MARINE YEASTS AS SOURCE OF SINGLE CELL PROTEIN AND IMMUNOSTIMULANT FOR APPLICATION IN PENAEID PRAWN CULTURE SYSTEMS

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SARLIN.P.J.

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

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
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2005

Dedicated to

My beloved parents

and

To the boundless love, sacrifice and undaunted support of

Poly and Our kids

DECLARATION

I hereby do declare that the thesis entitled ***"Marine yeasts as source of single cell protein and immunostimulant for application in penaeid prawn culture systems"****, is a genuine record of research work done by me under the supervision of Dr. Rosamma Philip, Senior Lecturer, School of Marine Sciences, Cochin University of Science and Technology, Cochin – 682016, and that no part of this work has previously formed the basis for the award of any degree, diploma associate ship, fellowship or other similar title of any University or Institution*

Sarlin. P.J.

Cochin- 682016
April, 2005.

CERTIFICATE

This is to certify that the thesis entitled ***"Marine yeasts as source of single cell protein and immunostimulant for application in penaeid prawn culture systems"*** *is an authentic record of research work carried out by Ms.Sarlin. P.J. under my supervision and guidance in the school of Marine Sciences, Cochin University of Science and Technology in partial fulfillment 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
April, 2005.

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Chapter 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Shrimp aquaculture started in 1970s as an activity and developed rapidly with a huge increase in number of hatcheries and farms. Shrimp farming provides roughly 30% of the shrimp supplied to the world market (Rosenberry, 1996). Feed is a major concern for shrimp farmers, representing up to 60% of the total variable production costs (Akiyama *et al.*, 1992; Sarac *et al.*, 1993). Good growth performance of shrimp in ponds is associated with survival during larval stages (Castille *et al.*, 1993). Looking for the production of good quality post larvae, research on larval nutrition has traditionally focused on the establishment of suitable feeding protocols for the different developmental stages (Loya-Javellana, 1989; Gallardo *et al.*, 1995).

In aquaculture, the primary dietary animal protein source is fishmeal, but its availability is limited and supply varies because of reductions in fish stocks. Yeasts are a rich source of proteins and B-complex vitamins. They have been used as a supplement in animal feeds to compensate for the amino acid and vitamin deficiencies of cereals, and are recommended as a substitute for other ingredients in fish. In addition, they are considered a cheap dietary supplement as they are easily produced on an industrial level from a number of carbon-rich substrate by-products such as citrus pulp, molasses, paper industry wastes and fruit waste as well as from hydrocarbons. Yeast nutritional value varies according to origin, and many researchers have indicated that yeast grown in alkanes could be of better quality than that produced using carbohydrates. The use of yeasts also highlights the profitability of using beer and liquor industry by-products in animal nutrition.

This variability can seriously affect aquaculture sustainability and profitability, and as a response, research in identifying alternative dietary protein sources has increased (Kissil *et al.*, 2000; Naylor *et al.*, 2000).

Marine yeasts are reported to be truly versatile agents of biodegradation. Utilization of this potential in the biotransformation of cheap raw materials or waste matter into value added products will be a highly rewarding endeavour. Biotransformation and the partial conversion of raw material into yeast biomass (single cell protein) is highly significant due to the nutritional quality of yeast and its possible utilization as animal or aquaculture feed. Advantages of microbial protein as food are high productivity, high proportion of cell mass as protein, a good profile of desirable aminoacids, good performance in feeding livestock and no toxic or carcinogenic components. Yeast products are frequently used as feed ingredients in aquaculture because of the nutritional value of these products, which include protein, lipids, B-vitamins etc. (Mahnken, 1991; van der Meeren, 1991).

Yeasts are unicellular fungi, nucleated, non motile, spherical, ovoid or elliptical in shape. They are found in fresh water, marine and terrestrial environments. Yeasts, specifically the *Saccharomyces* genus are often referred to as the "oldest plant cultivated by man" (Russel *et al.*, 1986). As unicellular fungi, yeasts have historically been recognized for their fermentative capabilities. Traditionally, yeasts have been used by the food industry principally for their production of ethanol and carbon dioxide, which are important to the brewing, wine distilling and baking industries. Today, yeasts are acquiring increasingly more attention for their other uses. High protein and

vitamin content of yeasts make them attractive as natural supplements for improving the nutritional profile of human food and animal feeds.

Cellular dimensions vary according to the species, but, generally, yeasts range from 2.5 to 10.5μ in width and 4.5 to 21μ in length (Reed and Peppler, 1973). Since yeasts are eukaryotic, they have a more highly organized cellular structure than bacterial cells (prokaryotes), possessing a distinct nucleus and subcellular organelles such as an endoplasmic reticulum and mitochondria. Yeasts have 14 pairs (haploid) of chromosomes compared to the fruit fly with 8, the honeybee with 16, man with 46, and bacteria-prokaryotic organisms-with 1 (Lehninger, 1975).

There are approximately 590 species of yeasts, grouped to about 83 genera.

Yeasts are aerobically grown on a variety of substrates specifically for use in the human food and feed industries. These products are designated by several terms, including dried yeast, dried inactive yeast, microbial protein, dried *Torula* yeast, debittered brewer's dried yeast, and single cell protein (SCP). The Germans were among the first to capitalize on SCP technology for increasing their reserves of human food and feed. During World War I, they grew *S. cervisiae* in aerated tanks of molasses and later, they propagated *C. utilis* on waste such as sulfite liquor. Today, a number of large-scale SCP production facilities are in operation world wide.

Yeast populations have been observed to decrease with increased distance from land (Ahearn *et al.,* 1968) and certain yeast

species frequently collected in the seawater were obtained in highest quantities in the vicinity of heavily polluted areas (Fell and van Uden, 1963). Yeast populations appear to be highest in coastal waters where nutrients are most abundant, at greater depths only 25% of the samples yielded yeasts probably due to decreased O_2 tension and high concentration of Hydrogen sulphide. Even though yeasts are the most commonly used source of Single Cell Protein (SCP) in animal feeds works on marine yeasts are very much limited.

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

1. Isolation of yeasts from the coastal and offshore waters of the east and west coast of India.
2. Characterization and identification of the strains.
3. Screening of marine yeasts for utilization as feed supplement in aquaculture.
4. Optimization of media components and physico-chemical conditions for yeast biomass production.
5. Testing the efficacy of the selected marine yeasts as feed supplement for *Fenneropenaeus indicus*.
6. Assessment of the immunostimulant property of marine yeasts to *Fenneropenaeus indicus*.

The results of the present study are presented in Six chapters after giving a General Introduction to the topic in Chapter 1. Isolation and characterization of the yeasts isolated from marine environment are presented in Chapter 2. The third chapter deals with the screening of marine yeast for utilization as feed supplement in aquaculture. Optimisation of the physico-chemical parameters of the selected

strains are presented in chapter 4. Testing the efficacy of the selected marine yeast as growth promoter in *Fenneropenaeus indicus* are presented in chapter 5. Testing the utilization of whole cell marine yeasts as immunostimulants are presented in chapter 6.

Summary, list of references cited and appendices follow this.

Chapter 2

ISOLATION OF YEASTS FROM MARINE ENVIRONMENT AND THEIR IDENTIFICATION

2.1 INTRODUCTION

The discovery of marine yeasts goes back to 1894, when Fischer separated red and white yeast from the Atlantic Ocean and identified them as *Torula* sp. and *Mycoderma* sp. Following Fischer's discovery, many other workers such as Hunter (1920), Bhat and Kachwalla (1955), Suehiro (1960) and Uden van and Fell (1968) isolated marine yeast from different sources *viz.* seawater, marine deposits, sea weeds, sea fish, marine mammals and sea birds.

Morphological and cultural characteristics of these yeasts were studied on wort-agar, wort-gelatin and wort fluid. Kohlmeyer and Kohlmeyer (1979) named 177 species, which were found in the water, sediment, algae, animals or detritus of the sea. Of those only 26 species were regarded as obligate marine forms. The most important genera of true marine yeast are *Metchnikowia*, *Kluyveromyces*, *Rhodosporidium*, *Candida*, *Cryptococcus*, *Rhodotorula* and *Torulopsis*.

2.1.1. Ecology and distribution

Yeasts are distributed in almost every part of the aquatic environment, such as oceans, seas, estuaries, rivers and lakes. Yeasts are ubiquitously present on the surface of fruits and cereal grains, in honey, in the exudates of trees, soil and aquatic environments. Yeasts found in aquatic environments are generally asporogenous and oxidative or weakly fermentative (Phaff *et al.*, 1978).

Oceans and seas

As early as 1894, Fischer working on SMS Moltke found yeasts in Atlantic water samples regardless of the distance from land. During

the Charriot expedition to the Atlantic, two yeast species were identified (TsiKlinsky, 1908). ZoBell and Feltham (1934) observed yeasts on most of their plates inoculated with samples of marine materials collected from land as well as from the open ocean.

Kriss and Novozhilova (1954) made a transect in the black sea with stations situated at 0.5, 2, 10, 30, 50 and 60 miles from the shore. From these spots water samples were collected and yeasts were separated by membrane filter technique. It was found that yeasts were found in all regions in the surface layers, while in the inshore areas, yeasts could be found at all depths also. Bhat and Kachwalla (1955) isolated yeasts from water samples collected from two to six miles off the coast of Bombay. They obtained species like *Saccharomyces italicus, S. chevalieri, S. rosei, Debaryomyces hansenii, Pichia quilliermondi, Candida tropicalis, Torulopsis glabrata, Torulopsis candida, Rhodotorula* sp., *Cryptococcus* sp. etc.

During the cruise of the R/V Vitiyaz in 1957-1958, *Debaryomyces globosus* was isolated from a depth of 400m in the central Pacific. Kriss (1961) found that yeasts were observed not only in the oxygenated zone but also in the H_2S zone of the black sea. Further studies of Kriss revealed that the distribution of yeast in seawater is characterized by microzonation. Fell *et al.,* in 1960 obtained a total of 179 yeast isolates from 45 sampling stations in the course of a qualitative yeast survey in Biscayne bay, Florida. Of these, *Candida tropicalis* and *Rhodotorula rubra* were the predominant species. Fell (1967) found living yeasts in the Indian Ocean from the surface down to a depth of 200m. van Uden and ZoBell (1962) obtained yeasts from 45 out of 62 samples collected from algal and

coral growths in the Torres strait region. Species like *Metschnikowia reukaufii, Pichia farinose, Kluyveromyces aestuarii, Candida marina, Torulopsis torresii* and *Torulopsis maris* were obtained. Presence of some salt-tolerant yeast has been reported in the open ocean by uden Van and Fell (1968).

Roth *et al.* (1962) and Fell (1965) made a quantitative study of yeasts distribution in the coastal areas of Southern Florida and in the Gulf stream of Florida. Freshwater influx and heavy recreational bathing directly affected viable yeast count in these areas. *Candida tropicalis* and *Rhodotorula rubra* were predominant in the inshore region.

In coastal waters, upto several thousand yeast cells per liter of water were found (Roth *et al.*, 1962; Meyers *et al.*, 1967). In heavily polluted waters there could be considerably more. Yeasts are known to be the normal components of the biota of the world oceans (Kriss *et al.*, 1967). Yeasts of the Indian Ocean were studied by Fell and van Uden (1963); D'Souza (1972); and Godinho *et al.* (1978).

Species of the genera *Cryptococcus* and *Rhodotorula* were predominant among yeasts isolated from deep-sea waters from Loma Trough, off San Diego, California. In samples collected off La Jolla California, total yeast count varied from 0 –1920 viable cells/L. (Uden van and Branco, 1963). Fell (1967) collected yeasts from 16 stations during a cruise of R/V Anton Brunn in the Indian Ocean. The highest population of yeast was found in the Somali current and the species isolated were grouped according to their distribution. 1. Ubiquitous species like *Rhodotorula rubra* and *Candida atmospherica*, which is seen in all water masses. 2. Widely distributed species which occurred

in all water masses except Red Sea water and it is represented by *Candida polymorpha* and *Rhodotorula glutinis*. 3. Species of restricted distribution like *Sporobolomyces hispanicus, Sporobolomyces odorus* and *Rhodotorula crocea.*

Filamentous fungi and yeasts are common in marine environments (Norkrans, 1966; Litchfield and Floodgate, 1975; Ahearn and Meyers, 1976; Hagler *et al.*, 1979; Kohlmeyer and Kohlmeyer, 1979; Phaff *et al.*, 1978). Allen and Leda (1981) and Kirk and Gordon (1988) studied the yeasts from marine and estuarine waters with different levels of pollution in the state of Rio de Janeiro, Brazil. He found that yeast counts in clean seawater generally range from a few to several hundred per liter, but in the presence of enrichment, like pollution or algal blooms, can reach thousands per liter or more. In addition there is a shift from a prevalence of strictly aerobic yeasts in clean water to a presence of fermentative yeasts in polluted waters.

Estuaries

In littoral zones of Crimea, Florida and California coasts, yeast population densities were generally higher than in adjacent open seas. (Kriss *et al.*, 1952). Taysi and uden N. Van (1964) found that the higher number of yeasts was obtained from regions where there is relatively light pollution. It is found that with increase in distance from the estuaries the number of species decreased. Ecological observations showed that estuaries had more dense yeast populations than adjacent oceanic zones. Total colony counts and number of species decreased with distance from the estuaries. The species common to both estuaries and oceanic regions were the genera *Debaryomyces* and *Rhodotorula.*

Estuaries probably take an intermediate position with yeast population fluctuating between high levels in inland waters and low levels in non-estuarine regions. There are evidences that estuarine waters contain not only more yeast cells/volume, but also more species than adjacent sea (uden Van, 1967).

The apparent dominance of some yeast species in estuaries and their apparent absence in open oceans may be due to a variety of reasons. One obvious possible source of yeast in estuaries is sewage pollution and terrestrial run-off. In fact two ecological groups encountered were yeasts like *Rhodotorula glutinis* which were wide spread in estuaries, the open oceans and inland waters and intestinal yeasts like *Candida tropicalis* and *Candida intermedia* from terrestrial substrates that were dominant in estuaries but rare in open seas. (Cook and Matsura, 1963).

Yeasts are encountered in many flowing waters and are particularly common in rivers, which carry sewage (Rheinheimer, 1965). Numerous yeasts were identified from polluted water and sewage (Cook and Matsura, 1963; Ahearn *et al.*, 1968; Hagler and Mendonca, 1981).

Elevated yeast densities have been observed at nutrient rich haloclines in estuaries (Norkrans, 1966). *Candida* and *Rhodotorula* were the main isolated yeasts from this Swedish estuary. Yeasts are often isolated from waters enriched in nutrients, while yeasts populations in unpolluted waters are low (Hagler *et al.*, 1979). Hagler *et al.* (1979) reported that *Candida* and *Rhodotorula* were the most frequently isolated genera from a polluted estuary. 112 yeast isolates were obtained from 31 samples of decaying vegetation in the

rhizosphere of the mangrove plants, from 11 sites in Chapora, Mandovi and Zuari estuaries of Goa (D'Souza and D'Souza, 1979).

Sediments

Fell *et al.* (1960) in his study at Florida reported that yeasts are abundant in silty muds than in sandy sediments. Fell and van Uden (1963) found that yeasts are confined to the upper 2cm of the substrate at a depth of 540m, in the Gulf stream. In shallow Florida waters, however where strong wave action and rapid settling of sediments prevail, yeasts were found in depths upto 9cm. The author concluded that the availability of oxygen is the limiting factor for growth process of yeasts within the sediments.

Meyers *et al.* (1971) observed very high concentrations of viable cells of *Spartina alteriniflora* in marshes of Lousiana coast than adjacent water samples. Hagler *et al.* (1982) studied the densities of some yeasts in intertidal sediments of a polluted subtropical estuary in Rio de Janeiro, Brazil. Highest yeast densities were found at the most polluted site, and in the upper 2cm of sediments.

Yeasts also have been found in marine sediments. They occur particularly in the top most centimeters and, according to Suehiro (1963), they are more frequent in black zone than in sandy sediments. Several hundred living yeast cells per cm^3 were found in the damp mud from the Kiel Fjord (Hoppe, 1970).

Relatively high yeast densities upto 2000 viable cells/g have been reported for marine sediments, with most of the population contained in the top few centimeters (Fell *et al.*, 1960; Lazarus and Koburger, 1974). The prevalent isolates from estuarine, littoral and

deep water marine sediments of Florida and the Bahamas have been mostly oxidative yeasts, including *Rhodotorula* and *Cryptococcus*, typical of sea water (Fell *et al.*, 1960; Lazarus and Koburger, 1974; Voltz *et al.*, 1974). *Candida steatolytica* have been reported from an aquatic source by Hedrick (1976). Yeasts are found in sediments of eutrophic lakes in numbers upto several thousands per gram (Niewolak, 1977). Niewolak (1977) found upto 82 yeast cells per ml in slightly polluted North Poland lakes, but upto 2310 per ml in heavily polluted waters.

The *Cryptococcus vishniacii* (yeasts of basidiomycetous affinity), isolated from the soil samples of Dr. W.V.Vishniac's 1973 expedition, is, peculiar to the dry valleys of Antarctica, constituting the only heterotrophic biota demonstrably indigenous to the most severe cold desert on earth. (Vishniac and Hempfling, 1979a and b, Baharaeen *et al.*,1982).

Yeast *Rhodotorula rubra*, was not isolated from sediments of the most polluted sites (Hagler and Mendonca, 1981). This suggests that polluted littoral sediments are an unfavorable environment for strictly oxidative yeasts like *Rhodotorula* and *Cryptococcus* which are common in less polluted sediments. According to Cooke and Rayner (1984), many types of yeast, which are found in lakes, have been brought in by wind and rain from the flowers and leaves of land plants. The most important species identified were *Candida nigens, C. steatolytica, C.maris* and *C. albicans*. Prabhakaran and Gupta (1991), studied yeasts from sediment samples of EEZ along the South West coast of India. They found that *Candida* was the dominant group of all the species and next in abundance was *Rhodotorula*.

Weeds and algae

Bunt (1955) examined microbes present in the decomposing giant kelp at Macquaria island in Antarctica and found that large amount of yeast were present in decomposing kelp tissue. According to Kriss (1959), the planktonosphere is richer in yeasts than other zones of the sea. Plankton catches from the black sea contained yeasts in 90% of the samples. Studies of Suehiro (1960) revealed that decomposing algae constitute a suitable substrate for yeast development. The predominat species of yeasts isolated from the marine algae were *Torulopsis* sp., *Candida albicans, Candida natalensis, Trichosporon cutaneum* and *Endomycopsis chodatii.* Suehiro *et al.* (1962) estimated that more than 50% of the algal biomass (phytoplankton) was transferred into yeast biomass. He also estimated that a mixed population of yeasts may be capable of degrading and assimilating a large proportion of organic material released from decaying phytoplankton, even in the absence of bacteria. Fell *et al.* (1973) isolated several *Rhodosporidium* sp. from the planktonic samples of the Pacific Ocean. Patel (1975) found that living algae contained lower counts of yeasts compared to counts in the surrounding seawater, but when decomposition starts, yeasts in the algal material increased to higher numbers than those found in the surrounding seawater.

Invertebrates

The studies on invertebrates showed that they are either devoid of yeasts or supports only a small density of population. Phaff *et al.* (1952) obtained yeasts from Mexico shrimp, *Penaeus sertiferus* and the yeast species isolated were *Trichosporon cutaneum, Rhodotorula*

glutinis, Candida parapsilosis, Pichia quilliermondi and *Pullularia pullulans.*

Siepmann and Hohnk (1962) sampled shrimp eggs, sponges and other invertebrate material collected from the North Atlantic Ocean and the species isolated were *Debaryomyces hansenii, Torulopsis, Candida* and *Trichosporon cutaneum.* Yeast populations from conch and spiny lobster on Bahama islands were studied by Voltz *et al.* (1974). Chresanowski and Cowley (1977) found *Rhodotorula glutinis* and *Torulopsis ernobii* in the gut of fiddler crab, *Uca pugilator.*

Fishes, Birds and Mammals

Of the various species of yeasts associated with fish, *Debaryomyces hansenii* is the most dominant species. This species is frequent in seawater, which may explain its high incidence in fish. Another important yeast species isolated from fish was *Metschnikowia zobelli.* High numbers of *Metschnikowia zobelli* were isolated from the gut contents of fish and it has been suggested that yeast flora of fish merely reflect their feeding habits (Fell and uden Van 1963). Ross and Morris (1965) reported that the greatest variety and highest number of yeasts were obtained from fish skin while gill counts gave only less numbers.

Shore droppings of birds yielded species like *Torulopsis glabrata* (Kawakita and uden Van 1965). They suggested that birds like gulls introduce yeasts to water bodies the world over. *Candida tropicalis* was found in the stomach of marine mammals like dolphin and porpoise (Morri, 1973).

2.1.2 Isolation and classification

2.1.2.1 Isolation

Kriss (1959) found that the number of yeasts countable by direct microscopic observation were higher than those obtained by plate counting. This disparity can partly be explained by the presence of non-viable yeast cells. Another explanation is that numerous yeast cells may be attached to organic or inorganic particles and will produce only one colony on the membrane filter.

Woods (1982) used various antibiotics containing media for the enumeration of yeasts and moulds in foods and the comparative efficiency was worked out. The ability of media to suppress bacterial growth and to prevent excessive growth of fungal colonies was the two main factors considered. Broad-spectrum antibiotics are both more effective in preventing bacterial growth and less harmful to yeast cells (Mossel *et al.*,1970; Flannigan, 1974; Beuchat, 1979; Thomson, 1984). Various compounds have been added to media to inhibit the growth of moulds, including dichloran (King *et al.*, 1979) and propionate (Bowen and Beech, 1967). Oxytetracycline glucose yeast extract agar (OGYE) has been recommended for the selective isolation and enumeration of yeasts and moulds from food stuffs (Mossel *et al.*, 1970).

Yeasts can be maintained on agar slopes of malt extract agar. Yeasts of certain genera *Bensingtonia, Bullera, Cryptococcus, Leucosporidium, Rhodosporidium, Rhodotorula* and *Sporobolomyces*, generally survive longer on potato glucose agar.

It was concluded that Rose Bengal Chloramphenicol agar is the medium of choice for samples heavily contaminated with moulds. Malt extract agar containing oxytetracycline was recommended for samples where the main concern is enumeration of yeasts.

2.1.2.2 Classification of yeasts

Yeasts are at present classified on the basis of their morphology and biochemical characteristics. The workers of the Dutch school were responsible for much of their pioneering work on the classification of yeast species known upto 1950. These workers classified all the yeasts available to them on the basis of cellular morphology, spore shape and number and nature of conjugation process and at species level based on the ability to ferment and assimilate 6 sugars, to use ethanol and nitrate and to hydrolyze arbutin. The distinction between some species was rather fine as judged by these criteria.

Wickerman and Burton (1948) and Wickerman (1951) at about the same time, introduced a number of refinements to the Dutch system, especially the use of a much larger number of carbon compounds. These included additional hexoses, di-, tri-, and tetrasaccharides, 2 polysaccharides and a number of pentoses, polyhydric alcohols and organic acids. They also introduced tests for vitamin requirements. The ability to use nitrite as well as nitrate at depressed temperature and on media of high sugar or salt content are also used.

Difficulties both major and minor accompany the use of these methods. One is question of the stability of the biochemical criteria for eg; *Candida* and *Torulopsis* are separated solely on the ability of the

former to produce pseudo hyphae, for differentiation into species until it was observed that the same species may produce two or more forms simultaneously or at different stages of growth. It has now become evident that different strains of the same species may differ in their ability to produce pseudomycelium and the value of this criterion in distinguishing the two genera approaches the vanishing point.

Another problem is the instability of physiological characters. Scheda and Yarrow (1966) observed enough variability in the fermentation and carbon assimilation patterns of a number of *Saccharomyces* sp. causing difficulties in the assignment of these yeast strains to different species. Another difficulty lies in the relationship of the biochemical tests to metabolism of the organisms. It was not originally sufficiently appreciated that the various carbon compounds are not necessarily assimilated independently but may be metabolized by common pathways. Thus yeasts, which can use a compound can use a structurally related one by the same metabolic pathway, Barnett (1968) noted that there was a small percentage of yeasts that were exceptions to this rule. In general the conclusions were valid, that the effective number of criteria for the number of substrates reduced distinguishing yeast species metabolized by such linked mechanisms. The metabolism of most or all of the compounds used involves a few distinct central pathways and depends on the cell's ability to convert the substrates into intermediary metabolites of one of these pathways.

For taxonomic tests, yeasts are usually incubated at 25°C, although optimum temperatures for growth are higher for some yeasts and lower for others (Watson, 1987). Certain yeasts, such as four

species of *Leucosporidium*, only grow at temperatures below 25°C. On the other hand, certain strains of, for example, *Pichia jadinii* (*Candida utilis*) or *Saccharomyces cerevisiae*, may give faster sustained growth at 30°C than at 25°C. However, many types of yeast grow only very slowly at 30°C, but quite fast at 25°C. Growth tests may often be done successfully at 25°C with yeast which, under certain conditions, could be isolated from a natural habitat only at lower temperature. This is probably because a minority of the cells of such yeasts grow at 25°C (Buhagiar and Barnett, 1971).

As per Barnett *et al.* (1990) the chief characteristics used to classify yeasts are 1) Microscopic appearance of yeasts 2) Mode of sexual development 3) Certain physiological (especially nutritional) activities 4) Certain biochemical features 5) Comparisons of genomes in terms of base sequences, by DNA-DNA, or DNA-RNA reassociation.

1. Microscopical appearance

Taxonomists examine yeast cells microscopically and consider their size and shape, how they reproduce vegetatively (by multipolar, bipolar or unipolar filaments) and the form, structure and mode of formation of ascospores and teliospores. If any of these are to be found the shape of cells often indicate the mode of vegetative reproduction. Budding and fission may also be seen in trichosporon and used in classification i.e. whether the filaments are psuedohypahe or true septate hypahe, whether the septae have pores and the occurrence and arrangement of blastoconidia.

2. Sexual reproduction

Some yeasts reproduce sexually by ascospores, others by teliospores and yet others by basidia. For ascosporogenous yeasts, taxonomic importance is given to whether asci are formed a. from vegetative cells b. from two conjugating cells or c. from a mother cell, which has conjugated with its bud.

For yeasts with asci borne on filaments, the arrangement of asci whether in chains or bunches may be used to distinguish between genera. The number of ascospores in each ascus, their shape and whether the ascospore walls are smooth or rough are factors, which are used in classification.

3. Physiological features

Physiological factors used for classifying yeasts are chiefly the ability to 1) Ferment certain sugars anaerobically 2) Grow aerobically with various compounds such as a sole source of carbon or nitrogen 3) Grow without an exogenous supply of vitamins 4) Grow in the presence of NaCl or glucose 5) Grow at 37°C 6) Grow in the presence of cycloheximide 7) Split fat 8) Produce starch like substances 9) Hydrolyze urea 10) Form citric acid.

4. Biochemical characteristics

Studies of certain biochemical characters may influence taxonomic decisions, for e.g. the chemical structure of cell walls (Phaff 1971), particularly of the cell wall mannans (Gorin and Spencer, 1970, Ballaou, 1974). Another example comes from observations on the kind of ubiquinone (coenzyme Q) present in different yeasts.

2.2 MATERIALS AND METHODS

2.2.1 Samples used

Yeasts were isolated from sea water samples collected during cruise No. 147 (Fig.2.1), 156 (Fig.2.2) and 157 (Fig.2.3) of Fisheries and Oceanographic Research Vessel (FORV) Sagar Sampada of Department of Ocean Development, Government of India from the South West Coast and East coast of India upto 200m depth in the Exclusive Economic Zone.

Samples from 22 stations were used for the study. The stations were designated as SS1 to SS22 (Fig.2.1, 2.2. and 2.3).

Water samples collected from the coastal waters off Cochin were also used for the study. These stations were designated as S1 to S8 (Fig.2.4).

2.2.2 Medium used

Malt extract agar

Malt extract	-	30g
Peptone	-	5g
Agar	-	20g
Sea water	-	1000ml
pH	-	7

Antibacterial compounds

Oxytetracycline 100mg/1000ml was added to prevent the growth of bacteria.

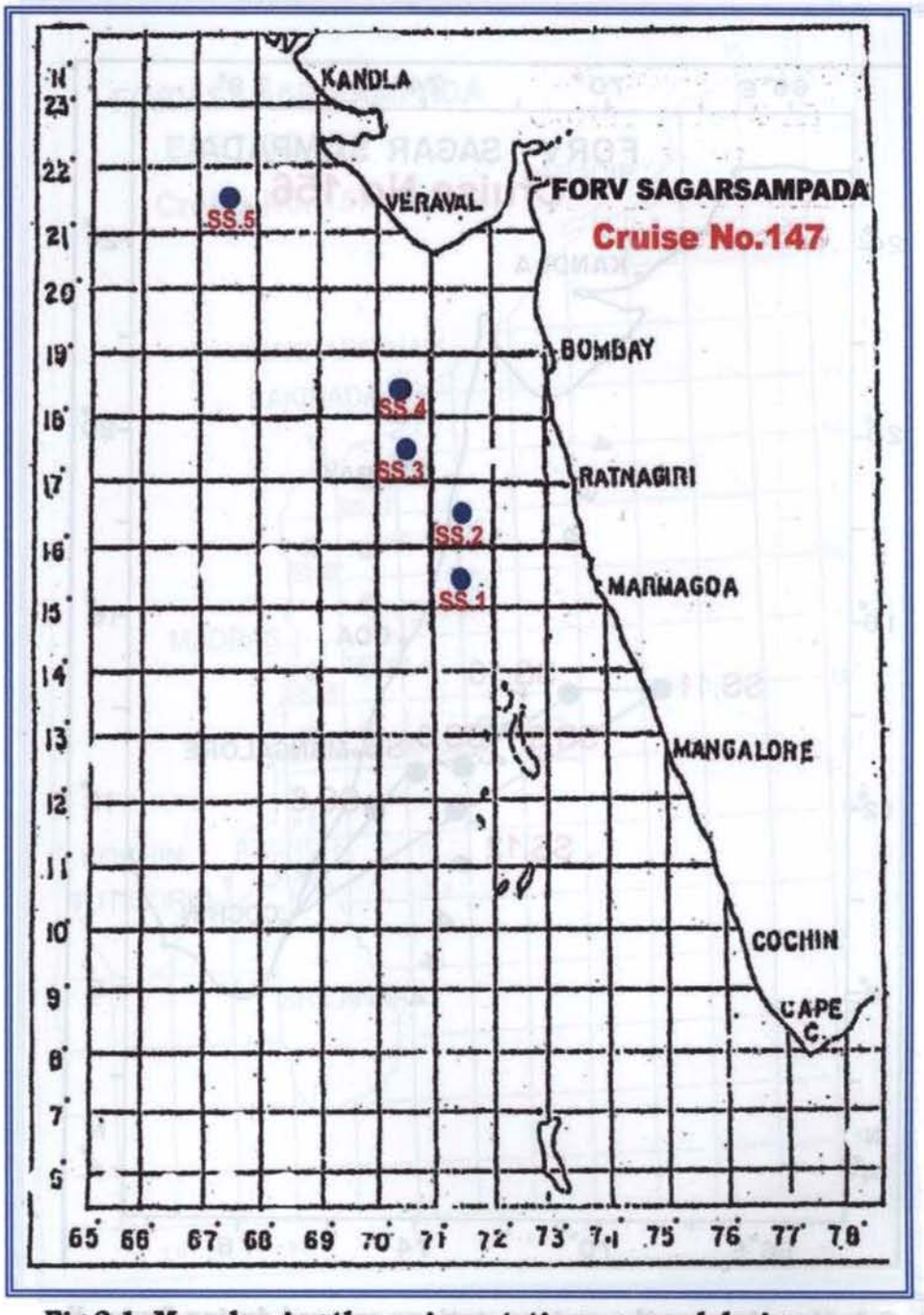


Fig.2.1. Map showing the various stations covered during Cruise No. 147 of FORV Sagar Sampada in the North West Coast of India (SS.1 –SS. 5)

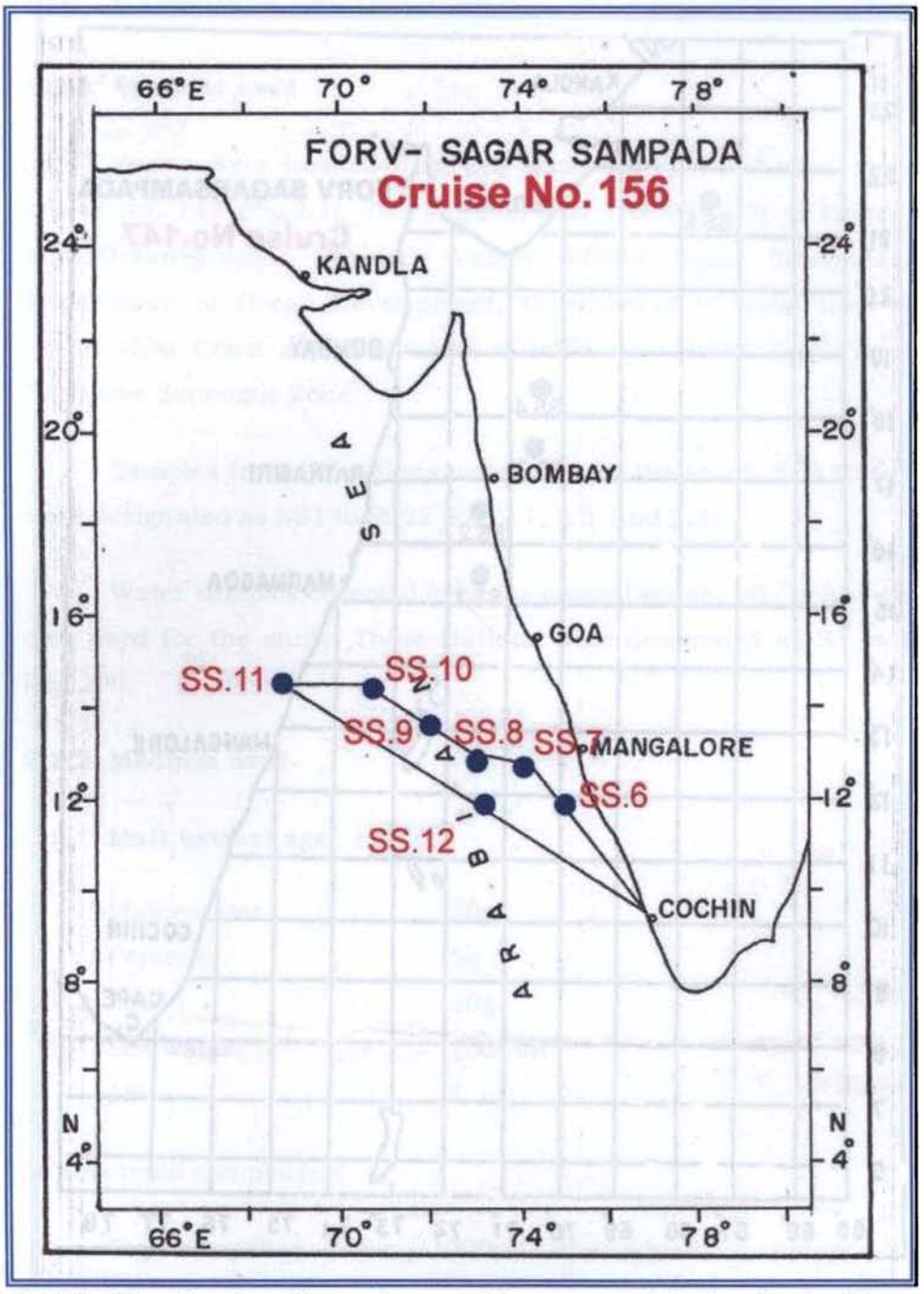


Fig.2.2. Map showing the various stations covered during Cruise No. 156 of FORV Sagar Sampada in the West Coast of India (SS.6 – SS.12)

