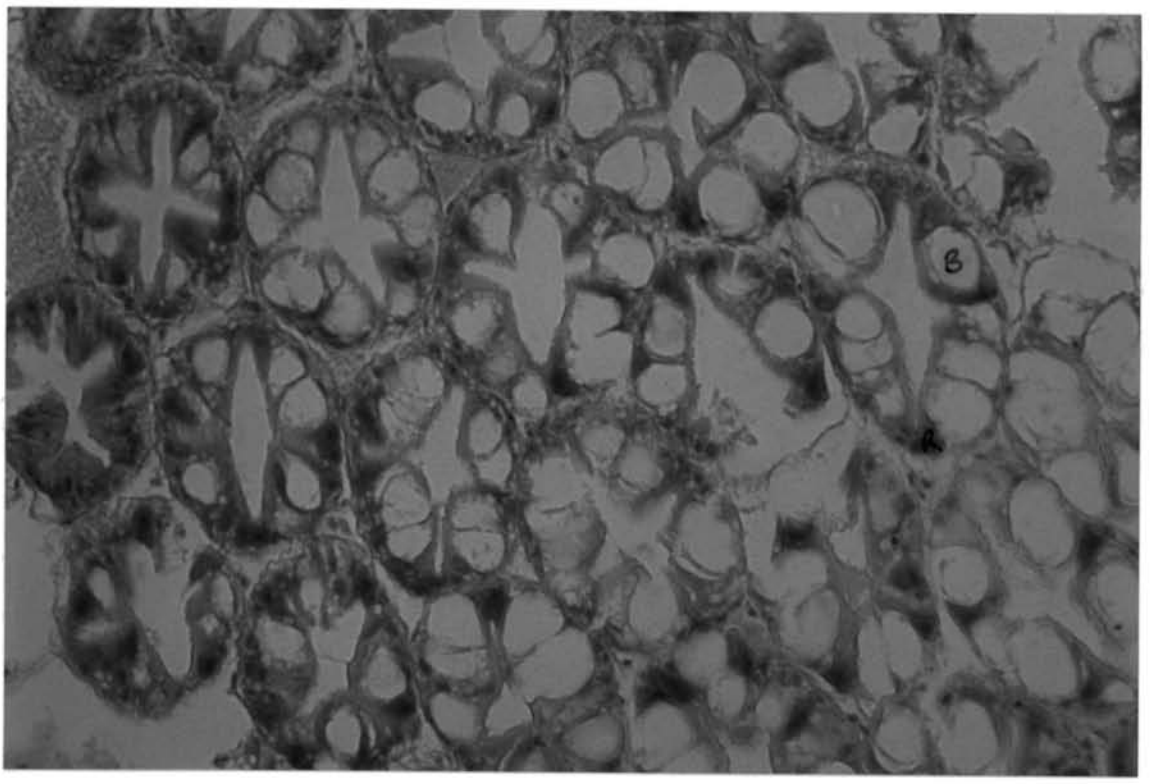


Fig 6.12a. CS of Hepatopancreas of *Penaeus indicus* maintained on Control diet. Hematoxylin-Eosin stain. 360x

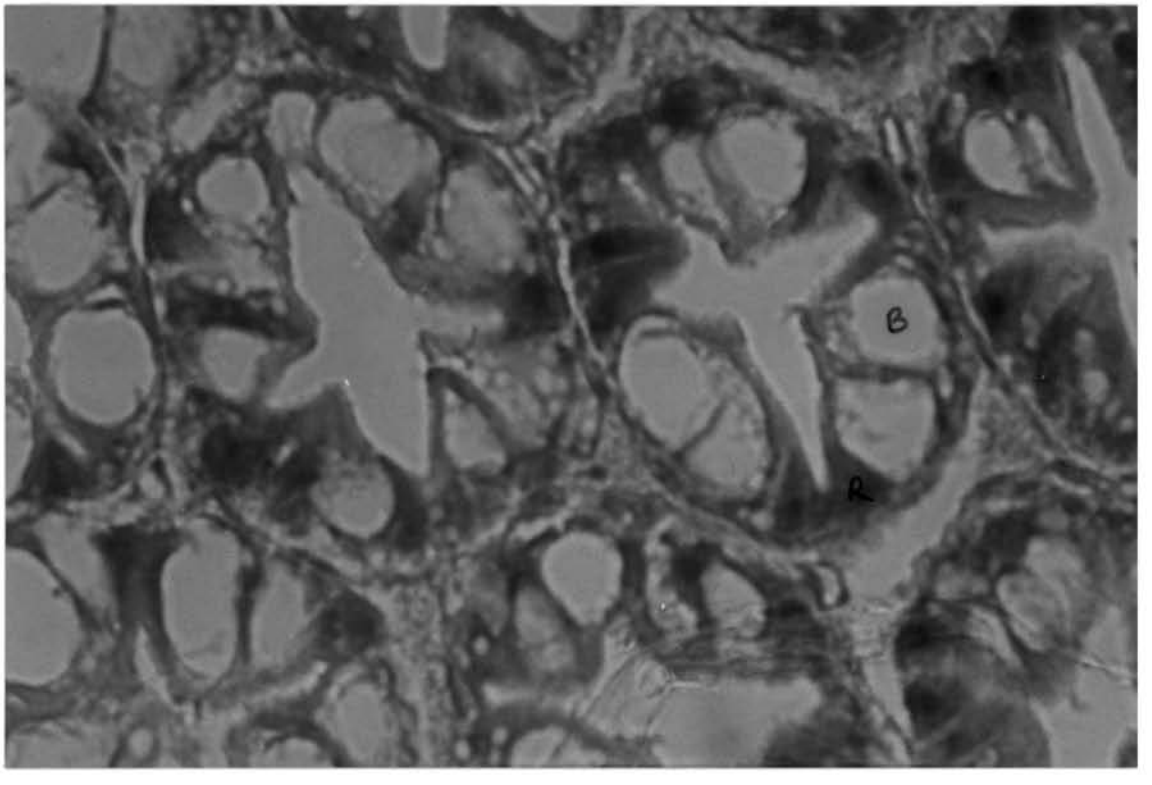


Fig. 6.12b CS of Hepatopancreas of *Penaeus indicus* maintained on Control diet. Hematoxylin-Eosin stain. 720x

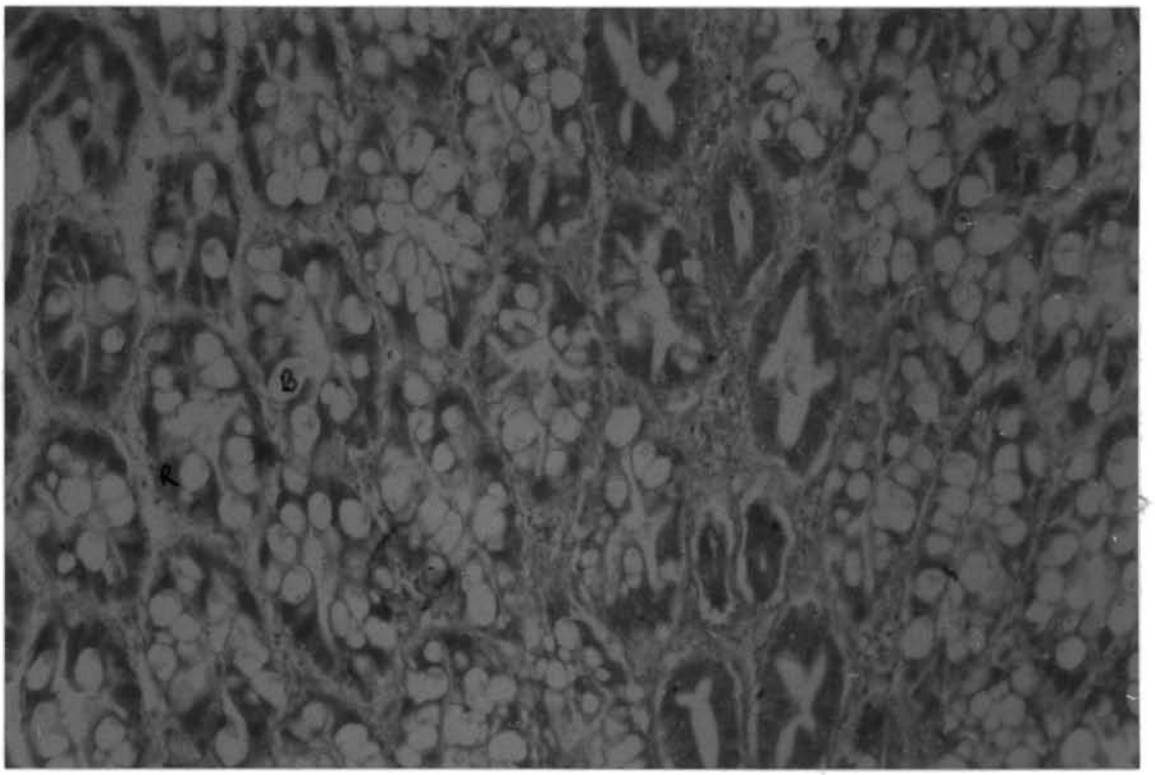


Fig.6.13a CS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet F111 Hematoxylin-Eosin stain. 360x.

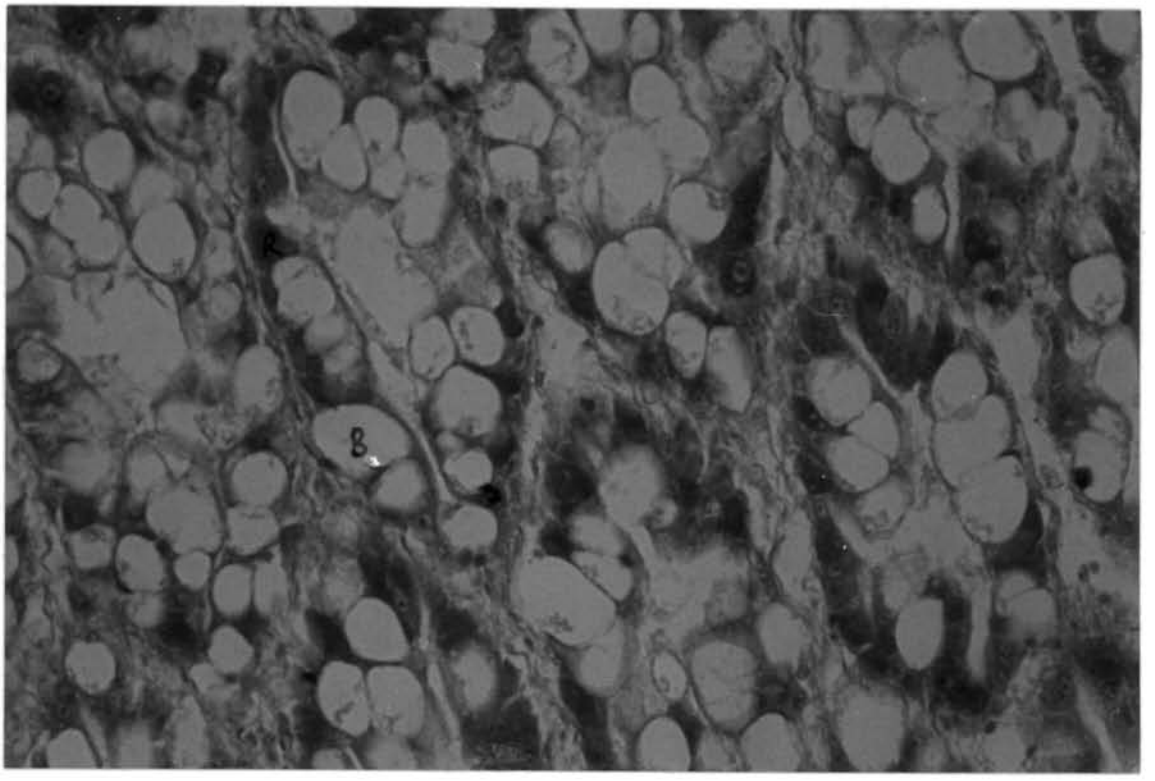


Fig 6.13b CS of Hepatopncreas of *Penaeus indicus* maintained on experimental diet F111 Hematoxylin-Eosin stain. 720x.

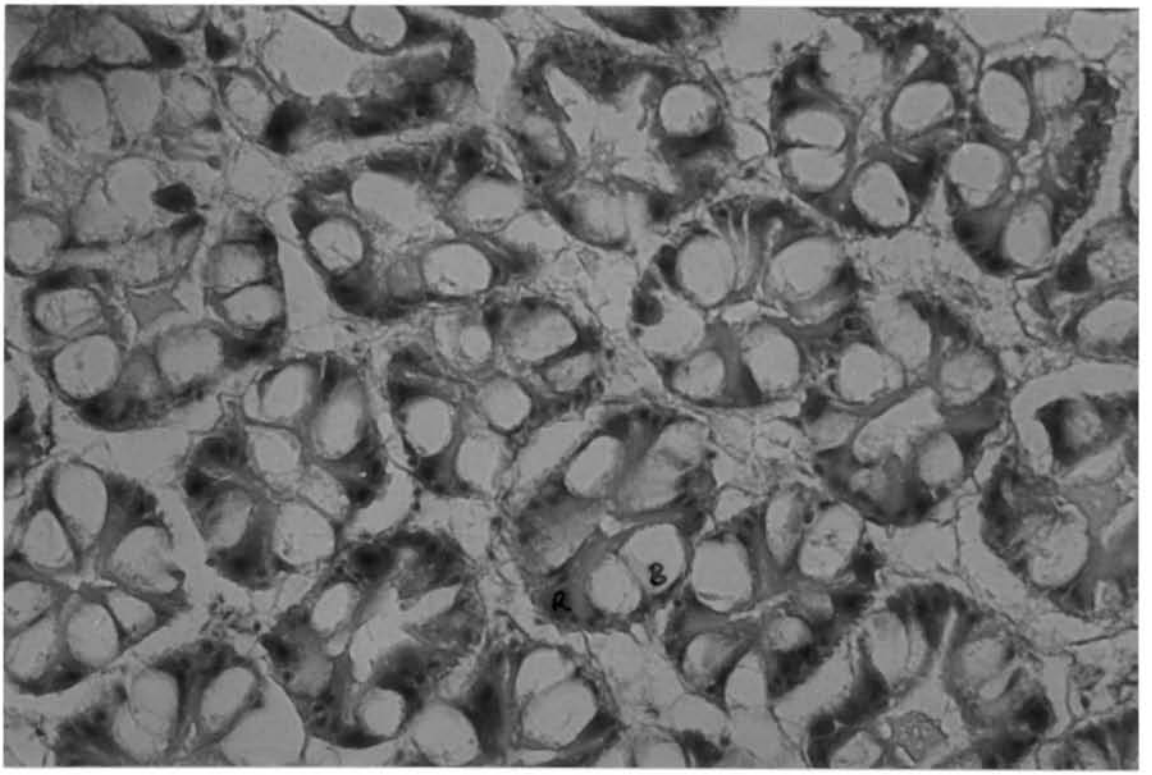


Fig6.14aCS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet F124. Hematoxylin-Eosin stain. 360x

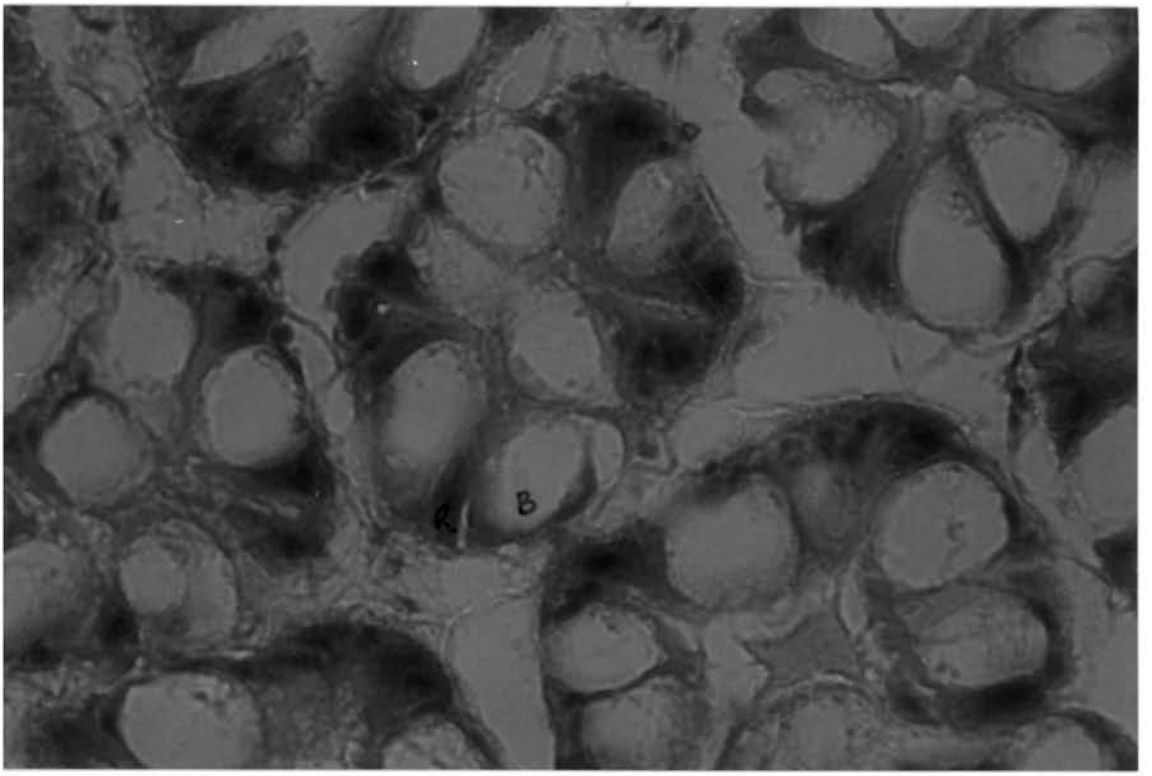


Fig.6.14bCS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet F124. Hematoxylin-Eosin stain. 720x

Fig.6.15a CS of Hepatopancreas of *Penaeus indicus* maintained on experimental feed F134. Hematoxylin-Eosin stain. 360x

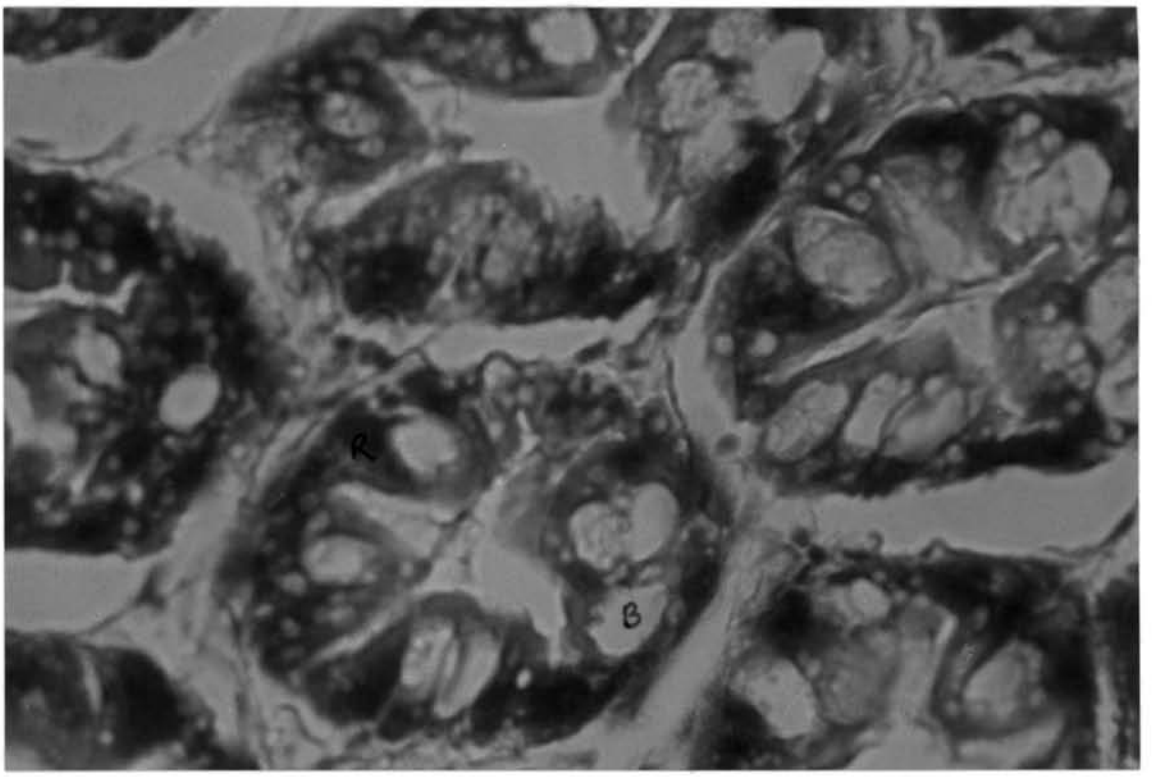


Fig.6.15b CS of Hepatopancreas of Penaeus indicus maintained on experimental diet F134. Hematoxylin-Eosin stain. 720x