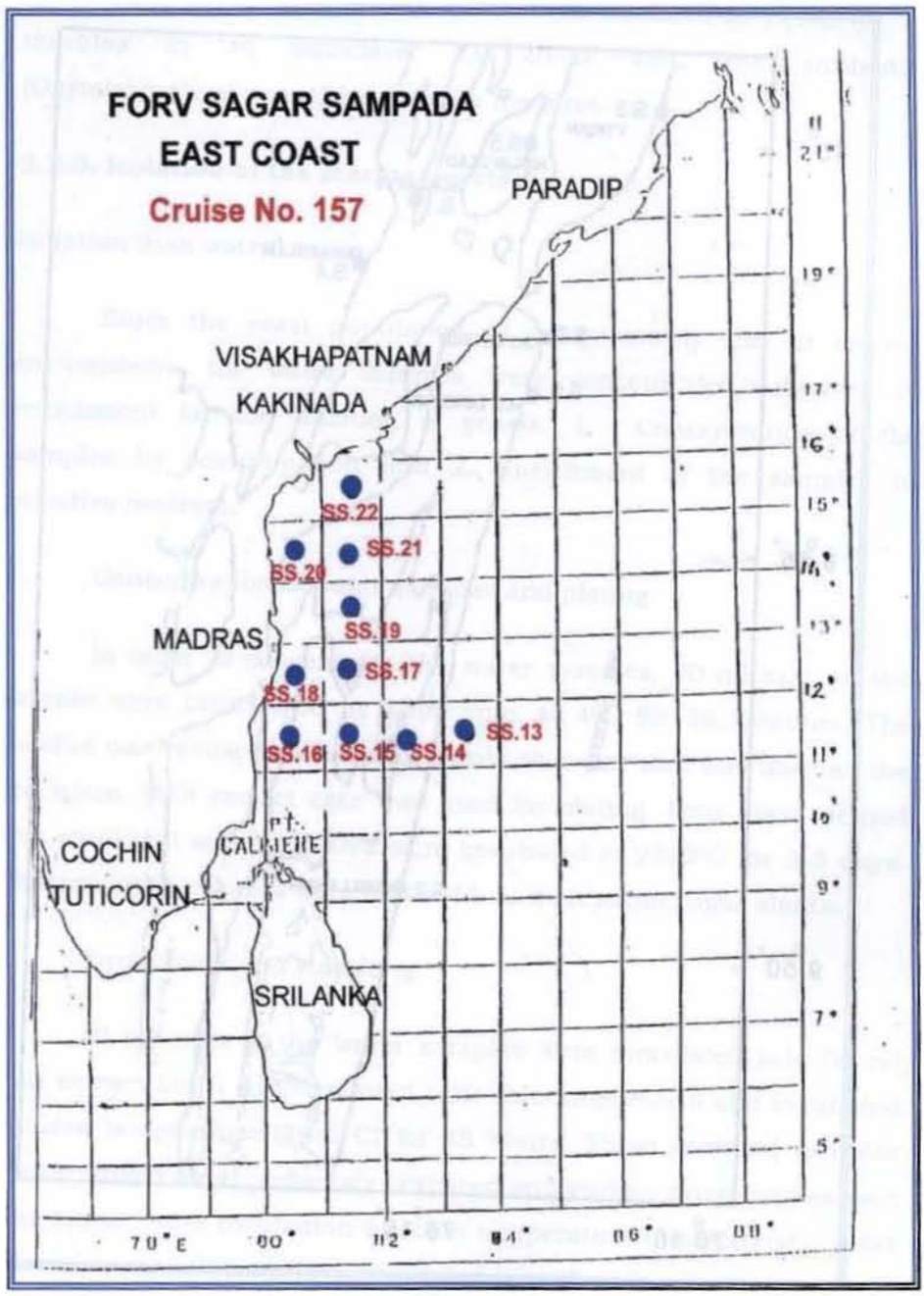


Fig.2.3. Map showing the various stations covered during Cruise No. 157 of FORV Sagar Sampada in the East Coast of India (SS.13 – SS.22)

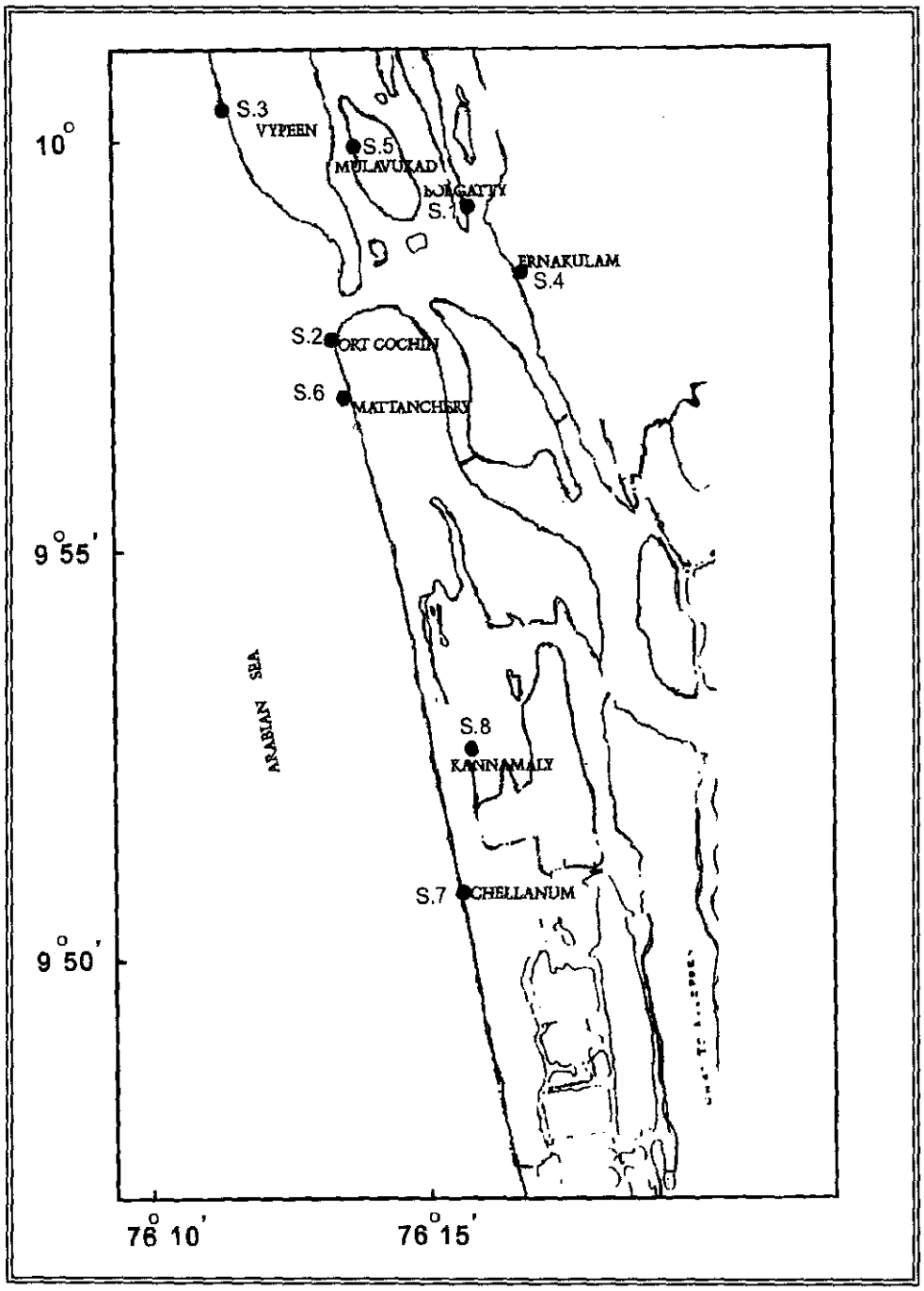


Fig.2.4. Map showing the various stations in the coastal waters of Kochi (S.1-S.8)

Malt extract agar was prepared and sterilized at 121°C for 15 minutes in an autoclave. At about 45°C the antibiotic, (Oxytetracycline) was added into the medium.

2.2.3. Isolation of the marine yeasts

Isolation from water

Since the yeast population is comparatively less in marine environment, the water samples were concentrated/subjected to enrichment for the isolation of yeasts. 1. Concentration of the samples by centrifugation and 2. Enrichment of the samples in selective medium.

1. Concentration of water samples and plating

In order to concentrate the water samples, 50 ml each of the sample were centrifuged at 7500 rpm at 4°C for 15 minutes. The residue was resuspended in 1ml sterile seawater and was used as the inoculum. Malt extract agar was used for plating. Pour plate method was employed and the plates were incubated at 28±2°C for 3-5 days. Characteristic colonies were isolated into malt extract agar slants.

2. Enrichment and streaking

10 ml each of the water samples were inoculated into 50 ml malt extract broth supplemented with chloramphenicol and incubated at room temperature (28±2°C) for 48 hours. These enriched samples were streaked on to previously prepared and surface dried malt extract agar plates. After incubation at room temperature characteristic yeast colonies were isolated into malt extract agar slants.

Preservation

All yeast isolates were repeatedly streaked on malt extract agar plates for purification and stocked in malt extract agar vials overlaid with sterile liquid paraffin.

2.2.4 Identification and characterization

The isolated yeast strains were identified as per Barnett *et al.* (1990). For this the microscopic appearance of the cell, mode of sexual reproduction and certain biochemical and physiological characteristics were studied.

1. Microscopic appearance of yeast cells

a. Non-filamentous vegetative cells

Young growing yeast cultures (1 day old) were inoculated into sterile malt extract broth and incubated at 28±2°C for 3 days. Wet mount preparations of the cultures were observed under oil immersion objective for the following characteristics. a) Whether the yeast reproduce by budding, splitting or both. b) If the yeast form buds, where do they occur on the mother cell c) What are the shapes and sizes of the vegetative cells?

b. Microscopic examination for filamentous growth

Slide cultures of isolated yeasts were prepared. For this, malt extract agar plates were prepared. In each plate four sterile cover slips dipped in malt extract agar (1% agar) was kept on the medium surface at 45° angle position by gently piercing the agar. These slides were

examined microscopically daily or once in two days for upto about 2 weeks. Observations were done to ascertain whether or not there is filamentous growth. If so, what kind of cells grows from filaments?

2. Assessing the ability to use nitrogen compounds for aerobic growth

The test of ability to use nitrate as a sole source of nitrogen is a valuable aid to identify yeasts. A mineral basal medium supplemented with glucose as carbon source and KNO_3 as the sole nitrogen source was employed for the test.

Beijerinck medium (composition)

KH_2PO_4	-	2g
$MgSO_4.7H_2O$	-	0.5g
Ca_2HPO_4	-	0.5g
Glucose	-	20g
KNO_3	-	1g
NaCl	-	20g
Distilled Water	-	1000 ml

Cultures were inoculated into the medium and incubated at 28±2°C for one week. Ability to use nitrate as the sole nitrogen source was determined by observing the growth and turbidity.

3. Assessing the ability to use sugars anaerobically/aerobically

Marine oxidation fermentation (MOF) medium was used for testing the ability of yeast to metabolize dextrose aerobically (oxidative) or anaerobically (fermentative). When dextrose is utilized, acid is

produced which changes the colour of the medium from pink to yellow. The pH indicator in the medium is phenol red. Yellow colouration at the slope region indicates an oxidative reaction, where as the whole tube turning yellow indicates a fermentative reaction.

4. Urea hydrolysis

Composition of the medium

Yeast extract	-	0.1g
KH_2PO_4	-	9.1g
Na_2HPO_4	-	9.5g
NaCl	-	20g
Urea	-	20g
Agar	-	20g
Phenol red	-	4ml of 0.25% solution
Distilled water	-	1000ml
pH	-	6.8

The above ingredients except urea were dissolved in 950ml of distilled water and autoclaved at 15 lbs for 15 minutes. Urea was sterilized using solvent ether and dissolved in 50ml sterile distilled water. This urea was added to the basal medium, dispensed into test tubes (3ml each) and slants were prepared. Cultures were inoculated and after incubation a change of colour in the medium from yellow to pink was noted as urea hydrolysis.

5. Starch, lipid and gelatin hydrolysis

Nutrient agar supplemented with starch (1%), gelatin (2%) and lipid (1%) were prepared separately. Plating was done and spot inoculation was performed on the various media plated. After

incubation at room temperature (28±2°C) for 3-5 days observations were made. Starch agar plates were flooded with iodine solution and the appearance of a halo zone around the colonies was noted for a positive result. Gelatin agar plates were flooded with mercuric chloride solution and the appearance of a halo zone around the colonies was noted for a proteolysis. Lipid agar plates were noted for a clear zone around the colonies, which indicated lipolysis.

6. Production of starch like substances

Certain yeasts produce starch like substances during metabolism. For testing this property, a mineral basal medium supplemented with glucose was used.

Composition of medium			Trace metal mix	
NH_4Cl	-	5g	$FeCl_3$	- 16mg
NH_4NO_3	-	1g	$MnCl_2$	- 18mg
Na_2SO_4	-	2g	$Co(NO_3)$	- 13mg
K_2HPO_4	-	3g	$MgSO_4$	- 25mg
KH_2PO_4	-	1g	$ZnSO_4$	- 4mg
NaCl	-	20g	$CuSO_4$	- 0.01mg
Yeast extract	-	100mg	$CaCl_2$	- 14.5mg
Thiamine HCl	-	1mg	Distilledwater	- 1000ml
Trace metal mix	-	5ml		
Glucose	-	20g		
Distilled water	-	1000ml		

The cultures were inoculated into the medium and were incubated for a week. Gram's iodine was added to each tube. The change of color to dark blue indicated the presence of starch like substances.

7. Cellulose hydrolysis

A mineral basal medium supplemented with Whatman filter paper strips was used as th e medium.

Composition of basal medium

KH_2PO_4	-	2g
$MgSO_4.\ 7H_2O$	-	0.5g
Ca_2HPO_4	-	0.5g
Glucose	-	20g
KNO_3	-	1g
NaCl	-	20g
Distilled Water	-	1000ml

Cultures were inoculated into the tubes and incubated for 2-3 weeks. Growth and turbidity of the cultures were noted and the cellulose strips were observed visually for signs of degradation. Final confirmation was done after adding Benedict's reagent, which shows a positive reaction for glucose, if the culture is cellulose degrading.

8. Pectin hydrolysis

Composition of the medium

Pectin	-	5g
$CaCl_2.\ 2H_2O$	-	0.2g
NaCl	-	20g
$FeCl_3.\ 6H_2O$	-	0.01g
Yeast extract	-	1g
Agar	-	20g
Distilled water	-	1000ml
pH	-	7

Pectin agar plates were prepared and the cultures were spot inoculated. After incubation for 4-5 days, the plates were flooded with 1% aqueous solution of Cetavlon and allowed to stand for 20-30 minutes. Clearing zone indicate positive nature of the test.

9. Diazonium Blue B (DBB) test.

A culture at least 10 days old, on malt - yeast-glucose-peptone agar is held at 55°C for several hours and then flooded with ice-cold DBB reagent. If the culture turns dark red within 2 minutes at room temperature the result is recorded as positive.

Malt-yeast-glucose-peptone agar (Wickerman, 1951).

Composition of the medium

Malt extract	-	3g
Yeast extract	-	3g
Peptone	-	5g
D-Glucose	-	10g
NaCl	-	20g
Agar	-	20g
Water	-	1000ml
pH	-	7

10. Myo-Inositol Test

A mineral basal medium supplemented with myo-inositol was used for preparing the broth. Cultures were inoculated into this broth and after one week if the broth shows turbidity then the test is recorded as positive.

Composition of mineral basal medium

KH_2PO_4	-	2g
$MgSO_4.\ 7H_2O$	-	0.5g
Ca_2HPO_4	-	0.5g
Glucose	-	20g
KNO_3	-	1g
NaCl	-	20g
Distilled Water	-	1000ml

Based on the various biochemical and physiological characteristics all the isolates were classified upto generic level.

2.3 RESULTS

Of the total isolates (260) the most predominant genera was *Candida* (116) followed by *Filobasidium* (55), *Leucosporidium* (24) etc. (Table 2.1). The station details showing the source of isolation of the yeasts is given in Table 2.2.

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis: Benedicts Reaction	Cellulose hydrolysis: Growth	Cellulose hydrolysis: Degradation	Urease	Assessing the ability to use nitrogen compounds for aerobic growth: Turbidity	Assessing the ability to use nitrogen compounds for aerobic growth: Nitrite estimation	Assessing the ability to use nitrogen compounds for aerobic growth: Starch estimation	Pectin	Myo-inositol	DBB*	Production of starch like substances: Turbidity	Production of starch like substances: Colour	Genera
S2	F	O alk	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S3	NF	F alk	-	+	+	-	+	+	-	+	+	-	-	-	-	+	-	*Williopsis*
S4	NF	F alk	-	-	+	-	+	-	-	+	++	-	-	-	+	+	-	*Williopsis*
S5	NF	F	+	-	+	-	+	•	-	+	-	•	•	-	-	+	-	*Lodderomyces*
S6	NF	F	-	+	+	-	+	•	-	+	-	•	•	-	+	+	-	*Lodderomyces*
S7	NF	F alk	+	+	+	-	+	+	+	+	+	-	-	-	-	+	-	*Leucosporidium*
S8	F	F	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	*Debaryomyces*
S9	NF	F	+	+	+	-	+	-	+	+	-	-	-	-	-	+	-	*Lodderomyces*
S10	F	F alk	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S11	NF	F	+	-	+	-	+	-	-	+	+	-	-	-	+	+	-	*Leucosporidium*
S12	F	F	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S13	NF	F	+	+	+	-	+	-	+	+	-	-	-	-	+	+	-	*Lodderomyces*
S15	NF	F	-	+	+	-	+	+	+	+	+	-	-	-	-	+	-	*Lodderomyces*
S16	NF	F	-	-	+	-	+	-	-	+	-	-	-	-	+	+	-	*Lodderomyces*
S17	F	F alk	-	+	+	-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S20	F	F	-	+++	+++	-	+	-	-	+	-	-	-	-	+	+	-	*Candida*
S27	NF	F	-	++	-	-	+	+	-	+	-	-	-	-	-	+	-	*Kluyveromyces*
S28	NF	F	-	-	+	-	+	+	-	+	-	-	-	-	+	+	-	*Kluyveromyces*
S30	F	F	+	+	+	-	+	+	-	+	-	-	-	-	-	+	-	*Aciculoconidia*
S34	NF	O	-	+	+	-	+	-	-	-	+	-	-	-	-	+	-	*Oosporidium*
S36	F	F	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S42	NF	O	-	-	+	-	+	-	+	+	++	-	-	-	-	+	-	*Rhodotorula*
S43	NF	O	-	-	+	-	+	-	-	+	+	-	-	-	-	+	-	*Rhodotorula*
S45	NF	F	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-	*Schizosaccharomyces*
S46	F	F alk	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	*Candida*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-inositol	DBB*	Production of starch like substances		Genera
						Benedicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S47	F	F	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	*Candida*
S48	NF	F		-	+	-	+	-	+	-	-	-	-	-	+	+	-	*Lodderomyces*
S49	F	F alk		-	-	-	+	-	-	-	-	-	-	-	-	+	-	*Aciculoconidia*
S50	F	F		-	+	-	+	+	+	++	-	-	-	+	+	+	-	*Cryptococcus*
S51	F	F			+	-	+		+		-		-		+	+	-	*Cryptococcus*
S52	F	F	-	-	+	-	+	-	-	-	-	-	-	-	+	+	-	*Cryptococcus*
S53	F	F		-	+	-	+	-	-	+	-	-	-	-	+	+	-	*Lodderomyces*
S54	F	F alk		-	-	-	+	-	+			-	-		+	+	-	*Filobasidium*
S55	NF	F		-	-	-	+	-	-		-	-	-	-	-	+	-	*Candida*
S56	F	F alk	-	-	+	-	+		+		-	-	-	-	+	+	-	*Pichia*
S57	NF	F alk		-	+	-	+	-	-	-	-		-	-	+	+	-	*Candida*
S59	F	F	-	-	+	-	+	-	-	-	-	-	-	-	+	+	-	*Geotrichum*
S61	F	F	-	-	-	-	+	-	-	-	-		-	-	-	+	-	*Candida*
S62	NF	F alk	-	-	+	-	+	+	+	-	-		-	-	-	+	-	*Candida*
S63	F	F alk	-	-	+	-	+	+	+	-	-		-	+	-	+	-	*Candida*
S64	F	F alk	+	+	+	-	+	-	-	+	-	-	-		+	+	-	*Dekkera*
S65	NF	F alk	-	-	+	-	+	-	-	-	-	-	-	-	+	+	-	*Candida*
S67	F	F	-	-	+	-	-	-	-	+	-		-	+	-	+	-	*Candida*
S68	NF	F	+	+	+	-	-	-	-	-	-		-	-	-	+	-	*Candida*
S69	F	O	-	-	+	-	-	-	-	-	-		-	+	+	+	-	*Lipomyces*
S70	NF	F	-	+++	-	-	+	-	-	+	-		-		+	+	-	*Hormoascus*
S71	F	F	-	-	-	-	+	-	+	+	++		-	-	+	+	-	*Hormoascus*
S72	F	F	-	+	+	-	+	+	+	+	-	-	-	+	+	+	-	*Candida*
S73	F	F alk	++	-	++	-	+	-	+	+	-	-	-	+	+	+	-	*Candida*
S75	F	F	-	-	-	-	+	-	+	+	+	-	-	+	-	+	-	*Hormoascus*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis: Beledicts Reaction	Growth	Degradation	Urease	Assessing the ability to use nitrogen compounds for aerobic growth: Turbidity	Nitrite estimation	Starch estimation	Pectin	Myo-inositol	DBB*	Production of starch like substances: Turbidity	Colour	Genera
S76	NF	F alk	+	-	+	-	+	-	-	+	-		-	-	-	+	-	*Candida*
S78	F	F alk		-		-	+	-	-	+			-	-	-	+	-	*Candida*
S80	F	O	-	-	-	-	+	-	+	+			-	-	-	+	-	*Rhodotorula*
S81	F	F	-		-	-	+	+	-	+			-		-	+	-	*Arxtoxyma*
S83	NF	F alk	-		-	-	+	+	-	+			-	-	-	+	-	*Candida*
S84	F	F	-		-	-	+	-	-	+	-		-	-	-	+	-	*Candida*
S87	F	O	-	-	-	-	+	-	-	+	-		-	-	-	+	-	*Debaryomyces*
S88	NF	O	+	+	+	-	+	-	+	+	-		-	-	-	+	-	*Debaryomyces*
S90	NF	F	+	+	+	-	+	-	+	+	-		-	-	-	+	-	*Candida*
S92	NF	F	+++	++	++	-	+	+	-	+	-		-	-	-	+	-	*Candida*
S93	NF	F		+		-	+	+	-	+			-	-	-	+	-	*Candida*
S96	F	F alk	-	+	-	-	-	-	-	+			-	+	-	+	-	*Lodderomyces*
S97	F	F	-	+	-	-	+	-		+	-		-	-	-	+	-	*Candida*
S100	F	F	-	-	-	-	-	-	-	+	-		-	-	+*	+	-	*Debaryomyces*
S103	NF	F alk	-		-	-	+	+	+	+	+	-	-	-	+*	+	-	*Leucosporidium*
S105	NF	F alk	-		-	-	+	+	+	+	+		-	+	+*	+	-	*Leucosporidium*
S107	NF	F	-		-	-	+	-	+	+	-		-	-	+*	+	-	*Lodderomyces*
S120	F	F	-	++	+	-	+	-	+	+	-		-	-	-	+	-	*Filobasidium*
S128	F	F	+	++	++	-	+	-	+	+	++		-	-	+*	+	-	*Filobasidium*
S123	F	F	-	-	-	-	+	-	+	+			-	-	-	+	-	*Leucosporidium*
S134	F	F		+	-	-	+	-	+	+	-		-	-	-	+	-	*Filobasidium*
S141	F	NF	+	+++	+++	-	-	-	+	+	-		-	+	-	+	-	*Filobasidium*
S144	NF	O alk	-	-	-	-	+	-	-	+	-		-	++		+	-	*Saccharomycopsis*
S145	NF	F	++	++	+	-	+	-	+	+	-		-	-	-	+	-	*Filobasidium*
S146	F	F alk	+	+++	-	-	+	-	+	+	+		-	-	-	+	-	*Leucosporidium*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-inositol	DBB*	Production of starch like substances		Genera
						Benedicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S151	F	F	+	+	+	-	+	-	+	+	+	-	-	+	-	+	-	*Leucosporidium*
S152	NF	F alk	+	++	+	-	+	-	-	+	-	-		-	-	+	-	*Candida*
S157	F	F	-	-	-	-	+	+		+	-	-		-	-	+	-	*Candida*
S165	F	F	+	+	+	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S166	F	F alk	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S167	F	O	+		+	-	+	-	-	+	+	-		+	-	+	-	*Candida*
S169	F	O alk	-	-	-	-	+	-	-	+	+	-		-	-	+	-	*Debaryomyces*
S170	F	F	-	+	+	-	+	-	-	+	-	-	-	+	-	+	-	***Dekkera***
S175	NF	F	-	-	++	-	+		+	+	+++		-	+	-	+	-	***Lodderomyces***
S181	NF	F alk	++	++	+++	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S184	F	F alk	+++	+++	++	-	+	-	+	+	-	-		+	-	+	-	*Filobasidium*
S185	N	F	+++	+++	-	-	+	+	+	+	+	-	-	-	-	+	-	*Candida*
S186	F	F alk	-	-	+	-	+	-	-	+	-	-	-	+	+*	+	-	*Candida*
S188	NF	F	++	++	+++	-	+	-	+	+	-		-	-	+*	+	-	*Candida*
S189	NF	F	+	+	-	-	+	-	+	+	-		-	+		+	-	*Candida*
S190	F	F	-	-	-	-	-		-	+	+		-	+	-	+	-	*Candida*
S195	NF	F	+	++	++	-	+	-	+	+	-		-	-	-	+	-	*Filobasidium*
S199	N	F	+++	+++	-	-	+	+	+	+	+	-	-	-	-	+	-	*Candida*
S200	NF	F	+	++	++	-	+		+	+	-		-	-	-	+	-	*Filobasidium*
S202	NF	F	++	++	+++	-	+		+	+	-		-	-	+*	+	-	*Candida*
S203	NF	F	+	+	-	-	+	-	+	+	-	-	-	+		+	-	*Candida*
S205	F	F		-	-	-	-	-	-	+	+	-	-	+	-	+	-	*Candida*
S206	F	F	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S223	F	F	-	+	-	-	+	-	+	+	-		-	-	-	+	-	*Filobasidium*
S224	F	Falk	++	+++	+	-	+	+	-	+	-	-	-	-	+	+	-	*Candida*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (FII / Non FII)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis: Benedict's Reaction	Cellulose hydrolysis: Degradation	Growth	Urease	Assessing the ability to use nitrogen compounds for aerobic growth: Turbidity	Assessing the ability to use nitrogen compounds for aerobic growth: Nitrite estimation	Assessing the ability to use nitrogen compounds for aerobic growth: Starch estimation	Pectin	Myo-inositol	DBB*	Production of starch like substances: Turbidity	Production of starch like substances: Colour	Genera
S225	F	Falk	-	++	+++	-	-	+	-	+	-	-	-	-	+	+	-	*Candida*
S226	F	F	+	++	+	-	+	+	-	+	-	-	-	-	+	+	-	*Candida*
S227	F	O	+	++	+	-	-	-	+	-	-	-	-	-	+	+	-	*Candida*
S232	NF	F alk	-	++	+	-	-	-	-	+	-	-	-	-	-	+	-	*Candida*
S238	F	F	-	+++	++	-	+	+	-	+	+	-	-	-	-	+	-	*Candida*
S246	F	F alk	+	-	-	-	+	+	-	+	++	-	-	-	+	+	-	*Candida*
S248	NF	F alk	++	+++	-	-	+	+	-	+	-	-	-	-	+	+	-	*Candida*
S250	NF	F	++	+++	+	-	-	+	+	-	-	-	-	-	+	+	-	*Filobasidium*
S251	F	F	-	++	-	-	-	-	-	+	++	-	-	-	-	+	-	*Candida*
S252	NF	F	+	+	-	-	-	-	-	+	++	-	-	-	+	+	-	*Candida*
S254	NF	F	++	+	+++	-	-	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S255	NF	F	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	*Candida*
S256	NF	F	-	-	+	-	-	+	-	+	+++	-	-	-	-	+	-	*Candida*
S257	NF	F	-	++	+++	-	+	+	-	+	-	-	-	-	-	+	-	*Candida*
S258	NF	F	-	+++	-	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S259	NF	F	-	++	-	-	-	-	-	+	++	-	-	-	-	+	-	*Candida*
S260	NF	F	+	+	++	-	-	+	-	+	+++	-	-	-	-	+	-	*Candida*
S262	NF	F	+	+	-	-	-	+	-	+	++	-	-	-	+	+	-	*Candida*
S263	NF	F alk	++	+	-	-	+	+	+	+	+	-	-	-	+	+	-	*Candida*
S268	NF	F	+	+	+	-	-	+	-	+	-	-	-	-	-	+	-	*Candida*
S270	NF	F	+	+	++	-	-	+	+	+	-	-	-	-	-	+	-	*Candida*
S274	NF	F	+	+	+	-	-	+	-	+	+	-	-	-	-	+	-	*Candida*
S275	F	F	++	+	++	-	-	+	+	-	-	-	-	-	+	+	-	*Filobasidium*
S276	F	F	++	+	++	-	-	+	-	-	-	-	-	-	-	+	-	*Candida*
S284	F	F	-	+	-	-	-	+	-	+	-	-	-	-	-	+	-	*Filobasidium*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-inositol	DBB[a]	Production of starch like substances		Genera
						Benedicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S285	NF	F	++	++	+	-	+	-	-	+	-	-	-	-	+	+	-	*Candida*
S286	NF	F	++	++	-	-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S287	NF	F	++	-	+	-	+	-	-	+	++	-	-	-	-	+	-	*Candida*
S288	NF	F	+	+	++	-	+	-	-	+	-	-	-	+	+	+	-	*Candida*
S289	F	F	-	-	++	-	+	+	-	+	++		-	-	-	+	-	*Candida*
S290	NF	F	-	+++	++	-	+	+	-	+	-	-	-	-	-	+	-	*Debaryomyces*
S291	NF	F alk	+	+	+	-	+	+	-	+	++	-	-	-	-	+	-	*Candida*
S292	F	F	++	++	++	-	+	-		+	++	-			-	+	-	***Dekkera***
S293	F	F	+	+		-	+	+	-	-	-	-	-	-	+	+	-	*Candida*
S294	F	F	++	++	-	-	+	+	-	+	+	-	-	-	-	+	-	*Debaryomyces*
S295	F	F	+	+++	++	-	+	-	-	+	-	-	-	-	+	+	-	*Candida*
S296	NF	F	-	++	+	-	+		-	+	++		-	-	-	+		*Candida*
S297	NF	F alk	++	-	+	-	-	-	+	+	++		-	+	-	+	-	*Leucosporidium*
S298	F	F	++	++	+++	-	+	-	+	+	++	-		-	-	+	-	*Leucosporidium*
S299	NF	F	++	+	-	-	+	-		+	-	-	-	-	-	+	-	*Leucosporidium*
S301	NF	O alk	-	-	-	-	-	-	-	+	++	-	-	-	-	+	-	*Candida*
S302	NF	F	-		++	-	+	+	-	+	-	-	-	+	-	+	-	*Candida*
S303	NF	F alk	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	***Torulaspora***
S304	F	F alk		-	-	-	+	-	+	+	+	-	-	+[a]	-	+		***Dekkera***
S305	F	F	-	-	++	-	+	+	-	+	-	-		+	-	+	-	*Candida*
S306	F	F	-	-	-	-	+	-	-	+	-	-	-	+	+	+	-	*Candida*
S307	F	O	+	++	+++	-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S308	F	F	++	+	+	-		-	+	+	++	-	-	-	+	+	-	*Leucosporidium*
S309	NF	F	++	++	-	-	-		+	+	-	-	-	-	-	+	-	*Filobasidium*
S311	NF	F	+	++	++	-	+	-	+	-	+	-	-	-	-	+	-	*Leucosporidium*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	7			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-Inositol	DBB*	Production of starch like substances		Genera
						Berfredicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S312	NF	F	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S313	NF	F	++	+	++	-	-	-	+	+	-	-	-	+	+*	+	-	*Filobasidium*
S314	NF	F	+	+	++	-	+	+	-	-	+	-	-	-	-	+	-	*Candida*
S315	F	F	+	++	++	-	+	+	-	-	+	-	-	-	-	+	-	*Candida*
S316	F	F	+	++	+	-	+	+	-	+	+	-	-	-	-	+	-	*Candida*
S317	F	F		+	+	-	-	-	+	+	-	-	-	-	-	+	-	*Schizosaccharomyces*
S318	F	F	-	++	-	-	-	-	+	+	-	-	-	-	-	+	-	*Schizosaccharomyces*
S321	F	F	+	+	++	-	-	-	-	+	-	-	-	+	-	+	-	*Candida*
S322	F	F	-	++	-	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S323	NF	F	++	+++	++	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S324	F	F	+	++	+	-	+	+	-	+	-	-	-	-	-	+	-	*Candida*
S325	NF	F	+	++	-	-	+	-	+	+	+	-	-	-	-	+	-	*Leucosporidium*
S326	F	F	+	+++	+	-	-	+	-	+	-	-	-	+	-	+	-	*Candida*
S327	F	F	+++	+	+	-	-	-	-	-	-	-	-	-	-	+	-	*Candida*
S328	F	F	++	++	++	-	+	+	+	+	-	-	-	+	+	+	-	*Filobasidium*
S329	F	F	+	+++	+++	-	+	+	-	+	+	-	-	-	-	+	-	*Candida*
S330	F	F	+	++	+++	-	+	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S331	NF	F	+	+	+++	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S332	NF	F	-	-	-	-	+	-	-	+	++		-	-	-	+	-	*Candida*
S333	NF	F	+	++	+	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S337	NF	F		-	-	-	-	-	+	+	-	-	-	+	+*	+	-	*Filobasidium*
S339	F	F		-	-	-	-		+	+	-	-	-	+	+*	+	-	*Filobasidium*
S340	F	F	-	-	-	-	-		+	+	-	-	-	-	-	+	-	*Filobasidium*
S341	F	O	-	-	+	-	+	-	+	+	-	-	-	+	-	+	-	***Rhodotorula***
S343	NF	F	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis: Benedicts Reaction	Cellulose hydrolysis: Growth	Cellulose hydrolysis: Degradation	Urease	Assessing the ability to use nitrogen compounds for aerobic growth: Turbidity	Assessing the ability to use nitrogen compounds for aerobic growth: Nitrite estimation	Assessing the ability to use nitrogen compounds for aerobic growth: Starch estimation	Pectin	Myo-inositol	DBB*	Production of starch like substances: Turbidity	Production of starch like substances: Colour	Genera
S344	F	F	+	++	+	-	-	-	+	+	++	-	-	+	-	+	-	*Leucosporidium*
S345	F	F	++	+	++	-	+	+	-	+	-	-	-	-	-	+	-	*Candida*
S346	NF	F	++	++	+++	-	+	+	-	+	-	-	-	-	-	+	-	*Candida*
S347	F	F	++	+	+	-	+	+	-	+	+++	-	-	+	-	+	-	*Candida*
S349	F	F	+	++	+	-	-	-	-	+	-	-	-	-	-	+	-	*Candida*
S351	F	F	+	+	+	-	-	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S352	NF	F	+	+	++	-	+	+	-	+	-	-	-	-	-	+	-	*Candida*
S353	NF	F	+	+	++	-	+	+	-	+	+	-	-	-	-	+	-	*Candida*
S354	NF	F	+	+	+++	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S355	NF	F	+	+	++	-	-	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S357	NF	F	+	++	+++	-	+	+	+	+	++	-	-	-	-	+	-	*Candida*
S359	F	F	+	++	++	-	+	+	-	+	-	-	-	-	-	+	-	*Candida*
S360	F	Falk	+	++	++	-	+	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S361	F	F	+	++	++	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*
S362	F	F	+	+	++	-	+	-	+	+	++	-	-	-	-	+	-	*Leucosporidium*
S363	NF	F	+	+++	+	-	-	-	-	+	-	-	-	-	-	+	-	*Candida*
S364	F	F	+	+	++	-	+	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S367	NF	F	+	+	+	-	+	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S369	NF	F	-	-	-	-	+	-	+	+	-	-	-	+	-	+	-	*Filobasidium*
S371	NF	F	-	-	-	+	+	+	+	+	++	-	-	-	-	+	-	*Leucosporidium*
S372	F	O	-	-	-	+	+	+	-	+	-	-	-	-	+	+	-	*Candida*
S373	NF	F	-	-	-	-	+	-	+	+	-	-	-	-	+	+	-	*Filobasidium*
S374	F	F	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	*Pichia*
S375	F	NR	-	-	-	-	+	-	+	+	+	-	-	-	-	+	-	*Leucosporidium*
S376	NF	F	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-	*Filobasidium*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-inositol	DBB*	Production of starch like substances		Genera
						Benedicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S377	F	NR	-	-	-	-	+	-	+	+	+	-	-	-	+	+	-	*Leucosporidium*
S379	F	F	++	++	-	-	+		+	+	-	-	-	+	-	+	-	*Filobasidium*
S380	F	O	++	++		-	+	-	+	-	-	-	-	-	-	+	-	*Filobasidium*
S381	F	F	+++	+++		-	+	+	+	-	-	-	-	-	-	+		*Filobasidium*
S382	NF	F	++	++		-	+	+	-	-	-	-	-	-	-	+		*Candida*
S383	F	F alk	-	++	-	-	+	-	-	+	-	-	-	-	-	+		*Candida*
S384	F	F	+	+++	+	-	+	+	-	-	-	-	-	-	-	+		*Candida*
S385	N	F	+	+++	+	-	+	+	-	-	-	-	-	-	-	+		*Candida*
S386	N	F alk	+	+	+	-	+	+	+	+	-	-	-	+	-	+		*Filobasidium*
S387	F	F alk	++	++	+	-	+	+	-	+	++	-	-	+	-	+		*Candida*
S388	F	F alk	+	++	+	-	+	+	-	+	-	-	-	+	-	+		*Candida*
S389	F	O	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	*Leucosporidium*
S390	F	Falk	+	++	-	-	+		-	+	++	-	-	+	-	+	-	*Candida*
S391	F	F	-	-	+	-	+		-	+	-	-	-	+	-	+	-	*Candida*
S392	F	F	-			-	+		+	+	-	-	-	-	-	+	-	*Filobasidium*
S393	F	F	-	-		-	+	-	-	+	+	-	-	-	-	+	-	*Candida*
S394	F	F	-	-	-	-	+	+	+	+	+	-	-	+	-	+	-	*Leucosporidium*
S395	F	F	-	+	-	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S398	F	O	-	-	++	-	+	-	-	+	-	-	-	+	-	+	-	*Candida*
S401	F	F	-	-	-	-	+	+	+	+	-	-	-	+	-	+	-	*Filobasidium*
S402	F	F	-	+	-	-	+	-	-	+	+	-	-	+	-	+	-	*Candida*
S403	F	F	+	+++	++	-	+	-	+	+	+		-		-	+	-	*Leucosporidium*
S404	F	Falk	-	+	-	-	+	+		+	+	-	-	-	-	+	-	*Candida*
S405	F	F	+	++	+	-	+	-		-	-	-	-	-	+	+	-	*Candida*
S406	NF	F	+	++	+	-	+	-	-	+	-	-	-	-	+	+	-	*Candida*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-inositol	DBB*	Production of starch like substances		Genera
						Benedicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S407	NF	F	-	+	-	-	+	+	-	+	-	-	-	-	+	+	-	*Candida*
S408	NF	F alk	-	-	-	-	+		-	-	+	-	-	-	+	+	-	*Mastigomyces*
S411	F	F	++	+++	+	-	+	-	+	+	+	-	-	-	-	+	-	*Filobasidium*
S412	F	F	++	+++	+	-	+	+	-	+	+	-		+	-	+		*Mastigomyces*
S413	F	F	++	+++	+	-	+	-	+	+		-	-	-		+		*Filobasidium*
S414	F	F	+	+++	++	-	+	+	+	-	-	-	-	+		+	-	*Filobasidium*
S415	F	F	+	+++	++	-	+	-	-	+	-	-	-	-	+	+	-	*Mastigomyces*
S418	NF	F	++	+++	+	-	+	-	-	+	-	-	-	+	-	+	-	*Mastigomyces*
S419	NF	F	+	++	+	-	+	-	-	+	-		-	-	+	+	-	*Mastigomyces*
S420	F	F	+	+	++	-	+	-	-	+	-	-	-	+	-	+	-	*Mastigomyces*
S421	NF	F		+	-	-	+	-	+	+	-	-			-	+		*Filobasidium*
S422	NF	F		+	+	-	+	-	+	+	-	-		-	-			*Filobasidium*
S423	NF	F		-	+++	-	+	-	-	+	++	-		-	-	-		*Candida*
S424	F	O	-	-	+	-	+		-	+	-	-	-	-		+	-	*Candida*
S425	NF	F alk	-	+++	++	-	+		-	+	+	-	-	-	-	+	-	*Dipodascus*
S430	NF	F	+	+	+	-	+	-	-	+	-	-	-	-		+	-	*Mastigomyces*
S431	NF	F	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	*Mastigomyces*
S432	NF	F	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	*Filobasidium*
S433	F	F	+	+	+	-	+	-	+	+	-		-	-	-	-	-	*Filobasidium*
S434	NF	F	-	++	+	-	+	+	+	+	-	-	-	-	-	+	-	*Filobasidium*
S435	F	F	+	++	+	-	+	-	-	+	-	-	-	-	-	+	-	*Mastigomyces*
S436	F	F	+	++	+	-	+	-	-	+		-		-	-	+		*Mastigomyces*
S437	F	F	+	+++	+	-	+	-	-	+	-	-		-	-	+		*Mastigomyces*
S438	F	F	+	++	-	-	+	-	+	+	-	-	-	-	-	+	-	*Filobasidium*
S439	F	F	+	+++	-	-	+	-	+	+	-	-	-	+	-	+	-	*Filobasidium*

(cont.....d.)

Table 2.1. Morphological / biochemical characteristics of the marine yeasts

Culture No.	Morphology (Fil / Non Fil)	MOF	Amylase	Gelatinase	Lipase	Cellulose hydrolysis			Urease	Assessing the ability to use nitrogen compounds for aerobic growth			Pectin	Myo-inositol	DBB*	Production of starch like substances		Genera
						Benedicts Reaction	Growth	Degradation		Turbidity	Nitrite estimation	Starch estimation				Turbidity	Colour	
S440	F	F	+	+++	-	-	+	+	+	+	-	-	-	-	+	+	-	*Filobasidium*
S441	F	F	-	++	+	-	+	+	+	+	++	-	-	-	-	-	-	*Leucosporidium*
S443	F	F	-	++	-	-	+	-	-	+	-	-	-	-	-	+	-	*Mastigomyces*
S444	NF	F	+	++	+	-	+	-		+	-	-	-	-	-	+	-	*Mastigomyces*
S445	NF	F	-	-		-	+		-	+	-	-	-	-	-	+	-	*Mastigomyces*
S446	NF	F	+	++	+	-	+	-	+	+		-	-	-	-	+	-	*Filobasidium*
S447	NF	F	+	++	+	-	+	-	-	+	-	-	-	-	-	+	-	*Mastigomyces*
S448	NF	O	-	-	-	-	+	-	+	+	++	-	-		-	-		*Leucosporidium*
S449	NF	F	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	*Filobasidium*
S450	NF	O	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	*Candida*

F-Filamentous, NF - Non-Filamentous, MOF - Marine Oxidation Fermentation Test, O - Oxidative , F - Fermentative,
alk - alkaline , * DBB - +^{w} Pale red , + deep red

Table 2.2. Details of the stations showing the source of isolation of yeasts

S.No.	Cultures	No. of isolates	Station No.	Location
1	**S 1 - S 18**	18	S 1	Bolgatty (Off Cochin)
2	**S 19 - S 25**	7	SS 1	Cruise No. 147 (West Coast)
3	**S 26 - S 30**	5	SS 2	Cruise No. 147 (West Coast)
4	**S 31 - S 33**	3	SS 3	Cruise No. 147 (West Coast)
5	**S 34 - S 39**	6	SS 4	Cruise No. 147 (West Coast)
6	**S 40 - S 47**	8	SS 5	Cruise No. 147 (West Coast)
7	**S 48 - S 56**	9	S 2	Fort Cochin (Off Cochin)
8	**S 57 - S 80**	24	S 3	Vypeen (Off Cochin)
9	**S 81 - S 91**	11	S 4	Ernakulam (Off Cochin)
10	**S 92 - S 98**	7	S 5	Mulavucadu (Off Cochin)
11	**S 100 - S 146**	47	S 6	Mattancherry (Off Cochin)
12	**S 148 - S 162**	15	S 7	Chellanum (Off Cochin)
13	**S 163 - S 246**	84	S 8	Kannamali (Off Cochin)
14	**S 247 - S 256**	10	SS 6	Cruise No. 156 (West Coast)
15	**S 257 - S 283**	27	SS 7	Cruise No. 156 (West Coast)
16	**S 284 - S 299**	16	SS 8	Cruise No. 156 (West Coast)
17	**S 300 - S 312**	13	SS 9	Cruise No. 156 (West Coast)
18	**S 313 - S 320**	8	SS 10	Cruise No. 156 (West Coast)
19	**S 321 - S 342**	22	SS 11	Cruise No. 156 (West Coast)
20	**S 343 - S 369**	27	SS 12	Cruise No. 156 (West Coast)
21	**S 370 - S 382**	13	SS 13	Cruise No. 157 (East Coast)
22	**S 383 - S 399**	17	SS 14	Cruise No. 157 (East Coast)
23	**S 400 - S 408**	9	SS 15	Cruise No. 157 (East Coast)
24	**S 409 - S 415**	7	SS 16	Cruise No. 157 (East Coast)
25	**S 416 - S 420**	5	SS 17	Cruise No. 157 (East Coast)
26	**S 421 - S 426**	6	SS 18	Cruise No. 157 (East Coast)
27	**S 427 - S 430**	4	SS 19	Cruise No. 157 (East Coast)
28	**S 431 - S 435**	5	SS 20	Cruise No. 157 (East Coast)
29	**S 436 - S 441**	6	SS 21	Cruise No. 157 (East Coast)
30	**S 442 - S 450**	9	SS 22	Cruise No. 157 (East Coast)

Lipomyces, Geotrichum and *Arxioma* were mainly obtained from the coastal waters. However, strains like *Candida, Filobasidium, Leucosporidium, Mastigomyces, Rhodotorula, Schizosaccharomyces, Kluyveromyces, Torulaspora, Oosporidium* and *Dipodascus* were mainly encountered in the offshore water samples (Table 2.3).

Table. 2.3. List of yeast strains isolated from the East and West Coast of India

S.No	Genera	No.of isolates	Isolates from coastal region(%)	Isolates from offshore region(%)
1	*Candida*	116	37	63
2	*Filobasidum*	55	20	80
3	*Leucosporidium*	24	33	67
4	*Mastigomyces*	15	0	100
5	*Lodderomyces*	11	100	0
6	*Debaryomyces*	7	71	29
7	*Rhodotorula*	4	25	75
8	*Dekkera*	4	50	50
9	*Hormoascus*	3	100	0
10	*Cryptococcus*	3	100	0
11	*Schizosaccharomyces*	3	0	100
12	*Kluyveromyces*	2	0	100
13	*Williopsis*	2	100	0
14	*Aciculoconidia*	2	50	50
15	*Pichia*	2	50	50
16	*Torulaspora*	1	0	100
17	*Saccharomycopsis*	1	100	0
18	*Lipomyces*	1	100	0
19	*Geotrichum*	1	100	0
20	*Arxioxyma*	1	100	0
21	*Oosporidium*	1	0	100
22	*Dipodascus*	1	0	100
	Total	**260**	**36.54**	**63.46**

Most of the isolates were found to be fermentative in nature. Filamentous growth was very common among the isolates.

More than 50% of the strains were capable of producing gelatinase, lipase and amylase. Strains belonging to Mastigomyces were found to be highly potent in enzyme production with more than 80% showing this capability (Table 2.4).

Table. 2.4. Comparison of marine yeasts based on their physiological / biochemical characteristics

S.No.	Genera	Total No.	Isolates capable of producing (%)				Isolates capable of producing (%)	Isolates capable of producing (%)
			Amylase	Gelatinase	Lipase	Urease	Myo-inositol	DBB
1	*Candida*	116	56	65	66	9	28	39
2	*Filobasidium*	55	54	78	53	100	25	36
3	*Leucosporidium*	24	80	54	50	92	25	50
4	*Mastigomyces*	15	9	87	80	0	25	33
5	*Lodderomyces*	11	29	55	82	55	20	82
6	*Debaryomyces*	7	0	43	29	14	36	0
7	*Rhodotorula*	4	50	0	75	75	0	100
8	*Dekkera*	4	0	75	75	25	25	50
9	*Hormoascus*	3	0	33	0	67	50	67
10	*Cryptococcus*	3	0	0	100	67	33	100
11	*Schizosaccharomyces*	3	0	67	33	100	33	0
12	*Kluveromyces*	2	0	50	100	0	0	0
13	*Williopsis*	2	50	50	100	0	50	100
14	*Aciculoconidia*	2	0	50	50	0	50	50
15	*Pichia*	2	0	0	50	50	0	100
16	*Torulaspora*	1	0	0	100	0	0	0
17	*Saccharomycopsis*	1	0	0	0	0	0	0
18	*Lipomyces*	1	0	0	100	0	100	100
19	*Arxioxyma*	1	0	0	0	0	100	0
20	*Oosporidium*	1	0	100	100	0	0	100
21	*Dipodascus*	1	0	100	100	0	0	0
22	*Geotrichum*	1	0	0	100	0	0	100
		260	**50**	**63.1**	**61.5**	**41.2**	**26.5**	**42.3**

Proteolytic and lipolytic forms were found to be 63.1 and 61.5% respectively. Urea utilization was restricted to 41.2%. Production of

myo-inositol was also observed in a few isolates (26.5%). Production of starch like substances was not observed in any of the isolates.

2.4 Discussion

Marine yeasts could be isolated both from coastal and offshore waters. However, the population was found to be very low and therefore concentration of the water sample as well as enrichment technique was employed for isolation. Fischer (1894) found yeasts in Atlantic water samples regardless of the distance from the land. Kriss and Novozhilova (1954) found that yeasts were present in all regions in the surface waters while in inshore areas yeasts could be found at all depths also. Several thousand yeast cells per liter of water were observed in coastal waters (Meyers *et al.*, 1967). The majority of yeasts are obligate aerobes that require oxygen for growth and reproduction. Therefore yeasts usually do not inhabit anaerobic waters and sediments. Near shore water samples of 250ml are generally adequate whereas open ocean samples of 1 liter or more are routinely required. Kriss (1961) noted that yeasts could be observed in the anaerobic zone of the black sea.

As many as 22 genera were isolated from the west and east coast of India. These isolates were comprised of mainly *Candida* followed by *Filobasidium, Leucosporidium, Mastigomyces, Lodderomyces* and *Debaryomyces* etc. Bhatt and Kachwalla (1955) had isolated *Saccharomyces, Debaryomyces, Pichia, Candida, Torulopsis, Rhodotorula, Cryptococcus* etc. off the coast of Bombay. As per Fell *et al.* (1960) *Candida* and *Rhodotorula* were the predominant species in Biscayne Bay, Florida. van Uden and ZoBell (1962) had isolated

Metschnikowia, Pichia, Kluyveromyces and *Torulopsis* apart from *Candida.*

This study and earlier studies show that *Candida* is well distributed in the marine environment. Whether they are indigenous to the marine environment or introduced from the terrestrial environment and got adapted to the marine conditions needs further investigation. The prevalent yeasts in estuarine, littoral and deep-water marine sediments of Florida and the Bahamas have been mostly oxidative including *Rhodotorula* and *Cryptococcus* (Voltz *et al.*, 1974). In the present study also *Rhodotorula* and *Cryptococcus* could be observed even though in small numbers. *Cryptococcus* is considered to be typical marine form indigenous to the most sever cold desert in earth, Antarctica (Baharaeen *et al.*, 1982). In the present study also Rhodotorula could be isolated from unpolluted environment away from the shore. Prabhakaran and Gupta (1991) found that *Candida* was the dominant group in the sediment samples of South West Coast of India.

Hydrolytic potential of the yeasts shows their degradation / mineralization capacity. This capacity in enzyme production would help them very much in utilization of wide range of substrates as nutrients for growth.

Chapter 3

SCREENING OF MARINE YEASTS FOR UTILIZATION AS FEED SUPPLEMENT IN AQUACULTURE

3.1 INTRODUCTION

In aquaculture operations, essential and expensive components of the feed are proteins. Fishmeal is used as a major protein source in most finfish and crustacean diets (Lovell, 1989) due to the high nutrient quality and digestibility of fishmeal protein. During the last few decades, there has been an increasing interest to use alternative plant and animal protein, as low cost substitutes of fish meal (Moore and Stanley, 1982; Kohler and Kruegger, 1986; Koshio *et al.*, 1992; Tidwell *et al.*, 1992, 1993 a & b and 1994). Among unconventional protein sources, Single Cell Protein (SCP) of microbial origin appears to be a promising candidate. Many workers have reported partial replacement of fishmeal with yeast, bacteria and soybean protein (Cho *et al.*, 1974; Bergstrom, 1979; Spinelli *et al.*, 1978). Bellamy, 1969 and Reese *et al.*, 1972 suggested the use of microbial proteins to supplement human and animal diets in the wake of the sweeping starvation and malnutrition problems faced by many developing countries. The very high protein content of SCP's makes them suitable for inclusion at high levels in pelleted fish rations (Nose, 1975).

The criteria for microorganisms to be used as food include many things like, 1) The organism must be non-toxic and grow rapidly on a simple non-specific medium. 2) It should have high nutritional or vitamin content and should be edible to humans and other animals. The organisms should also utilize the energy source without producing any side effects and any undesirable effects and 3) It should be easy to separate the cells from the medium.

SCP include microalgae, bacteria and yeast and are alternative to conventional protein sources that are frequently used as feed ingredients for fish due to their nutritional value (Sanderson and Jolly, 1994; Tacon, 1994). SCP basically comprises proteins, fats, carbohydrates and minerals such as phosphorous and potassium. Chemical analysis of microorganisms tested for SCP reveal that they are comparable in aminoacid content to the plant and animal sources with the exception of methionine which is low in some SCP sources. The substrates used for SCP manufacture include, 1) Molasses from sugar factories, 2) Spent sulphite liquor, the waste product of the paper industry, 3) The acid hydrolysate of wood, 4) Agriculture waste and 5) Waste from dairy industry.

Among the many groups of microorganisms used to produce SCP, yeasts are perhaps the most important one. Being one of the richest source of vitamins coupled with 45-65% protein content, yeasts has always surpassed any other microbes as SCP in its public acceptability. Yeasts have many advantages like,

1. Larger size and easy to harvest
2. Lower content of nucleic acid
3. Long history of use as food, hence so better public acceptance
4. Ability to grow on substrates of low pH, which reduce bacterial contamination of the medium.

The composition of yeast is given in Table 3.1. The protein component ranged from 29 to 63%, carbohydrate 21-39% and fat 1 to 23%. Protein content was found to be comparatively high in *Candida, Saccharomyces, Torula* and *Geotrichum. Phaffia* was reported to contain 23% fat (Sanderson and Jolly, 1994) and comparatively less

Table.3.1. Gross composition (% of dry weight) of yeasts

Strain	Dry Matter	Carbo-hydrate	Crude Fibre	Crude Protein	Nucleic Acids	Fat	Ash	NFE	Ether Extract	Reference
C.utilis			< 1.0	44.3	7.1		7.0		7.7	Han *et al.*, 1976
Geotrichum candidum		26.8		48		7.2				Dabrowski *et al.*,1980
Ethanol grown yeast				49.56		4.94	5.61	31.55		Frydrych and Heger, 1981
Yeasts				44-56	6-12	2-6	5-9.5			Reed, 1981
Candida sp.				34.9		2.1	9.1	53.8		Kamel and Kawano,1986
Torulopsis sp.				32.13		1.05	9.82	57		
Saccharomyces.				48-63		7	7.0-7.5	27-36		
Fresh yeast		±30		48-54		< 2				Coutteau *et al.*, 1992
Yeast		28.7	6.3	43.3		4.2	9.2			Chieh Lan and Sun Pan, 1993
Phaffia				22		23	3			Sanderson and Jolly, 1994
Candida utilis		23		42		6.1	13			Brown *et al.*,1996
Debaryomyces hansenii		21		23		5.9	11			
Dipodascus capitatus		25		32		4.4	9.3			
Dipodascus sp.		28		36		4.3	6.8			
Dipodascus sp.		30		35		4.5	4.7			
Dipodascus sp.		25		32		4.6	9.4			
Dipodascus sp.		28		32		2.5	7.9			
S.cerevisiae		39		29		7.7	6.4			
Torula yeast			0.62	46.11			10.2	37.19	1.51	Olvera-Novoa *et al.*, 2002

protein (22%). Carbohydrate expressed as NFE was maximum in *Torulopsis* and *Candida* (Kamel and Kawano, 1986).

Freshwater microorganisms and baker's yeast have been supplied as food for marine zooplankton cultures. However, it was found that the cells of these organisms get lysed, causing pollution of the cultures under high osmotic pressure of sea water (Kawano and Ohsawa, 1971). In order to solve these problems the utilization of marine microorganisms seems to be the best answer.

Yeasts are common in sea water (Hernandez-Saavedra *et al.*, 1992; Nell *et al.*, 1994) and may be natural components of the diet of oysters. Yeasts can be produced much more efficiently and economically than photosynthetic algae because of shorter generation time and the use of inexpensive culture media (Nell, 1993). Some yeasts like *Candida* spp. and *Saccharomyces cerevisiae* are also believed to have immunostimulatory properties by virtue of their complex carbohydrate components and nucleic acid content (Anderson *et al.*, 1955). Among SCP, yeasts have been the most used within aqua feeds (Tacon, 1994). Yeasts are a rich source of proteins and B-complex vitamins. They have been used as a supplement in animal feed to compensate for aminoacid and vitamin deficiencies of cereals, and are recommended as a substitute for soybean oil in diets for fowl (Gohl, 1991). Yeast products (primarily brewer's yeast and baker's yeast) are frequently used as feed ingredients in aquaculture because of the nutritional value of these products, which include protein, lipids, B-complex vitamins etc (Mahnken, 1991; van der Meeren, 1991). Yeast based diets are rich in proteins, lipids, attractants and

other nutrients. Some of the yeast species used as fishmeal substitutes are *Candida* sp., *Kluveromyces* sp. and *Phaffia* sp.

Hirata and Mori (1967) observed that rotifers could grow as well on a mixture of baker's yeast and *Chlorella* as on *Chlorella* alone. They also found that, when fed baker's yeast alone, the rotifer population grew as well as when fed *Chlorella* during the first week, but declined during the second week of culture, probably as a result of nutritional deficiency on a diet consisting of baker's yeast exclusively (Hirata, 1979). Many workers found yeast as an inexpensive and easily available alternative food for rotifers. (Furukawa and Hidaka, 1973; Hirata, 1974; Fushimi, 1975; Fukusho *et al.*, 1976; Mochizuki *et al.*, 1978; Hirata *et al.*, 1979; James *et al.*, 1983, 1987).

Successful incorporation of yeast and bacterial SCP into fish diets allows replacement of 25-50% of the fishmeal component (Andruetto *et al.*, 1973; Beck *et al.*, 1979; Mahnken *et al.*, 1980). For salmonids, diets containing hydrocarbon grown yeasts have worked well on fishmeal or other sources of protein with yeast (Andruetto *et al.*, 1973; Shimma and Nakada, 1974a, b; Brannon *et al.*, 1976; Gropp *et al.*, 1976; Shimma and Shimma, 1976; Shimma *et al.*, 1976; Roley *et al.*, 1977).

Another concern with SCP is their high concentration of nucleic acids, ranging from 5-12% in yeast and 8-16% in bacteria (Schulz and Oslage, 1976). In brewer's yeast, nucleic acid nitrogen is present mostly in the form of RNA and represents about 20-25% of the nitrogen (Rumsey *et al.*, 1991b).

Epifanio (1979) found that a diet consisting of 50% yeast *Candida utilis* (spray-dried) and 50% algae supported growth comparable to a 100% algal ration when fed to juvenile *Argopecten irradians, Mercenaria mercenaria* or *Mytilus edulis*, but was deficient for the American Oyster, *Crassostrea virginica*. The limited nutritional value of yeasts for bivalves has been attributed to their low digestibility (Epifanio, 1979; Nell, 1985) as well as a deficiency or imbalance of nutrients (Urban and Langdon, 1984). The supplementation of yeast-based diets with the deficient amino acids was shown to have beneficial effects on fish growth (Nose, 1974a and b; Spinelli *et al.*, 1979; Bergstrom, 1979; Mahnken *et al.*, 1980).

In rainbow trout, Tiews *et al.* (1979) succeeded to obtain growth rates equivalent to that of the control diet when fed yeast-based diets supplemented with methionine. However, other attempts using yeast as the sole source of dietary protein resulted in reduction of biogrowth parameters (Beck *et al.*, 1979; Mahnken *et al.*, 1980; Rumsey *et al.*, 1991a). In practice, yeasts have been incorporated in rations at levels of 30% resulting in a 25-50% replacement of the fish meal content (Tacon and Jackson, 1985; Tacon, 1994).

Several species of teleostean fishes including carp (Omae, 1979; Iida *et al.*, 1970) and eel (Ghittino, 1970) have been fed experimental diets containing hydrocarbon-grown yeasts. Only young carp have shown better growth on hydrocarbon grown yeast diets than on diets based on fishmeal (Omae, 1979). Experiment conducted with juvenile Tilapia (*Oreochromis mossambicus*), to evaluate an industrial SCP as an alternative component to fishmeal in complete diets, showed that

SCP could successfully replace upto 40% of the fishmeal in the practical diet (Davis and Wareham, 1988).

Baker's yeast, *Saccharomyces cerevisiae* chemically treated with sulfhydryl compounds to improve its digestibility were tested on juvenile *Mercenaria mercenaria* (Lavens *et al.*, 1989; Coutteau *et al.*, 1990). The development of techniques to improve the digestibility (Coutteau *et al.*, 1990) and the nutritional composition (Leger *et al.*, 1985) of yeast based diets provided the incentive to develop a product as a potential substitute for unicellular algae. Such a yeast-based diet has proven to be a valuable algal substitution in the larval culture of marine shrimp (Naessens-Foucquaert *et al.*, 1990). In juvenile Sydney rock oyster spat (Brown *et al.*, 1996), substitution with 86% (dry weight basis) live yeast produced a weight increase, 63-81% of those obtained on algal diet.

Rumsey *et al.* (1990) showed that the lower performance of fish fed diets containing high levels of brewer's yeast may be caused by intact yeast cells, as probably not all intracellular ingredients become available to the fish. Rumsey *et al.* (1991b) found that digestibility of intact brewer's yeast in rainbow trout is significantly lower than that of disrupted cells. In accordance to this finding, Rumsey *et al.* (1990) observed that brewer's yeast could replace 50% of total nitrogen in the diet of lake trout when the yeast cell walls were disrupted but a growth depression occurred when intact yeasts were used.

The efficacy of live yeasts as diet compliments in aquaculture diets have been tested by many workers (Coutteau *et al.*, 1991; Roques and Dussert, 1991; Coutteau *et al.*, 1993; Coutteau *et al.*, 1994) as substitutes for algae. *Phaffia rhodozyma* is a species of yeast,

containing astaxanthin, which is the most abundant carotenoid in the marine environment (Johnson and An (1991) .

Experiments were conducted to determine the effects of high dietary levels of brewer's dried yeast on the growth, feed conversion efficiency and uric acid metabolism of rainbow trout (*Oncorhynchus mykiss*). Growth was faster and feed conversion more efficient in fish when fed diets consisting of 25% yeast; the diets containing 50 and 75% yeast were unpalatable to trout. It was concluded that although feed intake was reduced at yeast levels greater than 25%, the fish did not appear to be adversely affected physiologically by high dietary levels of nucleic acid nitrogen (Rumsey *et al.*, 1991a).

Rumsey *et al.* (1991a) observed a feed intake depression in rainbow trout due to reduced acceptability of diets including more than 25% of brewer's yeast. Atack and Matty (1979) observed reduced feed intake in rainbow trout fed a brewer's yeast supplemented diet compared to a herring meal based diet. On the contrary, when rainbow trout was fed a diet including a yeast nucleic acid extract corresponding to a dietary level of 50% brewer's yeast, no negative effect on feed intake was observed (Rumsey *et al.*, 1992).

In contrast, it has been shown that common carps can utilize a high percentage of their dietary protein requirement from the yeasts *Candida tropicalis, Candida utilis and Candida lipolytica* with better results than those obtained with soybean or meat and bone meals. For carp larvae diets, it has been shown that from 62 to 88% of *Candida utilis* and *Candida lipolytica* can be used in combination with other materials such as fishmeal and other animal by-products (Atack *et al.*,

1979; Hecht and Viljoen, 1982; Dabrowski *et al.*, 1983; Alami-Durante *et al.*, 1991).

Experiments conducted in rainbow trout (*Oncorhynchus mykiss*) indicated that the nutritional significance of free dietary adenine versus nucleic acid bound adenine in yeast or yeast nucleic acid is an important consideration in evaluating the suitability of SCP's in fish feed formulations. Fish feed with increasing levels of yeast extract manifested significant increases in growth and nitrogen retention. No negative effects on feed intake were observed (Rumsey *et al.*, 1992).

Partial replacement of algal diets by bacteria (Martin and Mengus, 1977) and yeast (Epifanio, 1979; Urban and Langdon, 1984; Nell *et al.*, 1994) could significantly reduce bivalve production costs, particularly if these microorganisms could be purchased commercially as "off-the-shelf" dried or paste preparations. Recently, bacteria and yeast isolated from Oyster growing areas were tested as partial substitutes for live algae in the diet of Sydney rock oyster (*Saccostrea commercialis*) larvae and spat (Nell *et al.*, 1994).

Experiments conducted on diets for tilapia (*Oreochromis mossambicus*) to evaluate the effects of substituting animal protein with a mixture of plant feed stuffs including 25, 30, 35, 40 and 45% of the protein with torula yeast (*Candida utilis*), 20% with soybean meal and 15% with Alfalfa Leaf protein Concentrate (ALC). Feeding efficiency was compared against a diet with fishmeal as the sole protein source and the results showed that 30% yeast diet showed the best growth performance. The results suggests that it is possible to replace upto 65% of animal protein with a mixture of plant proteins,

including 30% torula yeast, in tilapia fry diets without adverse effects on fish performance and culture profit (Novoa *et al.*, 2002).

Experiments conducted on Nile tilapia (*Oreochromis niloticus*) evaluated the effects of three types of probiotics, two bacteria and one yeast on growth performance. Three diets were formulated containing the optimum protein level (40%) for tilapia fry: one supplemented at 0.1% with a bacterial mixture containing *Streptococcus faecium* and *Lactobacillus acidophilus*; a second supplemented at 0.1% with the yeast *Saccharomyces cerevisiae*; and third a control diet without supplements. Of the four treatments, the 40% protein diet supplemented with yeast produced the best growth performance and feed efficiency, suggesting that yeast is an appropriate growth stimulating additive in Tilapia cultivation (Lara-Flores *et al.*, 2003).

Feed is a major concern for shrimp farmers, representing upto 60% of the total variable production costs (Akiyama *et al.*, 1992; Sarac *et al.*, 1993). It must fulfill several characteristics including organoleptic properties such as odor, texture, flavor and physical properties such as particle size. In addition, the feed should contain all the essential nutrients for the cultured organism, and be readily available at low cost and highly digestible with the nutrients available for assimilation (Sudaryono *et al.*, 1995).

Although several attempts have been made to rear shrimp on artificial pelleted diets (Subrahmanyan and Oppenheimer, 1969; Kanazawa *et al.*, 1970; Cowey and Forster, 1971; Sick *et al.*, 1972) a formulation, which will promote optimal growth in these animals, have not been reported. A major obstacle in formulating a complete diet is the lack of information on the specific nutrient requirement of these

animals, particularly on the utilization of macronutrients such as protein, carbohydrate and lipids.

Mixing the probiotic with the feed ingredients is a method that could be used for application to shrimp, but the benefit of supplementing live yeast or another microorganism as a probiotic agent in a commercial shrimp diet will be effective only if it survives the manufacturing process. Pelleting is the most popular manufacturing process for shrimp feeds (Estrada and Valdez, 1993), it is preceded by a steam conditioning phase, particularly important for starch gelatinization, and activation of binders, to provide adequate feed water stability (Dominy and Lim, 1991), and followed by a short drying phase.

Supplementation with 0.1% live yeast did not significantly affect the stability in water of the extruded or pelleted diets. The feed stability in water is an important characteristic of shrimp feeds, due to the slow feeding habit of shrimp (Lee and Lawrence, 1997).

Growth appeared to increase with amount of protein in the diet. The protein requirement for optimal growth of *Penaeus monodon* has been reported to be between 35-61% (Lee, 1970; Ting, 1970; Chen and Liu, 1971; Deshimaru and Kuroki, 1975; New, 1976; Lin *et al.*, 1981; Alava and Lim, 1983; Shiau *et al.*, 1991).

Studies on the effects of dietary protein, carbohydrates and lipid levels on growth and survival of penaeid shrimp was studied by Andrews *et al.* (1972). They have reported that 28-32% was the optimal protein level and addition of starch to the diet enhanced the growth, and glucose caused a reduction in weight gain. Feeding trials

on *Macrobrachium rosenbergii* using diets with different levels of protein (15, 25 and 35%) showed that higher protein content produced larger prawns (Balazs and Ross, 1976). The use of dried prawn meal as a high quality reference protein in the nutrition of juvenile *Penaeus indicus* was tested and live weight gain at levels ranging from 21-53% crude protein was greatest with a 43% protein diet, but Protein Efficiency Ratio (PER) values declined with successive increases in dietary protein (Colvin, 1976).

Carbohydrates are not essential for shrimp brood stock. However, they can be a useful inexpensive source of energy with protein sparing and lipid-sparing effects. Complex sugars and polysaccharides are used more effectively than simple sugars (Deshimaru and Yone, 1978). Polysaccharides is most commonly used. Carbohydrates are also excellent binders in diet formulation.

Colvin (1976) supplemented a constant level of 5% plant oil in the experimental diets, in addition to the lipid present in other ingredients (fish meal) and reported that a diet containing 9.8% lipid gives better growth in juvenile *Penaeus indicus*. Kanazawa *et al.* (1977) reported poor growth with lipid-free diet, maximum growth with 12% lipid and reduced growth at 16% when powdered Pollack residual oil was included in diet of *Penaeus japonicus*. Read (1981) supplemented lipids at 3 and 4.5% levels in various diets containing selected lipid sources and found that a diet with a 3% mixture of fish oil and sunflower oil in the ratio of 2:1 gives better survival and growth in juvenile *Penaeus indicus*. High dietary lipid content of the yeast diets may have compensated for the deficiency in available calories of the

algae/yeast diets, which was hypothesized by Urban and Langdon (1984).