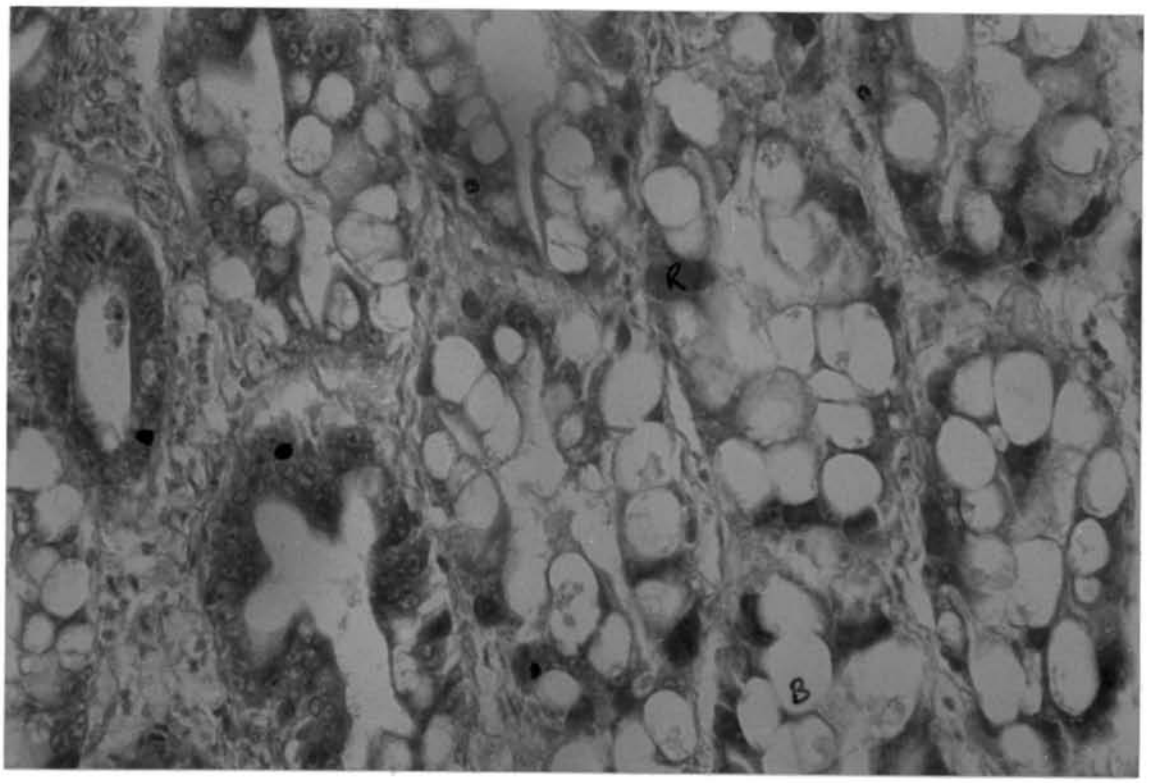


Fig.6.16aCS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet F163. Hematoxylin-Eosin stain. 360x

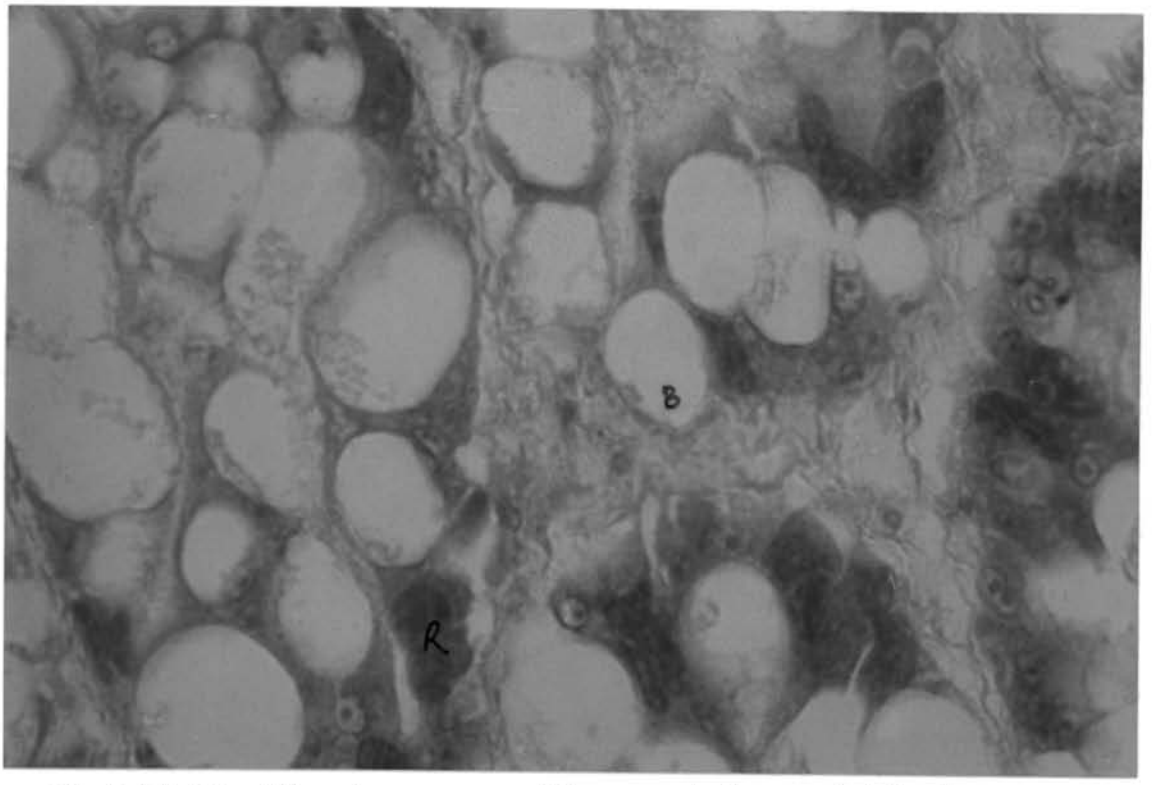


Fig. 6.16b CS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet F163. Hematoxylin-Eosin stain. 720x

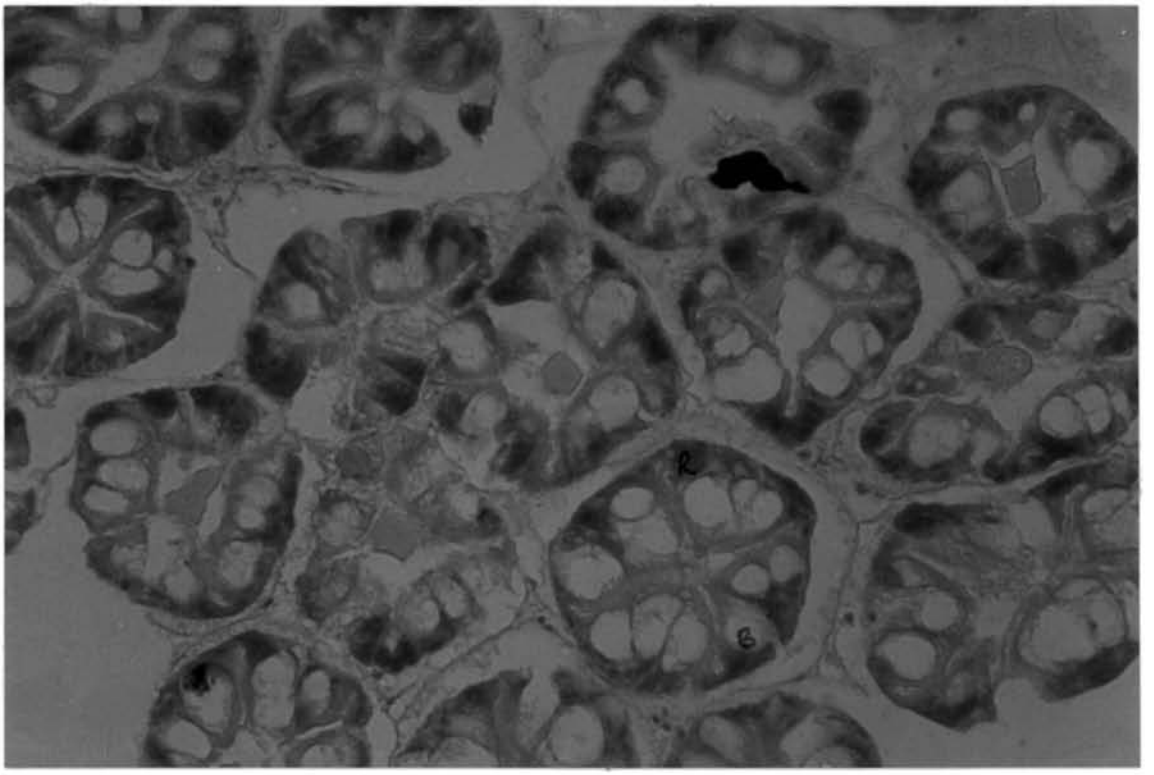


Fig.6.17aCS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet FCs (consortium) Hematoxylin-Eosin stain 360x

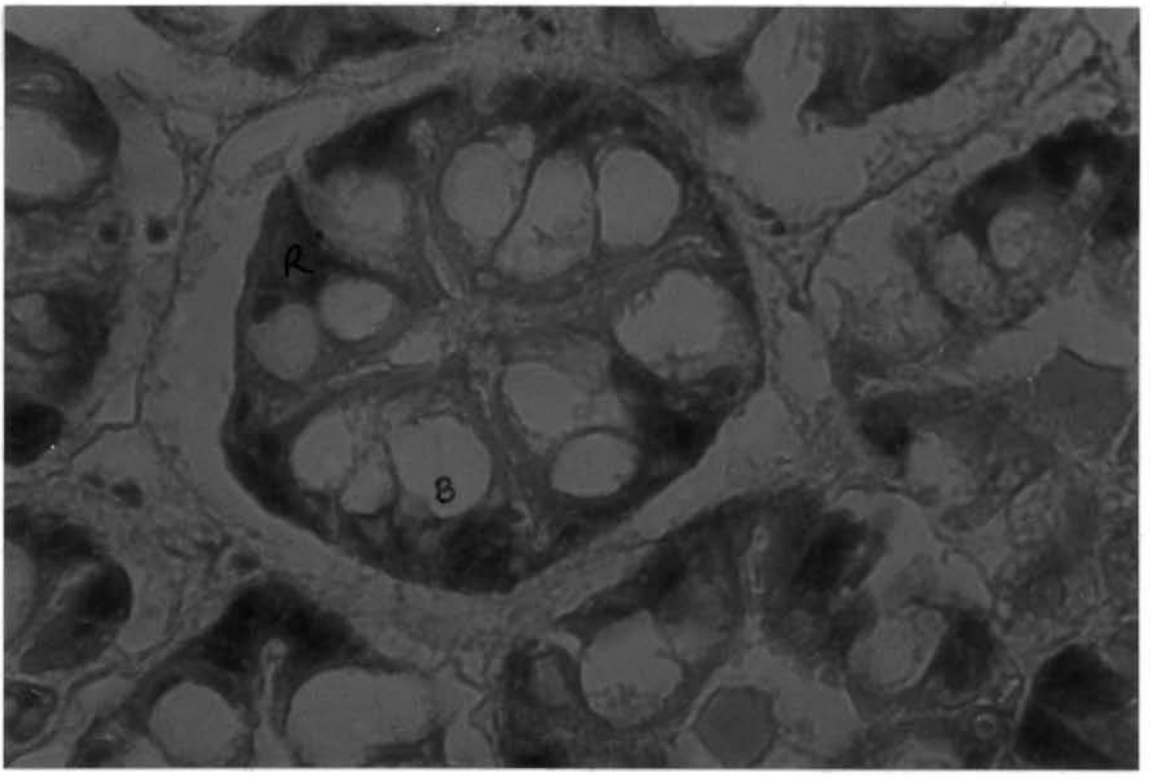


Fig.6.17bCS of Hepatopancreas of *Penaeus indicus* maintained on experimental diet FCs (consortium). Hematoxylin-Eosin stain. 720x

Fig.6.18 Hemocyte Count of hemolymph of *P. indicus* fed different experimental feeds

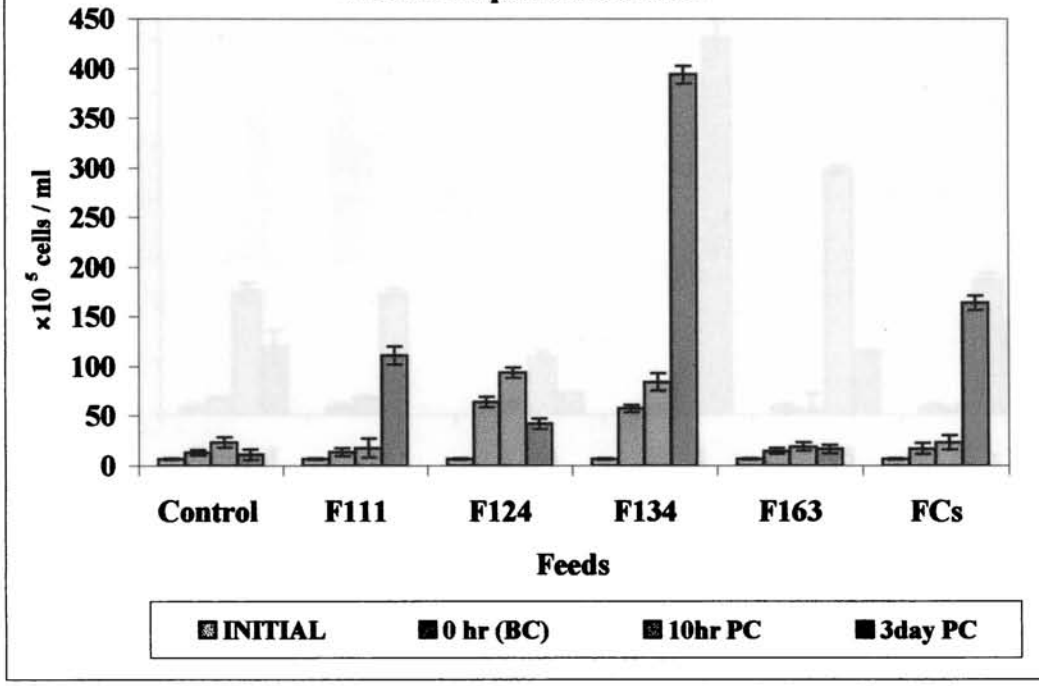
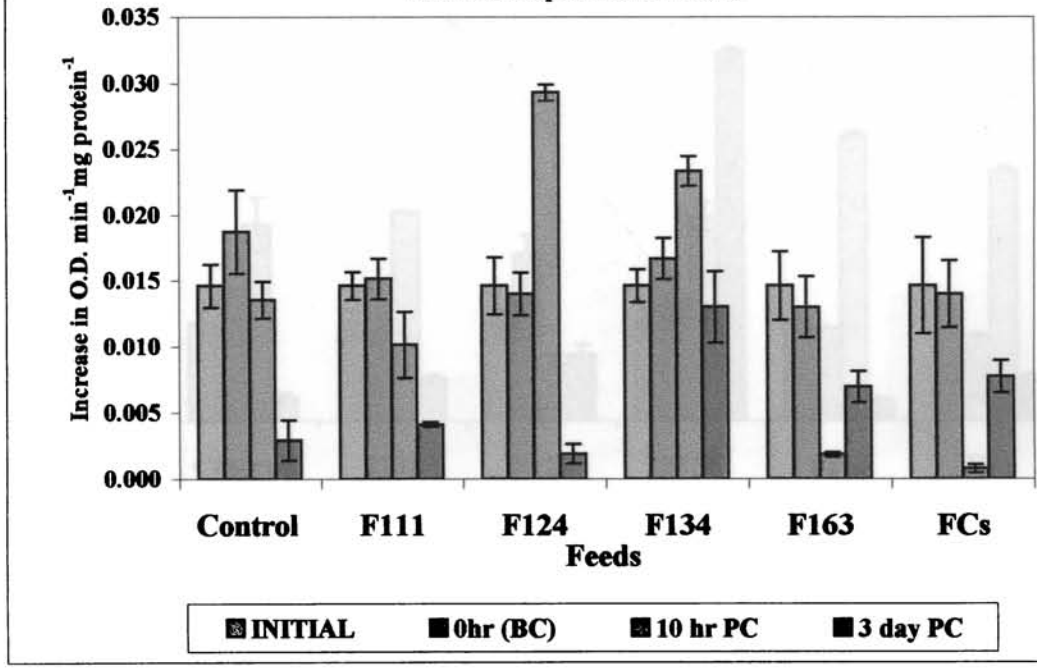


Fig. 6.19 Phenol oxidase activity of hemolymph of *P. indicus* fed different experimental feeds



Initial =Value at the begining of the feeding experiment
BC =Before Challenge (after the feeding expeirment for 28 days)
PC =Post Challenge
number of replicates =3

Fig.6.20 NBT activity of hemolymph of *P. indicus* prawns fed different experimental feeds

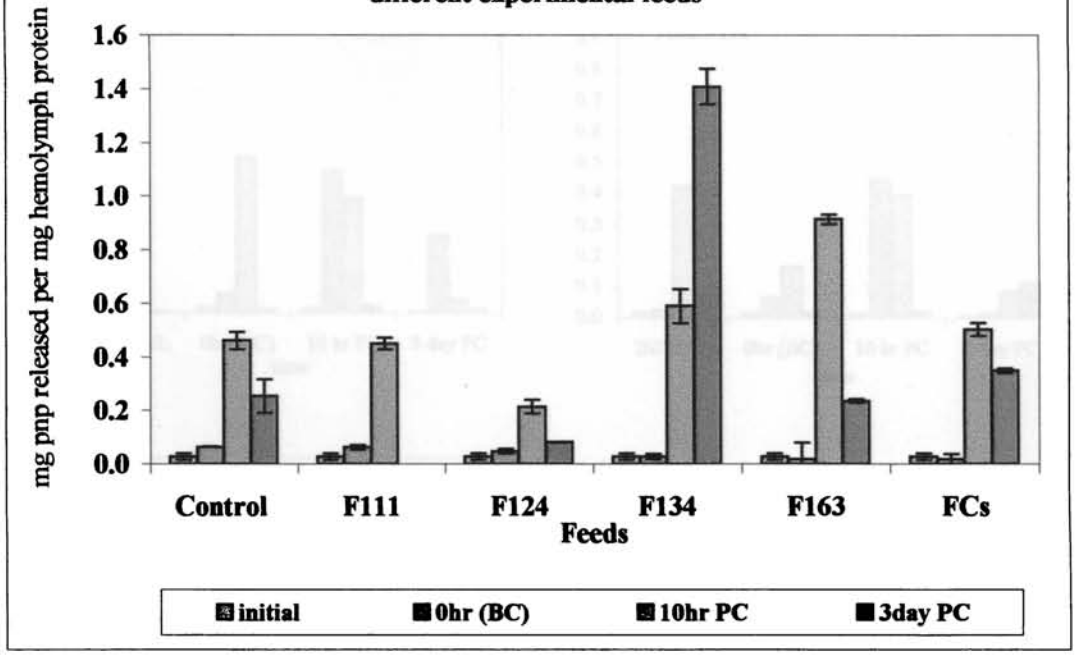
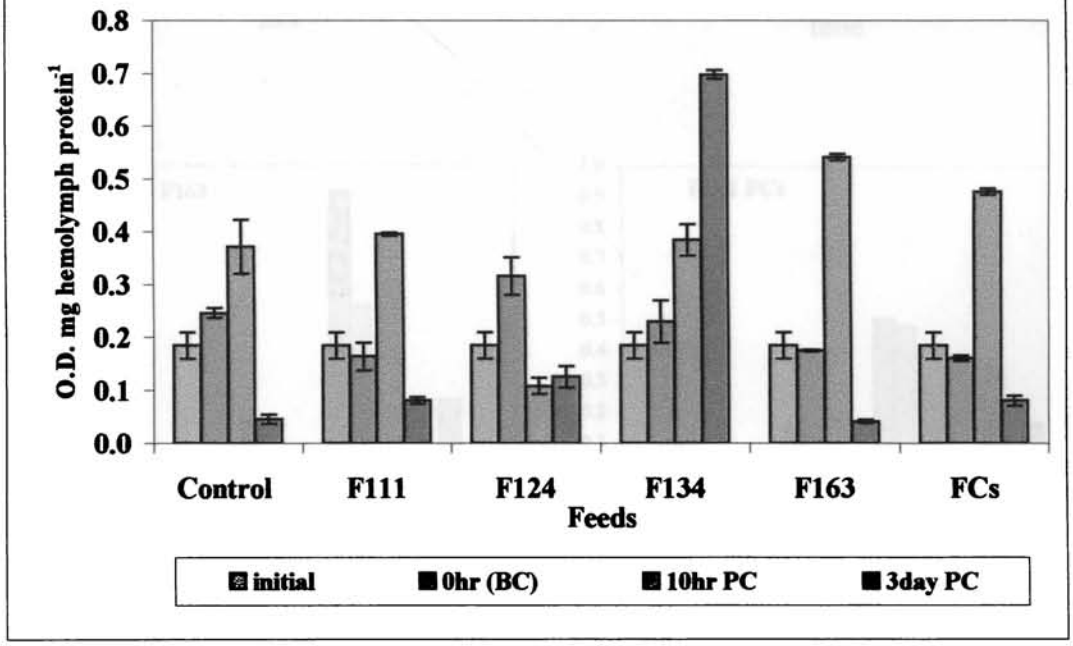