Shrimps do not have an absolute dietary lipid requirement (D'Abramo, 1989). Rather, the provision of sufficient lipid is based on satisfying the requirements for specific nutrients such as highly unsaturated fatty acids (HUFA), phospholipids and sterols, and for energy. Crustaceans have long been recognized as having limited ability to synthesize HUFA *de novo* (Chang and O'Connor, 1983; Mourente, 1996) and no ability to synthesize sterols *de novo* (Kanazawa *et al.*, 1988).

Fat-soluble vitamins A (or β-carotene), D and E were found to be essential to support shrimp growth (He *et al.*, 1992). Dietary levels of thiamine, riboflavin, niacin, vitamin B6, vitamin B12, choline, inositol and ascorbic acid have also been recommended for maximal growth in several shrimp species (D'Abramo and Conklin, 1992). Vitamin E has been shown to improve the percentage of normal sperm and the rate of ovarian maturation on *L.setiferus* (Chamberlain, 1988).

Studies on mineral requirements of shrimp brood stock are lacking, probably because of the following implications. Firstly, for minerals it is necessary to distinguish true dietary requirements and apparent physiological requirement, since the minerals can be absorbed from water. Secondly, the input through animal meals in artificial diets is high, and performing purified diets that would allow controlled mineral levels do not exist. In most studies, artificial diets with mineral mixes were formulated specially to fortify with Ca, P, Mg, Na, Fe, Mn and Selenium (Chamberlain, 1988; Alava *et al.*, 1993 a,b; Marsden *et al.*, 1997; Mendoza *et al.*, 1997; Xu *et al.*, 1994).

There is also a strong relationship between Ca and Mg levels in the diet, and deficiency of the latter led to the retardation of rainbow trout growth and renal calcinosis (Cowey *et al.*, 1977).

3.2 MATERIALS AND METHODS

3.2.1 Selection of strains

Representative strains (25 numbers) of various genera were selected for nutritional quality evaluation, which is an important criteria for the selection of a strain for SCP production. The following strains were subjected to proximate composition analysis i.e. S3, S8, S13, S28, S30, S42, S48, S50, S56, S69, S70, S81, S87, S100, S165, S169, S170, S186, S297, S303, S382, S394, S425, S434, and S437.

Preparation of yeast biomass

The selected 25 yeast cultures were swab inoculated onto malt extract agar plates, incubated at 28±2°C for 72 hrs and harvested with sterile saline. The cell suspensions were centrifuged at 7000 rpm for 20 minutes in a refrigerated centrifuge (Remi C-30, Mumbai) and the yeast biomass stored at 4°C in a refrigerator.

Proximate composition of the yeast biomass

Biochemical composition of the yeast biomass was analyzed to assess their nutritional quality. Protein was estimated by Microkjeldhal method (Barnes, 1959) and lipid by Phosphovanillin method following chloroform methanol extraction of the sample (Folch *et al.*, 1957) and Carbohydrate by Roe (1955).

Based on the nutritional quality analysis 14 yeast strains were selected for the feeding experiment.

3.2.2 Feeding experiment with *F.indicus* post larvae

Experimental animals

Post larvae (PL-21) of Indian white prawn, (*Fenneropenaeus indicus* H.Milne Edwards) of the size range 20-30 mg were brought to the laboratory from a commercial prawn hatchery in Kannamali, Kochi.

Experimental feed preparation

Powdered ingredients as given in table (3.2) were mixed well into a dough with 100ml water. This was steamed for 10 minutes in an autoclave and pelletised using a laboratory model pelletiser having 1mm die. Pellets were dried in an oven at 50°C for 18hrs. The pellets were broken into pieces of 4-5mm size. 14 different feeds were prepared incorporating the biomass of 14 yeast strains plus the control diet without the yeast biomass. Water stability of feed was checked by immersing pellets in seawater for 15 hrs and examining stability by visual observation. Feeds were stored in airtight polythene bags at -20°C in a freezer.

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 5hrs and moisture content by drying in an oven at 80°C to constant weight. Fiber content

was determined by acid and alkali treatment following AOAC (1990). The nitrogen free extract (NFE) was computed by difference (Crompton and Harris, 1969). (NFE=100 - (% protein + % lipid + % fiber + % ash).

Table 3.2 Composition of experimental diets

Ingredients	Control diet g	Experimental diet g
Prawn shell powder	10	10
Yeast[a]	-	20
Fish meal	30	30
Ground nut oil cake[b]	8	8
Soybean meal[c]	10	10
Maida[d]	8	8
Rice bran[e]	10	10
Vitamin and mineral mix[f]	2	2
Agar	2	2
Carboxy methyl cellulose	20	-
Water	100 ml	100 ml

[a]Biomass of 14 different yeast strains were incorporated in the 14 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 soybean
[d]Refined wheat flour
[e]Finely ground rice bran
[f]Vitamin and mineral mix (mg/g vitamin and mineral mix)

Thiamine	0.61 mg
Ribofalvin	0.48 mg
Pantothenic acid	2.42 mg
Pyridoxine	0.72 mg
Cyanocobalamine	0.02 mg
Biotin	0.02 mg
Retinol	0.13 mg
Menaptone	0.24 mg
Folic acid	0.13 mg
Niacin	2.42 mg
α tocopherol	2.42 mg
Banox	0.30 mg
Cholecalciferol	0.06 mg
Ascorbic acid	6.05 mg
K_2HPO_4	4.68 mg
$Ca_3(PO)_4$	6.36 mg
$MgSO_4.7H_2O$	7.12 mg
$NaH_2PO_4.2H_2O$	1.84 mg

Feeding schedule

Prawns were fed twice daily at 10 a.m. and 5 p.m with fourteen different feeds including control diet at the rate of 10-15% of the body weight per day. Pre-weighed experimental diets were placed in petridishes in the tank. Faecal matter was removed by siphoning twice daily.

Rearing facility

Fiber reinforced rectangular plastic (FRP) tanks of 30L capacity were used for the study. Water quality was monitored daily and was maintained as per Table (3.3). 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 1HP compressor through air stones. Physico-chemical parameters like salinity, nitrogen

and dissolved oxygen of the rearing water were estimated daily by following standard procedures (APHA, 1995).

Table 3.3. Rearing conditions and water quality parameters of the experimental system

Initial body weight (average)	: 20.55±9.4mg
Stocking density	: 25 PL/tank
Tank capacity	: 30L
Feeding level	: 10-15% body weight
Feeding frequency	: Twice daily
Feeding period	: 21 days
Water temperature	: 24-27°C
pH	: 7.5 - 8
Salinity	: 15 - 18ppt
NH_3	: 0.01 - 0.02mg/L
NO_3	: Below detectable
NO_2	: 0.00 - 0.01mg/L
Dissolved O_2	: 6-7mg/L

Design of experiment

The post larvae of *F. indicus* were maintained on prepared control diet for a period of one week. The larvae were then stocked into 30L rectangular fiberglass tanks containing 20L 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.

Measurements

The initial body weight of the prawns in each rearing tank was recorded. They were weighed on a precision balance after blotted free

of water with tissue paper. The mean weight of all the prawns in a tank was calculated (mean±0.01g). After 21 days, final weights of all the prawns were measured and mean weight was found. Parameters including individual increase in weight (production), food conversion ratio (FCR), specific growth rate (SGR), relative growth rate (RGR), gross growth efficiency (GGE), and protein efficiency ratio (PER) were determined based on the data collected during the experimental period.

The formula used for calculating the growth parameters are given below:

Production = Final weight - Initial weight

FCR = Food consumed / Live weight gain

SGR = (In final weight - In initial weight) x 100 / days of feeding experiment

RGR = (W2 - W1) / Mean weight/No of days

GGE ={(W2 -- W1) / Food consumed} x 100

PER = Live weight gain/ protein consumed in dry weight

Challenge experiment

After termination of the feeding experiment (21days) all treatment groups including the control, were maintained under the same rearing conditions. Challenge with white spot virus (WSSV) was performed through oral administration. For this, prawns were fed with white spot virus infected prawn flesh (*F. indicus* adult) in the morning (after a starvation period of 12 hrs.) 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 rates were recorded everyday for a period of 7 days. Mortality by WSSV infection was confirmed by checking the characteristic circular white spots on the carapace and other exoskeleton parts of the infected animal.

Data analysis

The data obtained in the feeding experiments were subjected to one-way analysis of variance (ANOVA). When a significant difference was found among the various treatments, Duncan's multiple range tests were done to bring out the difference between the treatment means. The statistical analysis was performed using the SPSS 7.5 package for windows.

3.3 RESULTS

3.3.1 Proximate composition of yeast biomass (SCP) and feed

Proximate composition of yeast biomass

Protein content of the yeast biomass of various strains belonging to different genera was found to be in the range 22-30% and the maximum was found in S169 (30.45%) belonging to *Debaryomyces* (Fig 3.1 and Table A-1). No significant difference could be observed between the genera in the biochemical composition. Lipid content of yeast biomass varied between 2 to 8.25% the maximum being in S28 (*Kluveromyces* sp.). There was no significant variation in the carbohydrate content in yeast biomass and it varied from 22.36 to

29.68% with a minimum in S48 (*Lodderomyces* sp.) and maximum in S70 (*Homoascus* sp.).

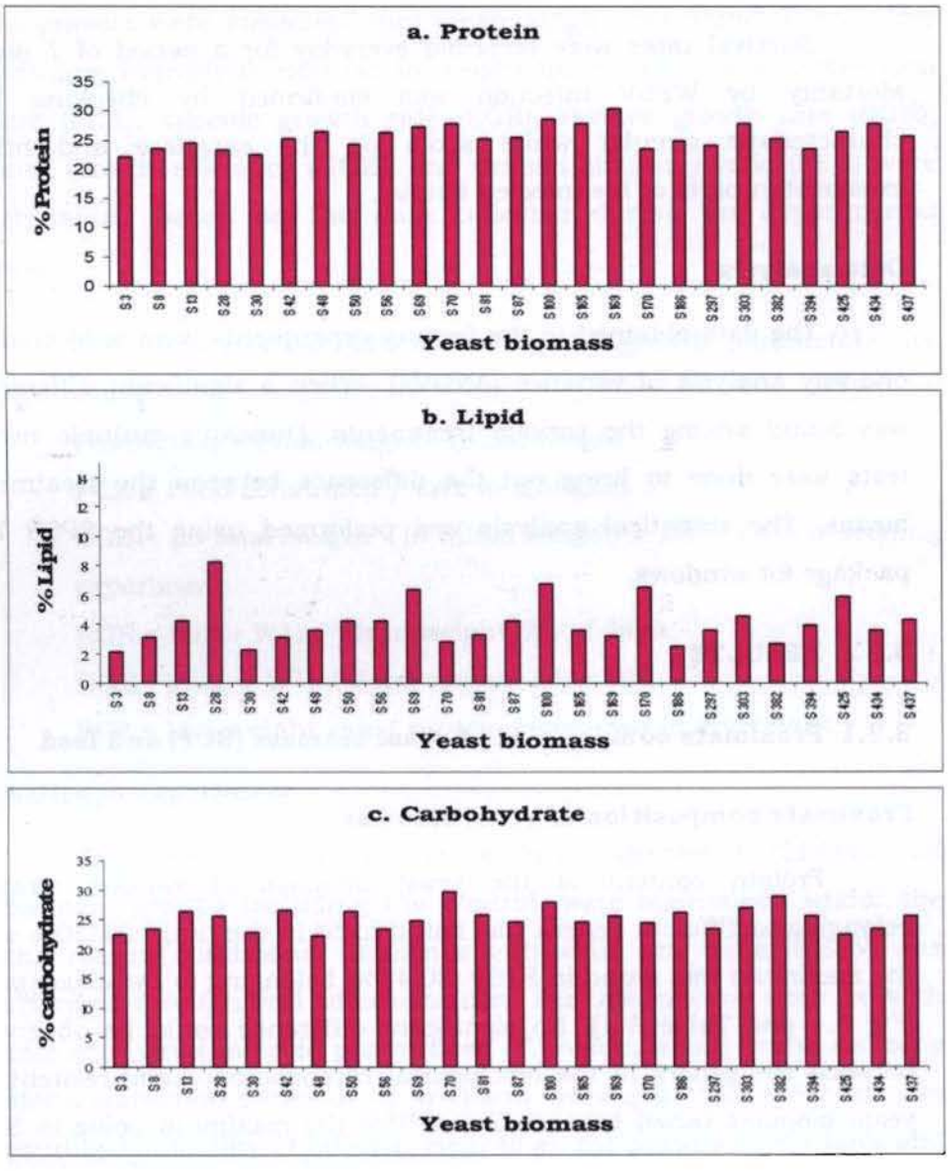


Fig.3.1. Proximate composition of yeast biomass

Proximate composition of feeds

Protein content of the feeds ranged from 40.2 to 55.4% with the maximum in F165 (55.4%) followed by F303 (53.9%). Lipid was maximum in F69 and F165 (11.2%) followed by F28, F87 and F434 (10.8%). Nitrogen Free Extract was maximum in the control feed C (36.8%). No significant variation could be obtained in the fiber content of various feeds and the value ranged from 1.9 to 2.2%. Ash content was higher in F434 (7.7%) followed by F186 and F303 (7.5%). Moisture content of the feeds ranged from 3.2 to 9.6%. (Table 3.4)

Table 3.4. Proximate composition of the experimental feeds
(% on dry weight basis)

Feed	Proximate composition (%)					
	Protein	**Lipid**	**Fiber**	**Ash**	**Moisture**	**NFE***
Control	40.2	8.1	2.0	7.2	5.8	36.8
F8	44.3	8.8	2.1	6.7	6.6	31.5
F28	47.6	10.8	2.2	6.8	5.2	27.4
F30	53.2	10.1	2.0	6.8	5.9	22.0
F69	50.2	11.2	2.0	5.7	9.6	21.3
F81	48.9	9.4	2.0	7.1	8.9	23.7
F87	47.4	10.8	2.1	6.2	6.7	26.8
F100	51.9	10.3	2.0	6.3	4.1	25.4
F165	55.4	11.2	2.0	5.4	5.1	20.9
F169	49.1	10.3	1.9	5.8	3.2	29.7
F170	48.4	10.2	2.1	6.9	5.2	27.2
F186	48.1	8.3	2.0	7.5	4.6	29.6
F303	53.9	10.0	2.0	7.5	5.9	20.7
F382	51.6	7.0	2.0	6.9	3.3	29.2
F434	49.8	10.8	2.0	7.7	5.9	23.7

* NFE - nitrogen free extract

3.3.2. Feeding Experiment

The data collected from the experiments were analyzed and the biogrowth parameters like production, food conversion ratio (FCR), specific growth rate (SGR), relative growth rate (RGR), gross growth efficiency (GGE), protein efficiency ratio (PER) were determined and are presented in Fig.3.2 a-f.

All the yeast biomass incorporated feeds supported better biogrowth parameters compared to the control feed. Performance of F8, F87, F165, F170, F186 and F303 was notable.

The experimental feed (F8 and F186) incorporated with the biomass of F8 and F186 gave the best performance in terms of biogrowth parameters followed by F165 and F100 (Table 3.5).

The highest production was recorded in prawns fed with feed F 165 (115.93 mg) followed by F186 (103.48mg) and the lowest was recorded for control feed (32.70mg) (Fig. 3.2a). Food conversion ratio (FCR) was found to be the best with feed F8 (0.61), followed by F186 (0.63) and F100 (0.68) (Fig.3.2b).

Specific growth rate (SGR) was found to be maximum for F186 (9.82) followed by F165 (8.59) and the lowest value recorded for control feed (3.24) (Fig.3.2c)

Gross growth efficiency (GGE) was found to be maximum in F8 (165.23) followed by F186 (158.52) and the lowest value was recorded for F30 (68.74) (Fig.3.2d). Relative growth rate (RGR) was highest for F186 (0.058) followed by F165 (0.057) and the lowest value for F30 (0.022) (Fig.3.2e).

Feed	*PRO (mg)
F8	93.98±0.18^{fg}
F28	47.25±1.23^{bc}
F30	39.43±2.32^{ab}
F69	78.04±2.67^{c}
F81	56.4±3^{cd}
F87	91.18±2.75^{fg}
F100	85.08±2.93^{ef}
F165	115.92±2.82^{g}
F169	73.03±2.63^{e}
F170	90.743±2.68^{fg}
F186	103.47±2.57^{g}
F303	103.09±2.37^{g}
F382	52.89±2.71^{cd}
F434	64.53±0.71^{d}
Control	32.69±2.43^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

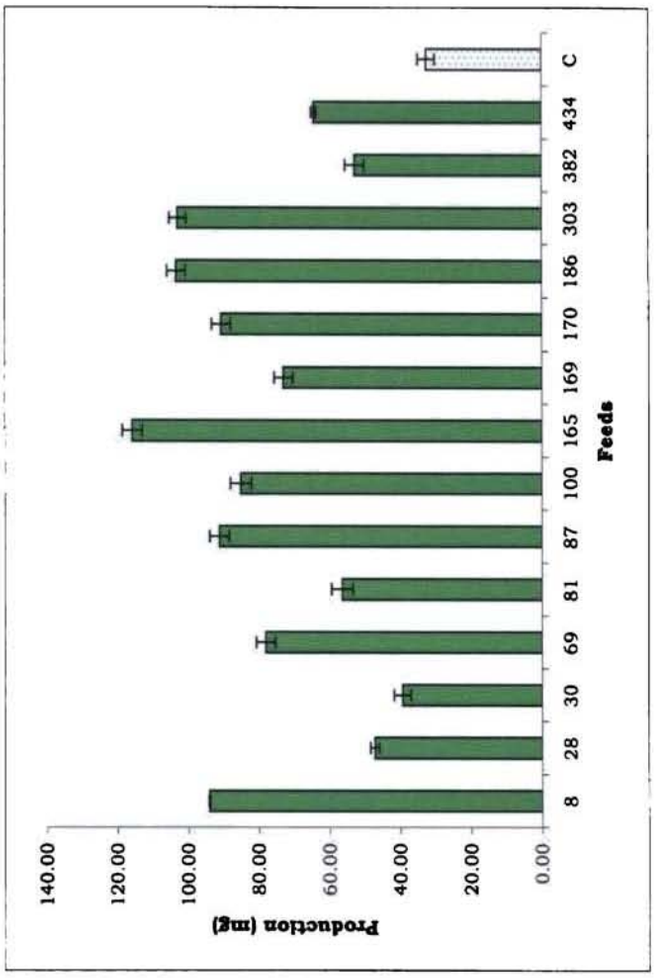
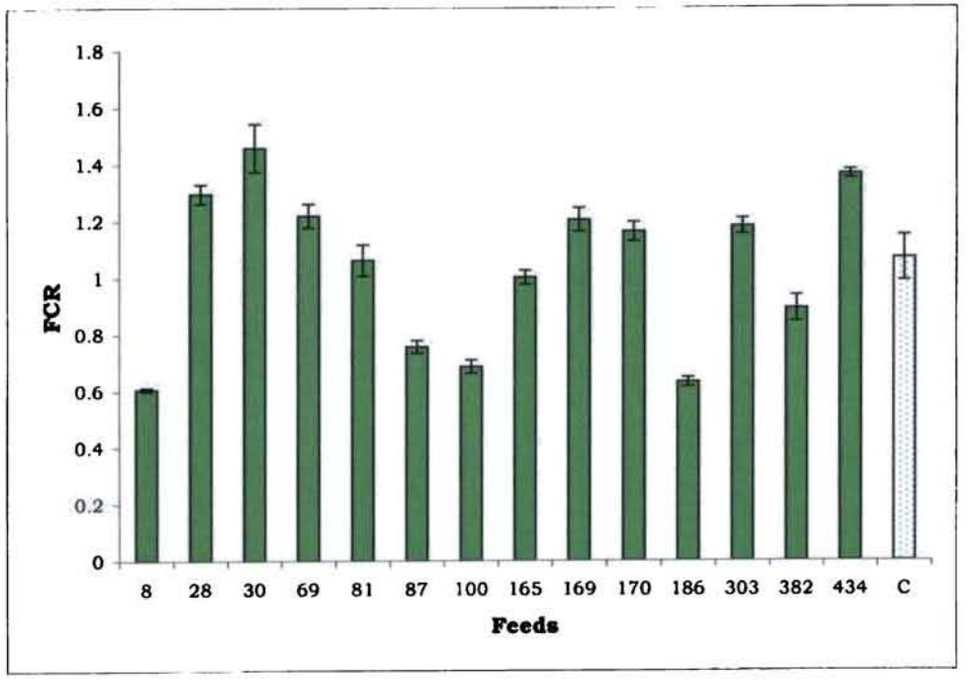


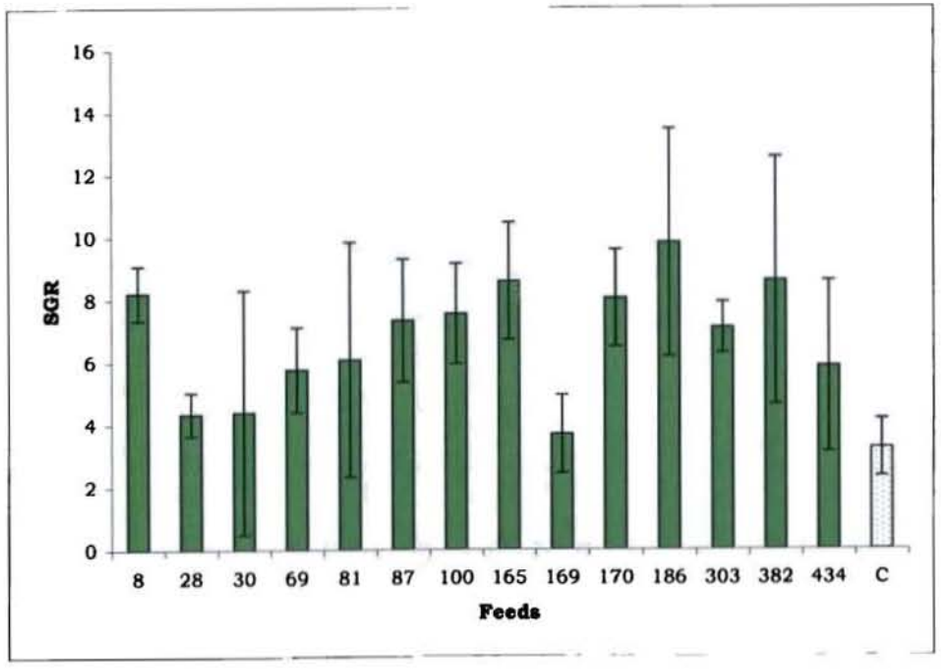
Fig.3.2a. Weight gain (Production) obtained in F.indicus post larvae when fed with various experimental feeds.



Feed	*FCR
F8	0.607±0.0056[a]
F28	1.296±0.034[g]
F30	1.458±0.085[h]
F69	1.219±0.042[f]
F81	1.062±0.055[e]
F87	0.756±0.022[c]
F100	0.686±0.024[b]
F165	1.0±0.024[e]
F169	1.203±0.042[f]
F170	1.162±0.034[f]
F186	0.631±0.016[ab]
F303	1.181±0.027[f]
F382	0.891±0.046[d]
F434	1.365±0.015[g]
Control	1.068±0.080[e]

* (mean± s.d.)
* Values with the same superscript does not vary significantly

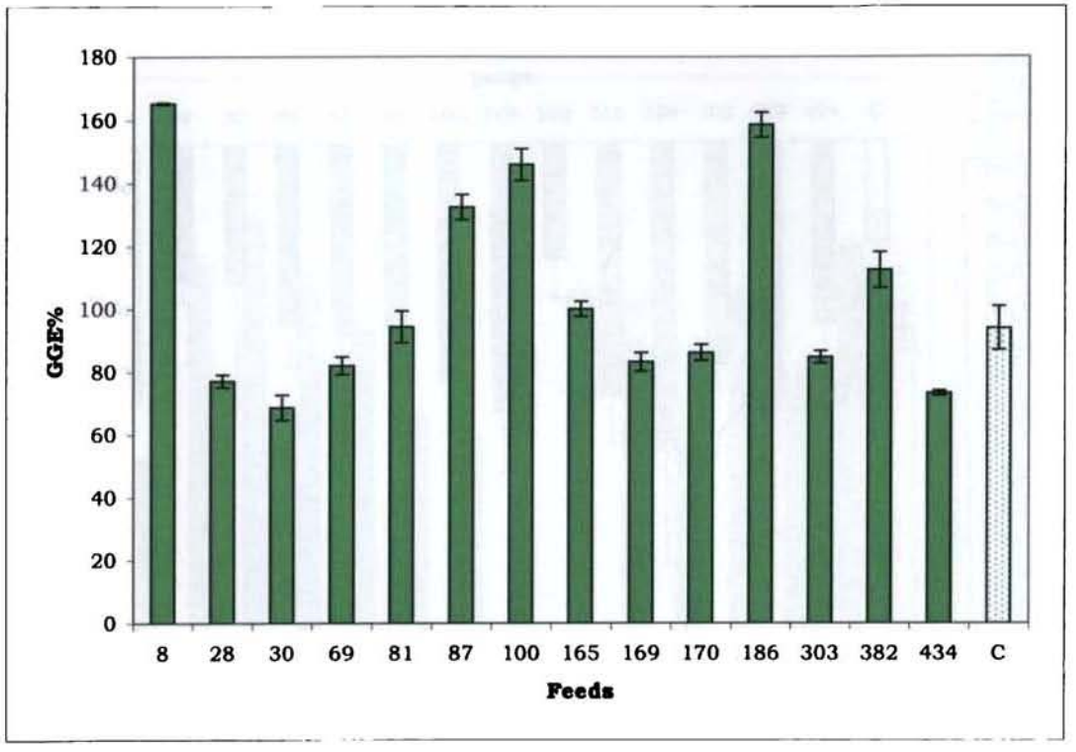
Fig.3.2b. Food Conversion ratio (FCR) obtained in F.indicus post larvae when fed with various experimental feeds.



Feed	*SGR
F8	8.21 ± 0.87^{bcd}
F28	4.33 ± 0.69^{abc}
F30	4.40 ± 3.90^{abc}
F69	5.76 ± 1.36^{abcd}
F81	6.08 ± 3.76^{abcd}
F87	7.34 ± 1.96^{abcd}
F100	7.55 ± 1.60^{abcd}
F165	8.59 ± 1.81^{cd}
F169	3.69 ± 1.25^{ab}
F170	8.04 ± 1.55^{bcd}
F186	9.81 ± 3.64^{d}
F303	$7.09\pm.81^{abcd}$
F382	8.59 ± 3.95^{cd}
F434	5.84 ± 2.73^{abcd}
Control	3.24 ± 0.91^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig.3.2c. Specific Growth Rate (SGR) obtained in F.indicus post larvae when fed with various experimental feeds.



Feed	*GGE
F8	165.23±0.31^{j}
F28	77.206±2.01^{bc}
F30	68.747±4.04^{a}
F69	82.113±2.81^{cd}
F81	94.378±5.03^{e}
F87	132.38±3.99^{g}
F100	145.88±5.03^{h}
F165	100.01±2.43^{e}
F169	83.14±2.99^{cd}
F170	86.127±2.54^{d}
F186	158.51±3.93^{i}
F303	84.725±1.94^{d}
F382	112.45±5.75^{f}
F434	73.271±0.81^{ab}
Control	93.956±6.99^{e}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig. 3.2d. Gross Growth Efficiency (GGE) obtained in F.indicus post larvae when fed with various experimental feeds.

Feed	*RGR
F8	0.06 ± 0.0^{cde}
F28	0.03 ± 0.01^{abcd}
F30	0.03 ± 0.03^{abc}
F69	0.04 ± 0.01^{abcde}
F81	0.04 ± 0.02^{abcde}
F87	0.05 ± 0.01^{bcde}
F100	0.05 ± 0.01^{bcde}
F165	0.06 ± 0.01^{de}
F169	0.03 ± 0.01^{ab}
F170	0.05 ± 0.01^{cde}
F186	0.06 ± 0.01^{e}
F303	0.05 ± 0.0^{bcde}
F382	0.05 ± 0.02^{cde}
F434	0.04 ± 0.02^{abcde}
Control	0.02 ± 0.01^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

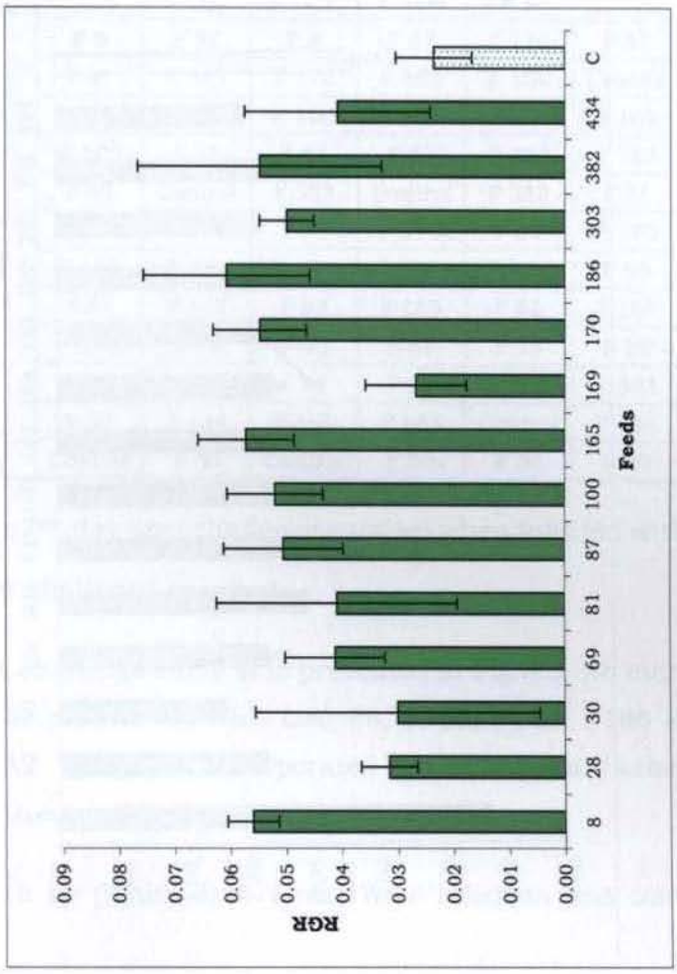
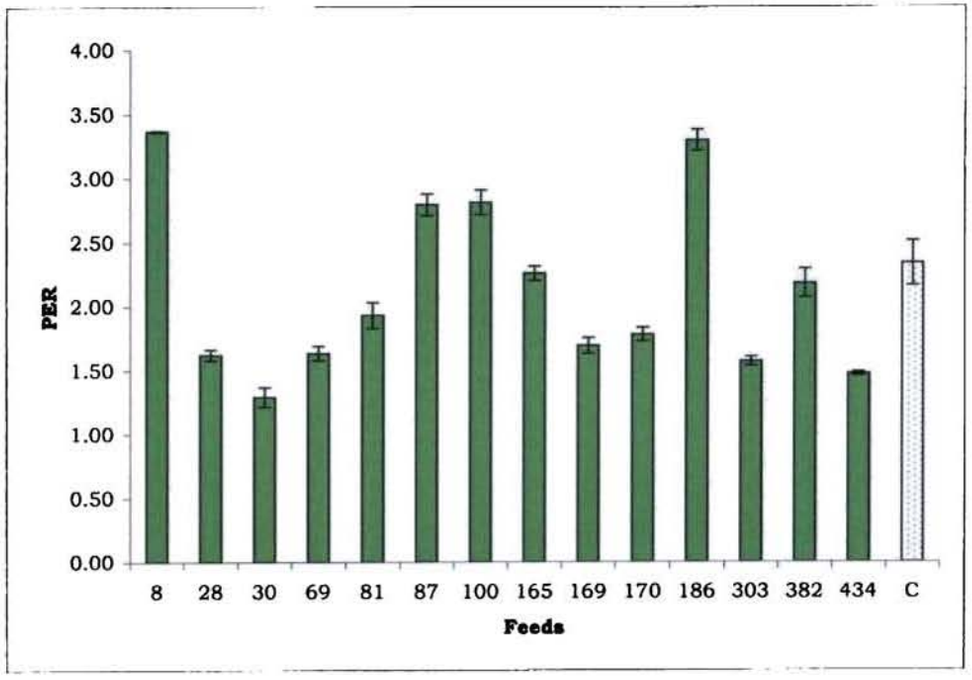


Fig.3.2e.Relative Growth Rate (RGR) obtained in F.indicus post larvae when fed with various experimental feeds.



Feed	*PER
F8	3.37±0.01^{i}
F28	1.62±0.04^{c}
F30	1.29±0.08^{a}
F69	1.64±0.06^{c}
F81	1.93±.10^{e}
F87	2.79±0.08^{h}
F100	2.81±0.10^{h}
F165	2.26±0.05^{fg}
F169	1.69±0.06^{cd}
F170	1.78±0.05^{d}
F186	3.30±0.08^{i}
F303	1.57±0.04^{bc}
F382	2.18±0.11^{f}
F434	1.47±0.02^{b}
Control	2.34±0.17^{g}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig. 3.2f. Protein Efficiency Ratio (PER) obtained in F.indicus post larvae when fed with various experimental feeds.

Protein efficiency ratio (PER) was found to be the best with F8 (3.37) followed by F186 (3.29) (Fig.3.2f).

Table. 3.5. Relative position of various feeds with respect to their performance in terms of bio-growth parameters and percentage survival in *F. indicus* post larvae maintained on experimental diets

Parameters	PRO	FCR	SGR	GGE	RGR	PER	*Survival
Experimental Feeds	F 165	**F 8**	**F 186**	**F 8**	**F 186**	**F 8**	**F 8**
	F 186	**F 186**	F 165	**F 186**	F 165	**F 186**	**F 100**
	F 303	**F 100**	F 382	**F 100**	**F 8**	**F 100**	F 169
	F 8	F 87	**F 8**	F 87	F 170	F 87	**F 186**
	F 87	F 382	F 170	F 382	**F 100**	Control	F 434
	F 170	F 165	**F 100**	F 165	F 87	F 165	F 165
	F 100	F 81	F 87	F 81	F 303	F 382	F 170
	F 69	Control	F 303	Control	F 382	F 81	F 69
	F 169	F 170	F 81	F 170	F 69	F 170	F 303
	F 434	F 303	F 434	F 303	F 434	F 69	F 382
	F 81	F 169	F 69	F 169	F 81	F 169	F 30
	F 382	F 69	F 30	F 69	F 28	F 28	F 87
	F 28	F 28	F 28	F 28	F 169	F 303	Control
	F 30	F 434	F 169	F 434	Control	F 434	F 81
	Control	F 30	Control	F 30	F 30	F 30	F 28

*Survival : 7th day post challenge survival when infected with WSV.

3.3.3 Post challenge survival

Post challenge survival is presented in Fig.3.3. No mortality was observed for prawns fed with feed F8, F165, F169, F186 and F434. (Fig.3.3). All the yeast incorporated feeds showed better survival compared to control feed.

Death by (White Spot Virus) WSV infection was confirmed by

the presence of white spots on the carapace of the infected prawns.

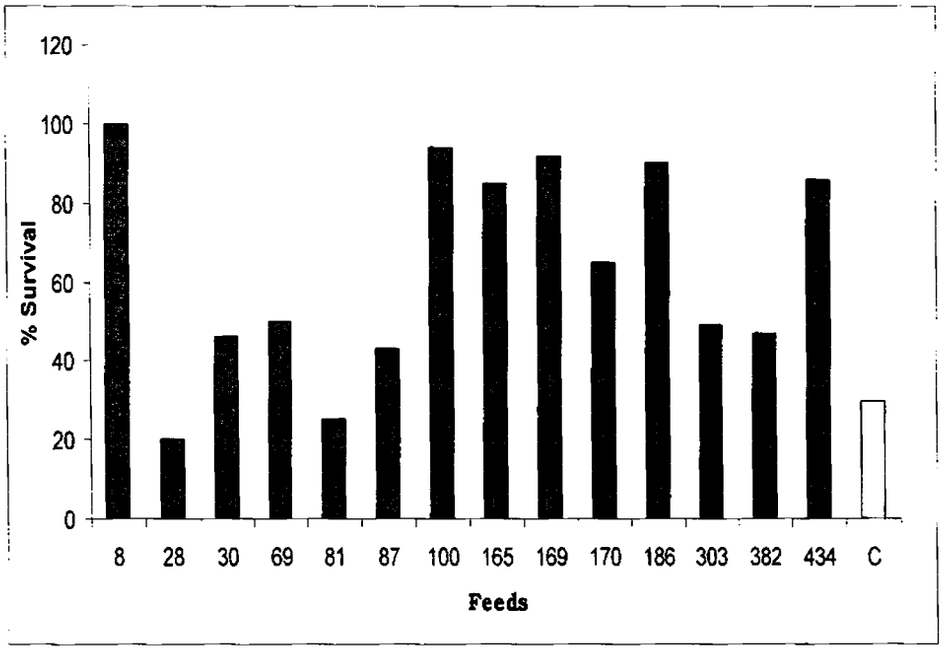


Fig.3.3. Percentage survival of F. indicus post larvae on seventh day post challenge with white spot virus.

3.3.4 Statistical analysis

Duncan's multiple range analysis of the various growth parameters effected by the different feeds showed that the performance of the yeast incorporated experimental feeds varied significantly from that of the control feed. Within the various yeast incorporated feeds itself significant variation could be observed. Generally the performance of F8, F186, F87 and F100 was found to be best compared to other feeds.

3.4 DISCUSSION

Protein content of yeast biomass of various genera was found to be in the range of 22-30% and the maximum was encountered in S169 (30.45%) belonging to *Debaryomyces*. Brown *et al.* (1996) noticed about 21% protein in *Debaryomyces hansenii*. Han *et al.* (1976) noticed 44.3% protein in *Candida utilis* and Brown *et al.* (1996) have recorded 42% in the same species. However, according to Kamel and Kawano (1986) *Candida* sp. contained only 34.9% protein. In this study *Candida* was found to contain lower protein content (25-28%) when compared to earlier studies. As per Brown *et al.* (1996) various yeast strains of *Dipodascus* sp. contained 25-30% protein which is in agreement with the value recorded (26.5%) for *Dipodascus* isolated in the present study. In *Torula* yeast 46% protein was recorded by Olivera Novoa *et al.* (2002) whereas in the present study only 28% could be noticed. *Saccharomyces* sp. was found to contain larger quantity of protein (48-83%) by Kamel and Kawano (1986) whereas Brown *et al.* (1996) noticed only 29% protein.

Lipid content of yeast biomass was found to be in the range 2 to 6.78% which is found to be almost similar with the earlier reports (Kamel and Kawano, 1986 and Brown *et al.* 1996) where a range of 1.05 to 7.7 was noted. However, Sanderson and Jolly (1994) have reported a very high content of fat (23%) in *Phaffia*.

As per the literature, carbohydrate content in yeasts ranged from 21 to 39%. In the present study the amount of carbohydrate varied from 22.36 to 29.68%. This high carbohydrate content make yeast SCP a good carbon source for animal feeds.

The protein content of the feeds ranged from 44-55%. This range was found acceptable for optimum growth in penaeid prawns as shown by various earlier workers. Dietary protein has been reported as the most essential nutrient for the growth of prawns (Andrews *et al.*, 1972; 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 basal diet (Ali, 1989) for optimal growth in shrimp. The protein quantity of a feed ingredient depends on several variables, digestibility and content of essential aminoacids, which are also crucial to biological value of the protein.

Juveniles or adult penaeids have been shown to attain optimum growth on diets containing 22-60% protein (Hanson and Goodwin, 1977). In the present study, a part of protein in the feed is contributed by yeast protein. The nutritional value of the microorganisms used in aquaculture depends on their digestibility and assimilation characteristics and the target animal. In the present study most of the yeast incorporated feeds supported better growth.

Yeast was examined as a replacement for fish meal in rainbow trout diets by Dabrowski *et al.* (1980). Yeast and bacteria have been evaluated as food for bivalve aquaculture. Protein is the major constituent of both yeast and bacteria (25 to 49% on dry weight basis).

In the present study, the quantity of lipid in the diet was not found to have much effect on the growth parameters. However, the influence of the qualitative composition of the lipids in the various yeasts cannot be ruled out. Microorganisms contain a diverse range of

fatty acid composition and are rich sources of useful unsaturated fatty acids like PUFA (Brown *et al.*, 1996). 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). Lipid content in the feeds ranged from 7 to 10.8. Qualitative composition was not estimated and therefore the role of lipids in the performance of the feeds cannot be explained. Among the lipid components in the diet 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 growth in *Penaeus monodon* larvae that were fed lipid enriched *Artemia nauplii.* A qualitative analysis of the lipids in yeast biomass is essential to comment on its role as nutritional parameters.

Various studies with *Penaeus japonicus* have demonstrated that dietary phospholipids enhance growth and survival of larvae (Teshima *et al.*, 1982; Kanazawa *et al.*, 1985) and growth and stress resistance in post larval/ juvenile stages (Sandifer and Joseph, 1976; Levin and Sulkin, 1984; Kanazawa *et al.*, 1979a, b; Camara *et al.*, 1997; Kontara *et al.*, 1977). Watanabe *et al.* (1974) have reported that yellowtail fed diets with alternative protein sources replacing fish meal, 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 *e t al.* (1998).

Deshimaru and Yone (1978) and Abdul 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.* Manomaitis (2001) determined that the crude protein requirement of newly released juveniles of red claw to be at least 40%. He also concluded that a diet of 30% should be utilized for 9 to 19 week red claw.

The nutritional value of brewer's yeast *S. cerevisiae* has been studied in lake trout (Rumsey *et al.*, 1990), rainbow trout (Rumsey *et al.*, 1991a and b) and sea bass (Oliva-Teles and Goncalves, 2001) by comparing growth performance, feed efficiency, liver uricase and nitrogen retention. Based on these studies it was found that brewer's yeast could replace upto 25-50% of fishmeal protein without adversely affecting growth of these species.

In the present study, the yeast incorporated feeds were found to be giving better performance in terms of various growth parameters in shrimps compared to the control feed. Yeast wise variation could be noted in the performance, which may be due to the variation in the protein, lipid, and carbohydrate and vitamin profile of the yeast biomass. Desired nutritional components in the required proportion are essential for better growth performance in prawns. This is evidenced by the prawns fed on various yeast incorporated feeds. Atack *et al.*, 1979 tested the utilization of SCP's in comparison with other protein sources for Mirror Carp, *Cyprinus carpio* and found bacteria supported highest growth followed by casein, herring meal,

petro yeast, soybean and algal diet. However, in their study, protein levels in the various feeds were below optimum requirements of carp and therefore higher growth could have been due to the higher protein content of bacterial diet. Numerous workers have shown that protein levels in the diet markedly effects growth (Ogino *et al.*, 1976; Matty and Smith, 1978).

Production (weight gain) was found to be more than twice for some of the yeast diets when compared to the control (free of yeast) and it was found to be significantly different for all the animals fed on yeast diets. The study showed that *Candida sake* S165, *Debaryomyces hansenii* S8, *Candida utilis* S186 and *Debaryomyces hansenii* S100 supported better growth in prawns compared to other strains. Atack and Matty (1978 and 1979) while evaluating Single Cell Proteins in the diet of Rainbow trout found that petro yeast and brewer's yeast differed in their efficacy as a feed supplement. Moo-Young *et al.* (1978) has reported that *Chaetomium cellulolyticum* is potentially more attractive feed supplement than yeast based products because of its higher content of sulphur amino acids and lower content of nucleic acids.

The study shows that marine yeasts can serve as potential feed supplements in aquaculture. However, a preliminary screening of the yeasts before application is essential since they vary in their nutritional value and to avoid toxic/pathogenic effects, if any.

Chapter 4

OPTIMIZATION OF THE PHYSICO-CHEMICAL PARAMETERS OF THE SELECTED MARINE YEASTS

4.1 INTRODUCTION

Growth of aquatic microorganisms is influenced by various physico-chemical factors such as incubation temperature, pH and salt concentrations, which 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 (Rheinheimer, 1980).

The batch fermentation kinetics of a novel thermotolerant strain of yeast *Kluveromyces marxianus* was evaluated between 30°C and 48°C. The most significant effects of elevated temperature were reductions in overall biomass and ethanol yields (Hughes *et al.*, 1984). Low temperatures, such as 8°C is close to the minimum temperature for growth in *Debaryomyces hansenii* and *Saccharomyces cerevisiae* (Sa-Correia and van Uden, 1983; Prista and Madeira- Lopes, 1955). For the determination of the optimum growth conditions of *Candida* sp., the influence of temperature, pH and salinity were determined by Kamel and Kawano (1986). Maximum growth was obtained at 37°C and the growth declined with the increase or decrease in temperature. Yeasts usually have optimum growth temperature from 25-30°C. Optimum growth was reached at pH values of 4 and 5. No significant difference in growth was found when salinity varied from 0–40 ‰.

An incubation temperature of 20-25°C is often used for the growth of mesophilic yeasts (Deak *et al.*, 1988; Hocking, 1996; Yarrow, 1998). The combined effects of temperature, pH and NaCl on growth of *Debaryomyces hansenii* was analyzed by flow cytometry and predictive microbiology by Sorensen and Jakobsen (1997). Growth was determined by flow cytometry at different combinations of

temperatures (10-30°C), pH (4.7-6.0) and NaCl concentrations (1-12% w/v). Growth occurred at all environmental conditions. The lag phase was not affected by changing temperatures in the range 20-30°C, and it decreases when temperature decreased in the range 10-20°C. An increase in temperature in the range 10-25°C increased the maximum specific growth rate with optimal growth in the range 25-30°C. At high salt concentrations the lag phase of the organism was only markedly prolonged at lower temperatures. Increasing salt concentrations decreased the maximum specific growth rate, the effect being less pronounced at low temperature. Changes in pH (4.7-6) had little effect on growth of *Debaryomyces hansenii.*

The effects of temperature (8 and 22°C), pH (2.5-6.0) and NaCl concentration (0.4-8% w/v) on the growth of 13 strains of yeasts representing five genera: (*Debaryomyces, Pichia, Zygosaccharomyces, Candida* and *Saccharomyces)* were studied. At 22°C, twelve of the 13 yeast species tested were able to grow at 8% NaCl, and all 13 species were able to grow at pH 2.5. The NaCl concentration permitting growth at this pH ranged from 0.8% (*Debaryomyces* sp.) to 8% (eight species). From this it was evident that there was a synergistic effect between NaCl and pH at the lower temperature (Betts *et al.*, 1999).

The effect of Na+ and K+ on growth and thermal death of *Debaryomyces hansenii* and *Saccharomyces cerevisiae* were compared under stress conditions and the results showed that at the supraoptimal temperature of 34°C, both the cations stimulated growth in *Debaryomyces hansenii,* while K^+ had no effect and the Na^+ inhibited growth of *S. cerevisiae* (Almagro *et al.*, 2000).

PH

The effects of temperature, pH and xylose concentration on the fermentation parameters of *Candida shehatae* and *Pichia stipitis* were evaluated by du Preez *et al.* (1985). The optimum pH was in the region of pH 4 - 5.5, with an optimum fermentation temperature of 30°C. The prevalence of *Kluyveromyces apiculata* in the acidic environments of fruits is well known (Recca and Mrak, 1952., Spencer *et al.*, 1992). Growth of *Zygosaccharomyces bailii* at pH 2.5-3.0 has been reported by Thomas and Davenport (1985), Cole and Keenan (1986) and Warth (1986a and b). Yeasts are known to acidify their growth media, largely as a result of the activity of the plasma-membrane H±ATPase (Vallejo and Serrano 1989).

Environmental pH is particularly significant in determining the growth of yeast (Pitt, 1974, Cole and Keenan, 1986) and it may also affect their responses to high concentrations of salt or sugar (Tokuoka, 1993) although such interactions have not been examined in depth. It is generally reported that most yeasts prefer a pH range, 3.0-7.0 (Walker, 1977., Miller, 1979., Deak, 1991).

NaCl

English (1954) showed that *Zygosaccharomyces rouxii* grew in 46% glucose in a pH range 2.0-8.7. Onishi (1957b) found that growth of osmophilic yeasts in NaCl free medium occurred over the range pH 3.0-7.0, whereas in the presence of 18% NaCl, growth was limited to the pH range 4.0-5.0. Smittle (1977) reported that increasing concentrations of salt shifted the pH optimum for growth of *Zygosaccharomyces bailii* and *Z. acidifaciens* to lower values. For most yeasts, tolerance of NaCl is decreased at the extremes of pH (Onishi,

1957a and b., Norkrans, 1996). The greater tolerance of *Debaryomyces hansenii* and *Saccharomyces cerevisiae* to NaCl at pH 5.0-7.0 compared with other pH values has been reported by Hobot and Jennings (1981). The mechanisms by which some yeast species tolerate high salt and high sugar (low water activity) environments have been the subject of considerable study (Onishi, 1963., Witter and Anderson, 1987., Tokuoka, 1993).

Growth of *D. hansenii, Zygosaccharomyces rouxii* and *Pichia anomala* at low water activities (a_w 0.89-0.91) produced by NaCl at concentrations of 15 or greater is well known (Onishi, 1963., Norkrans, 1996., Tokuoka and Ishitani, 1991., Tokuoka, 1993).

The growth of *Zygosaccharomyces rouxii* in the presence of 16% (w/v) NaCl, a condition which prevents contamination by the *Lactobacillus* sp., is significantly slower than that in the presence of 10% (w/v) NaCl (Tomita, 1976).

In food systems where *Z. bailii* grew at water activity a_w of 0.90 (16% NaCl) when the pH was 3.6 but only grew at a_w 0.95 (8% NaCl) when the pH was reduced to 3.2 (Meyer *et al.*, 1989). In *Debaryomyces hansenii* where growth was observed at 10% NaCl at pH 3-7, but only occurs at a maximum of 2.5% NaCl at pH 2.0 (Praphailong and Fleet, 1997). In food systems, *Zygosaccharomyces bailii* grew at water activity a_w 0.90 (16% NaCl) when the pH was 3.6 but only grew at a_w 0.95 (8% NaCl) when the pH was reduced to 3.2 (Meyer *et al.*, 1989).

Hamada *et al.* (1991) reported the continuous production of *Zygosaccharomyces rouxii* cells using a chemostat culture supplemented with 10% (w/v) NaCl to reduce microbial

contamination, but cells grown in such a glucose-limited culture displayed significantly lower fermentative activity than those cultivated in batch culture. *Debaryomyces hansenii* grows in the presence of high concentrations of sodium chloride and it has been reported that some strains are able to grow in the presence of 20% (w/v) NaCl. (Seiler and Busse, 1990., Besancon *et al.*, 1992) and *Zygosaccharomyces rouxii* is recognized for its tolerance of high concentrations of sugars (Tokuoka, 1993).

Debaryomyces hansenii is very salt tolerant and it has been reported that some strains are able to grow in the presence of 20% (w/v) NaCl (Besancon *et al.*, 1992) and *Zygosaccharomyces rouxii* is recognized for its tolerance of high concentrations of sugars (Tokuoka, 1993). *Debaryomyces hansenii*, and its anamorph *Candida formata*, are cryotolerant marine yeasts which can tolerate salinity levels upto 24%, whereas *Saccharomyces cerevisiae* growth is inhibited when salinity reaches 10%. In recent years considerable effort has been put in the study and understanding of the mechanisms mediating salt tolerance in cell-walled eukaryotic organisms (Haro *et al.*, 1993). *Saccharomyces cerevisiae*, moderately tolerant yeast, has been used as a model in these studies.

Baker's yeast cells grown in the presence of 3% NaCl were reported to have a high leavening ability in dough without NaCl (Oda and Tonomura, 1993). Although the reason for the high leavening ability is still not well understood, the physiological state of the cells grown in the presence of NaCl is probably different from that of cells grown in the absence of NaCl. Watson (1970) suggested that *S. cerevisiae* cells exposed to high NaCl concentrations need large

amounts of ATP yielding substrates (glucose) to maintain the intracellular NaCl concentrations lower than the extracellular concentration.

According to Guerzoni *et al.* (1993), *Yarrowia lipolytica* can grow at a low a_w of 0.89 (10% NaCl + 10% Sucrose). The growth of *Saccharomyces cerevisiae* and *Kluveromyces marxianus* at concentrations up to 7.5% NaCl and 10% NaCl. (Hobot and Jennings, 1981., Besancon *et al.*, 1992., Vivier *et al.*, 1993, 1994). *D. hansenii* is a spoilage yeast usually found contaminating brine food and low water activity products. This is isolated from salty water and solar networks and is considered a halotolerant yeast (Kreger-van Rij, 1984., Tokuoka, 1993). Lipid saturation is important for salt tolerance in *Zygosaccharomyces rouxii* (Yoshikawa *et al.*, 1995), while adaptation to low temperature results in an increase in the degree of lipid unsaturation in several yeast species (Arthur and Watson, 1976). Norkrans (1996) reported that *Debaryomyces hansenii* isolated from sea water and solar salt works was characterized by a high resistance to salt. The halophilic yeast *Zygosaccharomyces rouxii* is a well known species that is tolerant towards highly concentrated salt and sugars (Ushio *et al.*, 1996). Foods which contain high levels of salt and sugar will select for yeasts capable of growth under low water activity, such as *Debaryomyces hansenii* in the case of high salt (Pitt and Hocking, 1997) and *Zygosaccharomyces rouxii* in the case of high sugar (Board, 1994).

Studies conducted by Praphailong and Fleet (1997) showed that *S. cerevisiae* did not grow at salt levels higher than 7.5% within a 14 day storage period. In *D. hansenii* where growth was observed at 10%

NaCl at pH 3-7, but only occurred at a maximum of 2.5% NaCl at pH 2.0. (Praphailong and Fleet, 1997).

Increasing the salt concentration results in increased lag phase. For example, the lag time for *Debaryomyces hansenii* in YM medium was 16.4 hr at a salt level of 1% (w/v) when the temperature was 10°C and the pH was 5.3 (Sorenson and Jakobsen, 1997).

Praphailong and Fleet (1997) found the maximum salt level (w/v) allowing growth of *Zygosaccharomyces bailii* within 7-14 days, to be 50% at pH 5.0 whereas Betts *et al.* (1998) found, growth of *Zygosaccharomyces bailii* in 8% (w/v) salt at pH 5.0 but after 21 days.

Debaryomyces (Torulaspora) hansenii, and its anamorph *Candida formata*, is a cryotolerant, marine yeast, which can tolerate salinity levels up to 24%, whereas *Saccharomyces cerevisiae* growth is inhibited when salinity reaches 10% (Lepingle *et al.*, 2000).

The pH, temperature and inoculum ratio for the production of β-galactosidase by *Kluyveromyces marxianus* CDB 002 were optimized using sugar-cane molasses (100g/l) in a lactose free medium. The culture medium used for the study of the pH and the temperature was composed of yeast extract (10g/l), peptone (20g/l) and sugar cane molasses (100g/l). Maximum cell growth was observed at 28 to 30°C and decreased as the temperature increased. Little influence of the pH was noted on cell growth, production of ethanol and rate of substrate consumption (Furlan *et al.*, 2001).

Effects of pH, temperature and salt concentration on growth of *Zygosaccharomyces rouxii* and formation of fusel alcohols from

branched-chain aminoacids were studied by Jansen *et al.* (2003). Results showed that maximum fusel alcohol production occurred at low pH (3.0-4.0) and low NaCl concentrations (0 - 4%) at 25°C. The highest cell densities were obtained in plates incubated at 30°C.

Nutritional factors in medium formulation

Nutritional ingredients in a fermentation medium are indispensable for growth of microorganisms. Fermentation media are composed of carbon sources, inorganic metal salts and buffering agents such as $CaCO_3$ and special growth factors. Complex nutrients are preferred as they often support higher yields and for economic reasons, chemically defined media are rarely used in fermentation media. The major carbon/nitrogen source of fermentation media are soybean meal, molasses, corn steep liquor, sulphite waste liquor, cotton seed meal, yeast extract, peptone etc. Calcium chloride, ammonium phosphate, phosphates of potassium etc. can be incorporated for enhanced production of growth. Of all the factors used, carbon and nitrogen sources are of particular importance in the medium since microbial cells are composed largely of these elements.

Molasses as substrate

The modern history of SCP began in late 1950 when petroleum industry began experiments on the use of microorganisms for removal of paraffin wax and sulphur containing fractions from crude oil. In 1964, the British Petroleum Company announced success in cultivating a yeast *Candida lipolytica* on broad petroleum fractions such as gas oils and slack waxes with the simultaneous removal of paraffin component from crude fractions. It was found that these

organisms contained over 50% of high quality protein and so what started as a by-product became the primary objective of research to produce SCP. In addition, they are considered a cheap dietary supplement as they are easily produced on an industrial level from a number of carbon rich substrates such as citrus pulp, molasses, paper industry wastes, fruit wastes and hydrocarbons. Nutritional value of yeasts varies according to origin, and many researchers have indicated that yeast grown in alkanes could be of better quality than that produced on carbohydrates. The use of yeasts also highlights the profitability of using beer and liquor industry by-products in animal nutrition.

Beet and Cane molasses are by-products of the sugar industry. These molasses are the concentrated syrups or mother liquors recovered at any one of several steps in the sugar-refining process, and different names are applied to the molasses depending on the particular step from which it was recovered. Of these, blackstrap molasses prepared from sugar cane normally is the cheapest and the most used sugar source for industrial fermentations. When molasses is used as a fermentation medium component, it contains 50% fermentable sugars.

Beet molasses are produced by procedures resembling those for sugar cane. However, beet molasses may be limiting in biotin for yeast growth so that a small amount of cane blackstrap or other source of biotin should be added for growth of these microorganisms. "Hydrol" is a molasses resulting from the manufacture of crystalline dextrose from corn starch. It contains approximately 60% sugar, but it also contains

a relatively high salt concentration that must be considered if this molasses is to be used as a medium component.

Carbon sources for fermentation media can be simple or complex carbohydrates, sugar alcohols or other alcohols, organic acids, proteins, peptides, aminoacids and even hydrocarbons. These carbon sources are usually used in a crude form, although semi-purified sugars, sugar alcohols, polysaccharides, or hydrocarbons may be required for specific fermentations. Crude source of simple sugars include beet and cane molasses, corn molasses or "hydrol", whey, sulfite waste liquor, cull fruits, cannery wastes and so forth. Polysaccharides such as starches are supplied by corn, wheat, rye, milo, rice, potatoes, sweet potatoes and other agricultural products. Cellulosic byproducts also are usable as carbon sources, but they usually require costly saccharification by a procedure such as acid hydrolysis. Hydrocarbon substrates are usually mixture of various hydrocarbon components and are relatively inexpensive. The pure hydrocarbon compounds or hydrocarbon fractions, however, are more costly.

Several of the better crude nutritive sources for fermentation media are in themselves complex mixtures of nutrients, supplying carbon and nitrogen compounds as well as microbial growth factors. Specific examples are molasses, corn steep liquor and sulfite waste liquor. The overall composition of the various molasses differs according to the specific geographic areas of production.

Cane molasses is the final run-off syrup from sugar manufacture. Total residual sugars in molasses can amount to 50-60% (w/v), of which 60% is sucrose. In addition to sucrose there are

both growth promoting components (Jones *et al.*, 1981) and inhibitors, e.g. hydroxymethyl furfural (Glacet *et al.*, 1985).

An attempt was made to improve the salt tolerance of the thermotolerant flocculating yeast, *Saccharomyces cerevisiae* strain by maintaining a high concentration of KCl in the medium (Morimura *et al.*, 1997). Among the selected strains adaptive mutant K 211 had the highest cell viability and ethanol productivity in a molasses medium containing 25% (w/v) total sugar at 35°C.

The optimal medium for pullulan production by *Rhodotorula bacarum* was found to be 8.0% glucose, 2.0% soybean cake hydrolysate, 0.5% $MgSO_4.7H_2O$, 0.06% $(NH_4)_2SO_4$, pH 7.0 (Chi and Zhao, 2003).

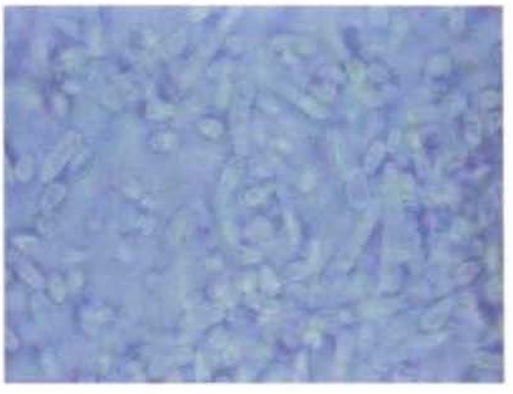
Maximum production of cephalosporin C by *Acremonium chrysogenum* was achieved by employing wheat rawa with 1% (w/w) soluble starch, 1% (w/w) yeast extract at 30°C. (Adinarayana *et al.*, 2003).

4.2. MATERIALS AND METHODS

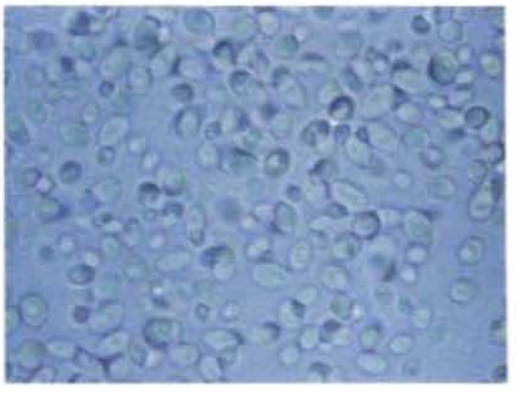
4.2.1 Yeasts used for the study

Four yeast cultures were selected for the study based on their performance in the feeding experiment with *Fenneropenaeus indicus* (Fig.4.1 and Fig. 4.2 a-d).

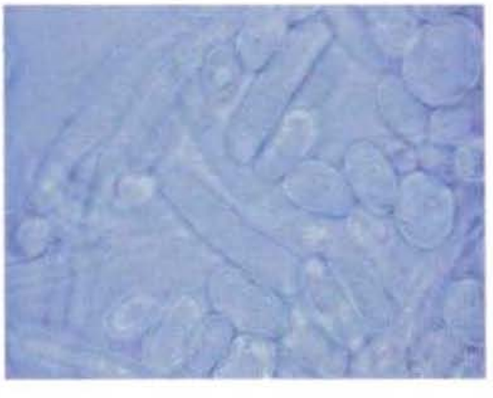
1. *Debaryomyces hansenii* (S8)
2. *Debaryomyces hansenii* (S100)
3. *Candida sake* (S165)
4. *Candida tropicalis* (S186)



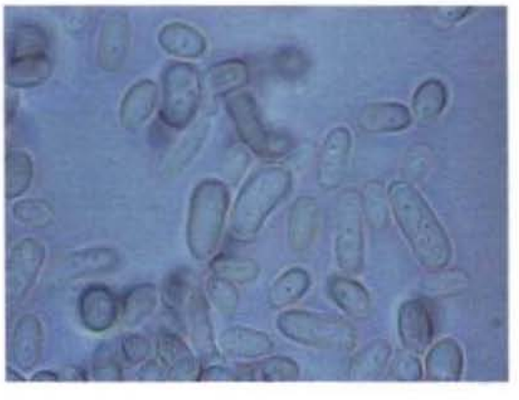
S8 Debaryomyces hansenii



S100 Debaryomyces hansenii



S165 Candida sake



S186 Candida tropicalis

Fig. 4.1 Selected marine yeasts used for the study 400X

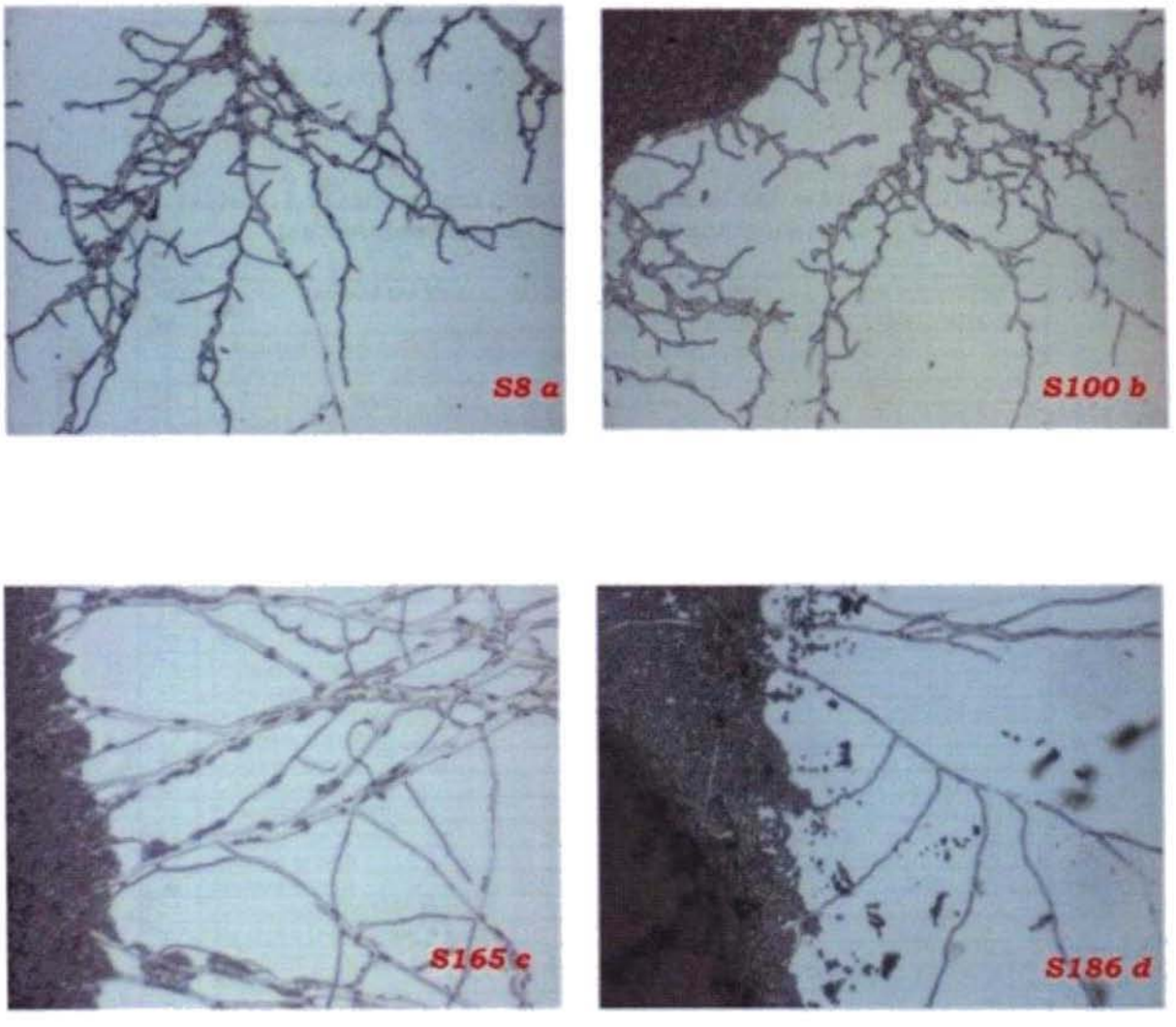


Fig.4.2 a-d. Slide cultures of the selected marine yeasts used for the study 10X

Taxonomical characteristics of the selected strains (as obtained from IMTECH Chandigarh, India) are presented in Table.4.1.

Table.4.1.Taxonomical characteristics of the selected strains (as obtained from IMTECH Chandigarh, India)

Sl No.	Name of the Test	8	100	165	186
		MTCC 4361	MTCC 4363	MTCC 4364	MTCC 4366
1	D-Glucose fermentation	+	+	+	+
2	D-Galactose fermentation	-	-	+	?
3	Maltose fermentation	-	-	+	+
4	Me alpha-D-glucoside fermentation	-	-	-	-
5	Sucrose fermentation	+	+	+	+
6	Alpha, alpha-Trehalose fermentation	-	-	-	?
7	Melibiose fermentation	-	-	-	-
8	Lactose fermentation	-	-	-	-
9	Cellobiose fermentation	-	-	-	-
10	Melezitose fermentation	-	-	-	-
11	Raffinose fermentation	-	-	-	-
12	Inulin fermentation	-	-	-	-
13	Starch fermentation	-	-	-	-
14	D-Galactose growth	+	+	+	+
15	L-Sorbose growth	+	?	-	-
16	D-Glucosamine growth	-	-	-	-
17	D-Ribose growth	?	?	?	-
18	D-Xylose growth	+	+	+	+
19	L-Arabinose growth	+	+	-	-
20	D-Arabinose growth	+	+	-	-
21	L-Rhamnose growth	+	+	-	-
22	Sucrose growth	+	+	+	+
23	Maltose growth	+	+	+	+
24	Alpha, alpha-Trehalose growth	+	+	+	+
25	Me alpha-D-glucoside growth	+	+	+	+
26	Cellobiose growth	+	+	+	+
27	Salicin growth	+	+	?	?
28	Arbutin growth	+	+	-	+
29	Melibiose growth	-	+	-	-
30	Lactose growth	-	-	-	-
31	Raffinose growth	+	+	-	?
32	Melezitose growth	+	+	+	+
33	Inulin growth	+	+	-	-
34	Stach growth	-	-	-	-
35	Glycerol growth	+	+	+	+
36	Erythritol growth	-	-	-	-
37	Ribitol growth	+	+	+	+
38	Xylitol growth	+	+	-	-
39	L-Arabinitol growth	+	+	-	-
40	D-Glucitol growth	+	+	+	+

(Cont.....d)

Table.4.1.Taxonomical characteristics of the selected strains(as obtained from IMTECH Chandigarh, India)

Sl No.	Name of the Test	8 MTCC 4361	100 MTCC 4363	165 MTCC 4364	186 MTCC 4366
41	D-Mannitol growth	+	+	+	+
42	Galactitol growth	-	+	-	-
43	Myo-inositol growth	-	-	-	-
44	D-Glucono-1,5-lactone growth	-	-	+	+
45	2-Keto-D-gluconate growth	+	+	+	+
46	5-Keto-D-gluconate growth	-	-	-	+
47	D-Gluconate growth	-	-	+	+
48	D-Glucuronate growth	-	-	-	-
49	DL-Lactate growth	-	-	?	-
50	Succinate growth	+	+	-	?
51	Citrate growth	+	?	-	-
52	Methanol growth	-	-	-	-
53	Ethanol growth	+	+	+	+
54	Growth at 25 deg C	+	+	+	+
55	Growth at 30 deg C	+	+	+	+
56	Growth at 35 deg C	?	?	?	?
57	Growth at 37 deg C	+	+	+	+
58	Growth at 42 deg C	?	+	-	+
59	0.01% Cycloheximide growth	-	-	-	-
60	0.1% Cycloheximide growth	-	-	-	-
61	50% D-Glucose growth	+	+	-	-
62	60% D-Glucose growth	?	-	-	-
63	Stach formation	?	?	?	?
64	Acetic acid production	-	-	-	-
65	Urea hydrolysis	-	-	-	-
66	Diazonium Blue B reaction	-	-	-	-
67	Pink colonies	-	-	-	-
68	Budding cells	+	+	-	+
69	Polar budding	?	?	?	?
70	Splitting cells	-	-	-	-
71	Filamentous	+	-	+	+
72	Pseudohypahe	+	-	-	-
73	Septate hyphae	-	-	+	+
74	Arthroconidia	-	-	-	-
75	Ballistoconidia	-	-	-	-
76	Symmetric ballistoconidia	-	-	-	-
77	Ascospores	-	-	-	?
78	Ascospores, round, conical, reniform	-	-	-	?
79	Ascospores cap, hat,saturn,walnut-shaped	-	-	-	?
80	Ascospores needle-shaped or whip like	-	-	-	?