Fig. 6.21 Alkaline phosphatase activity of hemolymph of *P. indicus* fed different experimental feeds



Initial =Value at the begining of the feeding experiment
BC =Before Challenge (after the feeding expeirment for 28 days)
PC =Post Challenge
number of replicates =3

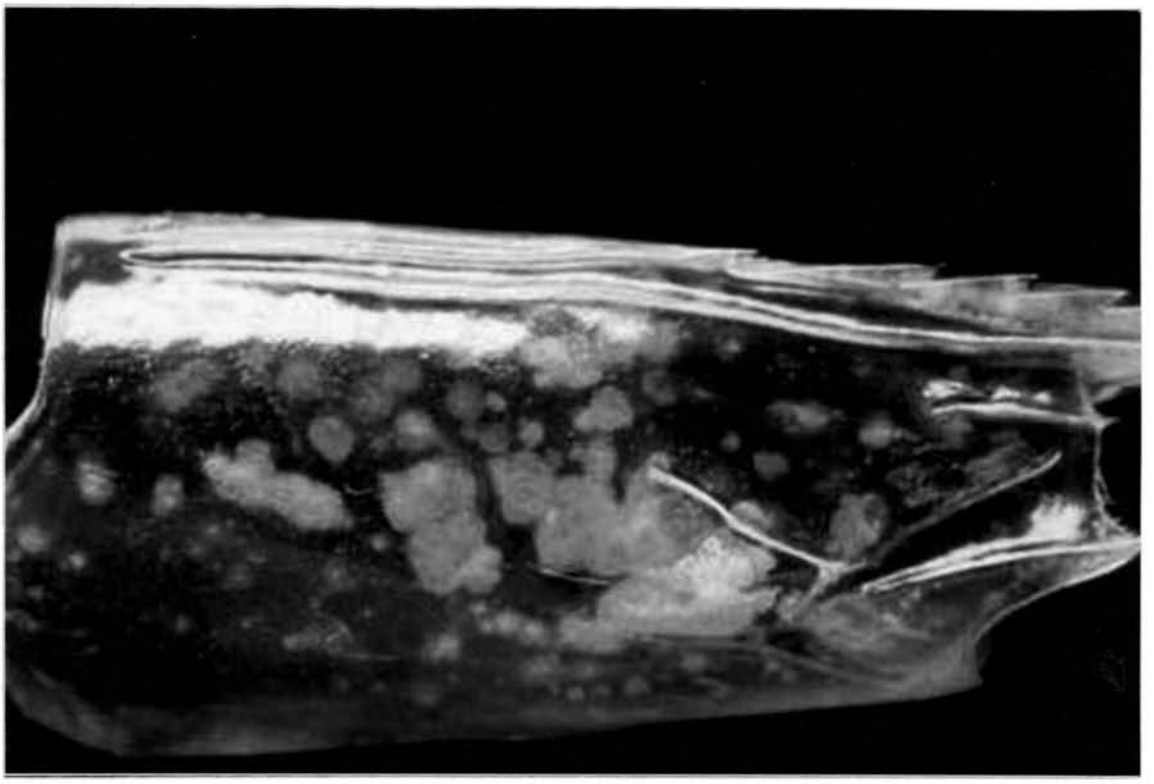


Fig 6 23 Carapace of *Penaeus indicus* infected with white spot virus

Fig.6.24 Percentage Survival of *P. indicus* post challenge with WSSV when fed on various experimental diets.

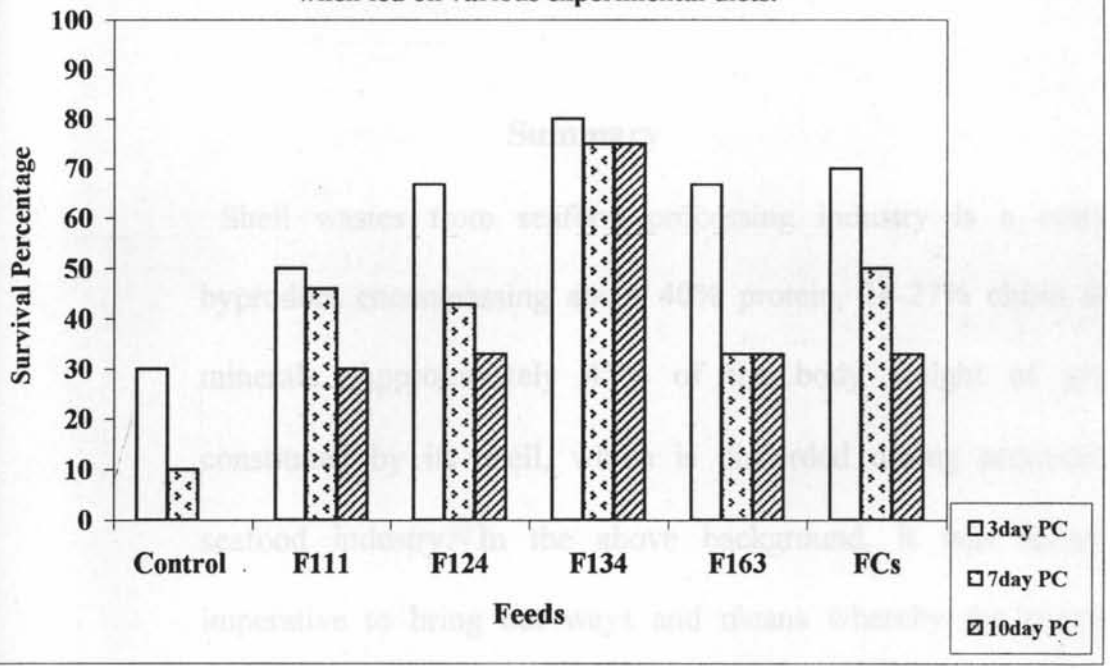
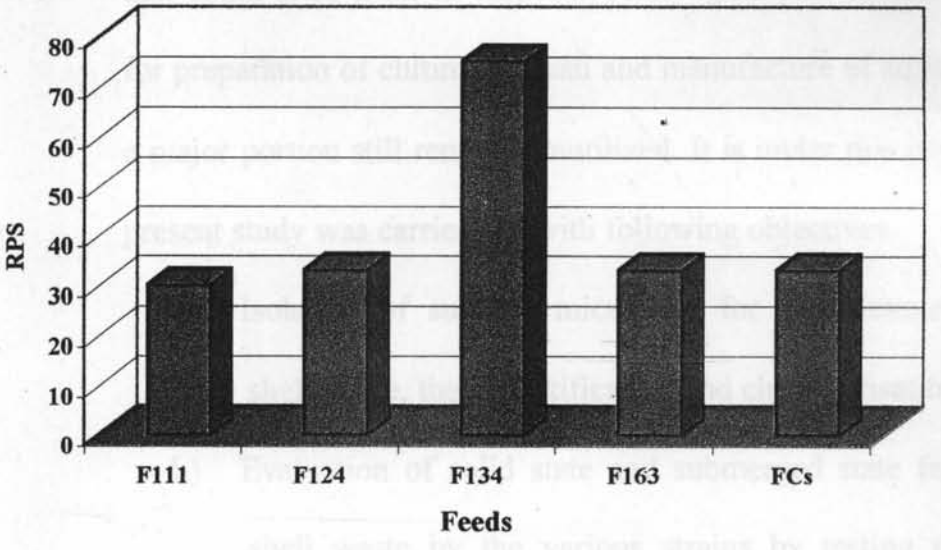


Fig. 6.25 Relative Percentage Survival after challenge with WSSV 10 day post challenge



RPS = 1- (% specific mortalities in treatment group) x 100
% specific mortalities in control group

Summary

Shell wastes from seafood processing industry is a nutrient-rich byproduct encompassing about 40% protein, 14-27% chitin and 33% minerals. Approximately 45% of the body weight of prawns is constituted by its shell, which is discarded during processing from seafood industry. In the above background, it was found highly imperative to bring out ways and means whereby the recovery and recycling of this unutilized nutrient rich "waste" could be judiciously used for commercial manufacturing of aquaculture feeds, which could subsequently mitigate the possible deleterious environmental impacts that could arise from its disposal. Although a part of this is being used for preparation of chitin, chitosan and manufacture of aquaculture feeds, a major portion still remains unutilized. It is under this purview that the present study was carried out with following objectives.

a) Isolation of suitable microflora for the biotransformation of shell waste, their identification and characterisation

b) Evaluation of solid state and submerged state fermentation of shell waste by the various strains by testing the nutritional quality of the product

c) Screening of the fermented products for its efficacy as an aquaculture feed and segregation of corresponding potential strains

d) Optimization of the physico-chemical parameters of shell waste fermentation for the selected strains

e) Application of the optimized fermented product as a dietary ingredient for *P. indicus* and testing its growth in terms of biogrowth parameters

f) Testing the presence of immunostimulants in the fermented products through feeding trials in *P. indicus* and post challenge assessment of haemotological parameters using white spot virus (WSSV) as pathogen.

The salient findings of the study is summarized as follows:

- Bacillus species were found to be the predominant group associated with the transformation of prawn shell waste. Other groups of importance were Coryneformes, Vibrio and Streptococcus .

- After screening for chitinoclastic properties the positive strains (30) were segregated into two

    1. Chitinoclastic and Proteolytic (25 nos.)
    2. Chitinoclastic and non-proteolytic (15 nos.)

- Raw prawn shell waste contained 38 % protein, 5.36 % lipid and 1.86% carbohydrate. Fermentation resulted in substantial increase in the nutritive value of the product. The maximum protein content obtained was 59.15 % in FP111 (SmF), lipid 8.97% in FP124 (SmF) and carbohydrate 3.29% in FP163 (SSF).
- Eventhough there was not much variation in protein enrichment between SSF and SmF, generally SmF was found to be better for the most of the strains. Lipid enrichment was also found to be better in SmF compared to SSF.
- The performances of proteolytic forms were found to be better in terms of nutritional quality of the fermented products. 20 strains were selected based on the nutritional profile of the fermented products.
- Significant increase in growth parameters could be obtained for *P. indicus* post larvae when fed with fermented product incorporated feeds compared to the control feed.
- Production (weight gain), Food Conversion Ratio (FCR), gross growth efficiency (GGE) and Protein efficiency ratio (PER) was found to be maximum for *P. indicus* fed on feed F134. Feed F124 was next in the order of performance in terms of bio-growth parameters.

- Duncan's multiple range test showed that the performance of the feeds in terms of bio-growth parameters differed significantly.
- Post challenge survival of *P. indicus* post larvae with white spot virus was found to be maximum with feed F111.
- Proximate composition of the fermented products did not show a significant positive correlation with any of the bio-growth parameters. However the post challenge survival percentage showed a significant positive correlation with lipid content ($p<0.1$).
- Based on the results of the feeding experiment with *P. indicus* post larvae four bacterial strains (3 *Bacillus* spp. and 1 *Serratia* sp.) for further study. *Bacillus* spp. C124 and C134 were selected based on growth performance and *Bacillus* sp. C111 based on post challenge survival data. *Serratia* sp. C163 was selected due to its pigmented nature.
- Characterisation of the selected strains for growth and enzyme production was done in nutrient broth at different physico-chemical conditions. Generally all the strains preferred pH 7, 1.5 to 2 % NaCl concentration, 0.5 to 1 % colloidal chitin and 0.8% calcium carbonate for maximal growth and enzyme production with a few exceptions.

- Optimization of shell waste fermentation conditions showed that nutritional enrichment was maximum with an initial pH of 6, moisture content 1:1.75 to 1:2 (substrate: moistening media), NaCl concentration 1.5 to 2 %, incubation temperature 35°C, inoculum 2x 10^8 cells /5 gms substrate and an incubation time of 20 days.

- Shell waste was fermented under optimal conditions by the four selected strains and by the consortia (mixed cell suspension of all the four strains). The feeding experiment conducted with the optimized fermented product incorporated feeds showed that the performance of feed F111 and F134 was significantly greater than that of the other feeds including control feed.

- Weight gain, FCR, SGR, RGR, GGE, and PER of the shrimps fed in feeds F111 and F134 were significantly high compared to the other four feeds. Feed F124 is next in the order of performance and differed significantly from F163, FCs and control feed. Feeds F163 and FCs presented very poor performance and was comparable to the control feed with raw prawn shell waste.

- Examination of the feeds for immunostimulant property showed that feed F134 contained some immunostimulatory substance, which is evidenced by better survival and hematological profile exhibiting an immune boost up post challenge with white spot virus.

- Post challenge survival on 10^{th} day was found to be significantly high for *P. indicus* maintained on feed F134 (75%) compared to other feeds. The value for the other four fermented product incorporated feeds remained at the same level (30%) and the control recorded 100 % mortality.
- A 10^{th} hour post challenge boost up could be recorded with various hematological parameters such as phenol oxidase activity (PO), Nitro Blue Tetrazolium (NBT) activity and alkaline phosphatase activity. However feed F134 was found to support an elevation in the various parameters (except phenol oxidase) on the 3^{rd} day post challenge.
- Histological analysis of the hepatopancreas do no show any structural or functional abnormalities with feeds F111, F124, F134, F163 and control feed. However, feed FCs showed disruption of the basal membrane and damage of tubules indicating the possible presence of toxic components.
- The results showed that the shell waste could be transformed by microorganisms into a potential feed ingredient for aquaculture applications. Thus the shell waste can be a valuable raw material for aquaculture feed industry and thereby the disposal and consequent environmental problems can be avoided. The fermentation is carried out without any input of nutrients or other chemical additives. In addition to this, the raw being very cheap and abundant, the technology would be cost-effective and the feed cost can be reduced

considerably. The growth promoting and immunostimulant property exhibited by the fermentation products generated by certain strains show its efficacy as a dietary ingredient for prawns.

References

Aakre, R., Wergeland, H.I., Aasjord, P.M. and Endersen, C., 1994. Enhanced antibody response in Atlantic salmon (*Salmo salar* L.) to *Aeromonas salmonicida* cell wall antigens using a bacterin containing β-1, 3-M-glucan as adjuvant. Fish Shellfish Immunol. 4: 47-61.

Abdel-Rahman, S.H., Kanazawa, A. and Teshima, S., 1979. Effects of dietary carbohydrates on growth and the levels of the hepatopancreatic glycogen and serum glucose of the prawn. Nippon Suisan Gakkaishi, Bull. Jap. Soc. Sci. Fish., 45: 1491-1494.

Abdullah, A.L., Tengerdy, R.P. and Murphy, V.G., 1985. Biotech. Bioeng., 27: 20.

Adams, A., Auchinachie, N., Bundy, A., Tatner, M. and Horne, M.T. 1988. The potency of adjuvanted injected vaccines in rainbow trout (*Salmo grairdneri*) and bath vaccines in Atlantic salmon (*Salmo salar* L.) against furunculosis. Aquaculture., 69: 15-26.

Aidoo, K.E., Hendry, R. and Wood, B.J.B. 1982. Solid-substrate fermentations. Adv. Appl. Microbiol., 28 : 201-237.

Aiso, K., Simidμ, U. and Hasuo. K., 1968. Microflora in the digestive tract of inshore fish in Japan. J. Gen. Microbiol., 52: 361-364.

Akiyama, D.M., Coelho, S.R., Lawrence, A.L. and Robinson, E.H., 1989. Apparent digestibility of feedstuffs by the marine shrimp *Penaeus vannamei* Boone. Nippon Suisan Gakkaishi, Bull. Jap. Soc. Sci. Fish., 55: 91-98.

Akiyama, D.M. and Dominy, W.G., 1989. Penaeid shrimp nutrition for the commercial industry. In : D.M. Akiyama (Editor), Proceedings of the People's Republic of China Aquaculture and Feed workshop, Singapore, Sept. 17-30. American Soybean Association, Singapore. pp. 143-163.

Akiyama, D.M. and Dominy, W.G., 1991. Penaeid shrimp nutrition for the commercial feed industry. American Soyabean Association and Oceanic Institute, Waimanalo, USA, 50p.

Alasheh, S. and Duvnjak, Z., 1995. Appl. Microbiol Biotechnol., 43: 25.

Alava, V.R. and Lim, C. 1983. The quantitative dietary protein requirement of *Penaeus monodon* juveniles in controlled environment. Aquaculture, 30: 53-61.

Aleksieva, P., Grigorog, I., Djerova, A., Sheremetska, P. and Tchorbanov, B. 1984. submerged cultivation of a fungal mutant strain *Humicola lutea* 120-5 producing acid proteinases, Acta Biotechnol., 4: 179-185.

Alexander, M., 1971. Microbial ecology. John Wiley and Son. Inc., New York.

Ali, S.A., 1982. Relative efficiencies of palletized feeds compounded with different animal proteins and the effect of protein level on the prawn, *Penaeus indicus*. Proc. Symp. Coastal Aquaculture, Part 1. CMFRI, Cochin, pp. 321-329.

Ali, S.A. and Mohamed, M.H., 1985. Utilization of prawn waste and mantis shrimp for compounding feeds for the culture of penaeid prawns. Proc. Symp. Harvest and Post Harvest Technology of Fish, Society of Fisheries Technologists (India), Cochin: 615-618.

Ali, S.A., 1989. Studies on the evaluation of different sources of protein, carbohydrate and mineral requirements for juvenile penaeid prawn, *Penaeus indicus* (H.Milne Edwards). Ph.D. Thesis. Cochin University of Science and Technology.

Amin, G. and Allah, A.M.K., 1992. By-products formed during conversion of sugar beets to ethanol by *Zymomonas mobilis* in conventional submerged and solid-state fermentations. Biotech. Lett., 14(12): 1187-1192.

Anderson, C. G., de Pablo, N. and Romo, C., 1978. Antartic krill (*Euphausia superba*) as a source of chitin and chitosan, In : Proceedings of the First International Conference on Chitin/ Chitosan, (eds R. A. A. Muzzarelli and E. R. Pariser), MIT Sea Grant Report 78-7, Cambridge, MA, pp. 54-63.

Anderson, D.P., 1992. Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. Ann. Rev. Fish. Dis., 2: 281-307.

Anderson, D.P. and Siwicki, A.K. 1994. Duration of protection against Aeromonas salmonicida in brook trout immunostimulated with glucan or chitosan by injection or immersion. Progressive Fish-Culturist., 56: 258-261.

Anderson, D.P., Siwicki, A.K. and Rumsey, G.L., 1995. Injection or immersion delivery of selected immunostimulants to trout demonstrate enhancement of nonspecific defense mechanisms and protective immunity, In: Shariff, M., Subasinghe, R. P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture

1. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 413-426.

Andrews, J.W., Sick, L.V. and Baptist, G.J., 1972. The influence dietary protein and energy levels on growth and survival of penaeid shrimp. Aquaculture, 1: 341-347.

AOAC (Association of Official Analytical Chemist), 1990. Official Method of Analysis. 15th Edn. Association of Official Analytical Chemist Washington, DC., 1094p.

APHA (1995).Standard Methods for the examination of Water and Wastewater. 19th Edition.

Aribisala, O. A. and Gooday, G. W., 1978. Properties of chitinase from *Vibrio alginolyticus* as assayed with the chromogenic substrate 3, 4, dinitrophenyl tetra-N-acetylchito-tetraoside. Biochem. Soc. Trans., 6: 568-569.

Ashford, N. A., Hattis, D. and Murrey, A. E., 1977. Industrial prospects for chitin and protein from shellfish wastes. MIT Sea Grant 77-3, No. 77-703. Zle.

Ashida, M. et al., 1982. In: Proceedings of the third International Colloquim on Invertebrate Pathology (Paynes, C.C. and Burges, H.D., eds), pp. 81-86, University of Sussex.

Ashmin, M., 1997. Selection of microorganisms for the transformation of shell fish chitin waste. M.Sc. Dissertation, Cochin University of Science and Technology, Cochin. pp.64.

Aunstrup, K., Andersen, O., Falch, E.A. and Nielsen, T. K., 1979. Prodcution of microbial enzymes. In: Microbial Technology, 2nd Ed. Academic Press, Vol. 1: 281-309.

Austin, P. R., C. J.Brine, J. E. Castle and Zikakis, J. P., 1981. Chitin: new facets of research. Science, 212: 749-753.

Bachere, E. 1998. Shrimp immunity and disease control: an integrated approach. In: Fifth Asian Fisheries Forum, Thailand. p. 20.

Bailey, J.E. and Ollis, D.F., 1977. Biochemical Engineering Fundamentals, Chapter 8., McGraw-Hill, New York.

Bajracharya, R. and Mudgett, R.E., 1979. Biotech. Bioeng., 21: 551.

Balagopalan, C., 1996. Improving the nutritional value of cassava by solid state fermentation : CTCRI experiences. J. Sci. Indus. Res., 55: 479-482.

Balakrishnan, K. and Pandey, A., 1996. Production of biologically active secondary metabolites in solid state fermentation. J. Sci. Indus. Res., 55: 365-372.

Balasubramanya, R.H. and Bhatawdekar, S.P., 1998. Bioenrichment of agricultural wastes with a free-living nitrogen fixing bacterium and a mushroom fungus.Indian-J.Microbiol., 28(3-4): 220-225.