4.2.2 Preparation of inoculum

The yeast isolates were streaked onto malt extract agar slants and after incubation for 3-4 days at room temperature (28±2°C), the cells were harvested using sterile physiological saline (0.9% NaCl in D.W). The optical density of the cell suspension was adjusted to 1 O.D (approximately $4X10^8$ cells/ml) and this cell suspension was used as inoculum. 1 ml was inoculated to 100 ml medium.

4.2.3 Measurement of growth

The yeast growth was estimated by measuring the optical density at 540nm in a Hitachi Model 200-20 UV Visible Spectrophotometer.

4.2.4 Selection of a suitable substrate as Carbon source for growth

Various substrates such as glucose, sucrose, rice water and molasses were tested as Carbon sources. Four different media (M_1, M_2, M_3 and M_4) were prepared incorporating these substrates.

	M_1			M_2	
Glucose	-	20g	Sucrose	-	20g
KNO_3	-	2g	KNO_3	-	2g
Sea water	-	1000ml	Sea water	-	1000ml
pH	-	5.5	pH	-	5.5

	M_3			M_4	
Rice water	-	100ml	Molasses	-	50ml
KNO_3	-	2g	KNO_3	-	2g
Sea water	-	900ml	Sea water	-	950ml
pH	-	5.5	pH	-	5.5

0.1ml aliquots of the inoculum was transferred into 10ml media. Incubated at room temperature (28±2°C), for 48 hours and the growth were measured by recording the optical density at 540 nm in a Hitachi Model 200-20 UV-Visible spectrophotometer.

Based on the results medium containing molasses (M_4) was selected for further study.

4.2.5. Optimization of the concentration of molasses in medium

Concentration of molasses in the medium was estimated and presented in terms of total sugars present. 50g of molasses was added into 100ml seawater. This solution was considered as the stock. Total sugars of the molasses solution were estimated by Anthrone Method (Roe, 1955). Then media with different concentrations of molasses (total sugars -1 to 10 mg/ml) were prepared. After sterilization, the selected marine yeasts were inoculated and they were incubated at room temperature (28±2°C), for 48 hours. Growth was estimated at 540nm.

4.2.6 Optimum peptone concentration

Molasses media (M_4) with different peptone concentration (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the

cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540 nm.

4.2.7 Optimum yeast extract concentration

Molasses media (M_4) with different yeast extract concentration (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540 nm.

4.2.8 Optimum Magnesium sulphate concentration

Molasses media (M_4) supplemented with different concentrations of magnesium sulphate (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540 nm.

4.2.9 Optimum Calcium chloride concentration

Molasses media with different calcium chloride concentrations (0, 0.25, 0.5, 0.75 and 1%) were prepared. Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540 nm.

4.2.10 Optimum Potassium dihydrogen phosphate concentration

Molasses media supplemented with different concentrations of potassium dihydrogen phosphate (0, 0.1, 0.2, 0.3 and 0.4%) were prepared. Inoculation was done and the cultures were incubated at

room temperature (28±2°C). After 48 hours growth was measured at 540 nm.

4.2.11 Effect of salinity

Molasses medium (M_4) was prepared in seawater of different strength (0, 10, 15, 20, 25, 30, 35 and 40ppt). Inoculation was done and the cultures were incubated at room temperature (28±2°C). After 48 hours growth was measured at 540 nm.

4.2.12 Effect of pH

Molasses media (M_4) with different pH (4,5,6,7,8,9 and 10) were prepared using various buffers as given below.

Buffers used	**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

All the four yeast strains were inoculated and incubation was done at room temperature (28±2°C) for 48 hours.

4.3 RESULTS

4.3.1 Selection of suitable C source for media preparation

Molasses was found to be the best carbon source supporting maximum growth followed by rice water and sucrose. All the strains tested preferred molasses as substrate. Maximum growth was

exhibited by *Debaryomyces hansenii* (S100) followed by *Candida sake* (S165). Growth was found to be almost double of that found in the other media. (Fig.4.3)

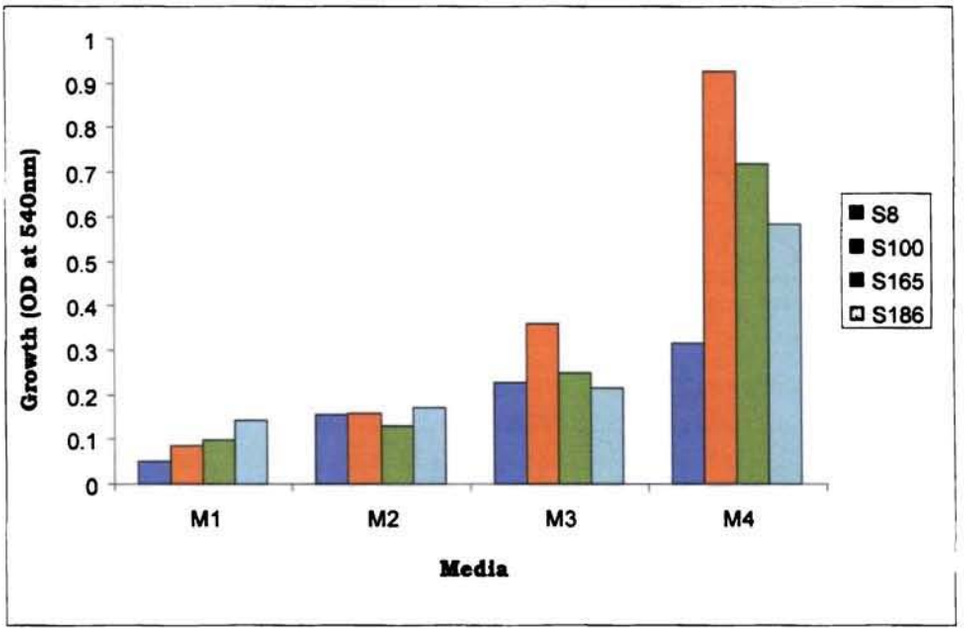


Fig.4.3 Growth of selected marine yeasts in media with different C sources

4.3.2 Effect of molasses concentration on growth

Molasses concentration is expressed as total sugars/ml in the medium. Growth of marine yeasts was found to be influenced by the concentration of molasses in the medium. Maximum growth was observed at a concentration of 9mg/ml for all the four strains tested. A gradual increase in growth could be observed with the increase of

molasses concentration. However, the presence of total sugars more than 9mg/ml was found to have adverse effect and resulted in lesser growth (Fig.4.4).

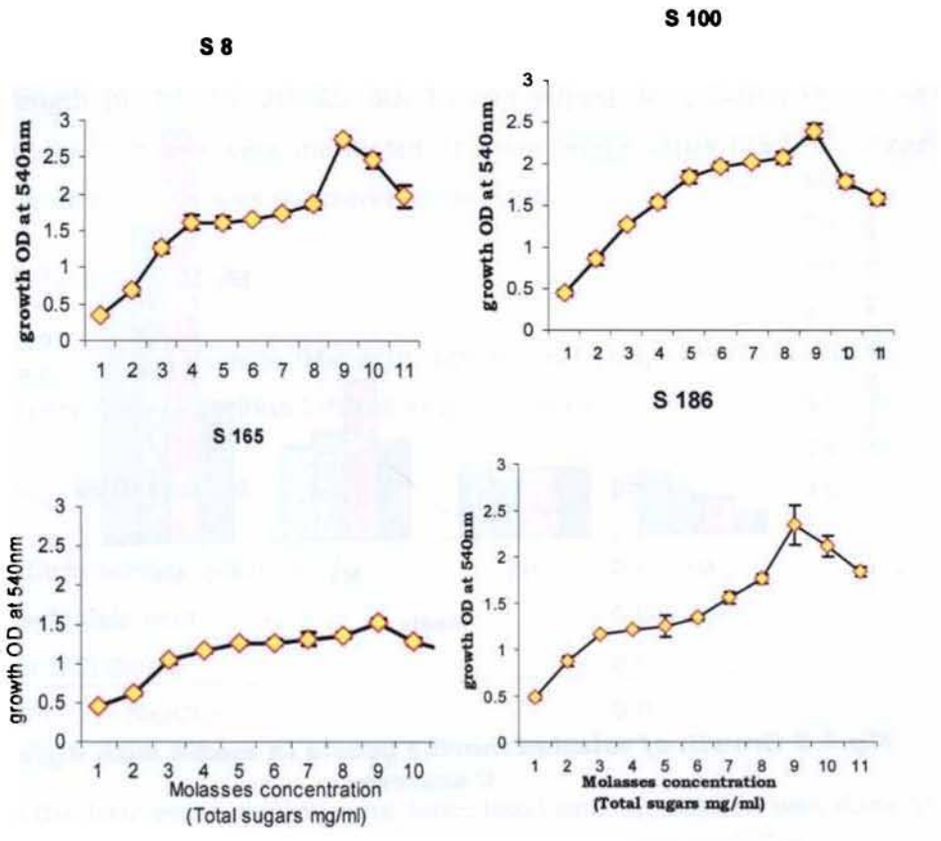


Fig. 4.4. Effect of molasses concentration in the media on the growth of selected yeasts

4.3.3 Effect of peptone concentration on growth

Increase in growth was not much pronounced in the presence of peptone and with the increase of peptone in the medium. However, a slight increase in growth could be observed with the increase in the

concentration of peptone showing a maximum at 0.75% for all the strains. Growth was found to be decreasing at higher concentrations of peptone (Fig. 4.5).

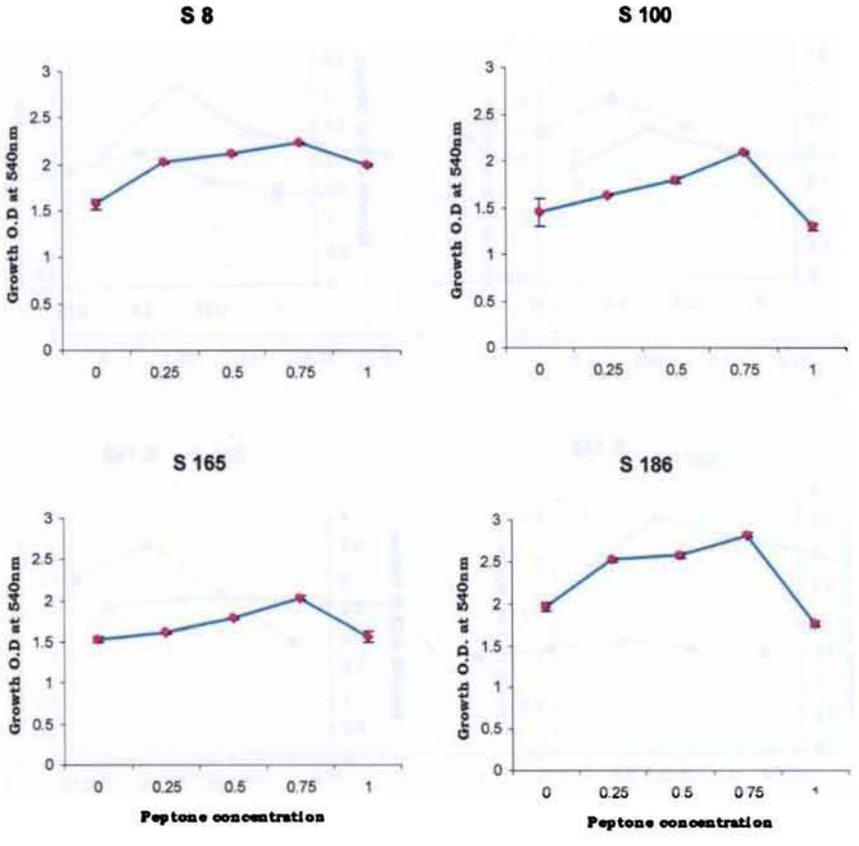


Fig.4.5. Growth of yeast strains at various peptone concentrations in the molasses medium

4.3.4 Effect of yeast extract concentration on growth

Considerable increase in growth could be observed when yeast extract was introduced into the medium. Growth was found to be maximum at a concentration of 0.5% yeast extract in the medium.

Growth was found to decrease with further increase in yeast extract concentration in the medium (Fig. 4.6).

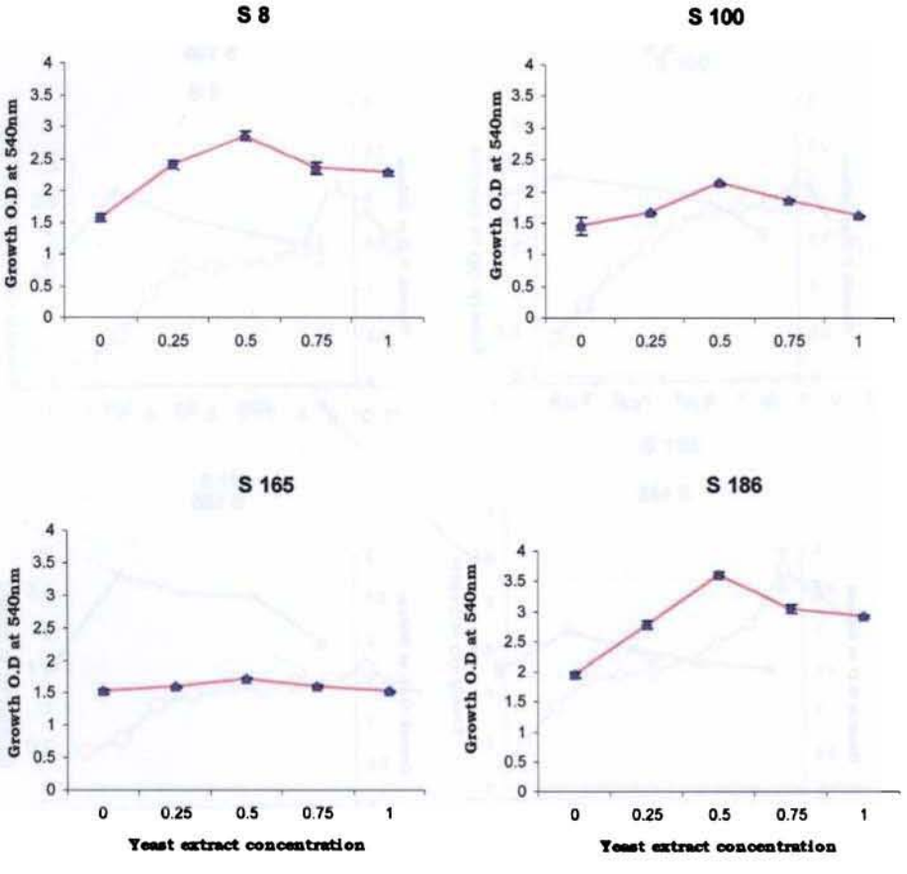


Fig.4.6. Growth of yeast strains at various yeast extract concentrations in the molasses medium

4.3.5 Effect of magnesium sulphate concentration on growth

Generally growth was found to be maximum at a concentration of 0.25% MgSO4 in the medium. There was a significant variation in

growth for S165 with the increase or decrease in MgSO4 concentration (Fig.4.7).

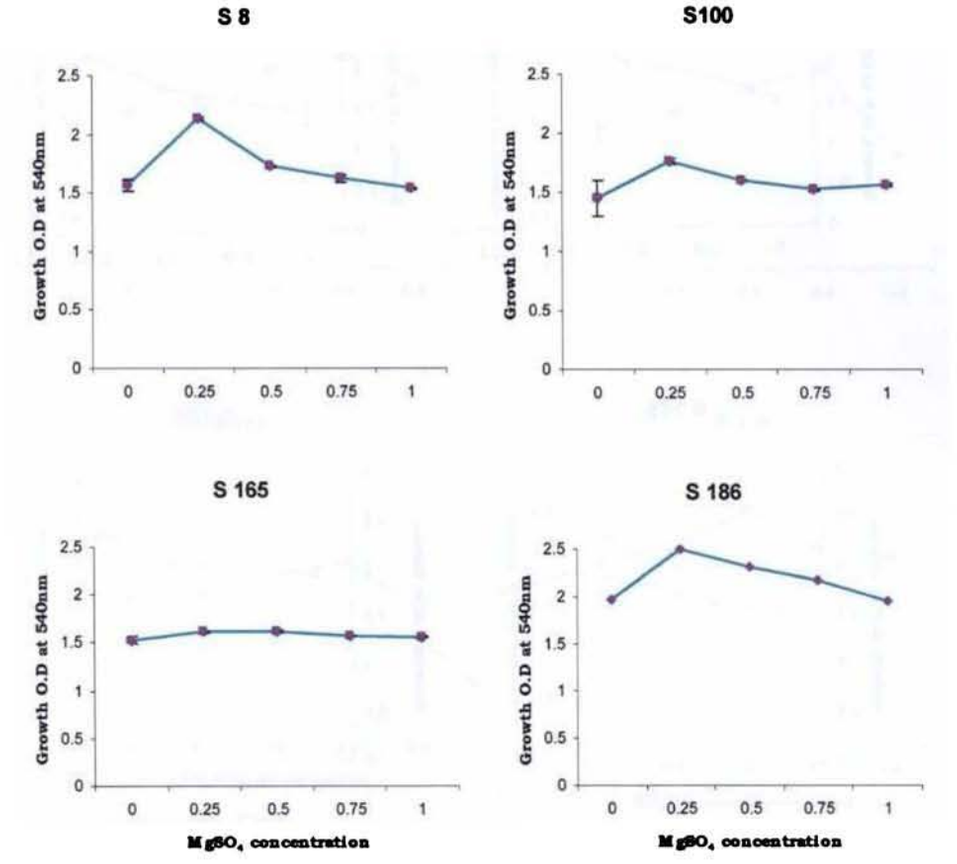


Fig.4.7 Growth of yeast strains at various magnesium sulphate concentration in the molasses medium

4.3.6 Effect of calcium chloride on growth

The optimal calcium chloride concentration for growth was found to be 0.15% for all the strains (Fig.4.8).

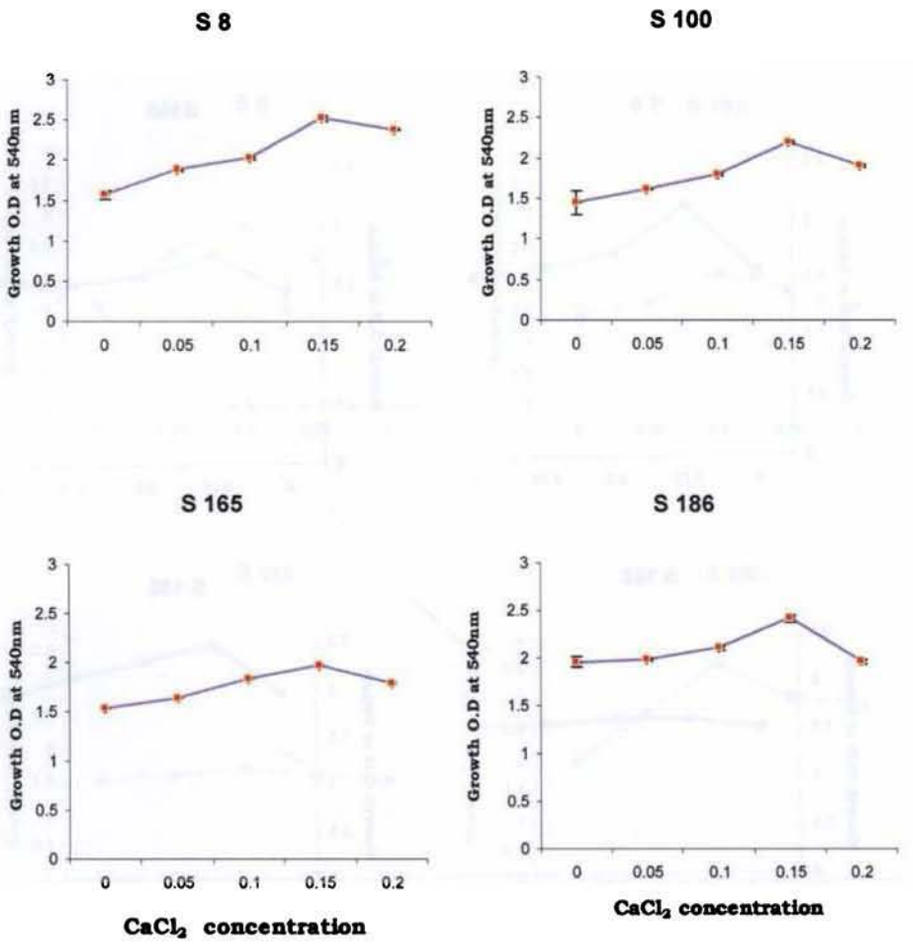


Fig.4.8 Growth of yeast strains at various calcium chloride concentration in the molasses medium

4.3.7 Effect of potassium dihydrogen phosphate on growth

The optimum potassium dihydrogen phosphate concentration for growth was found to be 0.3% for S8, S100 and S165. For S186, a concentration of 0.2% was found to be optimal for growth (Fig.4.9).

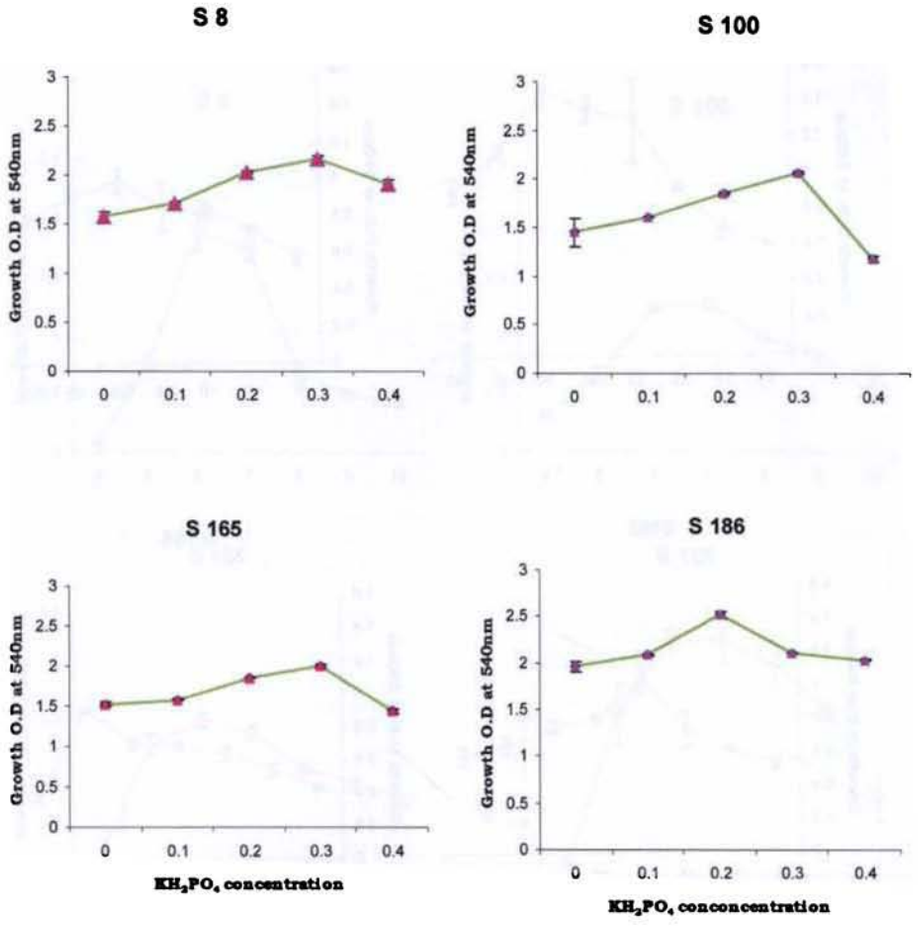


Fig.4.9 Growth of yeast strains at various KH_2PO_4 concentration in the molasses medium

4.3.8 Effect of salinity on growth

A salinity of 30ppt was preferred by S8 and S186 whereas S100 showed maximum growth in 25ppt and S165 in 20ppt. (Fig. 4.5) Considerable increase in growth was observed with the increase in salinity to the optimum level. (Fig.4.10)

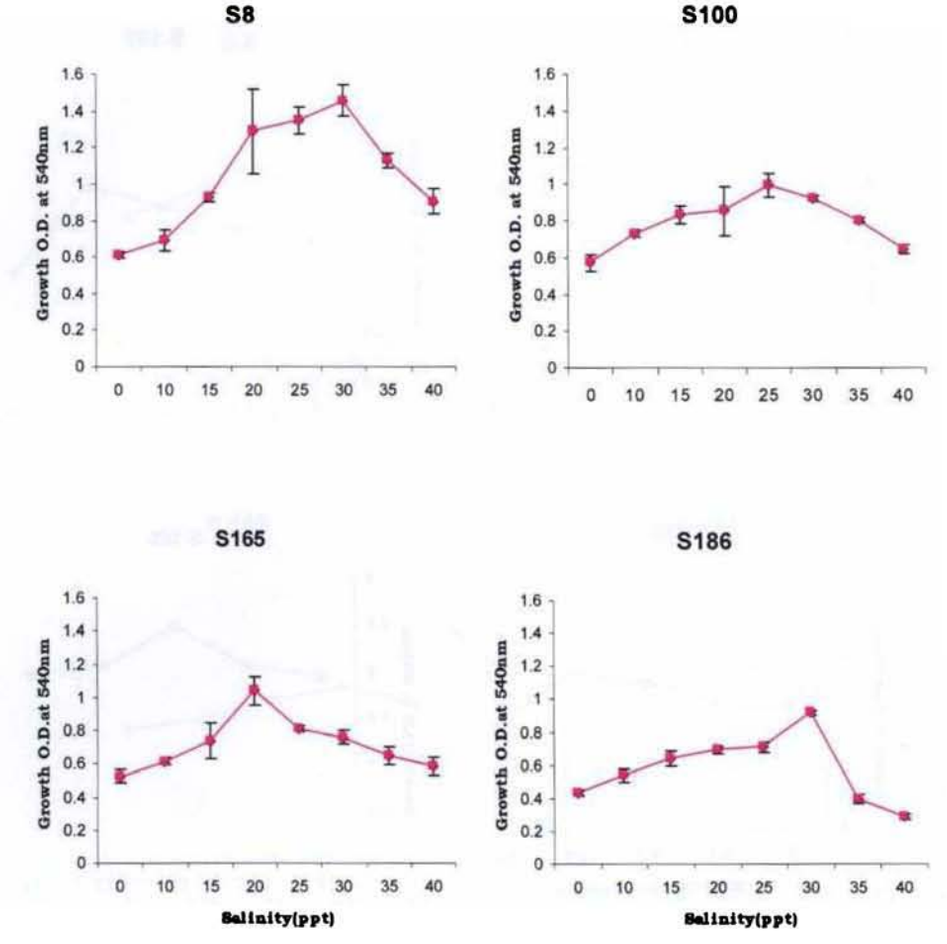


Fig. 4.10 Effect of salinity on the growth of selected yeasts

4.3.9 Effect of pH on growth

Generally growth was found to be optimum at pH 6 for most of the strains. There was no much reduction in growth at pH 7. Growth was meager at pH 4 and increased considerably at pH 5 reaching

maximum at pH 6. Drastic reduction in growth could be observed at alkaline conditions (Fig.4.11).

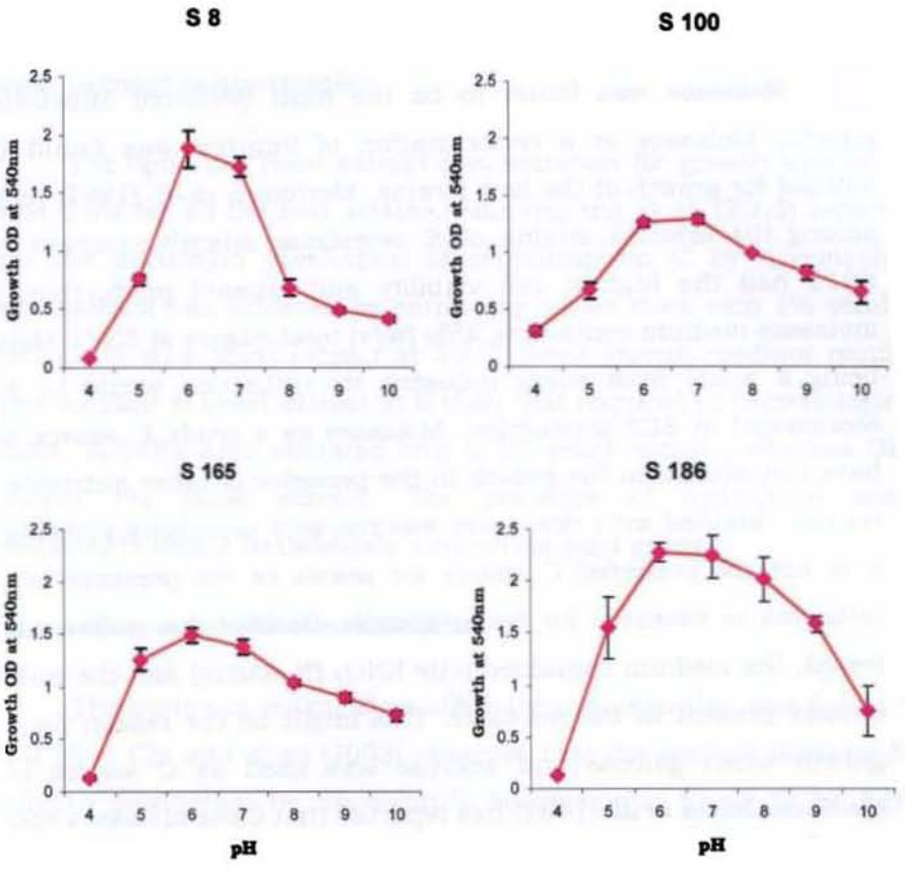


Fig. 4.11 Effect of pH on the growth of selected yeasts

4.4 DISCUSSION

4.4.1 Process Optimization

Results obtained in the present study suggest that optimum concentrations of nutrients enhance growth resulting in better

biomass production. Optimization of parameters was essential to achieve maximum yield as growth.

Molasses

Molasses was found to be the most preferred substrate for growth. Molasses at a concentration of 9mg/ml was found to be optimal for growth of the four strains. Morimura *et al.* (1997) reported among the selected strains of *S. cerevisiae,* adaptive mutant strain K211 had the highest cell viability and ethanol productivity in a molasses medium containing 25% (w/v) total sugars at 35°C. Molasses being a waste from sugar industry, its utilization would be highly economical in SCP production. Molasses as a crude C source would have contributed to the growth in the presence of other nutrients. The results obtained with rice water was not very promising showing that it is not the preferred C source for yeasts or the presence of other nutrients is essential for better growth. Besides the carbon sources tested, the medium contained only KNO_3 (N source) and the nutrients already present in the seawater. This might be the reason for lesser growth when glucose and sucrose was used as C source in the medium. Jones *et al.* (1981) has reported that Cane molasses contains 60% sucrose in addition to growth promoting components.

Peptone concentration

Among the four strains used for the study the optimum peptone concentration was found to be 0.75%. The pH, temperature and inoculum ratio for the production of β-galactosidase by *Kluyveromycees marxianus* CDB002 in a lactose free medium was studied by Furlan *et al.* (2001). The culture medium used composed of yeast extract (10g/l), peptone (20g/l) and molasses (100g/l). Generally

for bacteria a concentration of 0.5 to 1 % is incorporated in media. In the present study with yeasts also the optimal concentration falls within this range.

Yeast extract concentration

The optimum yeast extract concentration for growth was found to be 0.5% for all the four strains. Adinarayana *et al.* (2003) reported that the maximum production of cephalosporin C by *Acremonium chrysogenum* was achieved by employing wheat rawa with 1% soluble starch, 1% w/w yeast extract at 30°C. Yeast growth medium require more amount of yeast extract in it than that required in bacteriological media. ZoBell's agar contains only 0.1% yeast extract, whereas GPY contain 1% yeast extract. The presence of hydrolyzed yeast components would be definitely supporting good growth.

Magnesium sulphate concentration

The optimum magnesium sulphate concentration was found to be 0.25%. Chi and Zhao (2003) observed that the optimal medium for pullulan production by *Rhodotorula bacarum* was found to be 8.0% (w/v) glucose, 2.0% (w/v) soybean cake hydrolysate, 0.5% (w/v) $MgSO_4.7H_2O$, and 0.06% $(NH_4)_2SO_4$, pH at 7.0.

Calcium chloride concentration

Calcium chloride can control pH of the fermentation media and has its own effect on growth. In the present investigation, presence of 0.15% calcium chloride in the medium was most favourable for growth.

Phosphate concentration

Phosphate can enhance or suppress the production of growth at different concentrations. According to Lounes *et al.*, 1996 vegetative growth increased with the initial phosphate concentration up to 5mM, a further increase of phosphate supply showed no significant effect on cell yield. In the present study the optimum phosphate concentrations was found to be in the range of 0.2 to 0.3%.

Salinity

Among the four strains used for the study, two strains (S8 and S186) showed 30ppt as their optimal salinity growth. Other two strains (S100 and S165) showed maximum growth at 25ppt and 20 ppt respectively. Strains which showed a preference for 30ppt seawater shows their indigenous marine nature. *D. hansenii* is salt tolerant and it has been reported that some strains are able to grow in the presence of 20%(w/v) NaCl (Besancon *et al.*, 1992 ; Seiler and Busse (1990). Similar observations were made by Pitt & Hocking (1997) that the *D. hansenii* can grow under low water activity. The greater tolerance of *Debaryomyces hansenii* and *Saccharomyces hansenii* to NaCl at pH 5.0 – 7.0 compared with other pH values has also been reported by Hobot and Jennings (1981). *Debaryomyces hansenii* is a cryotolerant marine yeast which can tolerate salinity levels up to 24%.

PH

An optimal pH of 6 was found to be very much favourable for growth by majority of the strains. Environmental pH is particularly

significant in determining the growth of yeasts (Pitt, 1974; Cole and Keenan, 1986). Most yeast grows within the range of pH 3.0-7.0 (Walker, 1977; Miller, 1979; Deak 1991). The effects of temperature, pH and NaCl concentration on the growth of 13 strains of yeasts representing five genera: *Debaryomyces, Pichia, Zygosaccharomyces, Candida* and *Saccharomyces* revealed that there was a synergistic effect between NaCl and pH at lower temperatures. The pH of seawater is about 7.8 and therefore Marine yeasts usually prefer a higher pH for growth compared to those isolated from fruits and terrestrial environments, which prefer a pH of 4.5-5 for maximal growth (Spencer *et al.*, 1992).

Chapter 5

TESTING THE EFFICACY OF THE SELECTED MARINE YEASTS AS FEED SUPPLEMENT FOR *FENNEROPENAEUS INDICUS* IN CULTURE SYSTEMS

5.1. INTRODUCTION

Several studies have proved that the quality of the algal and yeast proteins is reasonably good (Dirr and Soden, 1942; Goyco *et al.*, 1959; Kondratiev *et al.*, 1966; Dam *et al.*, 1965; Lee *et al.*, 1967). Shacklady and Gatumel (1972) found in rats a mixture consisting of 50% maize protein and 50% yeast protein had 55% higher net protein utilization value than the two components alone. Production of unicellular algae is laborious and is a major constraint for the culturing of aquatic filter feeders. Due to small particle size and high protein content, yeasts are considered as a substitute for micro-algae. In addition, yeasts have high protein content and can be produced on the basis of various raw materials, independently of the climate and at relatively low production costs (Kihlberg, 1972).

Yeast of the genus *Candida* is most commonly used for the production of microbial protein from cellulose hydrolysates. Because of its large cell size, fast growth rate and ability to assimilate a variety of sugars derived from straw hydrolysate, *Aureobasidium pullulans* has attracted our attention as a possible organism for SCP production from grass straw (Han *et al.*, 1976).

Feeding experiments on mirror carp with yeast, bacterial, algal and soybean meal proteins showed that the major portion of protein requirement could be met by bacterial and yeast proteins (Atack *et al.*, 1979). Rainbow trout fed with various novel proteins such as herring meal, soybean, petroleum yeast, brewer's yeast, bacteria, algae etc. exhibited the highest specific growth rate with the fishmeal and petroyeast diets. Maximum Protein Efficiency Ratio was obtained with petroyeast. The petroyeast also had good physical characteristics,

producing a hard water-soluble pellet (Atack *et al.*, 1979). Bivalve molluscs grew as fast or faster than controls when fed diets containing 50% yeast (*Candida utilis*). Growth of soft tissue in *Crassostrea virginica*, decreased with the amount of yeast in the diet (Epifanio, 1979). The abundance and availability of proteins of vegetable origin has increased research emphasis on their potential utilization. As a protein source, SCP of yeast or bacterial origin appear especially attractive because the protein content and amino acid composition of these organisms compare well with those of fishmeal (Spinelli *et al.*, 1979).

It was found that *Geotrichum candidum* single cell protein could replace 100, 75 or 50% of fishmeal in a pelleted diet when fed to rainbow trout (Dabrowski *et al.*, 1980). Yeasts were found to be a suitable substitute for fishmeal upto 40% level in rainbow trout. Up to 25% substitution in diet was judged to be acceptable for marine coho-salmon (Mahnken*et al.*, 1980).

Most of the SCP produced for animal feed is derived from yeast, and as such appears to have a much higher protein and lower fiber content (Plavnik *et al.*, 1981) and is therefore more suitable monogastric animals. Working with two filamentous fungi, Alexander *et al.* (1979) reported that digestibility of SCP is lower than casein, and attributed this to the resistance of rigid cell walls to enzymatic digestion. Volesky *et al.* (1975) also reported reduced growth in rat diets with 20% fungal SCP. Cardoso and Nicoli (1981) developed a fungal SCP of comparable proximate and aminoacid profile to the *Chaetomium* SCP used in these trials. These workers indicated an

apparent nitrogen digestibility of 64% relative to a casein standard of 90%.

A mixed single cell protein biomass, containing the microorganisms *Hansenula anomala, Candida kruzei* and *Geotrichum candidum* grown on malt whiskey spent wash, was incorporated at increasing levels of substitution for casein in semi purified diets of 40% crude protein content for rainbow trout. The SCP could replace upto 50% of casein without any adverse effects on the growth, but N-utilization was reduced by 8-13% (Murray and Marchant, 1986). Yeasts have generally been able to partially replace the protein components of trout diets at low to intermediate levels of substitution, although total replacement of the major protein source in trout diet has resulted detrimental effects on growth and dietary nitrogen utilization. Factors responsible for the poor performance of the yeast as the sole source of protein for trout include the high nucleic acid content (Tacon and Cook, 1980), deficiency of the sulphur amino acids required by trout (Nose, 1974a,b) and a low content of some essential minerals, particularly calcium (Arai *et al.*, 1975). The mixed SCP biomass was of rather poor quality for trout, it had similar values to those reported elsewhere for brewer's yeast (Windell *et al.*, 1974; Atack and Matty, 1979) and hydrocarbon-grown yeast (Nose, 1974a, b; Arai *et al.*, 1975) but had inferior values to those reported for alkane yeast and bacterial SCP's (Atack and Matty, 1979; Kaushik and Laquet, 1980) as the sole dietary N source for trout. Methionine deficiency and essential amino acid imbalance reduced efficiency in growth and feed utilization with diets containing SCP (Mahnken *et al.*, 1980; Davies and Wareham, 1988).

The use of yeasts for sustaining laboratory cultures of *Artemia* for genetic and morphological studies were described by several workers (Bond, 1937; Weisz, 1946; Bowen, 1962; Bowen *et al.*, 1985) i.e. yeast products included as a protein source in mixed diets for the production of brine shrimp biomass, e.g. baker's yeast (Talloen, 1978; James and Makkeya, 1981), *Kluyveromyces* (Lavens *et al.*, 1987; Lavens and Sorgeloos, 1991), brewer's yeast and methanol yeasts (Robin *et al.*, 1987). Most trials with various species of marine yeasts led to poor results (Johnson, 1980; Nimmanit and Assawamunkong, 1985).

James *et al.* (1987) reported high production yields with marine yeast, *Candida* when culturing *Artemia* in batch cultures without water renewal. Preliminary trials in 51 aquaria by Blanco Rubio (1987) indicated that *Torula* yeast (*Candida utilis*) might be a promising food for cultivating *Artemia*.

In rainbow trout, the replacement of casein by a mixed SCP biomass (including predominantly yeasts) in semipurified diets, although not affecting growth, negatively affected N utilization (Murray and Marchant, 1986). Rumsey *et al.* (1992) also observed an increase of N retention in rainbow trout fed with increasing levels of yeast extract.

Feeding a diet consisting solely of fresh baker's yeast (*Saccharomyces cerevisiae*) under controlled conditions led to poor growth (Coutteau *et al.*, 1990). However, removing or permeabilizing the yeast cell wall by an enzymatic treatment could improve the growth performance. In this way Coutteau *et al.* (1990) could reveal that the ineffectiveness of untreated baker's yeast is mainly due to its

low digestibility. The development of techniques to improve the digestibility (Coutteau *et al.*, 1990) and the nutritional composition of (Leger *et al.*, 1985) yeast based diets provided the incentive to develop a product as a potential substitute for unicellular algae. Such a yeast-based diet has proven to be a valuable algal substitute in the larval culture of marine shrimp (Naessens-Foucquaert *et al.*, 1990).

Rumsey *et al.* (1991a and b) found that digestibility of intact brewer's yeast in rainbow trout is significantly lower than that of disrupted cells. In accordance to this finding, Rumsey *et al.* (1990) observed that brewer's yeast could replace 50% of total nitrogen in the diet of lake trout when the yeast cell walls were disrupted but a growth depression occurred when intact yeasts were used. Experiments showed the lower performance of fish fed diets containing high levels of brewer's yeast may be caused by intact yeast cells as probably not all intracellular ingredients become available to the fish.

In brewer's yeast nucleic acid nitrogen is present mostly in the form of RNA and represents about 20-25% of the nitrogen (Rumsey *et al.*, 1991a). In humans and most monogastric animals, an excess of dietary nucleic acid supply is toxic, as the capacity of excretion of the uric acid formed is limited, leading to deposits of uric acid in the body and to possible disorders of metabolism (Schulz and Oslage, 1976; Tuse, 1984). However, no such effect was found in fish due to their very active liver uricase (de la Huiguera *et al.*, 1981; Rumsey *et al.*, 1991a)

Urban and Pruder (1992) reported a growth rate of upto 82% of that obtained for the algal diet when replacing 75% of the algal ration with *Torula* yeast for *M. mercenaria*.

Kiessling and Askbrandt (1993) observed significant growth reduction in rainbow trout fed diets with increasing levels of SCP (*Brevibacterium lactofermentum* and *Bacterium glutamaticum*) when the bacterium exceeded 4% of the diet. In another study, Davies and Wareham (1988) also observed growth depression when more than 10% of the fishmeal protein was replaced with the bacterium *Micrococcus glutamicus* in diets for tilapia (*Oreochromis mossambicus*). Apparently SCP sources other than yeast provide lower nutritional value in fish diets. Vazquez-Juarez *et al.* (1993) observed yeasts isolated from the intestines of wild rainbow trout when introduced into the digestive tracts of domestic rainbow trout, produced significant increase in the growth of the cultured trout.

Partial replacement of fishmeal by brewer's yeast (*Saccharomyces cerevisiae*) in diets for sea bass juveniles was studied by Oliva-Teles *et al.* (2001). Results of this study showed that brewer's yeast can replace 50% of fishmeal protein with no negative effect in fish performance and the inclusion of up to 30% brewer's yeast in the diet improved feed efficiency. There was no beneficial effect when the brewer's yeast diets were supplemented with methionine.

Lara-Flores *et al.* (2003) evaluated the effects of three types of probiotics, two bacteria and one yeast on growth performance in Nile tilapia. Three diets were formulated containing the optimum protein level (40%) for tilapia fry: one supplemented at 0.1% with a bacterial mixture containing *Streptococcus faecium* and *Lactobacillus acidophilus*; a second supplemented at 0.1% with the yeast *Saccharomyces cerevisiae*; and a third, a control diet without

supplements. Two additional diets were formulated to contain 27% protein to serve as a stress factor, and were supplemented at 0.1% with either the bacterial probiotic mix or the yeast. The two bacterial strains used in the present study were effective in stimulating fish performance, though the yeast produced the best results, being the most viable option for optimising growth and feed utilization in intensive tilapia culture. Feed utilization was highest in Tilapia fry fed with the yeast-supplemented diets, meaning the nutrients were more efficiently used for growth and energy. Based on the results, use of a 0.1% supplement of yeast in tilapia fry feeds is recommended to stimulate productive performance.

5.2 MATERIALS AND METHODS

5.2.1 Microorganisms used

Based on the results of the preliminary feeding experiment on *F. indicus* post larvae, three yeasts were selected for further study. In addition, Baker's yeast *S. cerevisiae* (MTCC36) obtained from Institute of Microbiology (IMTECH) Chandigarh was also included in the study for comparison. The selected marine yeasts (3 Nos.) were identified by IMTECH, Chandigarh: (Table.5.1). These yeasts are deposited at Microbial Type Culture Collection (MTCC) at IMTECH and the following numbers were assigned.

Table 5.1 List of yeast strains used for production of SCP

Culture No.	Species	MTCC Number
S 8	*Debaryomyces hansenii*	MTCC 4361
S 100	*Debaryomyces hansenii*	MTCC 4363
S 186	*Candida tropicalis*	MTCC 4366
S 36	*Saccharomyces cerevisiae*	MTCC 36

Preparation of yeast biomass

The selected four yeast cultures were swab inoculated onto malt extract agar plates, incubated at 28±2°C for 72 hrs and harvested with sterile saline. The cell suspensions were centrifuged at 7000 rpm for 20 minutes in a refrigerated centrifuge (Remi C-30, Mumbai) and the yeast biomass stored at 4°C in a refrigerator.

5.2.2 Proximate composition of the yeast biomass

Biochemical composition of the biomass of 3 yeast cultures (S8, S100 and S186) were analysed already. Biomass of S36 (*S.cerevisiae*) was also subjected to proximate composition analysis.

5.2.3 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 5hrs and moisture content by drying in an oven at 80°C to constant weight. Fiber content was determined by acid and alkali treatment following AOAC (1990). The nitrogen free extract (NFE) was computed by difference (Crompton and Harris, 1969). (NFE = 100- (% protein + % lipid + % fiber + % ash).

5.2.4 Feeding experiment with *Fenneropenaeus indicus* juveniles

Experimental animals

Juveniles of Indian white prawn, (*Fenneropenaeus indicus* H. Milne Edwards) of the size range 0.10- 0.12g were brought to the laboratory from a commercial prawn hatchery in Kannamali, Kochi.

Experimental feed preparation

Powdered ingredients as given in table 5.2 were mixed well into dough with 100ml water. This was steamed for 10 minutes in an autoclave and pelletised using a laboratory model pelletiser having 1mm die. Pellets were dried in an oven at 50°C for 18hrs. The pellets were broken into pieces of 4-5mm size. Four different feeds were prepared incorporating the biomass of 3 marine yeast strains and the baker's yeast (*S. cerevisiae*) plus the control feed (without the yeast biomass). Water stability of feed was checked by immersing pellets in seawater for 15 hrs and examining stability by visual observation. Feeds were stored in airtight polythene bags at -20°C in a freezer. (Fig. 5.1). A commercially available feed (CF) was also used for the study.

Table. 5.2 Composition of experimental diets

Ingredients	Control diet g	Experimental diet g
Prawn shell powder	10	10
Yeast[a]	-	15
Fish meal	30	30
Ground nut oil cake[b]	8	8
Soybean meal[c]	10	10
Maida[d]	8	8
Rice bran[e]	10	10
Casein	5	-
Vitamin and mineral mix[f]	2	2
Agar	2	2
Carboxy methyl cellulose	10	-
Water	100 ml	100 ml

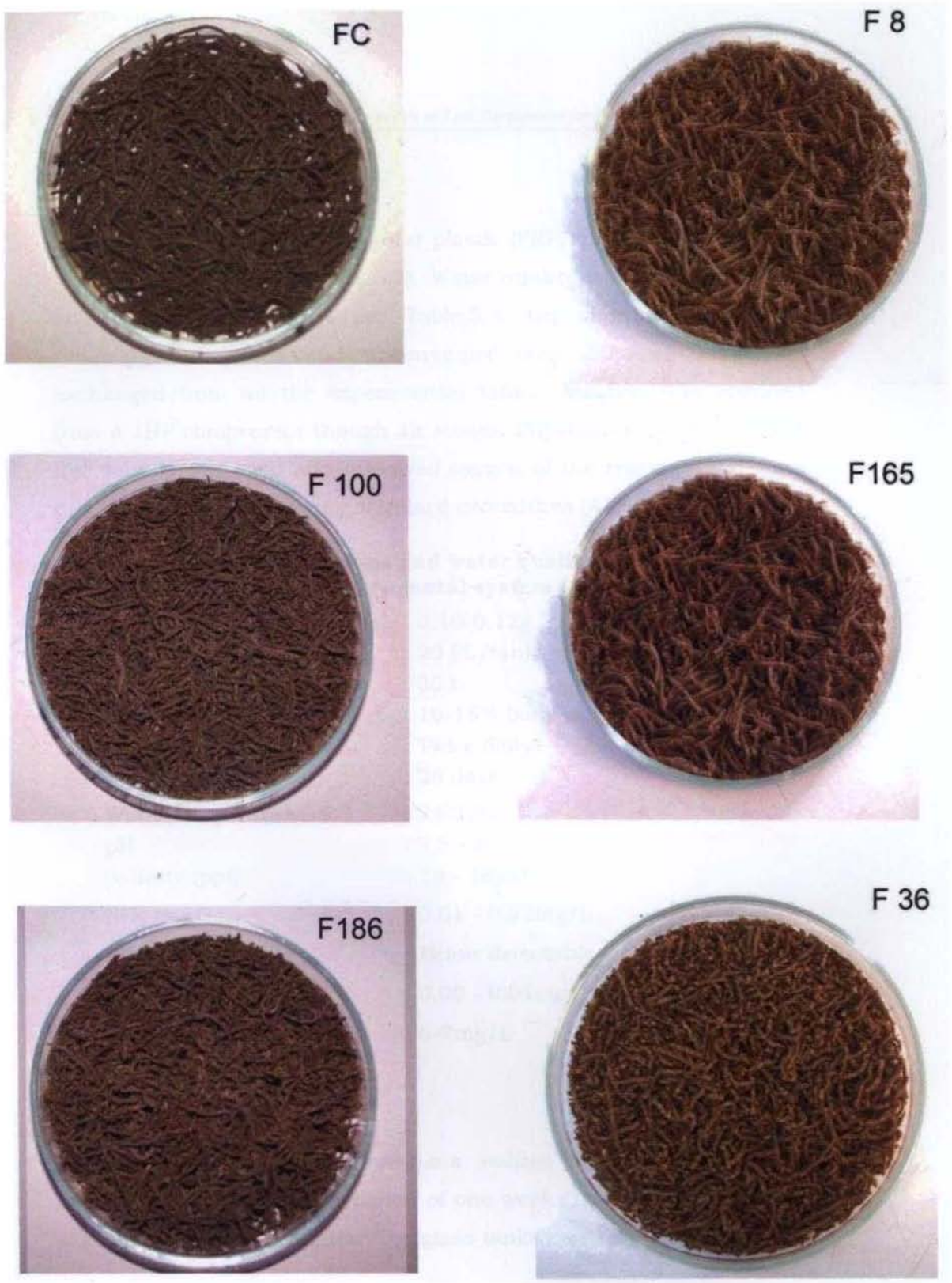


Fig. 5.1 Experimental diets prepared by incorporating the yeast biomass

Rearing facility

Fiber reinforced rectangular plastic (FRP) tanks of 30L capacity were used for the study (Fig.5.2). Water quality was monitored daily and was maintained as per Table.5.3. 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 1HP compressor though air stones. Physiochemical parameters like salinity, nitrogen and dissolved oxygen of the rearing water were estimated daily by following standard procedures (APHA, 1995).

Table 5.3 Rearing conditions and water quality parameters of the experimental system

Initial body weight (average)	: 0.10-0.12g
Stocking density	: 20 PL/tank
Tank capacity	: 30 L
Feeding level	: 10-15% body weight
Feeding frequency	: Twice daily
Feeding period	: 28 days
Water temperature	: 24-27°C
pH	: 7.5 - 8
Salinity (ppt)	: 15 - 18ppt
NH_3 (mg/L)	: 0.01 - 0.02mg/L
NO_3 (mg/L)	: Below detectable
NO_2 (mg/L)	: 0.00 - 0.01mg/L
Dissolved O_2	: 6-7mg/L

Design of experiment

Juveniles of *Fenneropenaeus indicus* were maintained on prepared control diet for a period of one week. The prawns were then stocked into 30L rectangular fiberglass tanks containing 20L seawater

with 20 individuals per tank and reared on the experimental diets for 28 days. Feeding trials were conducted using triplicate tanks for each treatment.

Feeding schedule

Six different feeds were given to the prawns including four yeast diet, one commercial feed and one control diet. Preweighed 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 preweighed filter paper and dried to a constant weight in an electric oven at 80°C for 24hrs.

Measurements

The initial body weight of the prawns in each rearing tank was recorded. They were weighed on a precision balance and after they were blotted free of water by tissue paper. The mean weight of all the prawns in a tank was calculated. After 28 days, final weight of all the prawns were measured and mean weight was found. Parameters including individual increase in weight (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 based on the data collected during the experimental period.

The formula used for calculating the growth parameters are given in 3.2.2.

Fig. 5.2 Culture facilities used for the various feeding experiments

Fig. 5.3 Close-up view of single experimental tank

Fig. 5.4 Post larvae of Fenneropenaeus indicus used for the feeding experiment

Data analysis

The data obtained in the feeding experiments were subjected to one-way analysis of variance (ANOVA). When a significant difference was found among the various treatments, Duncan's multiple range tests were done to bring out the difference between the treatment means. The statistical analysis was performed using the SPSS 7.5 package for windows.

5.2.5 Histology

Histological analysis of hepatopancreas of the experimental animals was carried out to examine whether the yeast 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 histological analysis was done as given below.

Fixation and staining of hepatopancreas sections

The hepatopancreas tissues were fixed in Davidson's fixative. 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 tissue became transparent after which they were transferred to benzene for 15 min and then xylene saturated with paraffin wax for 6 hours. 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 7.5 µ thickness, heat fixed onto albumin coated glass 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 Haematoxylin (Mayer's) and Eosin (Scott's) stains 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 40x lens magnifications.

5.3 RESULTS

5.3.1 Proximate composition of yeast biomass

Composition of S8, S100 and S186 are given in 3.3.1. Composition of *Saccharomyces cerevisiae* S36 is given below.

Culture No.	Protein	Lipid	Carbohydrate
S36	27.52	4.23	25.8

5.3.2 Proximate composition of feeds

Composition of feeds F8, F100 and F186 are described in chapter 3.3.1. and the composition of the other feeds are given below:

Feed no.	Protein	Lipid	Fiber	Ash	Moisture	NFE
F 36	45.2	8.2	1.9	5.2	6.3	33.2
CF	48.5	7.2	2.1	6.2	7.3	28.71
Control	47.2	7.9	2.0	5.8	7.2	29.9

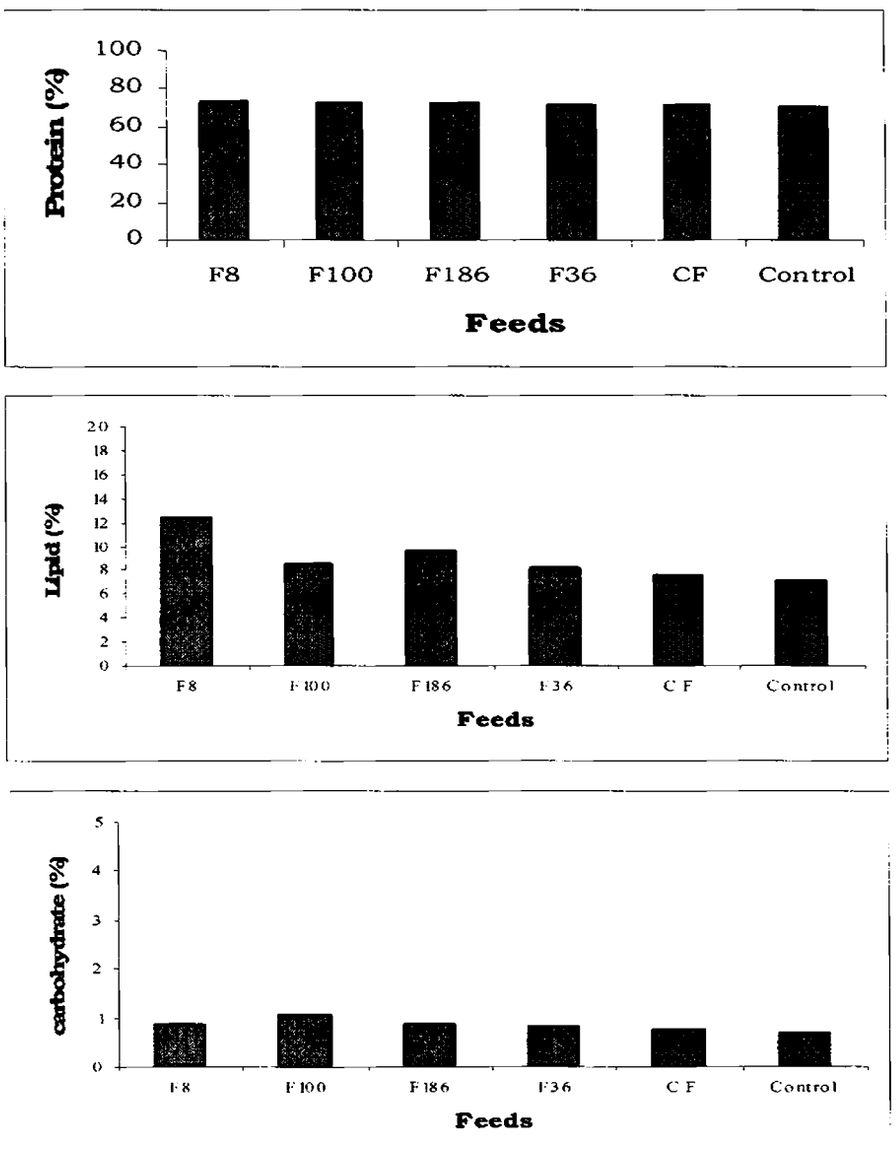


Fig.5.5 Proximate composition of flesh of Fenneropenaeus indicus maintained on different diets

5.3.3 Proximate composition of prawn flesh

Prawns fed on various diets exhibited almost the same protein content and the percentage of protein in flesh at the beginning of the experiment was maintained without much change. (Fig. 5.5) Protein content was found to be maximum in prawns fed with F8 (73.9%). The percentage of lipid was found to be maximum in prawns fed with feed F8 (12.45%) and carbohydrate with feed F100 (1.07%) (Fig. 5.5).

5.3.4 Biogrowth parameters

The data collected from the experiments were analysed and the biogrowth parameters like production, food conversion ratio (FCR), specific growth rate (SGR), relative growth rate (RGR), gross growth efficiency (GGE), protein efficiency ratio (PER), consumption per unit weight per day (CUD) were determined and are presented in Fig. 5.6a to 5.6g.

All the three marine yeast incorporated feeds were superior in performance in terms of the observed biogrowth parameters in prawns compared to both the control feeds and commercial feed. Of the three yeast diets, the feed F8 incorporated with the biomass of S8 (*Debaryomyces hansenii*) gave the best performance in terms of biogrowth parameters followed by F186 and F100 (Table 5.4).

The highest production was recorded in prawns fed with feed F8 (1.07gms) followed by F186 (0.83gms) and the lowest was recorded for control feed (0.42) (Fig.5.6a). Food conversion ratio (FCR) also was found to be the best with feed F8 (1.35), followed by F 186 (1.68) (Fig.5.6b)

Table 5.4. Relative position of various feeds with respect to their performance in terms of bio-growth parameters and percentage survival in *F. indicus juveniles* maintained on experimental diets

Parameters	PRO	FCR	SGR	GGE	RGR	PER	CUD
Experimental Feeds	**F 8**	**F 8**	**F 8**	**F 8**	**F 8**	**F 8**	**F8**
	F 186	**F 186**	**F 186**	**F 186**	**F 186**	**F 186**	**F 186**
	F 100	F 100	F 100	F 100	F 100	F 100	F 100
	C F*	C F	C F	C F	C F	F 36	C F
	F 36	F 36	F 36	F 36	F 36	C F	F 36
	Control	Control	Control	Control	Control	Control	Control

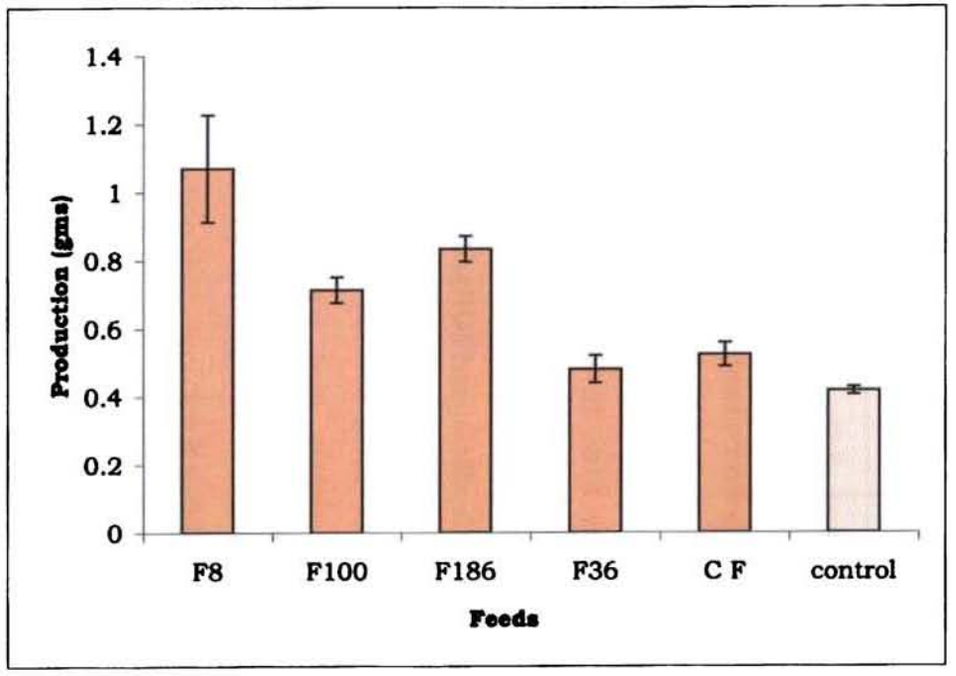
* Commercial Feed

Specific growth rate (SGR) was maximum for prawns fed diet F8 (7.719) followed by F186 (7.454) and the lowest value recorded for control feed (5.12) (Fig.5.6c).

Gross growth efficiency (GGE) was found to be maximum with F8 (75.48) followed by F186 (59.48) and the lowest value was recorded for control feed (29.27) (Fig.5.6d).

Maximum Relative growth rate (RGR) highest value was recorded for F8 (0.05) followed by F186 (0.049) and the lowest value in control feed (0.037) (Fig.5.6e).

Protein efficiency ratio (PER) was found to be the best with F8 (1.70) followed by F186 (1.23) (Fig.5.6f). Consumption per unit weight per day (CUD) was found to be best with F8 (0.07) and F186 (0.08) (Fig.5.6g).



Feed	*PRO(gms)
F8	1.070 ± 0.157^{c}
F100	0.713 ± 0.038^{b}
F186	0.83 ± 0.04^{b}
F36	0.48 ± 0.04^{a}
C F	0.52 ± 0.04^{a}
Control	0.42 ± 0.01^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig.5.6a Weight gain (Production) obtained in F.indicus juveniles when fed with various experimental feeds.

Feed	*FCR
F8	1.35 ± 0.22^{a}
F100	2.0 ± 0.135^{b}
F186	1.68 ± 0.040^{ab}
F36	2.96 ± 0.314^{cd}
Higashi	2.6 ± 0.474^{c}
control	3.43 ± 0.206^{d}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig.5.6b Food Conversion Ratio (FCR) obtained in F.indicus juveniles when fed with various experimental feeds.

Feed	*SGR
F8	7.719±0.58^{d}
F100	7.010±0.342^{c}
F186	7.454±0.317^{cd}
F36	5.847±0.464^{b}
C F	6.024±0.277^{b}
Control	5.121±0.114^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig.5.6c Specific Growth Rate (SGR) obtained in F.indicus juveniles when fed with various experimental feeds.

Feed	*GGE
F8	75.48±12.21^{c}
F100	50.2±3.46^{b}
F186	59.48±1.34^{b}
F36	34.08±3.46^{a}
C F	39.25±6.79^{a}
Control	29.27±1.82^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

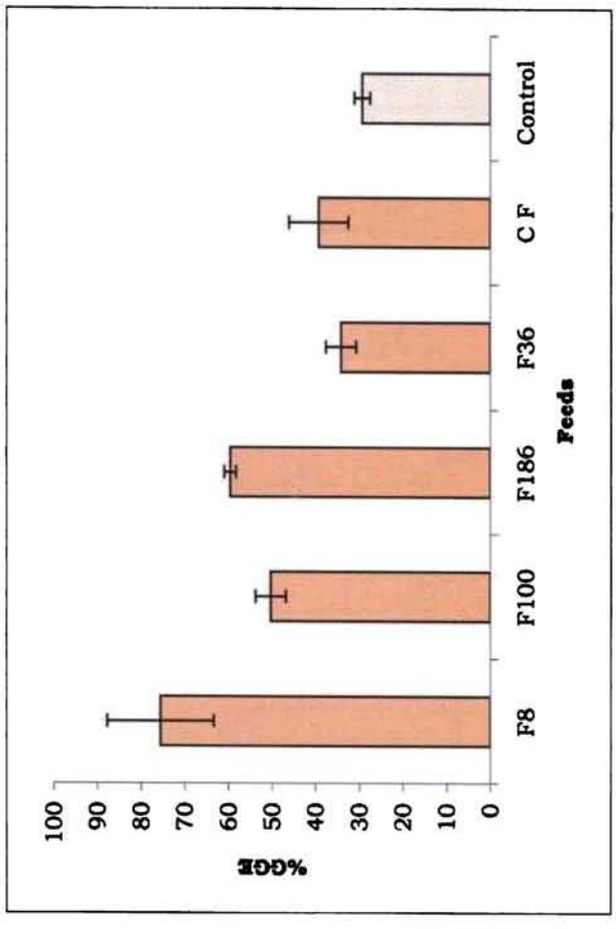
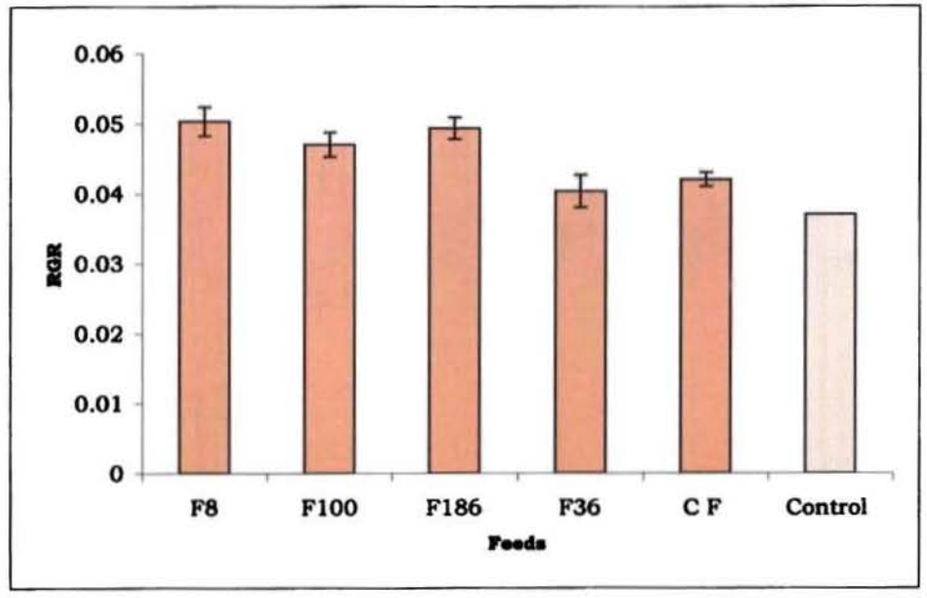


Fig. 5.6d Gross Growth Efficiency (GGE) obtained in F.indicus juveniles when fed with various experimental feeds.



Feed	*RGR
F8	0.05 ± 0.002^{d}
F100	0.047 ± 0.002^{c}
F186	0.049 ± 0.002^{cd}
F36	0.040 ± 0.002^{b}
C F	0.042 ± 0.001^{b}
Control	0.370 ± 0.000^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig.5.6e Relative Growth Rate (RGR) obtained in F.indicus juveniles when fed with various experimental feeds.

Feed	*PER
F8	1.70 ± 0.276^{d}
F100	0.97 ± 0.07^{b}
F186	1.23 ± 0.029^{c}
F36	0.75 ± 0.077^{ab}
C F	$0.81\pm.137^{ab}$
Control	0.73 ± 0.045^{a}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

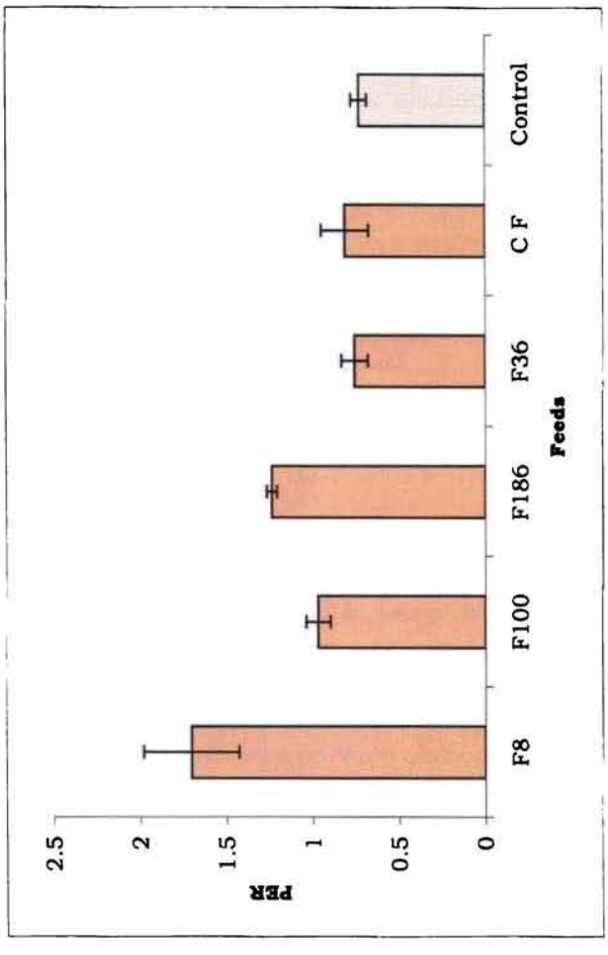
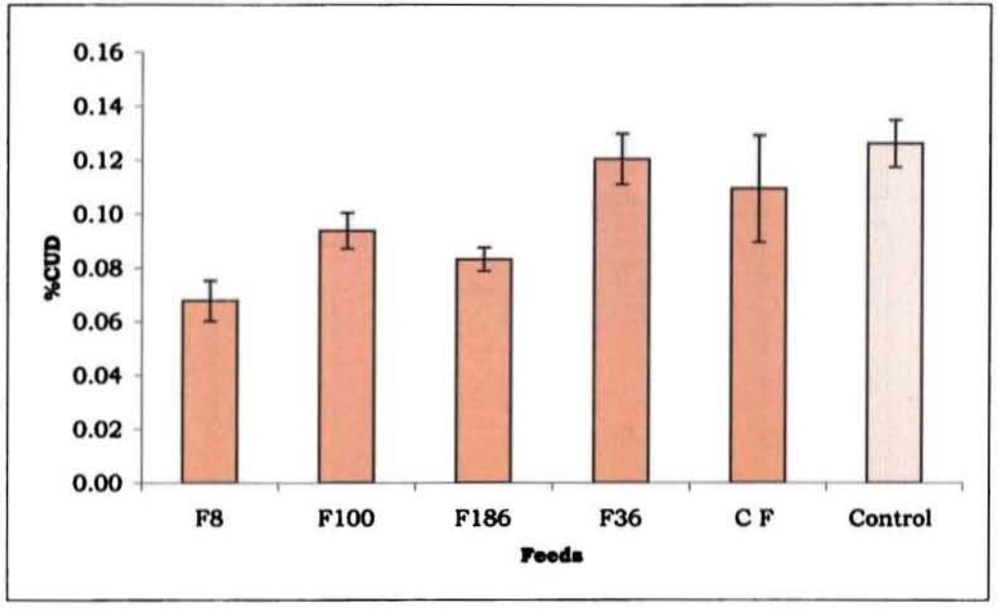


Fig.5.6f Protein Efficiency Ratio (PER) obtained in F.indicus juveniles when fed with various experimental feeds.