Balazs, G.H., Ross, E. and Brooks, C.C., 1973. Preliminary studies on the preparation and feeding of crustacean diets. Aquaculture, 7: 299-313.

Balazs, G.H. and Ross, E., 1976. Effect of protein source and level on growth and performance of captive freshwater prawn *Macrobrachium rosenbergii* Proc. World Maricult. Soc., 5: 1-14.

*Baldensperger, J., Lemer, J., Hannibal, L. and Quinto, P.J., 1985. Biotech. Lett., 7: 743.

Barboza-Corona, J.C., Contreras, Velasquez-Robledo, Bautista-Juslo, Gomez-Romirez, Cruz-Camarillo and Ibarra, 1999. Selection of chitinolytic strains of *Bacillus thuringiensis*. Biotechnol. Lett., 21:1125-1129.

*Barnes, H., 1959. Apparatus and methods in Oceanography. George Allen Unwin, New York. 125p.

Barnes, T.G., Eggins, H.W. and Smith, E.L., 1972. Int. Biodeter. Bull., 8: 112.

Barracco, M.A., Duvic, B. and Soderhall, K., 1991. The β-1,3 glucan-binding protein from the crayfish, *Pacifastacus leniusculus* when reacted with a β-1,3-glucan, induces spreading and degranulation of crayfish granular cells. Cell Tissue Res. 266: 491-497.

Barrios-Gonzalez, J., Castillo, T.E. and Mejia, A., 1993. Development of high penicillin producing strains for solid-state fermentation. Biotechnol.Adv., 11(3): 525-537.

Bau, H.M., Villaume, C., Lin, C.F., Evrard, J., Quemener, B. and Nicolas, J.P., 1994. Effect of a solid-state fermentation using *Rhizopus oligosporus* sp. T-3 on elimination of antinutritional substances and modification of biochemical constituents of defatted rapeseed meal. J. Sci. Food-Agric., 65(3): 315-322.

Bautista, M.N. and Subosa, P.F., 1997. Changes in shrimp feed quality and effects on growth and survival of *Penaeus monodon* juveniles. Aquaculture, 151: 121-129.

Bautista, M.N., 1986. The response of *Penaeus monodon* juveniles to varying protein/energy ratios in test diets. Aquaculture, 53: 229-242.

Beg, S., Zafar, S. I. and Shah, F. H., 1986. Rice husk biodegradation by Pleurotus ostreatus to produce a ruminant feed. Agric. Wastes, 17(1): 15-21.

*Benecke, W., 1905. Uber *Bacillus chitinovorous* einen chitin zergenden spaltpilze. Bot. Zeitung Abt., 1, 63: 227-242.

Benton, A. G., 1935. Chitnivorous bacteria. A preliminary survey. J. Bact., 29: 522-534.

Bertolin, T.E., Dillon, A.J., Santin, J.A., Rarrizi, T.M. Tonial, T.M. and Vieira, M.L.X. 1996. Protein enrichment of rice husk by semi-solid fermentation. Int. Biodeterior. Biodegrad., 37 (1-2): 118.

Beuchat, L.R., 1983. Indegineous fermentation of foods. In: Biotechnology. (Reed, G. ed.), Vol. 5. Ver;lag Chemie, Weinheim, Germany.

*Bhalla, T.C. and Joshi, M., 1994. World J. Microbiol. Biotechnol., 10: 116.

Bhushan, B. and Hoondal, G.S., 1998. Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. Biotechnol. Lett., 20(2): 157-159.

*Bianchi, A. J. M., 1971. Distribution de certaines bacteries heterotrophes aerobies dans les sediments profonds de Mediterrance Orientale. Mar. Biol., 11: 106-117.

*Billy, C., 1969. Etude d'une bacterie chitinolytique anaerobie *Chlostridium chitinophilum* n. sp. Ann. Inst. Pasteur (Paris), 116: 75-82.

Bird, J.N. and Savage, G.P., 1990. Carotenoid pigmentation in aquaculture. Proc. Nutr. Soc. NZ, 15: 45-56.

Blazer, V.B., 1992. Nutrition and disease resistance in fish. Ann. Rev. Fish Dis., 2: 309-323.

Boer, W.D., Gerards, S., Gunnewiek, P.J.A.K. and Modderman, R., 1999. Response of the chitinolytic microbial community to chitin amendments of dune soils. Biology and Fertility of Soils, 29(2): 170-177.

Boeye, A., Wayenbergh M. and Aerts, M., 1975. Density and composition of heterotrophic bacterial populations in North Sea sediments. Mar. Biol., 32: 263-270.

Bogwald, J., Dalmo, R.A., Leifson, R.M., Stenberg, E and Gildberg, A., 1995. The stimulatory of a muscle protein hydrolysate from Atlantic cod, *Gadus morhua* L., on Atlantic salmon, *Salmo salar* L., head kidney leucocytes. Fish Shellfish Immunol., 2: 251-261.

Boonyaratnapalin, S., 1990. Shrimp larval diseases. In: New, M.B., de Saram, H., Singh, T. (Eds). Technical and economical aspects of shrimp farming. Proceedings of the Aquatech '90 Conference, Kuala Lumpur, Malaysia.

Boonyaratnapalin, S., Boonyaratnapalin, M., Supamattaya, K. and Toride, Y., 1995. Effects of peptidoglycan (PG) on growth, survival, immune response and tolerance to stress in black tiger shrimp, *Penaeus monodon*. p. 469-477. In: Diseases in Asian Aquaculture II, Shariff, M., Arthur, J.R. and Subasinghe, R.P., eds., Asian Fisheries Society, Manila, Philippines.

*Bough, W., Salter. W., Wu, A. and Perkins, B. 1978. Biotechnol. Bioeng., 20: 1931-1943.

Boyer, J.N. and Kator, H.I., 1985. Method for measuring microbial degradation and mineralization of ^{14}C-labeled chitin obtained from the blue crab, *Callinectes sapidus*. Microb. Ecol., 11: 185-192.

Bravo, O., Ferrer, A., Aiello, C., Ledesma, A. and Davila, M., 1994. Growth of *Chaetomium cellulolyticum* in solid state fermentation of sugar cane bagasse treated with water and alkali at several liquid/ solid ratios. Biotechnol. Lett., 16: 865-870.

Brine, C. J. and Austin, P. R., 1981. Chitin variability with species and method of preparation. Comp. Biochem. Physiol., 69B: 283-286.

Brine, C., 1982. Chitin-protein interactions. In: Chitin and chitosan. Proceedings of the Second International Conference on Chitin and Chitosan.(S. Hirano and S. Tokura, eds.). The Japanese Society of Chitin and Chitosan, Tokyo, Japan. pp. 105-110.

Brock, T. D., 1997. Biology of microorganisms. Edition. 8. (Edn. M. T.Madigan), Prentice –Hall, New Jersey.

Broussignac, P., 1968. Chitosan, a natural polymer not well known by the industry. Chim. Ind. Genie Chim., 99: 1241-1247.

Brown, M.R., Barrett, S.M., Volkman, J.K., Nearhos, S.P., Nell, J.A. and Allan, G.L., 1996. Biochemical composition of new yeasts and bacteria

Brzeski, M.M., 1982. Concept of chitin/chitosan isolation from Antarctic krill (*Euphausia superba* Dana) shells on a technical scale. In: Chitin and Chitosan, Hirano, S. and Tokura, S., eds. Sapporo, Japan, 15-21.

Brzeski, M.M., 1987. Chitin and Chitosan-putting waste to good use. Infofish International, 5: 31-33.

Buchanan, R.E. and Gibbons, N.E., 1974. Bergey's Manual of Determinate Bacteriology. 8th edition. Williams and Wilkins Co., Baltimore.

Burke, V., 1933. Bacteria as food for Vertebrates. Science, 78: 194-195.

Burrel. R (1990) Immunomodulation by bacterial endotoxin ,Crit.Rev. Microbiol ., 17: 189-208

Bushan and Hoondal. 1998. Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. Biotechnologyletters., 20(2): 157-159.

Bustos, R. O. and Healy, M. G., 1994a. Proc. Icheme Research Event, London 4-6, Jan. 1994. pp. 126-128.

Bustos, R. O. and Healy, M. G., 1994b. Proc. 2nd Int. Symp. Environ. Biotechnol., Brighton. 4-6 Jul, 1994. pp. 13-15.

Camara, M.R., Coutteau, P. and Sorgeloos, P., 1997. Dietary phosphatidylcholine requirements in larval and postlarval *Penaeus japonicus* Bate. Aquaculture Nutrition, 3: 39-47.

Campoz Perez,J.J., Ellis,A.E. and Secombes,C.J. 1997. Investigation of factors influencing the ability of *Renibacterium salmonerum* to stimulate rainbow trout macrophage respiratory burst activity. Fish and Shellfish Immunology., 7: 555-566.

Canella, M., Bernardi, A. and Marghinott, D. 19894. Improvement of germinated sunflower meal by fermentation. J.Food Sci. Technol., 17: 314.

Cano-Lopez, A., Simpson, B. K. and Haard, N. F., 1987. Extraction of carotenoprotein from process wastes and selection of microorganisms. J. Food Sci., 43: 1158-1161.

Cardenas, W. and Dankert, J.R. 1997. Phenoloxidase specific activity in the red swamp crayfish *Procambarus clarkii*. Fish and Shellfish Immunol., 7: 283-295.

Carroad, P.A. and Tom, R.A., 1978. Bioconversion of shellfish chitin wastes: process conception and selection of microorganisms. J.Food Sci., 43: 1158-1161.

Carroad, P.A. and Montano, R. 1986. Shrimp heads a new source of protein. Infofish-Mark-Dig. No. 4. p.21.

Castell, J.D., Shinnhuber, R.O. and Wales, J.H., 1972. Essential fatty acids in the diet of rainbow trout (*Salmo gairdnerii*) growth, feed conversion and some gross deficiency symptoms. J. Nutr., 102: 77-86.

Catap, E. and Munday, B.L. 1999. Oral supplementation with B 1,3 glucan enhances macrophage respiratory burst in sand whitting, (*Sillago ciliata*) infected with *Aphanomyces invadans*. Fourth symposium on Diseases in Asian Aquaculture,1999. pp. 54-966.

Chakraborty and Srinivasan. 1993. Production of athermostable alkaline protease by a new *Pseudomonas* sp. by solid substrate fermentation. J.Microb. Biotechnol., 8(1):7-16.

Chan, J. G., 1970. The occurrence, taxonomy and activity of chitinolytic bacteria from sediment, water and fauna of Puget Sound. Ph.D. Diss. University of Washington, Seattle. 312 pp.

Chandramohan, D. and Thomas, I., 1984. Studies on chitinase activity and chitinoclastic bacteria in sediments, fishes and prawns. Proc. Symp. Coastal Aquaculture, Cochin, 12-18 Jan. 1980. Part 3, Finfish Culture Symp. Ser. Mar. Biol. Assoc. India (Cochin), pp. 839-845.

Chandrasekaran, M., 1985. Studies on the microbial spoilage of *Penaeus indicus*. Ph.D thesis. University of Cochin. 258 pp.

Chandrasekaran, M., 1994. Economic utilization of marine microrganisms employing solid state fermentation. In: Pandey, A., editor. Solid State Fermentation. New Delhi: Wiley Eastern, pp. 168-172.

Chandrasekaran, M., 1996. Harnessing marine microrganisms through solid state fermentation. J. Sci. Ind. Res., 55: 468-471.

Chang, P.S., Lo, C.F., Wang, Y.C. and Kou, G.H., 1996. Identification of whitespot syndrome associated baculovirus (WSBV) target organs in shrimp, *Penaeus monodon* by in situ hybridization. Diseases of Aquatic Org., 27: 131-139.

Channel, E. and Moo-Young, M., 1980. Solid state fermentations systems. Process Biochem., 15 (5); 24-28.

Chein, Y.H. and Jeng, S.C., 1992. Pigmentation of Kuruma prawn, *Penaeus japonicus* Bate, by various pigment sources and levels and feeding regimes. Aquaculture, 102: 333-346.

Chen, H, Y. and Jenn, J.S., 1992. Increased pellet water stability by the supplementation of phospholipid and its effect on shrimp growth. Asian Fish. Sci., 5: 211-217.

Chen, H. M. and Meyers, S. P., 1983. Ensilage treatment of crawfish waste for improvement of astaxanthin pigment extraction. J. Food Sci., 48: 1516-1555.

Chen, H.S., Hsu, M.F. and Jiang, S.T., 1997. Purification and characterization of an exo-N, N'-diacetylchitobiohydrolase-like enzyme from *Cellulomonas flavigena* NTOU1. Enzyme Microbial Technol., 20: 191-197.

Cheng, T.C., 1975. Functional morphology and biochemistry of molluscan phagocytes. Annals of the New York Academy of Sciences, 266: 343-379.

Chigaleichik, A.G., Pirieva, D.A. and Rylkin, S.S., 1976. Chitinase from *Serratia marcescens* BKM B-851. Prikl. Biokhim. Mikrobiol., 12: 581-586.

Citarasu, T., Immanuel, G. and Marian, M.P., 1998. Effect of feeding *Artemia* enriched with stresstol and cod liver oil on growth and stress resistance in the Indian white shrimp, *Penaeus indicus* postlarvae. Asian Fish. Sci., 12: 65-75.

Cjazkowska, D. and Ilnicka-Olejniczak, O., 1989. Acta Biotechnologica, 9: 35-42.

Clarke, D.J., Lawrence, A.L. and Swakon, D.H.D., 1993. Apparent chitin digestibility in penaeid shrimp. Aquaculture,109: 51-57.

Clarke, P. H. and Tracey, M. V., 1956. The occurrence of chitinase in some bacteria. J. Gen. Microbiol., 14: 188-196.

Cobb.B.F. III. and Vanderzant, C., 1971. Biochemical changes in shrimp inoculated with *Pseudomonas*, *Bacillus* and a Coryneform bacterium. J. Milk. Food Tech. 34: 533-540.

Cody, R.M., Davis, N.D., Lin, J. and Shaw, D., 1990. Screening microorganisms for chitin hydrolysis and production of ethanol from amino sugars. Biomass, 21: 285-295.

Coles, J.A. and Pipe, R.K., 1994. Phenoloxidase activity in the hemolymph and hemocytes of the marine mussel *Mytilus edulis*. Fish & Shellfish Immunol., 4: 337-352.

Condrey, R. E., Gosselink, J. G. and Bennet, H. J., 1972. Comparison of the assimilation of different diets by *Penaeus setiferus* and *P. aztecus*. U.S. Fish. Wildl. Serv. Fish Bull., 70: 1281-1292.

Cosio, I.G., Fisher, R.A. and Carroad, P.A. 1982. Bioconversion of shellfish chitin wastes: waste treatment, enzyme production, process design and economic analysis. J.Food Sci., 47 : 901 - 911.

Crestini, C., Kovac, B. and Giovannozzi-Sermanni,G., 1996. Production and isolation of chitosan by submerged and solid-state fermentation from *Lentinus edodes*. Biotechnol. Bioeng., 50(2): 207-210.

Crompton, E.W. and Harris, L.E.,1969. Applied Animal Nutrition, 2nd ed. Freeman and Co., San Francisco. pp. 45 - 50.

* Czajkowska, D. and Ilnicka, O., 1988. Acta Biotechnol., 8: 407.

D'Abramo, L.R. and Sheen, S.S., 1993. Polyunsaturated fatty acid nutrition in juvenile freshwater prawn *Macrobrachium rosenbergii*. Aquaculture, 115: 63-86.

D'Abramo, L.R., Bordner, C.E., Daggett, G.R., Conklin, D.E. and Baum, N.A., 1980. Relationships among dietary lipids, tissue lipids and growth in juvenile lobsters. Proc. World Maricult. Soc., 11: 335-345.

Dabrowski, K., Hassard, S., Quinn, J.P., Pitcher, T.J. and Flinn, A.M., 1980. Effect of *Geotrichum candidum* protein substitution in pelleted fish feed on the growth of rainbow trout (*Salmo gairdneri* Rich.) and on utilization of the diet. Aquaculture, 21: 213-232.

Dall, W. and Moriarity, D.J.W., 1983. Functional aspects of nutrition and digestion. In: The biology of Crustacea. Vol. 5. Internal anatomy and physiological regulation. Bliss, D.E. and Mantel, L.H. (eds.) Academic Press, New York, p. 215-251.

Dalmo, R.A., McQueen Leifson,R. and Bogwald,J. 1995. Microspheres as antigen carriers: studies on intestinal absorption and tissue localization of polystyrene microspheres in Atlantic salmon, *Salo salar* L. J. Fish Diseases. 18: 87-91.

Das, M.M., Mukhopadhyay, P.K. and Nanda, S.K., 1995. Evaluation of four formulated diets in *Macrobrachium malcolmsonii* juveniles. J. Aqua. Trop., 10: 129-137

Daubresse, P., Ntibashirwa, S., Gheysen, A. and Meyer, J.A., 1987. Biotechnol. Bioeng., 29: 962.

Deshimaru, O. and Yone, Y., 1978. Effect of dietary carbohydrate source on the growth and feed efficiency of prawn. Bull. Jpn. Soc. Sci. Fish., 44: 1161-1163.

Deshimaru, O., Kuroki, K., Mazid, M.A. and Kitamura, S., 1985. Nutritional quality of compounded diets for prawn *Penaeus monodon*. Bull. Japan. Soc. Sci. Fish., 51: 1037-1044.

Devaraja, T.N., Otta, S. K., Shubha, G., Indrani Karunasagar, Tauro, P. and Iddya Karunasagar. 1998a. Immunostimulation of shrimp through oral administration of vibrio bacterin and yeast glucan. Abstract In: International Conference on Fisheries and Food Security Beyond the Year 2000, Thailand., p. 100.

Devaraja, T.N., Otta,S.K., Shuba,G., Karunasagar,I., Tauro,P. and Karasu. 1998b. Immunostimulation of shrimp through oral administration of vibrio bacteria.. In Flegal T.We (de) Advances in shrimp biotechnology – National Genetic Engineering and Biotechnology, Bangkok. pp. 167-170

Dorta, B., Bosch, A., Arcas, J. and Ertola, R., 1994. Water balance on solid state fermentation without forced aeration. Enzyme Microb. Technol., 16: 562-565.

Doung van Qua, Simidu, U. and Taga, N., 1981. Purification and some properties of halophilic protease produced by a moderately halophilic marine *Pseudomonas* sp., Can. J. Microbiol. 27: 505-510.

*Durand, A. and Chereau, D., 1988. Biotechnol. Bioeng., 31: 476.

Durand, A., Armoux, P., Teilhard de-Chardin, O., Chereau, D., Boquien, C.Y. and Larios de Anda, G., 1983. Protein enrichment of sugar-beet pulp by solid state fermentation, in Production and feeding of single cell protein, Eds

Ferranti, M.P. and Feitcher, A. (Applied Science Publishers, London and New York). pp. 120-123.

Duvic, B. and Soderhall, K., 1990. Purification and characterization of a β-1,3-glucan binding protein from plasma of crayfish *Pacifastacus leniusculus*. J. Biol. Chem., 265: 9327-9332.

Engstad, R.E., Robertsen, B. and Frivold, E., 1992. Yeast glucan induces increase in activity of lysozyme and complement-mediated hemolytic activity in Atlantic salmon blood. Fish Shellfish Immunol., 2: 287-297.

Epremyan, A.S., Chestukhina, G.G., Azizbekyan, R.R., Neksya, E.M., Rdenskaya, G.N. and Stepano, V.M. 1981. Extracellular serine proteinase of *Bacillus thuringiensis*. Biokhimiya., 46: 920-929.

Erdal.,J.I. Evensen, O., Kaurstad,O.K Lillehaug.A., Solbakken, R and Thorud K. 1991 Relationship between diet and Immune response in Atlantic (Salmon Salmo Salar L.) after feeding various levels of ascorbic acid Aquaculture, 98: 363-379.

Erichsen, 1983. In: Food Microbiology: Advances and prospects. (Roberts, T.A. and Skinner, F.A., 1983. Edn.), Academic Press, New York.

Esterman, R.,1994. Biological functions of carotenoids. Aquaculture, 124: 219-222.

Ewing, W. and Haresign, W., 1989. Probiotics. UK, Chalcombe Publications, Great Britain.

Fao, 1998. State of world fisheries and aquaculture. Food and Agricultural Organisation of theUnited Nations, Rome., pp.125

Fardiaz, D. and Markakis, P., 1981. Degradation of phytic acid in oncom (fermented peanut press cake). J.Food Sci., 46: 523.

Felse, P.A. and Panda, T., 1999. Self directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Process Biochem. 34: 563-566.

Folch, J., Lees, M. and Sloane-stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497-509.

Forster, J.R.M., 1970. Studies in the development of a compounded diet for prawns with particular reference to *Palaemon serratus*. Ph.D thesis, University

College of North Wales, Marine Sciences Laboratories, Menai Bridge, Anglesey, 79 pp.

Forster, J.R.M. (1975) Studies on the development of compouded diets for prawns, p 229 - 248. In K.S. Price, Jr., W.N. Shaw and K.S. Danberg (eds.) Proceedings of the First International Conference on Aquaculture Nutrition. Delaware Sea Grant College Program and US/Japan Aqauculture Panel, College of Marine Studies, University of Delaware, Newark.

Forster, J.R.M. and Beard, T.A., 1973. Growth experiment with *Palaemon serratus* Pennant, fed with fresh and compounded foods. Fishery Invest. London, Ser. II, 27(7): 16.

Foster, A. B. and Hackman, R. H., 1957. Applications of ethylenediamino tetraacetic acid in the isolation of crustacean chitin. Nature, 180: 40-41.

Fox, C.J., 1993. The effect of dietary chitin on the growth, survival and chitinase levels in the digestive gland of juvenile *Penaeus monodon* (Fab.) Aquaculture, 109: 39-49.

George, M.R., John, K.R. and Sundaraj, V. 1999. Immunisation trials in penaeid shrimps using pathogenic Vibrio alginolyticus. In: Fourth Symposium on Diseases in Asian Aquaculture, Philippines. pp.

Gervais, P., Molin, P., Grajek, W. and Bensoussan, M., 1988. Influence of water activity of a solid sustrate on the growth rate and sporogenesis of filamentous fungi. Biotechnol. Bioeng., 31: 457-463.

*Ghildyal, N.P., Losane, B.K., Sreekantiah, K.R. and Murty, V.S., 1985. J. Food Sci. Technol., 22: 171-176.

Gialdroni-Grassi, G. and Grassi. C., 1985. Bacterial products immunomodulating agents. Internat. Arch. Allergy and Immunol., 76: (suppl. 1), 119-127.

*Gibbon, W.R., Westby, C.A. and Dobbs, T.L., 1984. Biotech. Bioeng., 26: 1098.

Gibson, R. and Barker, P.L., 1979. The decapod hepatopancreas.Oceanogr. Mar. Biol. Annu. Rev., 17: 285-346.

Gildberg, A., Bogwald, J., Johansen, A. and Stenberg, E., 1996. Isolation of acid peptide fractions from fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (*Salmo salar*) head kidney leucocytes. Comp. Biochem. Physiol., 114B: 97-101.

Girujie, O., Popov, S. and Ggacesa, S., 1992. Mikrobiologiia, 29: 41.

Gomes et al., 2000. Chitinolytic actinomycete from a Brazilian tropical soil active against phytopathogenic fungi. World of Microbiol. Biotechnol., 16: 109-110.

Gomez, K. A. & Gomez, A.A. (1984) Statistical Procedure for Agricultural Research. An IRRI Book, John Wiley & Sons. pp. 680

Gonzalez, F., Ester Farez-Vidal, M., Arias, J.M. and Montaya, E. 1994. J. Appl. Bacteriology., 77: 567-573.

Gonzalez-Blanco, P., Saucedo-Castaneda,G. and Viniegra-Gonzalez,G., 1990. Protein enrichment of sugarcane by-products using solid-state cultures of *Aspergillus terreus*. J.Ferment.Bioeng.. 70 (5): 351-354.

Goodrich, T.A. and Morita, R.Y., 1977. Incidence and estimation of chitinase activity associated with marine fish and other estuarine samples. Mar. Biol., 41: 349-353.

*Goodrich, T.D. and Morita R.Y. 1977. Mar. Biol ..., 41: 35-360.

Goswami, U. and Goswami, S.C., 1982. Formulation of cheap prawn diets and their biological evaluation of some penaeid prawns. Proc. Symp. Coastal Aquaculture, Part 1. CMFRI, Cochin, pp. 211-215.

*Grajek, W., 1988. Biotechnol. Bioeng., 32: 255.

Gray, T.R.G. and Baxby, P., 1968. Chitin decomposition in soil. II. The ecology of chitinoclastic micro-organisms in forest soil. Trans. Br. Mycol. Soc. 51(2): 293-309.

Guary, J.C., Kayama, M., Murakami, Y. and Ceccaldi, H., 1976. The effects of a fat-free diet and compounded diets supplemented with various oils on molt, growth and fatty acid composition of prawn, *Penaeus japonicus* Bate. Aquaculture, 7: 245-254.

Gujral, G.S., Bisaria, R., Madan, M. and Vasudevan, P., 1987. J. Fermen. Technol., 65: 101.

Gumbira-Sa'id, E., Mitchell, D.A., Greenfield, P.F. and Doelle, H.W., 1992. A packed bed solid–state cultivation system for the production of animal feed: cultivation, drying and product quality. Biotech. Lett., Vol. 14 (7): 623-628.

Hackman, R. H., 1954. Chitin. I. Enzymatic degradation of chitin and chitin esters. Austr. J. Biol. Sci., 7: 168-178.

Haddadin, M.S., Abdulrahim, S.M., Al-Khawaldeh,G.Y. and Robinson,R.K., 1999. Solid-state fermentation of waste-pomace from olive processing J.Chem.Technol. Biotechnol., 74(7): 613-618.

Hall, G.M. and de Silva, S., 1992. Shrimp waste ensilation. Infofish, 2: 27-30.

* Han, Y.W. and Anderson, A.W., 1975. 30: 930.

* Han, Y.W., 1987. Biotech. Bioeng., 30: 672.

Hansen, M. E. and Illanes, A., 1994. Applications in crustacean wastes in biotechnology. In Fisheries Processing: Biotechnological applications. (Edited by A.M. Martin.) Chapman and Hall, London. pp: 174-205.

Hanson, J.A. and Goodwin, H.L., 1977. Shrimp and prawn farming in the western hemisphere. Dowden, Hutchinson and Ross Inc., Stroudsburg, Pennsylvnia, USA.

Hatakka, A.I. and Pirhonen, T.I., 1985. Cultivation of wood-rotting fungi on agricultural lignocellulosic materials for the production of crude protein Agric.Wastes., 12 (2): 81-97.

Healy, M. and Bustos, R., 1993. In: 'Chitin Enzymology"(Muzzarelli, R. A. A. ed.), pp. 461-468. European Chitin Soc. Italy.

Healy, M. G., Romo, C. R. Hansen, P., Montero, P., Villadsen, A., Borresen, T. and Cummins, A., 1992. Total Integrated Utilization of Marine Crustaceans including Antartic Krill and Arctic Shrimp. Project Submission to the European Commission's Life Sciences and Technologies for Developing Countries (STD-3).

Healy, M. G., Romo. C. R. and Bustos, R., 1994a. Bioconversion of marine crustacean shell waste. Resources, Conser. .. Recycl., 11: 139-147.

Healy, M., Bustos, R., Solomon, S. and Devine, C., 1994b. Int. Symp. /Worksh. Environ. Biotechnol. Waterloo, Ontario, Canada 4-8 Jul, 1994, Abstract FA-2.

Hesseltine, C.W., 1972. Solid state fermentation, Part I. Biotechnol. Bioeng., 14: 517-532.

Hesseltine, C.W., 1977. Solid state fermentations. Part I. Process Biochem., 12(5):24.

Hesseltine, C.W., 1981. A microbe's view of fermentation. Developments in Indus. Microbiol., 22: 1-18.

Hesseltine, C.W., 1987. Solid state fermentation- An overview. Intern. Biodeter., 23: 79-89

Hirano, S., Koishibara, Y., Kitaura, S., Taneko, T., Tsuchida, H., Murae, K. and Yamamoto, T., 1991. Chitin biodegradation in Sand Dunes. Biochem. System. Ecol., 19: 379-384.

Hock, C. H., 1940. Decomposition of chitin by marine bacteria. Biol. Bull., 79: 199-206.

Hock, C. H., 1941. Marine chitin-decomposing bacteria. J. Mar. Res., 4: 99-106.

Hodge, R.A., Manderson, G.J. and Yu, P.L., 1990. Semi-solid fermentation of apple pomace using yeasts. Ferment. Technol. Ind. Appl.; 292-95

Hood, M. A., 1973. Chitin degradation in the salt marsh environment. Ph.D Diss. Louisiana State University, Baton Rouge. 158 pp.

Hood, M. A. and Meyers, S. P., 1973. The biology of aquatic chitinoclastic bacteria and their chitinolytic activities. La Mer (Bulletin de la Societe franco Japonaise d' oceanographie). Tome 11 N'4. pp. 213-229.

Hood, M. A. and Meyers, S. P., 1977. Rates of chitin degradation in an estuarine environment. J. Oceanogr. Soc. Japan., 33: 328-334.

Hopkin, S.P. and Nott, J.A., 1980. Studies on the digestive cycle of the shore crab *Carcinus maenus* (L.) with special reference to the B cells in the hepatopnacreas. J.Mar. Biol. Assoc. UK., 60: 891-907.

Horowitz, S., Poseman, S. and Blumenthal, H.,J., 1957. The preparation of glucosamine oligosaccharides. I. Separation. J. Am. Chem. Soc., 79: 5046-5049.

Horst, M. N., 1989. Association between chitin synthesis and protein synthesis in the shrimp *Penaeus vannmei*. J. Crustacean Biology, 9(2): 257-265.

Hsu, S.C. and Lockwood, J.L., 1975. Powdered chitin as selective medium for enumeration of actinomycetes in water and soil. Appl. Microbiol., 29: 422-426.

Huang, C.C and Y.L.Song (1999). Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*).*Dev Comp.Immonol* (In press)

Hunt, S., 1970. Protein polysaccharide complexes in invertebrates. Academic Press, New York, pp. 1-329.

Isogai, H., Isogai, E., Fujii, N., Oguma, K., Chang, K.L., Deguchi, E., Takano, K., Hayashi, M. and Namioka, S., 1989. Biological effects of lipopolysaccharide from *Acromobacter stenohalis* on lymphocytes and macrophages. Jpn. J. Vet. Sci., 51: 1003-1010.

Itami, T., Yan, T. and Takahashi, Y., 1992. Studies on vaccination against vibriosis in cultured kuruma prawn *Penaeus japonicus*-I. Effect of vaccine concenrtration and duration of vaccination efficacy. J. Shimonoseki Univ. Fish., 40: 83-87.

Itami, T., Takahashi, Y. and Nakamura, Y., 1989. Efficacy of vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus*. J. Aquat. Anim. Health, 1: 238-242.

Itami, T., Takahashi, Y., Tsuchihira, E., Igusa, H. and Kondo, M., 1994. Enhancement of disease resistance of kuruma prawn *Penaeus japonicus* and increase in phagocytic activity of prawn hemocytes after oral administration of β-1,3-glucan (Schizophyllan). In : Chou, L.M. et al. (Eds.), The Third Asian Fisheries Forum, Asian Fisheries Society, Manila, Philippines, pp. 375-378.

Itami, T., Asara, M., Tokushige, K., Kubono, K., Nakagawa, A., Takino, N., Nishimura, H., Maeda, M., Kondo, M. and Tabahashi, Y., 1998. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived form *Bifidobacterium thermophilum*. Aquaculture, 164: 277-288.

Jacob, Z., 1991. Enrichment of wheat bran by *Rhodotorula gracilis* through solid-state fermentation. Folia-Microbiol., 36 (1): 86-91.

Jayalaksmy, B. and Natarajan, P., 1994. Growth, conversion efficiency and moulting frequency of *Macrobrachium idella* fed on different artificial feeds. J. Aquaculture Tropics, 9: 193-200.

Jeuniaux, C., 1959. The consequences of two different enzymes during the complete hydrolysis of chitin. Arch. Intern. Physiol. Biochim., 67:115-116.

Johansson, M.W. and Soderhall, K., 1985. Exocytosis of the prophenoloxidase activating system from crayfish hemocytes. J. Comp. Physiol., 156: 175-181.

Johnson, D.E., 1932. Some observations of chitin-destroying bacteria. J. Bact., 24: 335-340.

Johnson, E. L. and Peniston, Q. P., 1978. The production of chitin and chitosan, in Proceedings of The First International Conference onChitin/Chitosan,

(eds R.A.A. Muzzarelli and E.R. Pariser), MIT Sea Grant Report 78-7, Cambridge, MA, pp. 80-87.

Johnson, E. L. and Peniston, Q. P., 1982. Utilization of shellfish waste for chitin and chitosan production, in Chemistry and Biochemistry of Marine Food Products, (eds R. E. Martin, C. G. Flick, C. sE. Hebard and D.R. Ward), Avi Publishing, Westport; CT, pp. 415-428.

Johnson, E.A. and Ann, G.H., 1991. Astaxanthin from microbial sources. Crit. Rev. Biotechnol., 11: 297-326.

Jones, D.A., Kanazawa, A. and Ono, K., 1979. Studies on the nutritional requirements of the larval stages of *Penaeus japonicus* using microencapsulated diets. Mar. Biol., 54: 261-267.

Jones, G. E., 1958. Attachment of marine bacteria in zooplankton. U.S. Fish. Wildlife Ser. Spec. Sci. Report, 279: 77-78.

Jones, Z., 1991. Enrichment of wheat bran by *Rhodotorula gracilis* through solid state fermentation. Folia Microbiol., 36: 86-91.

*Jorgensen ,J.B.(1994). Stimulation of anti bacterial mechanisms in salmonid macrophages by yeast β-Glucan and bacterial lipopolysaccharid. Dr.Sc. thesis, The Norwegian college of fishery science, University of Tromso , Norway.

Joseph, J.D. & Meyers, S.P. (1975) Lipid fatty acid composition of shrimp meals and crustacean diets. Feedstuffs, 47, 35.

Joseph, J.D. & Williams, J.E. (1975) Shrimp head oil : A potential feed additive for mariculture. Proc. Symp. World Mariculture. Soc. 6 , 147 - 152.

Kahlon,S.S. and Dass,S.K., 1987. Biological conversion of paddy straw into feed Biol.Wastes, 22(1): 11-21.

Kanazawa, A. (1985) Nutrition of penaeid prawns and shrimps. Proc. Of the First Intl. Conf. on the culture of penaeid prawns/shrimps. Illoilo city, Philippines, 1984. pp. 123 - 130.

Kanazawa, A.and Teshima,S., 1977. Biosynthesis of fatty acid from acetate in the prawn. *Penaeus japonicus*. Mem. Fac. Fish., Kagoshima Univ., 26: 49-53.

Kanazawa, A., Tokiwa, S. and Kayama, M., 1977a. Essential fatty acids in the diet of prawn-I. Effects of linoleic and linolenic acids on growth. Nippon Suisan Gakkaishi, Bull. Jap. Soc. Sci. Fish., 43: 1111-1114.

Kanazawa, A., Teshima, S. and Tokiwa, S., 1977b. Nutritional requirement of prawn-VII. Effect of dietary lipids on growth. Nippon Suisan Gakkaishi, Bull. Jap. Soc. Sci. Fish., 43: 849-856.

Kanazawa, A., Teshima, S., Endo, M. and Kayama, M., 1978. Effects of eicosa-pentaenoic acid on growth and fatty acid composition of the prawn, *Penaeus japonicus*. Mem. Fac. Fish., Kagoshima Univ. 27: 35-40.

Kanazawa, A., Teshima, S., Tokiwa, H., and Ceccaldi, J., 1979a. Effects of dietary linoleic and linolenic acids on growth of prawn. Oceanological Acta., 2; 41-47.

Kanazawa, A., Teshima, S. and Endo, M., 1979b. Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. Comp. Biochem. Physiol. 63b: 295-298.

Kanazawa, A., Teshima, S. and Sakamoto, M., 1985. Effects of dietary lipids, fatty acids and phospholipids on growth and survival of prawn (*Penaeus japonicus*) larvae. Aquaculture, 50: 39-49.

Kaneko, T. and Colwell, R. R., 1975. Adsorption of *Vibrio parahemolyticus* onto chitin and copepods. Appl. Microbiol., 29: 269-274.

Kant, S., 1996. Potentiality of single cell protein (SCP) for aquafeed. Fishing Chimes, 35-37.

Karunandaa, K., Fales, S.L., Varga, G.A. and Royse, D.J., 1992. J. Sci., Food Agric., 60: 105.

Kawakami, H., Shinohara, N. and Sakai, M., 1998. The non-specific immunostimualtion and adjuvant effects of *Vibrio anguillarum* bacterin, M-glucan, chitin or Freund's complete adjuvant in yellowtail *Seriola quinqueradiata* to *Pasteurella piscicida* infection. Fish Pathol., 33: 287-292.

Keil-Dlouha, V., Misrahi, R. and B. Keil., 1976. The induction of collagense and a proteinase by their high mol. wt. substrates in *Acromobacter iophagus*. J. Mol. Biol., 107: 293-305.

Kerem, Z. and Haddar, Y., 1993. Enz. Microb. Technol., 15: 785.

Kim,J.H., Hosobuchi,M., Kishmoto,M., Seki,T. and Ryu,D.D.Y. 1985. Cellulase production by a solid state culture system. Biotechnol. Bioeng., 27:1445-1450.

Kirk, J.R., 1979. Nutritional changes upon processing. In: Food Science and Nutrition. Current Issues and Answers. (Clydesdale, F.ed.), p.62. Prentice Hall, Inc., Englewood Cliffs, N.J.

Kitabayashi, K., Kurata, H., Shudo, K., Nakamura, K. and Ishikawa, S. 1971. Studies on formula feed for Kuruma prawn. 1. On the relationship among glucosamine, phosphorus and calcium. Bull. Tokai Reg. Fish Res. Lab. 65 : 91 - 107.

Kitao, T. and Yoshida, T., 1986. Effect of an immunopotentiator on *Aeromonas salmonicida* infection in rainbow trout (*Salmo gairdneri*). Vet. Immunol. Immunopathol. 12: 287-291.

Klappach,G., Weichert, D., Zakordonets, D.A., Suprun, S.M. and Sholkevich, Y.G., 1991. Improvement of the nutritional value of rye products by solid-state fermentation with *Fusaria*. Acta-Biotechnol., (1991) 11(2): 109-114.

Kodama, H., Hirota, Y., Mukamoto, N., Baba, T. and Azuma, I., 1993. Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Dev. Comp. Immunol. 17: 129-140.

Kohler, C.C. and Kruegger, S.P., 1986. Use of pressed Brewer's grain as feed for freshwater prawn. J. World Maricult. Soc., 16: 181-182.

Kono, M., Matsui, T. and Shimizu, C., 1987. Effect of chitin, chitosan and cellulose as diet supplements on the growth of cultured fish. Nippon Suisan Gakkaishi, 53(1): 125-129.

Kontara, E.K.M., Coutteau, P. and Sorgeloos, P., 1997a. Effect of dietary phospholipid on requirements for and incorporation of n-3 highly unsaturated fatty acids in post larval *Penaeus japonicus* Bate. Aquaculture, 158(3-4): 305-320.

Koshio, S., Kanazawa, A. and Teshima, S., 1992. Nutritional evaluation of soyabean protein for juvenile fresh water prawn *Macrobrachium rosenbergii*. Nippon Suisan Gakkaishi, Bull. Jap. Soc. Sci. Fish., 58(5): 965-970.

Kovac, B., Batic, M. and Raspor, P., 1991. Bioconversion of food industry wastes. Recent-Adv.Biotechnol., (1991) 515-16

Kreag, R. and Smith, F., 1973. Seafood solid waste in Oregon: Disposal or recovery? Special Report 395. Oregon State University Extension, Marine Advisory Program, Corvallis. OR.

Kume, T., Matsuhashi, S., Hashimoto, S., Awang, M.R., Hamdani, H. and Saito, H., 1993. Radiation Phys. Chem., 42: 727.

Kumlu, M., Fletcher, D.J. and Fisher, C.M. 1998. Larval pigemntation, survival and growth of Penaeus indicus fed the nematode *Panagrellus redivivus* enriched with astaxanthin and various lipids. Aquaculture Nutr., 4: 193-200.

Kunitate, 1989. Purification and characterization of a serine protease form Bacillus thuringiensis. Agric. Biol. Chem., 53(12): 3251-3256.

Kunitz, M., 1947. Soybean trypsin inhibitors-II. J. Gen. Physiol., 30: 290-310.

Lafon, M., 1941. Sur la composition du tegument des crustaces. Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales. 135: 1003-1006.

Lambertsen, G. and Braekkan, O.R., 1971. Method of analysis of astaxanthin and its occurrence in some marine products.J.Sci. Food. Agric. 22: 99-101.

Lan, C.C. and Pan, B.S., 1993. In-vitro digestibility simulating the proteolysis of feed protein in the midgut gland of grass shrimp (*Penaeus monodon*). Aquaculture, 109: 59-70.

Latscha, T., 1990. The role of astaxanthin in shrimp pigmentation. In: Advances in Tropical Aquaculture, Act. Coll. 9 (Barret, J. ed.), pp. 319-325. IFREMER, Plouzane, France.

Laukevics, J.J., Apsite, A, F., Viesturs, U.E., and Tengerdy, R.P., 1984. Solid substrate fermentation of wheat straw to fungal protein. Biotechnol. Bioeng., 26: 1465-1474.

Lear, D. W., 1961. Occurrence and significance of chitinoclastic bacteria in pelagic waters and zooplankton. Bact. Proc., 61: 13.

Lear, D.W., 1963. Occurrence and significance of chitinoclastic bacteria in pelagic waters and zooplankton. In: Symposium on Marine Microbiology. (ed.) C. C. Thomas, Springfield, Illinois. pp. 594-610.

Lecadet, M.M., Lescouruat, M. and Kleier, A., 1977. Characterization of an intracellular protease isolated from sporulating cells and able to modify homologous RNA polymerase. European J. Biochem., 79: 329.

Lee, D.J., Roem, J.N., Yu, T.C. and Shinuber, R.O., 1967. Effect of W3 fatty acid on the growth of rainbow trout, *Salmo gairdneri*. J. Nutr., 92: 93-98.

Lee, D.L., 1971. Studies on protein utilization related to growth in *Penaeus monodon*. Aquaculture, 1; 1-13.

Leonard,C., Soderhall,K. and Ratcliffe,N.A. 1985. Studies on prophenoloxidase and protease activity of *Blaberus craniifer* haemocytes. Insect Biochem ., 15: 803-810.

Levin, J., 1985. In: Blood Cells of Marine Invertebrates: Experimental systems in cell biology and comparative physiology (Cohen, W.D., ed.), pp. 145-163.

Levine, D.M. and Sulkin, S.D., 1984. Nutritional significance of long chain polyunsaturated fatty acids to the zoeal development of the brachyuran crab, *Eurypanopeus depressus* (Smith). J. Exp. Mar. Biol. Ecol. 81: 211-223.

Lewis, D., Laird, B., Mards, J. and Munson, D., 1982. Viral disease control study develop shrimp culture immunization techniques. Aquaculture Magazine, May-June. pp. 12-15.

Liao, I.C., Su, M.S., Cheng, C.F., Her, B.Y. and Kojima, T., 1996. Enhancement of the resistance of grass prawn *Penaeus monodon* against *Vibrio damsela* infection by β-1,3-glucan. J. Fish. Soc. Taiwan. 23: 109-116.

Liener, I.E., 1980. Toxic constituents of plant foodstuffs, 4th Edn. (Liener, I.E., Edn.) pp. 484-527. Academic Press, New York, NY.

Lim, C. and Dominy, W. 1990. Evaluation of soyabean meal, as a replacement for marine animal protein in diets for shrimp (*Penaeus vannamei*). Aquaculture, 87(1): 53-63.

Lim, C., Sukhawngs, S. and Pascaul, F.P., 1979. Aquaculture, 17: 195.

Lim, C., Suraniranat, P. and Platon, R., 1978. A preliminary study on the evaluation of casein, shrimp meal, squid and spirulina as protein sources for *Penaeus monodon* (Fabricius) post larvae. Aquaculture Department, Southeast Asian Fish Development Center. Q. Res. Rep., 2(2): 13-18.

Lim, C., Suraniranat, P. and Platon, R.R., 1979. Evaluation of various protein sources for *Penaeus monodon* post larvae, Kalikasan, Philipp. J. Biol., 8(1): 29-36.

Lin, C.S., Chang, B.G., Su, M.S. and Shitada, K. 1982. Requirement of white fish meal protein in diet of grass shrimp, *Penaeus monodon*. China Fisheries Monthly, 337: 13-15.

Lin, D.S., Ilias, A.M., Connor, W.E., Caldwell, R.S., Cory, H.T. and Daves, Jr.G.D., 1982. Composition and biosynthesis of sterols in selected marine phytoplankton. Lipids, 17: 818-824.

Liston, J., 1957. The occurrence and distribution of bacterial types on flat fish. J. Gen. Microbiol., 16: 205-216.

Little, J. E., R. E. Sjogren and Carson, G. R., 1979. Measurement of proteolysis in natural waters. Appl. Environ. Microbiol., 37: 900-908.

Lonsane, B., Ghildyal, N.P., Budiatman, S. and Ramakrishna, S.V., 1985. Engineering aspects of solid state fermentation. Enzyme Microb. Technol., 7: 258-265.

Lonsane, B.K. and Ramesh, M.V., 1990. Adv. Appl. Microbiol., 35: 1-56.

Lopez, C., Carballal, M.J., Azavedo, C. and Villalba, A., 1997. Enzyme characterisation of the circulating haemocytes of the carpet shell calm, *Ruditapes decussates* (Mollusca: bivalvia). Fish & Shellfish Immunol., 7: 595-608.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randell, R.J. 1951. Protein measurement with Folinphenol reagent. J. Biol. Chem., 193 : 265 – 275.

Lynch, J.M., 1985. Straw as a fermentation raw material. Brit. Crop-Prot. Counc. Monograph-32 Biotechnol. Appl. Agric., pp. 35-41.

Lynn, K.R., 1990. Chitinases and chitobiases from the American lobster (*Homarus americanus*). Comp. Biochem. Physiol., 96B: 761-766.

Mac Ginite, G. E., 1932. The role of bacteria as food for bottom animals. Science, 76: 490.

Mac Farlane, H. and Path, M.R.C., 1977. Malnutrition and immunity. In: N. Catsimpoolas (Editor). Immunological Aspects of Food. AVI Publishing Co., Westport, CT. pp. 372-387.

Maita, M., Satoh, K., Fukuda, Y., Lee, H.K., Winton, J.R. and Okamoto, N., 1998. Correlation between plasma component levels of cultured fish and resistance to bacterial infection. Fish Pathol., , 33(3): 129-133.

Makino, K., Koshikawa, T., Nishihara, T., Ichikawa, T. and Kondo, M., 1981. Studies on protease from marine bacteria. 1. Isolation of marine *Pseudomonas* sp. 145-2 and purification of protease. Microbios., 31: 103-112.

Makino, K., Ogaki, J., Nishihara, T., Ichikawa, T. and Kondo, M., 1983. Studies on oprotease from marine nbacteria. I. Properties of extracellular protease from marine Pseudomonas sp.145-2. Microbios., 36: 7-20.

Manu-Tawiah, W. and Haard, N.F., 1987. Recovery of carotenoprotein from exoskeleton of snow crab. Can. Inst. Food Sci. Technol. J., 20: 31.

Margolis, L., 1953. The effect of fasting on the bacterial flora of the intestine of fish. J. Fish. Res. Bd. Can., 10: 62-63.

Martin, B.J., 1980. Growth and fatty acids of *Palaemon serratus* fed with compounded diets containing different proportions of linoleic and linolenic acids. Aquaculture, 19: 325-337.

Matsuo, K. and Miyazano, I., 1993. The influence of long-term administration of peptidoglucan on disease resistance and growth of juvenile rainbow trout. Nippon Suisan Gakkaishi, 59: 1377-1379.

Menesveta, P., Panichayakul, S., Piyatiratitivorakul, P. and Piyatiratitivorakul, S., 1983. Effect of different diets on survival of giant prawn larvae (*Macrobrachium rosenbergii* de Man). Proc. I. Int. Conf. on Warm water Crustacea, 317-319.

Menesveta, P., Panichayakul, P., Piyativativorakul, P. and Piyatirativorakul, S., 1984. Effect of different diets on survival of gaint prawn larvae (*Macrobrachium rosenbergii,* De Man). J. Sci. Thailand, 10: 179-187.

Merchie, G., Kontara, E., Lavens, P., Robles, R., Kurmaly, K. and Sorgeloos, P. 1998. Effect of vitamin C and astaxanthin on stress and disease resistance of postlaval tiger shrimp, *Penaeus monodon* (Fabricius). Aquaculture Res., 29: 579-585.

Meyers, S. P. 1986. Utilization of shrimp processing wastes. Infofish - Mar- Dig. no. 4, p. 18 -19.

Miki, W., 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem., 63: 141-146.

Millamena, O.M., Primavera, J.H., Pudadera, R.A. and Caballero, R.V., 1986. The effect of diet on the reproductive performance pond-reared *Penaeus monodon* Fabricius Broodstock. pp. 593-596. In MacLean, J.L., Dizon, L.B. and

Hosillos, L.V., editors. The First Asian Fisheries Society, Manila, Phillippines.

Millamena, O.M., Bombeo, R.F., Jumalon, N.A. and Simpson, K.L., 1988. Effects of various diets on the nutritional value of *Artemia* sp. as food for the prawn *Penaeus monodon*. Mar. Biol., 98: 217-221.

Milstein, O., Vered, Y., Sharma, A., Gressel, J. and Flowers, H.M., 1986. Heat and microbial treatments for nutritional upgrading of wheat straw.Biotechnol. Bioeng., 28(3): 381-386.

Miranda, P.M. and Horowitz, D.L., 1978. Higher fibre diets and the treatment of diabetes mellitus. Ann. Int. Med., 88: 482-486.

Mitchell, D.A. and Lonsane, B.K., 1991. In General Principles of Solid State Fermentation"(H.W. Doelle and C. Rolz, eds.). Monograph 1. in press. Rapid Publications of Oxford, London.

Mitchell, D.A., 1992. Microbial basis of process. In: Doell, H.W., Mitchell, D.A., Rolz, C.F. editors. Solid State Cultivation. London: Elsevier, 17-28.

Miyamoto, S., H. Yamamoto and Seki, H., 1991. Chitin dynamics in freshwater environment. Biochem. Syst. Ecol., 19: 371-377.

Monreal, J. and Reese, E.T., 1969. The chitinase of *Serratia marcescens*. Can. J. Microbiol., 15: 689-690.

Moore, L.B. and Stanley, R.W., 1982. Corn silage as a feed supplement for grow-out of *Macrobrachium rosenbergii* in ponds. J.World Maricult. Soc., 13: 86-94.

Moo-Young, M., Daugulis, A.J., Chahal, D.S. and Mac Donald, D.G., 1979. Process Biochem., 38.

Moo-Young, M., Moreira, A.R. and Tengerdy, R.P. 1983. Fungal Technology. Principles of solid-substrate fermentation. In: The Filamentuous Fungi.Vol. 4 (Smith, J.E., Berry, D.R. and Kritiansen, B., eds.). Edward Arnold, London. pp. 117-144.

Morgan, W.T.J. and Elson, L.A., 1934. Biochem. J., 28: 988.

Moriarity, D.J.W. 1977. Quantification of carbon, nitrogen and bacterial biomass in the food of some penaeid prawns. Aust. J. Mar. Freshwater Res., 28: 113-118.

Moriarity, D.J.W. and Barclay, M.C. 1980. Carbon and Nitrogen content of food and the assimilation efficiencies of penaeid prawns in the Gulf of Carpentaria. Freeman, San Francisco, CA, 753p.

Mourente, G. and Rodriguez, A., 1997. Effects of salinity and dietary DHA (22: 6n-3) content on lipid composition and performance of *Peneaus kerathurus* postlarvae. Mar. Biol., 128: 289-298.

Mukhopadhyay, N. and Ray, A.K., 1999. Effect of fermentation on the nutritive value of sesame seed meal in the diets for rohu, *Labeo rohita* (Hamilton), fingerlings. Aquaculture Nutr., 5:229-236.

Murata, K., Ikehata, H. and Miyamoto, T. 1967. Studies on the nutritional value of tempeh. J. Food Sci., 32: 580-585.

Murchelano, R. A. and Brown, C., 1970. Heterotrophic bacteria in Long Island Sound. Mar. Biol., 7: 1-6.

Muzzarelli, R. A. A., 1977. Chitin. Pergamon Press. 305 pp.

Muzzarelli, R. and Biagini, G., 1993. In "Chitin Enzymology" (Muzzarelli, R. A. A.ed.), pp. 187-196. European Chitin Soc. Italy.

Naczk, M. and Shahidi, F. 1990. Chemical composition and chitin content of crustacean offal. Advances in Fisheries Tech. and Biotechnology for increased profitability. Voigt. M. N. Botta. J. R. eds. p. 299-304.

Naczk, M., Synowiecki, J. and Sikorski, Z. E., 1981. The gross chemical composition of Antartic krill shell waste. Food Chem., 7: 175.

Nagendra, P.G. and Chandrasekaran, M., 1995. Polystyrene-an inert carrier for L-glutaminase production by marine *Vibrio costicola* in solid state fermentation. World J. Microbiol. Biotechnol., 11: 683-684.

Nagendra, P.G., and Chandrasekaran, M., 1996. L-glutaminase production by marine *Vibrio costicola* under solid state fermentation using different substrates. J. Marine Biotechnol., 4: 176-179.

Nagendra, P.G., and Chandrasekaran, M., 1997. Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* in solid state fermentation using polystyrene as an inert support. Process Biochem., 32: 285-289.

Nair, K.G.R, Mathew, P.T., Madhavan, P. and Prabhu, P.V., 1987. Chitin as feed additive for broiler chicken. Ind. J. Poult. Sci., 22: 40-44.

Nair, V.C., and Duvnjak, Z., 1991. Phytic acid content reduction in canola meal by various microorganisms in a solid-state fermentation process: Acta-Biotechnol., 11(3): 211-218.

Nair, V.C., Laflamme, J. and Duvnjak, Z., 1991. Production of phytase by *Aspergillus ficuum* and reduction of phytic acid content in canola meal J. Sci. Food-Agric., 54(3): 355-365.

Negre-Sadarguez, G., Castillo, R., Petit, H., Sance, S., Martinez, R.G., Milicua, J.C., Choubert, G. and Trilles, J.P. 1993. Utilization of synthetic carotenoids by the prawn *Penaeus japonicus* reared under laboratory conditions. Aquaculture, 110: 151-159.

Newman, S.G., 1996. Non-specific immune stimulants to prevent shrimp disease. Fisheries World, 2: 4-8.

*Nicolini, L., Volpe, C, Pezzotti, A. and Carilli, A., 1993. Bioresources Technol., 45: 17.

* Nigam, P. and Prabhu, K.A., 1985. Int. Sugar J., 87: 17.

*Nigam, P. and Vogel, M., 1988. Biotech Lett. 10: 755.

*Nigam, P. and Vogel, M., 1990. Eur. Pat. DE 3812612 C2, 1st March, 1990.

*Nigam, P., 1989. In : Proceedings 52nd Annual Convention of Sugar Technologists association of India, G53.

Nigam, P., 1990. Investigation of some factors important for solid-state fermentation of sugarcane bagasse for animal feed production. Enzyme-Microb.Technol., 12(10): 808-811.

*Nigam, P., 1994. Process Biochem., 29: 337.

Nishimura, K., Nishimura, S., Nishi, N., Numata, F., Tpne, Y., Tokura, S. and Azuma, I., 1985: Adjuvant activity of chitin derivatives in mice and guinea-pigs. Vaccine, 3: 379-383.

Nishio,N., Tai,K. and Nagai,S. 1979. Hydrolase production of *Aspergillus niger* in solid state cultivation. Eur. J. Appl. Microbiol. Biotechnol., 8: 263-270.

Nitkowski, M. F., S. Dudley and Graikoski, J. T., 1977. Identification and characterization of lipolytic and proteolytic bacteria isolated from marine sediments. Mar. Pollut. Bull., 8: 276-279.

Ohno, A., Kojima, M., Phae, C.G., Ano, T. and Shoda, M., 1992. Production of antifungal peptide produced by *Bacillus subtilis* in solid-state fermentation. Biochem. Eng., 2001:158-61

Ohno, N., Emori, Y., Yadomae, T., Saito, K., Masuda, A. and Oikawa, S., 1990. Reactivity of *Limulus* amoebocyte lysate towards (1-3)-β-D-glucans. Carbohydr. Res., 207: 311-318.

Olivier, G., Evelyn, T.P.T. and Lallier, R., 1985. Immunity to *Aeromonas salmonicida* in coho salmon (*Oncorhynchus kisutch*) induced by modified Freund's adjuvant: its specific nature and the probable role of macrophages in the phenomenon. Dev. Comp. Immunol., 9: 419-432.

Olivier, G., Eaton, C.A. and Campbell, N., 1986. Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). Vet. Immunol. Immunopathol., 12: 223-234.

Opoku, A.R. and Adoga, P.A., 1980. Enz. Microb. Technol., 2: 241.

Oriol, E., Raimbault, M., Rousos, S. and Gonzalves, V., 1988. Water and Water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. Applied Microbiol. Biotechnol., 27: 498-503.

Page, G.I., Hayworth, K.M., Wade, Harris, A.M. and Bureau, D.P., 1999. Non-specific immunity parameters and the formation of advanced glycosylation end-products (AGE) in rainbow trout, *Oncorhynchus mykiss* (Walbaum), fed high levels off dietary carbohydrates. Aquaculture Res., , 30: 287-297.

Pan, B.S., 1989. Recovery and utilization of shrimp waste for flavourant. Paper presented at the Seafood Biotechnology Workshop, Aug. 31-Sept.1, St.John's, NF, Canada.

Pandey, A., Soccol, C.R. and Mitchell., 2000. New developments in solid-state fermentation: I-bioprocesses and products. Process Biochem., 35(10): 1153-1169.

Pascaul, F.P. and Destajo, W. H., 1978. Growth and survival of *Penaeus monodon* postlarvae fed shrimp head meal and fish meal as primary animal source of protein. Q. Res. Rep. II. (1): 26-30. Aquacult. Dep. Southeast Asian Fish. Dev. Cent.

Pascaul, F.P., Coloso, R.M., and Tamse, C.T., 1983. Survival and some histological changes in *P. monodon* juveniles fed various carbohydrates. Aquaculture, 31: 169-180.

Paterson, W.D. and Fryer, J.L., 1974. Immune responses of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* cells administered intraperitoneally in Freund's complete adjuvant. J. Fish. Res. Board Can., 31: 1751-1755.

Penaflorida, V.D., 1989. An evaluation of indigenous protein sources as potential component in the diet formulation for tiger prawn, *Penaeus monodon*, using essential amino Index (EAAI). Aquaculture,83:319- 330

Penaloza, W., Molina, M.R., Gomez, Brenes, R. and Bressani, R., 1985. Solid-state fermentation: an alternative to improve the nutritive value of coffee pulp. Appl. Environ.Microbiol., 49(2) 388-93.

Perceval, P., 1978. The economics of chitin recovery and production, In Proceedings of The First International Conference on Chitin/Chitosan, (eds R.A.A. Muzzarelli and E.R. Pariser), MIT Sea Grant Report 78-7, Cambridge, MA. pp. 45-53.

Petit, H., Sance, S., Negre-Sadargues, G., Castillo, R. and Trilles, J.P., 1991. Ontogeny of carotenoid metabolism in the prawn *Penaeus japonicus* Bate (1888) (Crustacea Penaeidae): a qualitative approach. Comp. Biochem. Physiol., 99B; 667-671.

Philip, R., 1987. Studies on the proteolytic bacteria in fish spoilage. Ph.D. Thesis, Cochin University of Science and Technology, Cochin. pp.299.

Pipe R.K., 1990 Hydrolytic enzymes associated with granular hemocytes of the marine mollusc *Mytilus edulis*. Histochemical J., 22:595-603.

Pipe R.K., 1992. Generation of reactive oxygen metabolity by the hemocytes of the mussel *Mytilus edulis*. Dev. Comp. Immunol. 16: 111-122.

Pipe, R.K., Coles, J.A and Farley, S.R 1995. Assays for measuring immune response in the mussel Mytillus edulis. Techniques in Fish Immunology. 4: 93 – 100.

Poole, T. L. and Warnes, C. E., 1981. Microbial mineralization rates of chitin in a freshwater habitat. Reprint from Abstracts of the Annual Meeting American Society for Microbiology.

Potter, L. F. and Baker, G. E., 1961. The role of fish as conveyors of microorganisms in aquatic environments. Can. J. Microbiol., 7: 595-605.

Poulicek, M. and Jeuniaux, C., 1991. Chitin Biodegradation in Marine Environments: a experimental approach. Biochem. System. Ecol., 19: 385-394.

Raa, J., 2000.The use of immuno-stimulants in fish and shellfish feeds.In: Cruz-Suarez, L.E., Ricque-Maire, D., Tapia-salazar, M., Olvera-Novoa, M.A. and civera-Cerecedo, R., (Eds.). Avances en Nutricion acuicola V. Memorias del V Simposium Internacional de Nutricion Acuicola. 19-22 Noviembre, 2000. Merida, Yucatan, Mexico.

Raa, J., Roerstad, G., Engstad, R. and Robertson, B., 1992. The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. In: Shariff, M., Subasinghe, R. P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture 1. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 39-50.

Rahmat, H., Hodge, R.A., Manderson, G.J. and Yu, P.L., 1995.World J. Microbiol. Biotechnol., 11: 168.

Ramesh, M.V.and Lonsane, B.K., 1987. Solid state fermentation for production of α-amylase by *Bacillus megaterium* 16M Biotechnol. Lett., 9(5): 323-328.

Ramesh, M.V. and Lonsane, B.K. 1990. Appl. Microbiol. Biotechnol., 33: 501.

Rathbun, B.L. and Shuler, M.L., 1983. Biotech. Bioeng., 23: 929.

Read, G.H.L., 1981. Response of *Penaeus indicus* (Crustacea: Penaeidea) to purified and compounded diets of varying fatty acid composition. Aquaculture, 24: 245-256.

Reddy, H.R.V., Ganapthi Naik, M. and Annappaswamy, T.S. 1999. Evaluation of the dietary essentiality of vitamins for *Penaeus monodon*. Aquaculture Nutr., 5: 267-275.

Rees, J.F., Cure, K., Piyatiratitivorakul, S., Sorgeloos, P. and Menasveta, P., 1994. Highly unsaturated fatty acid requirements of *Penaeus monodon* post larvae: an experimental approach on *Artemia* enrichment. Aquaculture, Amsterdam, 122: 193-207.

Reid, G.C., Woods, D.R. and Robb, F.T., 1980. Peptone induction and rifampicin insensitive collagenase production by *Vibrio alginolyticus*. J. Bacteriol., 142: 447-454.

Reid, J. D. and Ogrydziak, D. M., 1981. Chitinase over-producing mutant of *Serratia marcescens*. Appl. Environ. Microbiol., 41: 664-669.

Reinheimer, G., 1972. Bacteriological investigations in the northern Gulf of Oman. J. Mar. Biol. Assoc. India, 14: 365-371.

Reinheimer, G., 1980. Aquatic Microbiology. Second Edition. John Wiley and Sons.

Reissig J.L., Stromainger, J.L. and Leloir, L.F., 1955. A modified colorimetric method for the estimation of N-Acetylamino sugars. J. Biol. Chem., 217, 959-966.

Rengpipat, S. and Rukpratapom. 1998. Probiotics in Aquaculture: a case study of probiotics of larvae of the Black tiger shrimp (*Penaeus monodon*). In: Fifth Asian Fisheries Forum, Thailand. p. 193.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menesveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture, 167: 301-313.

Renu, S., 1991. L-glutaminase production by marine bacteria. Ph.D. Thesis. Kochi, India: Cochin University of Science and Technology.

Revah-Moiseev, S. and Carroad, P., 1981. Conversion of the enzymatic hydrolysate of shellfish waste chitin to single-cell protein. Biotech. Bioeng., 23: 1067-1074.

Rhishipal, R. and R. Philip. 1998. Selection of Marine Yeasts for the generation of Single cell protein from prawn-shell waste. Bioresource Technol., 65: 255-256.

Robersten, B., Engstad, R.E. and Jorgense, J.B., 1994. β-glucans as immunomodulants in fish. Modul. Fish Immune Responses, 1: 83-99.

Robertson, B., Rorstad, G., Engstad, R. and Raa, J., 1990. Enhancement of nonspecific disease resistance in Atlantic Salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. J. Fish Dis. 13: 391- 400.

Rodriguez, J.A., Echevania, J., Rodriguez, F.J., Sierra, N., Danial, A. and Martiner, O., 1985. Biotech. Lett., 7: 577.

Rodriguez, Leon, J.A., Sastre, L., Echevarria, J., Delgado, G. and Bechstedt, W., 1988. Acta Biotechnol, 8: 307.

Roe, J.H.1955. The determination of sugar in the blood and spinal fluid with anthrone reagent. J. Biol. Chem., 212 : 335 – 343.

Rojas-Avaelizapa, Cruz-Camarillo, Guerrera, Rodriguez- Vasquez, Ibarra. 1999. Selection and characterization of a proteo-chitinolytic strain of *Bacillus thuringiensis*, able to grow in shrimp waste media. World J.Microbiol.Biotechnol., 15: 299-308.

Rolz, C., Leon, R, de., Arriola, M.C. de, and Cabrera, S, de., 1986. Appl. Environ. Microbiol., 52: 607.

Rossi, J., Clementi,F., Gobbetti, M. and Ribaldi, M., 1988. Fungal protein production from citrus waste solid substrates: Biotechnol. Food. Ind., pp. 545-555.

Roussos, S., Hannibal., Aquiahuatl, M.A., Hernandez, M.R.T. and Marakis, S., 1994. J. Food Sci. Technol. (Mysore), 31: 316.

Rudall, K.M., 1963. The chitin-protein complexes of insect cuticles. Adv. Insect Physiol., 1: 257-313.

Rukyani, A., Ruchimat, T. and Sunarto, A. 1999. Earthen pond trials of lipopolysccharide (LPS) as an immunostimulant for shrimp- Book of abstracts – Fourth symposium on disease in Asian Aqiaculture ' Aquatic animal health for sustainability ' Nov22-26, 1999. Phillipines : OP 33.

Saegal, C., Leon, J.B., Maria, B., Roussos, S. and Raimbault, M., 1993. Biotech. Techniques, 7: 563.

Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture, 172: 63-92.

Sakai, M., Kamiya, H., Ishii, S., Atsuta, S. and Kobayashi, M., 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*, In: Shariff, M., Subasinghe, R. P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture 1. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 413-417.

Sakai, M., Yoshida, T., Atsuta, S. and Kobayashi, M., 1995. Enhancement of resistance to vibriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by oral administration of *Clostridium butyricum* bacterin. J. Fish Dis. 18: 187-190.

Salte, R., Thomassen, M.S. and Wold, K., 1988. Do high levels of dietary polyunsaturated fatty acids (EPA/DHA) prevent diseases associated with the membrane degeneration in farmed Atlantic salmon at low water temperatures? Bull. European Assoc. Fish Pathol., 8: 63-65.

Sandford, P. A., 1989. Chitosan: commercial uses and potential applications, in Chitin and Chitosan. Sources, Chemistry, Biochemistry, Physical Properties and Applications, Elsevier Applied Science, New York. pp. 51-70.

Sandifer, P.A. and Joseph, J.D., 1976. Growth responses and fatty acid composition of juvenile prawns (*Marcobrachium rosenbergii*) fed a prepared ration augmented with shrimp head oil. Aquaculture, 8: 129-138.

Santarem, M., Novoa,B. and Figueras,A. Effects of β-glucans on the non-specific immune responses of thebot (*Scophtalmus maximus*, L.). Fish and Shellfish Immunol., 7:429-437.

Saono, S., Hull, R.R. and Dhamcharee, B.A., 1986. A concise handbook of indigenous fermented foods in the Asca Countries (The Indonesian institute of sciences, Jakarta).

Sato, K., Nagatini, M., Nakamura, M. and Sato, M., 1983. J. Ferment. Technol., 61: 623.

Saul, S.J. and Sugumaran, M., 1987. Arch. Insect Biochem. Physiol., 5: 1-11.

Scholz, U., Diaz, G.G., Ricque, D., Suarez, L.E.C., Albores, F.V. and Latchford, J., 1999. Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. Aquaculture, 176: 271-283.

Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Techniques in Fish Immunology (ed. by Stolen, J.S., Fletcher, T.C., Anderson, D.P., Robertson, B.S. and van Muiswinkle, W.B.) pp. 137-154. SOS publications, Fair Haven, N.

Seki, H. and Taga, N., 1963. Microbiological studies on the decomposition of chitin in the marine environments-5. Chitinoclastic bacteria as symbionts. J. Oceanogr. Soc. Japan, 19: 38-41.

Seki, H and Taga, N., 1965. Microbiological studies on the decomposition of chitin in marine environment-V. Chitinoclastic bacteria in the digestive tracts of whales from the Atlantic Ocean. J. Oceanogr. Soc. Japan, 20: 272-277.

Senecal, K, J., Mandels, M. and Kaplan, D.L., 1992. Biological conversion of inedible biomass to food. Biotechnol. Nutr., (1992) 59-79.

Senez, J.C., Raimbault, M. and Deschamps, F., 1980. World Anim. Rev. 35: 36.

Senez, J.C., M. Raimbault and F. Deschamps., 1983. Protein enrichment of starchy substrates by solid substrate fermentation. In the use of Organic Residues in Rural Communities (C.A. Shackaldy, ed.). UNU, Tokyo, pp. 52-61.

Sera, H., 1968. Breakdown of chitin in the digestive tracts of black sea bream, *Acanthopagrus schlegeli* (Bleeker). Ph.D. Thesis, Department of Fisheries, Faculty of Agriculture, Kyoto University. 75 pp.

Sera, H. and Ishida, 1972. Bacterial flora in the digestive tracts of marine fish. III. Classification of isolated bacteria. Bull. Jap. Soc. Sci. Fish., 38: 853-858.

Shahidi, F., Synowiecki, J. and Naczk, M., 1989. Flavourants from shellfish waste. Unpublished data. Food Science Program, Memorial University, St. John's, NF, Canada.

Shakaranand, V.S., Ramesh, M.V. and Lonsane, B., 1992. Idiosyncrasies of solid-state fermentation systems in the biosynthesis of metabolites by some bacterial and fungal cultures. Proc. Biochem., 27: 33-36.

Shaw, B.G. and Shewan, J.M., 1968. Psychrophilic spoilage bacteria of fish. J. Appl. Bact., 31: 89-96.

Sheen,S.S. and Chen,J.C., 1993. The feasibility of extruded rice in shrimp feed to replace wheat flour for tiger prawn, *Penaeus monodon*. J. Fish. Soc. Taiwan, 20(1): 65-72.

Shewans, J.M., 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In: Proc. Conf. Handl. Proc. Mark. Trop. Fish., London, 5-9 July, 1976. Tropical Products Institute.

Shewbart, R.W. and Mies, W.L., 1973. Studies on nutritional requirements of brown shrimp-The effects of linolenic on growth of *Penaeus aztecus*. Proc. World Maricult. Soc., 4: 277-287.

Shiau, S.Y. and Chou, B.S., 1991. Effects of dietary protein and energy on growth performance of tiger shrimp *Penaeus monodon* reared in seawater. Nippon Suisan Gakkaishi, 57: 2271-2276.

Shiau, S.Y., Lin, S.F. and Lu, L.J., 1991. Effects of different types of wheat flour in feed for grass prawn *Penaeus monodon*. Nippon Suisan Gakkaishi, Bull. Jap. Soc. Sci. Fish., 57(4): 705-710.

Shiau, SY., Kwok, C.C. and Chou, B.S., 1991. Optimal dietary protein level of *Penaeus monodon* juveniles reared in seawater and brackish water. Nippon Suisan Gakkaishi, 57(4): 711-716.

Shimahara, K., Ohkouchi, K. and Ikeda, M., 1982. In S. Hirano and Tokura (Eds.) Chitin + chitosan. Proc. 2nd Int. Conf. Chitin + Chitosan Sapporo Jap. 10—

Shoby, B., 1996. Chitinase production by marine actinomycetes under solid state fermentation. M.Sc. Thesis, Kochi, India: Cochin University of Science and technology.

Sick, L.V., Andrews, J.W. and White, J.B., 1972: Preliminary studies of selected environmental and nutritional requirements for the culture of penaeid shrimps. Fish. Bull., Fish. Wildl. Ser., U.S., 70: 101-109.

Sick, L.V. and Andrews, J.M., 1973. Effects of selected dietary lipids, carbohydrates and proteins on the growth, survival and body composition of *Penaeus duorarum*. Proc. World Maricult. Soc. 4: 263-276.

Simpson, K. L., 1978. The recovery of protein and pigments from shrimp and crab meals and their use in salmonid pigmentation, in Proceedings of The First InternationalConference on Chitin/Chitosan, (eds. R.A.A. Muzzarelli and E.R. Pariser), MIT Sea Grant Report 78-7, Cambridge, MA. pp. 253-262.

Simpson, S. K. and Haard, N. F., 1985. J., App. Biochem., 7: 212-222.

Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K., 1989. Production of protein and cellulase by *Aspergillus niger* in solid state culture J. Appl. Microbiol. Biotechnol.,5(4): 451-456.

Singh, K., Rai, S.N., Neelakantan, S. and Han, Y.W., 1990. J. Anim. Sci., 60(8): 484-490.

Siwicki, A.K., Andersen, D.P., and Dixon, O.W., 1990. *In vitro* immunostimulant of rainbow trout (*Onchorhynchus mykiss*) spleen cells with levamisole. Dev. Comp. Immunol., 14: 231-237.

Siwicki, A.K., Andersen, D.P. and Rumsey, G.L., 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Vet. Immunol. Immunopathol. 41: 125-139.

Sizemore, R. K., L. H. Stevenson and Hebeter, B. H., 1973. Distribution and activity of proteolytic bacteria in estuarine sediments. In: Estuarine Microbial

Ecology. L. H. Stevenson and R. R. Colwell (Eds) Univ. South Carolina Press, Columbia.

Smail, T., Salhi, O. and Knaap, J.S., 1995. World J. Microbiol. Biotechnol., 11: 171.

Smith, E.L., de Lange, R.J., Evans, W.H., London, M. and Markalnd, F.S. 1968. J. Biol. Chem., 243: 2184.

Smith, R.E., Osothsilp, C., Bicho, P. and Gregory, K.F., 1986. Biotech. Lett., 8: 31.

Smith, V.J. and Soderhall, K., 1986. Cellular immune mechanisms in the crustacea. In: Lackie, A.M., (Ed.), Immune mechanisms in Invertebrate Vectors. Zoological Society of London Symposia, No. 56. Oxford Science Publications. pp. 59-79.

Soderhall, K. and Unestam, T., 1979. Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. Can. J. Microbiol., 25: 406-414.

Soderhall, K., 1982. Prophenoloxidase activating system and melanisation-recognition mechanism of arthropods?–A review. Dev. Comp. Immunol., 6: 601-611.

Soderhall, K. and Hall, L., 1984. Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish hemocyte lysate. Biochem. Biophys. Acta, 797: 99-104.

Soderhall, K. and Smith. 1986. the Prophenoloxidase activating system; the biochemistry of its activation and role in arthropod cellular immunity with special reference to crustaceans. In: Immunity in Inverteberates. (M. Brehelin ed.)., pp. 208-223. Berlin: Springer Verlag.

Soderhall, K. and Cerenius, L., 1992. Crustacean immunity. Ann. Rev. Fish Diseases., 2: 3-23.

Soderhall, K., Aspan, A., and Duvic, B., 1990. The proPO-systems and associated proteins; role in cellular communication in arthropods. Res. Immunol., 141: 896-907.

Soderhall, K., Cerenius, L. Johansson, M.W., 1994. The prophenoloxidase activating system and its role in invertebrate defense. Annals of the New York Academy of Sciences, 712: 155-161.

Sondhi, H.S., Singh, K. and Neelakantan, S.1990. Solid-state fermentation of urea supplemented wheat straw with *Neurospora sitophila.* Indian-J. Microbiol., 30(2):185-190.

Song, Y.L. and Sung, H.H. 1990. Enhancement of growth in tiger shrimp (*Penaeus monodon*) by bacterin prepared from *Vibrio vulnificus*. Bull. Eur. Ass. Fish Pathol., 10: 98-99.

Song, Y.L. and Sung, H.H. 1996. Enhancement of growth in tiger shrimp (*Penaeus monodon*) by bacterin prepared from *Vibrio vulnificus*. Bull. Eur. Assoc. Fish Pathol., 10: 98-99.

Song, Y.L., Liu, J.J., Chan, L.C. and Sung, H.H., 1997. Glucan-induced disease resistance in tiger shrimp (*Penaeus monodon*). In: Fish Vaccinology (R. Gudding, A., Lillehaug, P.J., Midtlyng, and F. Brown, eds.), Deve. Biol. Stand. Basel, Karger, 90: 413-421.

Sorgeloos, P. and Leger, P., 1992. Improved larviculture outputs of marine fish, shrimp and prawn. J. World Aquacult. Soc., 23: 251-264.

Spinder-Bath, M., Van-Wormhoudt, A. and Spindler, K.D., 1990. Chitinolytic enzymes in the integument and midgut-gland of the shrimp *Palaemon serratus* during the moulting cycle. Mar. Biol., 106: 49-52.

Stanier, R. Y., 1947. Studies nonfruiting myxobacteria. I. *Cytophaga johnsonnae*, n. sp. A chitin-decomposing myxobacterium. J. Bact., 53: 297.

*Steiner, M., 1931. Beitrage zur Kenntnis der Zellulose und des Chitinabbaues durch Mikroorganismen in stehenden Binnengewassern.75 Jahre Stelle Matutina, Festschrift, Feldkirch. 2: 367. (Abstract from Biol. Abstr. 1934, 8: 2815)

Storch, V. and Anger, K., 1983. Influence of starvation and feeding on the hepatopancreas of larval *Hyas araneus* (decapoda, Majidae). Helgol, Meeresunters., 36: 67-75.

Storch, V., 1984. The influence of nutritional stress on the ultrastructure of the hepatopancreas of terrestrial isopods. Symp. Zool. Soc., London, 53: 167-184.

Storch, V. and Burkhardt, P. 1984. Influence of nutritional stress on the hepatopancreas of Talitrus saltator (Peracarida, Amphipoda). Helgol, Meeresunters., 38: 65-73.

• Song, L. and Hsieh, Y.T., 1994. Immunostimulation of Tiger shrimp (*Penaues monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Dev. Comp. Immunol., 18(3): 201-209.

Storch, V., Juario, J.V. and Pascaul, F., 1984. Early effects of nutritional stress on the liver of milkfish, *Chanos chanos* (Forsskal), and on the hepatopancreas of the tiger prawn, *Penaeus monodon* (Fabricius). Aquaculture, 37: 209- 213.

Stredansky, M., Conti, E., Navarini, L. and Bertocchi, C., 1999. Production of bacterial exopolysaccharides by solid substrate fermentation. Process Biochem., 34: 11-16.

Stuart, L. S., 1936. A note on halophilic chitinovorous bacteria. Jour. Am. Leather Chem. Ass'n., 31: 119-120.

Su, M.S., Liu, K.F., Chang, C.F. and Liao, I.C., 1995. Enhancement of grass prawn *Penaeus monodon* postlarvae viability by β-1,3-glucan from *Schizophyllum commune*. J. Taiwan Fish Res., 3: 125-132 (In Chinese with English abstract)

Subasinghe, S., 1999. Chitin from shellfish waste-health benefits overshadowing industrial uses. Infofish International, 3: 58-64.

Sudarmadji, K. and Markakis, P. 1977. The phytate and phytase of soyabean tempeh. J. Sci. Food Agric., 28: 381.

Sudarmadji, K. and Markakis, 1978. Lipid and other changes occuring during the fermentation and frying of tempeh. Food Chem., 3: 165-170.

Sudaryono, A., Tsvetnenko, E. and Evans, L.H. 1996. Digestibility studies on fisheries by-products based diet for *Penaeus monodon*. Aquaculture, 143: 331-340.

Sugita, H., Kumazawa, J. and Deguchi, Y., 1996. Production of chitinase and β–N-acetylglucosaminidase by intestinal bacteria of Pinnipedian animals. Lett. Appl. Microbiol., 23: 275-278.

Sung, H.H. and Song, Y.L., 1996. Tissue location of *Vibrio* antigen delivered by immersion to tiger shrimp (*Penaeus monodon*). Aquaculture, 145: 41-54.

Sung, H.H., Song, Y.L. and Kou, G.H., 1991. Potential uses of bacterin to prevent shrimp vibriosis. Fish Shellfish Immunol. 1: 311-312.

Sung, H.H., Kou, G.H. and Song, Y.L., 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). Fish Pathol., 29(1): 11-17.

Suntory Ltd., 1990. Composition containing astaxanthin as oxidation inhibitor useful as vitamin E substitute and as food additive, medicament, etc. Japan Patent JP-198947, 11 pp.

Supamattaya, Pongmaneerat., 1998. The effect of Macrogard^{R} on growth performance and health condition of black tiger shrimp. Scientific Report, Prince of Songkhla Univ.

Suresh, P.V. and Chandrasekaran, M., 1999. Impact of process parameters on chitinase production by an alkalophilic *Beauveria bassiana* in solid state fermentation. Process Biochem., 34: 257-267.

Surinder, S.K. and Dass, S.K., 1987. Biol. Wastes, 22: 11.

Sutardi, A. and Buckle, K.A.,1985. reduction in phytic acid levels in soyabean during tempeh production, storage and frying. Food Sci., 50: 260.

Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S. and Suzuki, M., 1986. Effect of N-acetylchito-oligosaccharides on activation of phagocytes. Microbiol. Immunol., 30: 777-787.

Takahashi,Y., Kondo, M., Honda, Y., Mizuno, K., Itami, T., Inagawa, H. and Soma, G.I. 1999. Enhancement of disease resistance in kuruma shrimp, *P. japonicus*, against white spot syndrome after oral administration of a lipopolysaccharide extracted from *Pantoea agglomeraus*. •

Takeda, M. and Abe, E., 1962. Isolation of crustacean chitin. Decalcification by disodium ethylenediamine tetraacetate and enzymic hydrolysis of incidental proteins. Norisho Suisan Koshusho Kenkyu Hokoku, 11: 339-406.

Takeda, M. and Katsuura, H., 1964. Purification of king crab chitin. Suisan Daigaku Kenkyu Hokoku, 13: 109-116.

Takiguchi, Nagahata and Shimahara. 1988. Nippon Nogeikgaku kaishi, 59:253.

Tautorus, T.E. and Chalmers, W.T., 1984. Dev. Ind. Microbiol., 25: 621.

Tengerdy, R.P., 1998. Solid state fermentation for enzyme production. In: Advances in Biotechnology (Editor Pandey, A). Educational Piublishers and Distributors. p. 13-15.

Tengerdy, R.P., Murphy, V.G. and Wissler, M.D., 1983. Solid-state fermentation of cellulosic residues. Ann. N. Y. Acad. Sci., 413: Biochem. Eng., 3: 469-472.

• In Proceedings of the Fourth Symposium on Diseases in Asian Aquaculture, Philippines. (Abstract. No. 42).

Teshima, S., Kanazawa, A. and Sakamoto, M., 1982a. Microparticulate diets for the larvae of aquatic animals. Min. Rev. Data File Fish. Res. Kagoshima Univ., 2: 67-86.

Teshima, S., Kanazawa, A., Sasda, H. and Kawasaki, M., 1982b. Requirements of larval prawn, *Penaeus japonicus*, for cholesterol and soyabean phospholipids. Men. Fac. Fish. Kagoshima Univ., 31: 193-199.

Teshima, S., Kanazawa, A. and Yamashita, M., 1986a. Dietary value of several proteins and supplemental amino acids for larvae of prawn *Penaeus japonicus*. Aquaculture, 51: 225-235.

Teshima. S., Kanazawa, A. and Kakuta, Y., 1986b. Effects of dietary phospholipids on growth and body composition of the juvenile prawn. Nippon Suisan Gakkaishi, 52: 155-158.

Teunissen, Q.S.P., Faber, R., Booms, G.H.R., Latscha, T. and Boon, J.H., 1998. Influence of vaccination on vibriosis resistance of the giant black tiger *Penaeus monodon* (Fabricius). Aquaculture, 164: 359-366.

Thomas, I., 1982. Studies on Chitinoclastic bacteria of the coastal zones. Ph.D. Thesis, Cochin University of Science and Technology, Cochin. pp.215.

Tidwell, J.H., Webster, C.D., Clark, J.A. and D'Abramo, L.R., 1992. Evaluation of distillers grains with solubles as protein source in diets for the freshwater prawn, *Macrobrachium rosenbergii*. Aquaculture'92: Growing towards the 21st Century World aquaculture Society (USA). May 1992. pp. 216-217.

Tidwell, J.H., Webster, C.D., Yancey, D.M. and D'Abramo, L.R., 1993a. Partial and total replacement of fish meal with soyabean meal and distillers' by-products in diets for pond culture of the fresh water prawn (*Macrobrachium rosenbergii*). Aquaculture, 118: 119-130.

Tidwell, J.H., Webster, C.D. and Clark, J.A., 1993b. Evaluation of distillers dried grains with solubles as an ingredient in diets for pond culture of the freshwater prawn *Macrobrachium rosenbergii*. J.World Aquacult. Soc. 24: 66-70.

Tidwell, J.H., Webster, C.D., Goodgame-Tiu, L. and D'Abramo, L.R., 1994. population characteristics of *Macrobrachium rosenbergii* fed diets containing different protein sources under coolwater conditions. J. World Aquacult. Soc., 28(2): 123-132.

Timmis, K., G. Hobbs and Berkeley, R. C. W., 1974. Chitinolytic clostridia isolated from marine mud. Can. J. Microbiol., 20: 1284-1285.

Tom and Carroad. 1981. Effect of Reaction Conditions on Hydrolysis of Chitin by *Serratia marcescens* QMB 1466 chitinase. 646. J. Food Sci., 46.

Tominaga, Y., 1977. Enzymes dissolving the cell walls of Eumycetes. Osaka-Shiritsu Kogyo Kenkyusho Hokodu, 54: 108.

Tominaga, Y. and Stujisake, Y., 1975. Purification and some enzymatic properties of the chitosanase from *Bacillus* R-4 which lyses *Rhizopus* Cell Walls. Biochim. Biophys. Acta., 410: 145-155.

Torrisen, O., Tideman, E., Hansen, F. and Raa, J., 1981. Aquaculture, 26: 77-93.

Tripathi, J.P. and Yadav, J.S., 1992. Anim. Feed Sci. Technol., 37: 59.

Tristam, D.A. and Ogra, P.L. 1994. Immunology of the gastrointestinal tract. In: Viral infections of the Gastrointestinal tract. (A.Z. Kapikian ed.) New York; Marcel Dekker.

Tsugita, T., 1991. Chitin/chitosan and their applications. Marine Colloids Biotechnology. pp. 287-297.

Tsujibo, H., Yoshida, Y., Imada, C., Okami, Y., Miyamoto, K. and Inamori, Y., 1991. Purification, properties and partial amino acid sequence of chitinase from a marine *Alteromonas* sp. strain O-7. Nippon Suisan Gakkaishi, 57: 2127-2131.

Tsuru, D., Kirk, H., Yamamoto, T. and Fukumoto, J., 1967. Studies on bacterial protease of *Bacillus subtilis* var. amylosacchariticus. Agric. Biol. Chem., 31: 718-723.

Ulhoa, C.J. and Peberdy, J.F.1992. Purification and some properties of the extracellular chitinase produced by Trichoderma harzianum. Enzyme Micro. Technol., 14: 236-240.

Ulmer, D.C., Tengerdy, R.P., and Murphy, V.G., 1981. Biotechnol. Bioeng. Symp. Ser., 11: 449 – 461.

Unestam, T. and Soderhall, K., 1977. Soluble fragments from fungal cell walls elicit defence reactions in crayfish. Nature, 267: 45-46.

Vaitheswaran, S. and Ali, S.A., 1986. Evaluation of certain substances as growth promoting agents for the prawn *Penaeus indicus*. Indian J. Fish., 33: 95-105.

van de Braak, C.B.T. and van der Knaap. 1998. Morphological, antigenic and functional characteristics of *Penaeus monodon* hemocytes. In: Fifth Asian Fisheries Forum, Thailand. p. 310. (Abstract).

van Veen, A.G. and Steinkraus, K.H., 1970. Nutritive value and wholesomeness of fermented foods. J. Agric. Food Chem., 18: 576.

Vande Braak, C.B.T., Botterblom, M.H.A., vande Knaap, W.P.W. and Rombout, J.H.W.M. 1999. Morphological characterization of monoclonal antibodies to different phases of black tiger shrimp (*Penaeus monodon*) haemocytes. •

Vargaz-Albores, F., Hinojosa-Baltazar, P., Portillo-Clark, G. and Magallon-Barajas, F., 1998. Influence of temperature and salinity on the yellowleg shrimp, *Penaeus californiensis* Holmes, prophenoloxidase system. Aquaculture Res., 29: 549-553.

Veldkamp, H., 1955. A study of the aerobic decomposition of chitin by micro-organisms. Meded. Landbouw., Wageningen. 55: 127-174.

Venkitaramiah, A., Lakshmi, G.J. and Gunther, G., 1975. Effect of protein level and vegetable matter on growth and food conversion efficiency of brown shrimp. Aquaculture, 6: 115-125.

Venkitaramiah, N.N., Cook, D.W., Biesiot, P. and Lakshmi, G.J., 1978. Nutritional value of high marsh grass and shrimp shell waste from commercial brown shrimp (*Penaeus aztecus* Ives). Proc. World Maricult. Soc., 9: 217-224.

Viesturs, U.E., Strikausa, S.V., Leite, M.P., Berzins, A.J. and Tengerdy, R.P. 1987. Combined submerged and solid state substrate fermentation for the bioconversion of lignocellulose. Biotechnol. Bioeng., (1987) 30(2):282-288.

Waagbo, R., 1994. The impact of nutritional factors on the immune system in Atlantic salmon, *Salmo salar* L., a review. Aquaculture Fish. Manag., 25: 175-197.

• In Proceedings of the Fourth Symposium on Diseases in Asian Aquaculture, Philippines. (Abstract No. 31)

Waksman, S. A., Carey, C. L. and Reuszer, H. W., 1933. Marine bacteria and their role in the cycle of life in the sea. I. Decomposition of marine plant and animal residues by bacteria. Biol. Bull., 65: 57.

Wang, H.L. and Hesseltine, C.W., 1981. Use of microbial cultures: legumes and cereal products. Food Technol., 35: 79.

Wang, H.L. and Hesseltine, C.W., 1982. In Prescot and Dunn's Indust. Microbiol., edited by G. Reed. (AVI Pub. Co., Westport), 4th Edn. pp. 492-538.

Wang, H.L., Ruttle, D.K. and Hesseltine, C.W., 1968. Protein quality of wheat and soyabean after *Rhizopus oilgosporus* fermentation. J. Nutr., 96: 109.

Watanabe, T., Ogino, C., Koshishi, Y. and Matsunaga, T., 1974. Requirement of rainbow trout for essential fatty acids. Bull. Jap. Soc. Sci. Fish. 41: 493-499.

Watanabe, T., Uchida, M., Kobori, K. and tanak, H., 1994 Side directed mutagenesis of the asp-197 asp. 202 residue in chitinase a1 of Bacillus circulans w1-12. Biosci. Biotechnol, 1994. 58: 2283-2285.

Wee, K.L., 1991. Use of nonconventional feedstuffs of plant origin as fish feeds – is it practical and economically feasible? In: Fish Nutrition Research in Asia. Proc. 4th Asian Fish Nutrition Workshop. (De Silva, S.S.ed.). pp. 13-32. Asian Fisheries Society, Manila, Philippines.

Weichert, D., Zakordonets, L., Klappach, G., Charkevitch, E. and Koval, E.Z., 1991. Protein enrichment in plant by *Fusaria*. Acta-Biotechnol., 11(2): 115-119.

Whistler, R. and Miller, B., 1962. Chitin. J. Organic Chem., 27: 1161-1163.

White, A., Handler, P., Smith, E.L., Hill, R. and Lehman, I.R., 1979. Principles of Biochemistry. In : Jeffers, V., Macnow,A., LaBarbera, M. and Armstrong, T. (Editors), McGraw-Hill, New York, 1338 pp.

Wight, C.P., Dargulis, A.J., Lau R.H. and White, B.N. 1988. Conditons for production of extracellular protein by *Bacillus Brevis 47*. Biotechnol. Lett., 10(11): 769-774.

Wijeyaratne, S. C., Waki, T., Suga, K. and Ichikawa, K., 1979. Annual report of International Center for Microbial Engeineering, Osaka, Japan. 2: p. 213.

Wilke, C.R., Cysewski, G. R., Yang, R. D. and von Stockar, U., 1976. Utilization of cellulosic materials through enzymatic hydrolysis. II. Preliminary

assessment of an integrated processing scheme. Biotech. Bioeng., 18: 1315-1320.

Williams, O. B., L. L. Campbell, Jr. and Reese, H. B. Jr., 1952. The bacteriology of Gulf coast shrimp. II. Qualitative observations on the external flora. Texas. J. Sci., 4: 53-54.

Yadav, J.S., 1987. Influence of nutritional supplementation on solid-substrate fermentation of wheat straw with an alkaliphilic white-rot fungus (*Coprinus* sp.) Appl. Microbiol. Biotechnol., 26: 474-478.

Yadav, J.S., 1988. Biotechnol. Bioeng., 31: 414.

Yamada, S., Tanaka, Y., Smaeshima, Z.M. and Ito, Y. 1990. Pigmentation of prawns (*Penaeus japonicus*) with carotenoids. I. Effect of dietary astaxanthin, β-carotene and canthxanthin on pigmentation. Aquaculture, 87: 323-330.

Yang, J.K., Shih, I.L., Tzeng, Y.M. and Wang, S.L., 2000. Production and purification of protease from Bacillus subtilis that can deproteinize crustacean wastes. Enzyme Microbial Technol., 26: 406-413.

Yang. S. S., 1988. Protein enrichment of sweet potato residue with amylolytic yeasts by solid state fermentation. Vol. 32 : 886 -890. Biotechnol. Bioeng.,

Yano, T., Mangindaan, R.E.P. and Matsuyama, H., 1989. Enhancement of the resistance of carp *Cyprinus carpio* to experimental *Edwardsiella tarda* infection, by some β-1, 3-glucans. Nippon Suisan Gakkaishi, 55: 1815-1819.

Yano, T., Matsuyama, H. and Mangindaan, R.E.P., 1991. Polysaccharide-induced protection of carp, *Cyprinus carpio* L., against bacterial infection. J. Fish. Dis., 14: 577-582.

Yazawa, K., Akari,K., Okasaki,N., Watanabe,K., Ishikawa,C., Inoue,A., Numao, N. and Kondo, K. 1988. Production of eicosopentaenoic acid by marine bacteria. J. Biochem., 103: 5-7.

Yemm. E.W and E.C.Cocking., 1955. The determining of amino acids with ninhydrin. Analyst, 80 : 209-213.

Yepiz-Plascencia, G., Vargas-Alborez, F., Vega, F.J., Ruiz-Verdugo, L.M. and Romo-Figueroa, G., 1998. Comparison of shrimp high density lipoprotein and beta-glucan binding protein. In: Fifth Asian Fisheries Forum, Thailand. p. 284.

Yongmanitchai, W. and Ward, O.P., 1989. Omega-3 fatty acids: Alternative sources of production. Process Biochem., 1, 117-125.

Yoshida, T., Sakai, M., Kitao, T., Khlil, S.M., Araki, S., Saitoh, R., Ineno, T. and Inglis, V., 1993. Immunomodulatory effects of the fermented products of chicken egg, EF203, on rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 109: 207-214.

Young, M.E. and Carroad, P.A., 1981. Dependence of extracellular chitinase activity of *Serratia marcescens* QMB1466 on continous culture dilution rate. Can. J. Microbiol., 27: 142-144.

* Yu, P.N., Han, Y.W. and Anderson, A.W., 1976. Proc. West. Am. Aoc., Animal Sci., 27: 189.

Zamora, R.G. and Veum, T.L., 1979. The nutritive value of dehulled soyabeans fermented with *Aspergillus oryzae* or *Rhizopus oligosporus* as evaluated by rats.

Zandee, D.I., 1966. Metabolism in the crayfish *Astacus astacus* (L). III. Absence of cholesterol synthesis. Arch. Int. Physiol. Biochem. 74(3): 435-441.

Zobell, C. E. and Feltham, K., 1938. Bacteria as food for certain marine invertebrates. J. Mar. Res., 1: 312-327.

Zobell, C. E. and Rittenberg, S. C., 1938. The occurrence and characteristics of chitinoclastic bacteria in the sea. J. Bacteriol., 35: 275-287.

* Zvauya, R. and Muzondo, M., 1993. Chem. Mikrobiol. Technol. Lebensm, 15: 171.

*NOT VIEWED IN ORIGINAL

1033 ✓

FERMENTATION OF PRAWN SHELL WASTE AND APPLICATION OF ITS PRODUCT AS DIETARY INGREDIENT FOR PENAEUS INDICUS (H. MILNE EDWARDS)

SYNOPSIS OF THE THESIS

To be submitted to

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

In partial fulfillment of the degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

Under the
Faculty of Marine Biology

By

Beatrice Amar

SCHOOL OF MARINE SCIENCES,
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN 682 106

SCHOOL OF MARINE SCIENCES,
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN 682 106

SYNOPSIS OF DOCTORAL THESIS

Title of the thesis :**Fermentation of Prawn Shell Waste and application of its product as dietary ingredient for *Penaeus indicus* (H. Milne Edwards)**

Name of the candidate : Beatrice Amar

Supervising Teacher : Dr.Rosamma Philip
Senior Lecturer
Dept. of Marine Biology,
Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Cochin 682 016.

Introduction

Shellfish processing industry in India generates about 8.5 tonnes of shell waste per year according to FAO statistics (1998). Notably, this waste contains 20-35% protein, 15% chitin and 5% fat. Eventhough a part of this is used for chitin/chitosan preparation, feed manufacture and as manure, a major portion still remains unused. A method for the complete utilization of this waste is required as a means of recovery and recycling of this nutrient rich waste rather than mere disposal and pollution abatement. The present work envisages the fermentation of prawn shell waste into a more nutritious product with simpler components for application as a feed ingredient in aquaculture. This product would be a rich source of protein along with chitin, minerals, vitamins and N-acetyl glucosamine. The raw material being a waste from shrimp processing industry, utilization of this as a feed ingredient after nutritional enrichment through fermentation will definitely contribute to reduction in feed cost. Testing the potential of the shell fermentation product as an immunostimulant is also envisaged in this programme.

The thesis has six chapters as described below:-

Chapter 1

Introduction

This chapter gives a general introduction to prawn shell waste – its composition, quantities generated and present utilization status. Processing of prawn shell waste has gained interest of researchers for the extraction of chitin, chitosan and protein concentrates. But all these procedures utilizes large quantities of chemicals and prove to be cost intensive and contributes to pollution. Moreover total utilization of the waste is still wanting. A brief description of the various processing (chemical and bioprocessing) methods employed for chitin, chitosan and single cell protein preparations from shell waste is added. An introduction into the objectives and significance of the present work is also given.

Chapter 2.

Microflora associated with Prawn Shell Waste Transformation

This chapter deals with the isolation of microflora associated with prawn shell degradation. Isolation of bacteria from shell waste with different media and screening of the isolates for chitinoclastic and proteolytic property with

the aim of employing them for the bioconversion of prawn shell waste. The best media that supported the growth of chitinoclastic forms was noted. The isolated strains were identified upto the generic level. The most common form was *Bacillus* sp. followed by *Coryneformes* and *Vibrio sp.* Thirty highly chitinoclastic strains were selected for further study.

Chapter 3.

Fermentation of prawn shell waste - comparison between SSF and SmF and biochemical evaluation of product quality.

This chapter describes the methods adopted for fermentation of prawn shell waste with the selected highly chitinoclastic strains. The comparison of SSF and SmF for each selected strain in terms of enrichment of protein, lipid and carbohydrate in the fermented product was done. Detailed analysis of product quality is discussed. The general trend reveals that SSF favors carbohydrate enrichment and SmF favors lipid enrichment. The highest value for protein, lipid and carbohydrate in SSF are 67%, 4.8% and 3.29% respectively. For SmF the values are 59%, 5.18% and 2.4% for protein, lipid and carbohydrate respectively. Based on nutritional enrichment (especially protein) the preferred method (SSF/SmF) for each strain was finalized.

Chapter 4

Screening of the fermentation product for its efficacy as dietary ingredient for Indian White Prawn, *Penaeus indicus*.

This chapter describes the first feeding experiment as a means of preliminary screening of the fermented product as feed ingredient for *P.indicus* post larvae. Twenty feeds were prepared by incorporating twenty different fermented products with a basal diet formula. A control feed was prepared by substituting fermented product with raw prawn shell powder. The feed formulation and feeding experiment is explained in detail. Data collected during the feeding experiment was consolidated to give measures of feed efficiency in terms of biogrowth parameters such as Production , Food Conversion Ratio (FCR), Specific Growth Rate (SGR), Relative Growth Rate (RGR), etc. After 21 days, the animals were challenged with white spot virus (WSV) via diet. The survival during feeding experiment, before and after challenge was noted. The relative percentage survival data suggested a defense boosting imparted by the various fermented products. Statistical analysis of the various biogrowth parameters was done with Duncan's multiple range test.

Correlation of the biogrowth parameters with that of the proximate composition of the fermented product did not show a significant positive

correlation with any of the factors except for free fatty acids which showed a positive correlation with SGR and RGR. Survival percentage before and after challenge with WSV had a significant correlation with the lipid content of the fermented product. Based on the results, four strains were selected for further study.

Chapter 5

Process Optimization and Product Development

This chapter describes the process of optimization of fermentation so as to attain best enrichment of the fermented product in terms of protein, lipid and carbohydrate. The parameters studied were moisture, pH, inoculum size, temperature and incubation time. The best four strains selected on the basis of performance in the previous feeding trail were employed. Since the optimal requirement of the physicochemical conditions was found to be different for the various nutritional criteria (protein, lipid and carbohydrate), protein content of the final product was given more weightage for finalising the optimal conditions for fermentation. Accordingly a moisture level of 1:4, pH 6 and NaCl concentration of 1.5% was found to be optimum for the various strains.

Characterisation of the strains using nutrient broth at different physicochemical conditions was also done to compare the strain's performance in nutrient broth with that of prawn shell waste. The parameters studied were time course of growth and enzyme (protease and chitinase) production at different sodium chloride concentration. Besides this, the effect of pH, concentration of colloidal chitin and $CaCO_3$ on growth and enzyme (protease and chitinase) production was also studied. This is done to get information regarding the influence and importance of these parameters on growth and enzyme production as the shell waste also contain chitin, $CaCO_3$, NaCl, etc. The data is expressed in the form of graphs and the optimal requirement was found out for the various strains.

Chapter 6

Application of fermented product as a dietary ingredient for *Penaeus indicus* juveniles in culture systems

This chapter describes the final feeding experiment incorporating all the optimized fermented products as ingredient in feed for juvenile *Penaeus indicus*. Five different experimental feeds were prepared incorporating the fermented product with a basal diet (as given in chapter 3) and the control feed by incorporating raw prawn shell powder instead of the fermentation

product. The experiment was carried out for 28 days. Proximate composition of the fermented product showed a maximum of 50% protein, 10% lipid and 0.5 % total sugars and this pattern was reflected in the feed composition too. The feed efficiency in terms of Production, FCR, SGR, GGE, RGR, etc. were assessed from the data collected. F111 showed high Production , high GGE, RGR, SGR and lowest FCR followed by F134 in overall performance. Histological examination of hepatopancreas of individuals fed on experimental feed was done to assess toxic effects if any.

At the end of the feeding experiment, the animals were challenged with WSV orally by feeding them infected flesh to evaluate percentage survival and immune response. The survival was noted at definite intervals post challenge.

Immunostimulant property of the fermented product was also estimated simultaneously during the feeding experiment. For this haematological parameters such as total haemocytic count, Nitroblue tetrazolium reduction assay and enzyme assays such as alkaline phosphatase and phenol oxidase assays were carried out. These immunoassays were done at the beginning of the feeding experiment (initial), after 28 days of feeding experiment (before challenge) and post challenge with WSV (10hr, 3day, 4day). The immune

response varied with the feeds. Feed F134 was found to be having immunostimulant property as evidenced by the immunological assays.

Conclusion

Results of all the experimental studies are summarized in this chapter. Importance of the present study, its economic importance and future potential is being discussed.