Feed	*%CUD
F8	0.07 ± 0.008^{a}
F100	0.09 ± 0.007^{bc}
F186	0.08 ± 0.004^{ab}
F36	0.12 ± 0.009^{d}
C F	0.11 ± 0.020^{cd}
Control	0.13 ± 0.009^{d}

* (mean± s.d.)
* Values with the same superscript does not vary significantly

Fig. 5.6g. Consumption per unit weight per day (CUD) obtained in F.indicus juveniles when fed with various experimental feeds.

Statistical analysis

Duncan's multiple range analysis of the various growth parameters effected by the different feeds showed that the performance of the feed F8 and F186 were significantly different from other feeds including the control diets.

5.3.5 Histological examination of hepatopancreas

Histological analysis of the hepatopancreas did not show any structural or functional abnormalities with feeds F8, F100, F 186, F36 and control feed. Fig.5.7. shows 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. Large numbers of R and F cells are also seen showing that the hepatopancreas is in a healthy condition. Healthy F cells have a basic pyramidal or cylindrical shape.

Fig.5.8. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F36. Large number of vacuolated cells occupies the epithelium, these could be both R or B cells. Obliteration of the lumen of the tubules was found which was mainly the result of excessive enlargement and vacuolation of B cells.

Fig.5.9. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed commercial feed CF. The B cells are more in number and larger suggesting an active and healthy condition of digestive process. Active B cells which are comparable to those fed

on the control feed was found. Compression of lumen space is noticed in some tubules. Sloughing off of the cells was noticed in some tubules.

Fig.5.10. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F8. The B cells are more in number and larger suggesting an active and healthy condition of digestive process. The normal healthy structure comparable to that of the control is observed and no degenerative changes are noticeable in the tubules.

Fig.5.11. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F100. Almost normal condition was noticed in the tubules. They were not completely obliterated or damaged. Slight disruption of the basal membrane was observed.

Fig.5.12. shows the cross section of hepatopancreas tubules of the midgut-gland of shrimps fed feed F186. Normal structure of B cells and F cells are noticed. The homogeneity of inclusions on the basal vacuoles were found to be similar to those found in control tubules. Slight disruption of the basal membrane was also observed, no possible reason can be found for this at this point of study. There was no shrinkage in the size of the tubules. F cells retain their pyramidal shape, which is an indication of their healthy condition.

5.4 Discussion

Feeding experiment

Proximate composition of the flesh of prawns did not show any remarkable change when fed on various yeast feeds, commercial feed

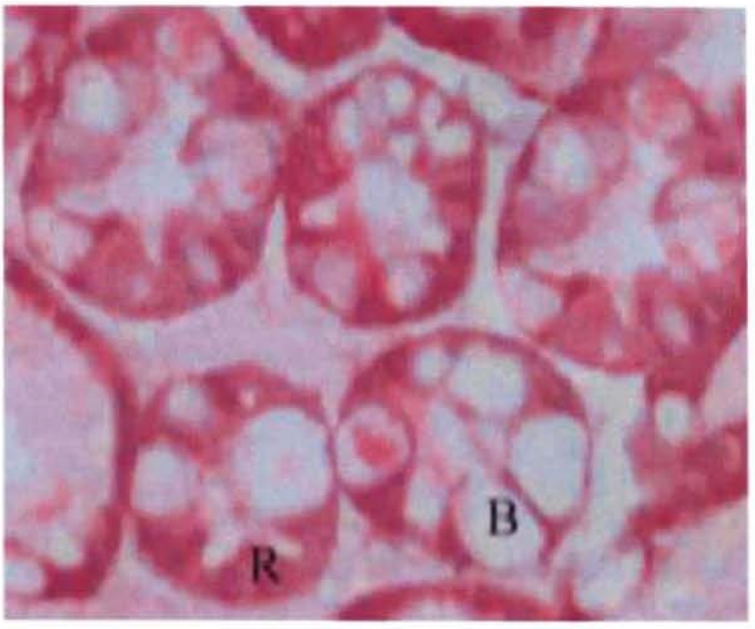


Fig. 5.7 C.S. of hepatopancreas of Fenneropenaeus indicus maintained on control feed. Haematoxylin-Eosin stain. 40 X

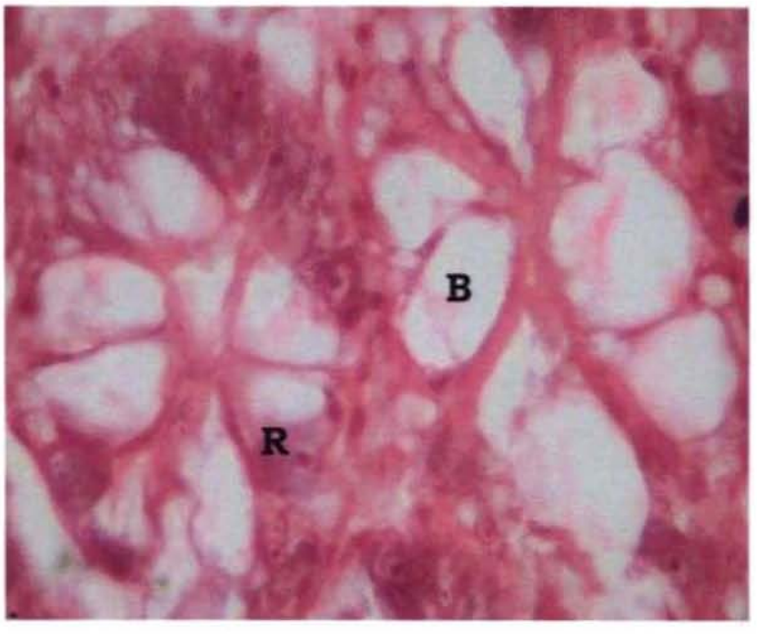


Fig. 5.8 C.S. of hepatopancreas of Fenneropenaeus indicus maintained on experimental feed F36. Haematoxylin-Eosin stain. 40x

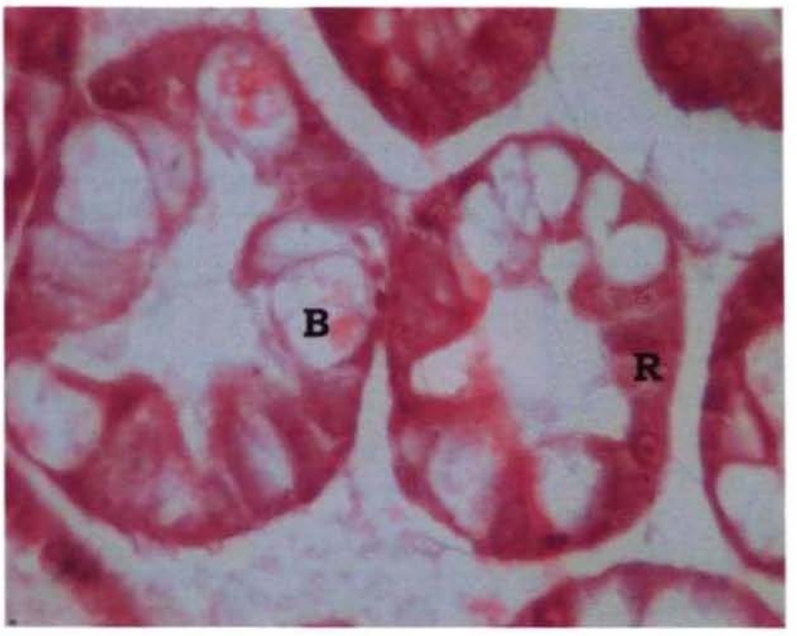


Fig. 5.9 C.S. of hepatopancreas of Fenneropenaeus indicus maintained on commercial feed CF. Haematoxylin-Eosin stain. 40X

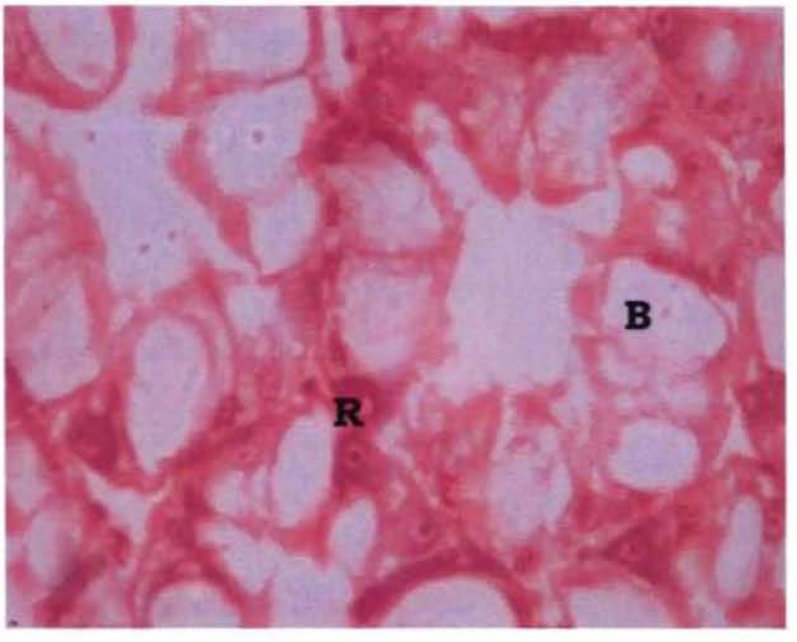


Fig. 5.10 C.S. of hepatopancreas of Fenneropenaeus indicus maintained on experimental feed F8. Haematoxylin-Eosin stain. 40X

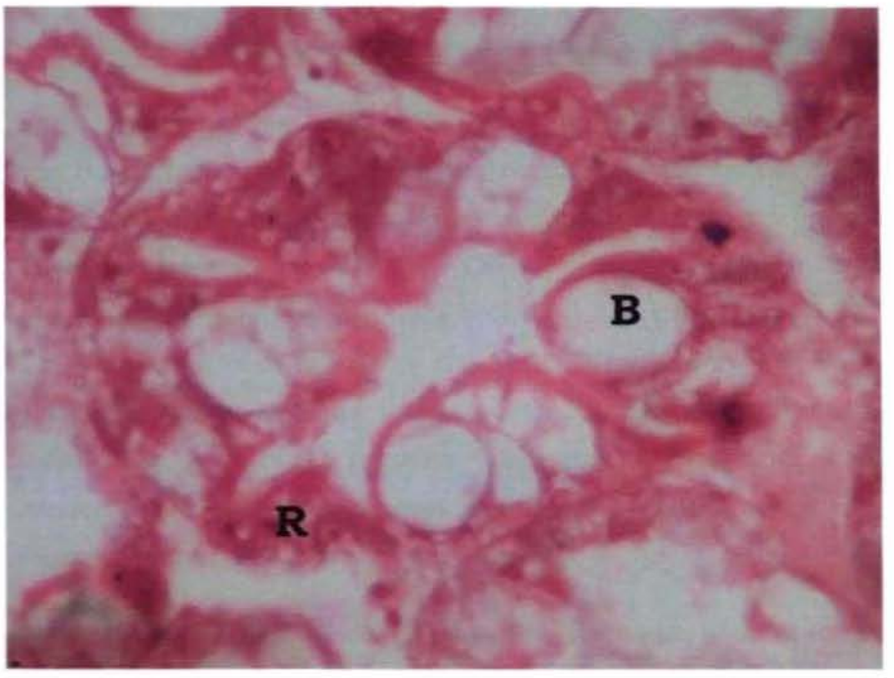


Fig.5.11 C.S. of hepatopancreas of Fenneropenaeus indicus maintained on experimental feed F100. Haematoxylin-Eosin stain. 40X

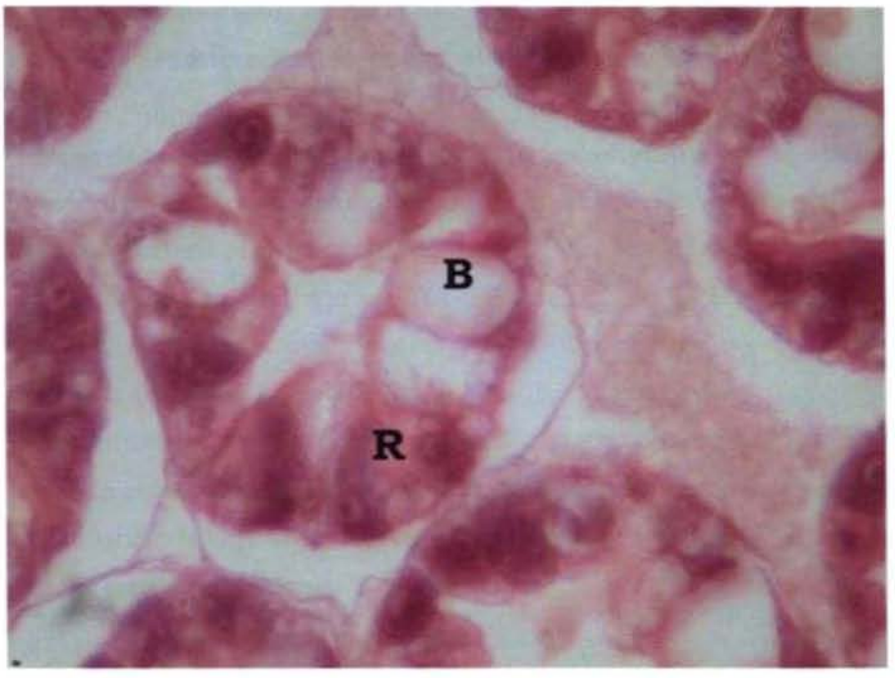


Fig.5.12 C.S. of hepatopancreas of Fenneropenaeus indicus maintained on experimental feed F186. Haematoxylin-Eosin stain. 40X

and the control feed. This shows that all the experimental feeds had the nutritional components in the required proportion and amounts. In the present study three marine yeasts (*Debaryomyces hansenii* S8, *Debaryomyces hansenii* S100, *Candida tropicalis* S186), a Baker's yeast, *Saccharomyces cerevisiae* MTCC 36, a commercial feed (CF) and a control feed were used for the study. The performance of marine yeast incorporated feeds was superior compared to other feeds. A detailed analysis of the proteins, fatty acids, carbohydrates, vitamins, nucleic acids and the mineral content is essential to elucidate the reason for this difference.

There is major concern regarding the availability of fishmeal for incorporation in fish diets (Hardy, 1996). Alternative protein sources such as plant feedstuffs are generally not well accepted due to non-palatability and amino acid imbalance. Single Cell Protein (SCP) which include micro algae, bacteria and yeast are alternative protein sources used in feed ingredients. Among SCP's yeasts have been the most used within aquafeeds (Tacon, 1994). Yeasts have immunostimulatory properties by virtue of their complex carbohydrates and nucleic acid contents (Anderson *et al.*, 1995). Another problem with SCP is their higher content of nucleic acids, 8 to 12% in yeasts (Schulz and Oslage, 1976) mostly in the form of RNA (Rumsey *et al.*, 1991a). Excess supply of dietary nucleic acids cause deposition of uric acid in the body (Schulz and Oslage, 1976). In fishes due to the presence of very active liver uricase, this problem is not usually observed. There are no reports related to this type of disorders in prawns. Investigations have to be undertaken in this area to find out the adverse effects of dietary nucleic acids in prawns, if any.

The application of single cell protein in aquaculture is a relatively recent practice and the interest in such practices are increasing rapidly. Single cell proteins (SCP) include micro algae, bacteria and yeast, and are alternative for conventional protein sources that are frequently used as feed ingredients for fish due to the nutritional values of their nutrients such as proteins, B-vitamins, pigments and complex carbohydrates, such as glucans (Sanderson and Jolly, 1994; Tacon, 1994). Among SCP, yeasts have been the most used within aquafeeds (Tacon, 1994). As a protein source, single cell proteins (SCP) of yeast or bacterial origin appear especially attractive because the protein content and aminoacid composition of these organisms compare well with those of fish meal (Spinelli *et al.*,1979). Most of the studies performed so far on the use of yeast as food source for crustaceans and fishes were related to the baker's yeast as main source. Studies on the use of marine yeast as main source of protein are limited. In the present study, marine yeasts with better nutritional value were selected and incorporated into the feed of *Fenneropenaeus indicus*.

Live micro algae and *Artemia nauplii* have been used as essential sources of nutrition in penaeid larviculture (Cook & Murphy, 1966; Simon, 1978; Tobias-Qunitio & Villegas, 1982). The culture and maintenance of these live food organisms, however, are tedious, labour intensive and expensive. Several attempts have been made to substitute non-living foodstuffs for live food using powdered Soya cake (Hirata *et al.*, 1975), microencapsulated diet (Jones *et al.*, 1979) and micro coated diets (Villegas & Kanazawa, 1980). Throughout this long period, however, reports on developing and using new live food organisms for shrimp larviculture were scarce.

Yeast was used as an inexpensive and easily available alternative food for rotifers (James *et al.*,1983 and 1987). Imada (1984) reported rotifers cultured successfully with baker's yeast contained high levels of vitamin B12. Experiments by Abdel Rahman *et al.* (1993) and Chatila, 1994 showed that yeast, *Candida utilis*, is an excellent food for rotifers and Artemia and thus it may be a suitable food for other filter feeders as it has many advantages, such as suitable size range (7-40μm), high nutritive value and simple culture methods. In the present study, the growth and survival of prawns maintained on yeast diets were much higher when compared to the control diet and the commercial feed. Yeast products (primarily brewer's yeast and baker's yeast) are frequently used as feed ingredients in aquaculture because of the nutritional value of these products, which include protein, lipids, B-vitamins etc. (Mahnken, 1991; van der Meeren, 1991).

Experiments by Hecht and Viljoen, 1982; Dabrowski *et al.*, 1983; Alami-Durante *et al.*, 1991 showed that common carps can utilize a high portion of their dietary protein from the yeasts *Candida tropicalis*, *C. utilis* and *C. lipolytica* with better results than those obtained with soybean or meat and bone meals. In this study also *C. tropicalis* was found to be a good feed supplement for prawns.

James *et al.* (1987) reported high production yields in Artemia when fed with yeast, *Candida*. Blanco Rubio (1987) also reported *Torula* yeast (*Candida utilis*) as a promising food for cultivating Artemia. Naessens-Foucquaert *et al.* (1990) reported that yeast-based diets has proven to be a valuable algal substitute in the larval culture of marine shrimp.

Among the three marine yeasts, *Debaryomyces hansenii* S8 supported the best biogrowth parameters, in terms of production, FCR, SGR, GGE, PER and CUD followed by S186 (*Candida tropicalis*) and S100 (*Debaryomyces hansenii*). Commercial feed was found to be better in efficiency compared to the Baker's yeast diet and the control diet.

Nell *et al.* (1996) evaluated the effect of *D. hansenii*, *C. utilis*, *S. cerevisiae* and *D. capitatus* as dietary supplement in oyster and found it to be inferior to the algal diet. Yeast cell wall constitutes 15-25% of the dry weight of the whole cell and consists of 80-90% complex, difficult to digest polysaccharides (Fleet, 1991). Low digestibility of yeast cell wall may be the reason for the comparatively low nutritional effect with respect to an algal diet. In the present study algal supplements were not included and the results obtained with marine yeast diets for *Fenneropenaeus indicus* were highly promising. Duncan's multiple range test (Statistical analysis) showed a significant increase ($P<0.05$) in the performance of all the three marine yeast feeds compared to the other diets. A detailed analysis of biochemical composition of these yeasts is essential to elucidate the reason for the food value of the marine yeasts. The limited digestibility of yeasts for bivalves has been attributed to their low digestibility (Epifanio, 1979) as well as deficiency or imbalance of nutrients (Urban and Langdon, 1984). The bivalve stomach is well equipped for the digestion of algal carbohydrates by the presence of various carbohydrases (including chitinase and laminarinase) (Reed, 1981). However the enzymes are not necessarily appropriate for an efficient digestion of the

polysaccharides composing the cell wall of intact yeast cells (Coutteau *et al.*, 1990).

Feed intake depression in rainbow trout could be observed by many workers when fed Brewer's yeast diet (Tacon and Cooke, 1980; Rumsey *et al.*, 1991a and b and Atack and Matty, 1979). On the contrary, Rumsey *et al.* (1992) and Oliva-Teles and Goncalves (2001) noted no negative effects on feed intake at 30% level inclusion of Brewer's yeast for rainbow trout and in sea bass respectively. In the present study also no reduced feed intake could be noticed.

Histology of hepatopancreas

Histopathological studies envisaged assessment of cellular damage, variations in the nature of inclusions, proliferation of specific types of cells and total damage of epithelia or any connected structure of this important organ. Hepatopancreas is essentially an organ, which constantly produces new cells, the growth taking place from the basic embryonic cells (Embryonalenzellen) situated at the distal end of the tubules.

Basically the hepatopancreas has four types of cells. About 75% of the total number of cells in a tubule is the R cells (Restzellen), which are essentially absorptive in nature. They contain numerous mitochondria, golgi bodies, small irregularly shaped vacuoles and an elaborate brush border. A significant feature of this cell is the presence of reserve food materials, especially lipid. The F cells, (Fibrillen-zellen) are characterized by a well-developed network of rough endoplasmic reticulum and large number of both membrane bound and free ribosomes. A good number of mitochondria and golgi bodies are also

seen. Functionally, these cells are secretary, the presence of elaborate endoplasmic reticulum indicating enhanced production of protein. The largest of the hepatopancreatic cells is the B cells (Blasenzellen) which contains a characteristically enormous vacuole, an undulating brush border on the luminal surface, an active apical complex and a compressed nucleus at the proximal end. (Gibson and Barker, 1979). This cell is secretary and often excretory also (by way of exocytosis of the large residual vacuole) in function (Gibson and Barker, 1979; Hopkin and Nott, 1980). Release of cellular contents from the B cells of decapod hepatopancreas has been reported to be holocrine, merocrine or apocrine. The R cells undergo variation, as a result of exposure to toxicants or when subjected to starvation.

The histological examination of the hepatopancreas was carried out in an attempt to evaluate the toxic effects, if any, in prawns fed yeast diets. 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. No histopathological alterations indicative of toxic effects could be observed in the prawns fed yeast diets. The study showed that the three marine yeasts used in the study could very well be used as feed supplement in aquaculture.

This study shows the potential of marine yeasts as a feed supplement in aquaculture. Yeasts are nutritionally rich with proteins, vitamins and carbohydrates. Besides being a nutritional source, yeasts serve as an immunostimulant also by virtue of its high carbohydrate (β, 1-3 glucan) and RNA content. Technology for mass production of the marine yeasts, storage and incorporation into diet has to be developed for application in culture systems.

Chapter 6

TESTING THE EFFICACY OF MARINE YEASTS AS IMMUNOSTIMULANT IN PENAEID PRAWNS

6.1. INTRODUCTION

Crustaceans contributed about 5.4% by weight (5.9 million t) and 15% by value (16.8 billion US dollars) to the world's supply of aquatic produce (less seaweeds) in 1993. 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 shrimps may grow to as much as 1.6 million tones by the turn of the century (Newman, 1996).

Shrimp aquaculture started in the 1970s as an industrial activity and developed rapidly with a huge increase in the number of hatcheries and farms. Shrimp farming provides roughly 30% of the shrimp supplied to the world market. The activity concerns tropical countries in South East Asia, Central and South America.The industry saw major growth during the 1980s, but now, production is regularly and seriously affected by problems linked to environment degradation and to infectious and non-infectious diseases. This situation has grown worse with the intensification of shrimp farming based on progress in zoo technology but with a lack of knowledge of penaeid physiology and often with little consideration of ecological aspects. The major constraint for aquaculture seems to be the loss due to diseases. In India, the loss of shrimp production during 1995-96 due to diseases is estimated to be over Rs.600 crores. The causative agents of infectious diseases in shrimp are mainly viruses and bacteria belonging to Vibrionaceae. These pathogens particularly hamper larval production and lead to profitability problems due to stock mortalities. They also lead to the over fishing of wild shrimp larvae and an

overexploitation of brood stock. Moreover, the local environment can be contaminated by the discharge of wastewater containing antibiotics, which can foster the development of drug-resistant bacteria. Finally, the practice of shrimp transfer at national or international levels has contributed to the spread of 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, haemolymph 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, 1999). Non-infectious diseases are often suspected to occur because of environmental degradation exacerbated by inappropriate management practices. The sustainability of the shrimp industry depends largely on disease control and the health status of shrimp. From this point of view, the immune system is a tool to assess shrimp health (Bachere *et al.*, 1995).

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. Till recently, disease management strategies were based mainly on chemotherapy. Aoki (1992) indicated that not less than 38 antimicrobials and 22 pesticides are used in Asian aquaculture. The lack of regulation for their use in most Asian countries has contributed to their extensive use. However, the emergence of drug resistance in pathogens, problems associated with drug residues in cultured fish, and awareness towards environmental problems associated with the use of chemotherapeutics have led to greater focus on alternate methods of disease management. 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 micro organisms dominate the harmful bacteria that cause disease (Ewing and Haresign, 1989).

Immunoprophylaxis for shrimp is being seriously considered by various investigators but the knowledge about immune system of shrimp is very limited. In addition to the hard exoskeleton which forms a structural and chemical barrier to pathogens and parasites, crustaceans need an efficient internal immune defence network to deal with opportunistic or pathogenic microorganisms which can gain entry into the body cavity either through wounds or during moult. Like in other animals, crustacean host defence is largely based on the activities of the blood cells or haemocytes.

Immunostimulants are aimed at enhancing the non-specific defence mechanisms in animals. A number of different biological and synthetic compounds have been found to enhance the non-specific defence 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 to both specific and opportunistic ones (Raa *et al.*, 1992).

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.

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 systems and make them more active. Nevertheless, some nutritional factors are so intimately interwoven with the biochemical

process 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 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).

However, there is a growing understanding of the importance of the 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 mixed into feed. (Siwicki *et al.*, 1994). These results show that immunomodulants provide protection when administered orally. This is promising news for aquaculture and aqua feed industry. However, there are still much uncertainties related to the dosage, time of administration, formulation of the feed, etc.

Even though an immune response against specific epitopes and/or immunoglobulins has not been detected in invertebrates, they are able to recognize and destroy invading microorganisms or parasites. Proteins involved in the recognition process of cell wall components from microorganisms, such as lipopolysaccharides (LPS) and β-1,3-glucans (BG), have been found in invertebrates.

Yeast may improve fish health as antagonists to pathogens and by immunostimulation (Andlid *et al.*, 1995). Rorstand *et al.* (1993) also

reported that a yeast glucan showed an adjuvant effect when included in vaccines against furunculosis in Atlantic Salmon (*Salmo salar* L.). One of the most promising areas of development for strengthening the defenses of fish is the administration of glucans as adjuvants or immunostimulants (Yano *et al.*, 1989; Nikl *et al.*, 1991; Chen and Ainsworth, 1992; Raa *et al.*, 1992).

Robertson *et al.* (1990) injected Atlantic Salmon (*Salmo salar*) with M-glucan, an yeast extract and showed reduced mortality when fish were challenged with *Vibrio anguillarum, V. salmonicida* or *Yersinia ruckeri*. Chen and Ainsworth, 1992 working with cat fish (*Ictalurus punctatus*), injected the baker's yeast-derived glucan alone or with *Edwarsiella tarda* bacterins to show increases in the non-specific defence parameters of phagocytic cell activity, the specific immune response and protection against disease.

The use of immunostimulants has gained a lot of interest as a valuable alternative to the use of antibiotics and vaccines in the fight against infectious diseases in shrimp farming (Subasinghe, 1997). A number of commercial products (yeast preparations, β-glucans, polysaccharides) are nowadays available, all claiming to have positive effects on disease resistance (Devresse *et al.*, 1997).

Profound changes in the immune responses are some of the earliest manifestations of malnutrition (Mac Farlene and Path, 1977). The relationship of diet and immune response in fish has been reviewed by Landolt (1989) and reported that nutritional factors play an important role in enhancing machinery.

Haemocytes

The circulating haemocytes of crustacean and other invertebrates are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Smith and Soderhall, 1983; Ratcliffe *et al.*, 1985; Soderhall and Smith, 1986; Johansson and Soderhall, 1989; Soderhall and Cerenius, 1992). The number of free haemocytes can vary and can, for instance, decrease dramatically during an infection (Persson *et al.*, 1987; Smith and Soderhall, 1983a; Smith *et al.*, 1984; Lorenzo *et al.*, 1999). Thus, new haemocytes need to be compensatory and proportionally produced, and it is commonly believed that haemocytes are released continuously, although at varying rates, from a specialized haematopoietic tissue. This tissue has been identified in several crustacean species (Ghiretti-Magaldi *et al.*, 1977; Hose *et al.*, 1992; Martin *et al.*, 1993; Chaga *et al.*, 1995).

Haemocytes play a central role in crustacean immune defense. Firstly, they remove foreign particles in the haemocoel by phagocytosis, encapsulation and nodular aggregation (Soderhall and Cerenius, 1992). Secondly, haemocytes take part in wound healing by cellular clumping and initiation of coagulation process through the release of factors required for plasma gelation (Johansson and Soderhall, 1989; Omori *et al.*, 1989; Vargas-Albores *et al.*, 1998), and carriage and release of the prophenoloxidase (proPO) system (Johansson and Soderhall, 1989; Hernandez-Lopez *et al.*, 1996). The haemogram consists of the total haemocyte count (THC) and the differential haemocyte count (DHC). For the differential count, three cell types are identified in penaeid shrimp : large granule haemocytes

(LGH), small granule haemocytes (SGH) and agranular haemocytes or hyaline cells (HC) (Tsing *et al.*, 1989; Martin and Graves, 1985; Rodriguez *et al.*, 1995; Van de Braak *et al.*, 1996).

For crustaceans, some information exists on the importance of THC in pathogen resistance. Persson *et al.* (1987) reported in *Pascifastacus leniusculus* a relationship between haemocyte number and its resistance to the parasite fungus *Aphanomyces astaci.* They demonstrated that a decrease in the haemocyte number of crayfish harbouring *A. astaci* as a latent infection resulted in an acute infection with incomplete melanization of fungus hyphae, leading to the death of the crayfish. Le Moullac *et al.* (1998) observed that *Penaeus stylirostris* with a low THC due to a hypoxia situation, became more sensitive to infections with highly virulent *Vibrio alginolyticus.*

For most crustacean species, the variation in total haemocyte and differential count values are high between individual animals. Consequently, they cannot be used to evaluate the physiological state of the animal.

Granular and semigranular cells can be cytotoxic and lyse foreign eukaryotic cells. This has been shown with both tumorous and non-tumorous cell lines as well as erythrocytes as target cells (Soderhall *et al.*, 1985). Proteins, which are part of or associated with the proPO system, such as proPO and peroxinectin, are present in the semigranular and granular cells and are not present in the hyaline cells.

The haemocyte count can vary greatly in response to infection, environmental stress and endocrine activity during moulting cycle

(Smith and Ratcliffe, 1980; Persson *et al.*, 1987; Smith and Johnston, 1992). Experimental injection of a fungal cell wall preparation or of a β-1,3-glucan causes a rapid decrease in the number of free haemocytes, followed by a slow recovery (Persson *et al.*, 1987b). Also after an injection of the crayfish parasite *Psorospermium haeckelii,* the number was significantly lower than after a control injection of saline, which, in contrast, gives a dramatic increase in the number of free haemocytes (Persson *et al.*, 1987b; Soderhall and Cerenius, 1992; Thornqvist and Soderhall, 1993).

ProPO system and recognition proteins.

Phenoloxidase (PO) is a key enzyme in the reactions related to biological melanin formation, which catalyzes the hydroxylation of phenols to o-diphenols and deprotonation of o-diphenols to o-quinones. In invertebrates, PO normally exists as its precursor, prophenoloxidase (proPO), and works in various aspects of life such as sclerotization, pigmentation, wound healing and defense reactions.

Shrimp immunology is a key element in establishing strategies for disease control i.e. shrimp culture (Bachere, 1998). Shrimps have been shown to possess a primitive immune system that relies mainly on pro-phenol oxidase system, phagocytosis, encapsulation, agglutination and the lysis activity of the haemocytes (Smith and Soderhall, 1986). Central to any active cellular or humoral response to microbial or parasitic invasion is the initial recognition of foreign matter by the host. Crustaceans accomplish this through a complex cascade of serine proteases and other factors in the haemocytes that are specifically triggered by foreign molecules. This is known as the proPhenol Oxidase system or proPO system, and is confined to the

semi granular cells of the haemolymph. Pro-PO is activated in a stepwise process by microbial cell wall components such as β-1,3 glucans of fungi, or the lipopolysaccharide (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 *et al.*, 1987; Duvic and Soderhall, 1990). The pro PO 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 active phenoloxidase, which is needed to synthesize bactericidal melanin.

In crustaceans, glucans have been shown to activate prophenol oxidase in the haemolymph (Unestam and Soderhall, 1977; Soderhall *et al.*, 1990; 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 juveniles and adult shrimp to WSSV infections (Su *et al.*, 1995; Liao *et al.*, 1996).

One of the mechanisms available to bivalve molluscs for killing invading pathogens involves the release of highly reactive oxygen metabolites (Pipe, 1992). A number of methods for assessing the release of oxygen metabolites are available including reduction of NBT or cytochrome-C for O^{2-}, oxidation of phenol red for H_2O_2 and chemiluminescence.

It has been recognized that defense reactions in many invertebrates are often accompanied by melanization. In arthropods, melanin synthesis is involved in the process of sclerotization and wound healing of the cuticle as well as in defense reactions (nodule

formation and/or encapsulations) against invading microorganisms entering the hemocoel (Soderhall, 1982; Ratcliffe *et al.*, 1985; Sugumaran, 1996). The enzyme involved in melanin formation is phenoloxidase and has been detected in the haemolymph (blood) or coelom of both protostomes and deutereostomes, as well as in the cuticle of arthropods (Soderhall and Cerenius, 1998). PO is a bifunctional copper containing enzyme, which catalyses both the o-hydroxylation of monophenols and the oxidation of phenols to quinones (Sugumaran, 1996). Thus, this enzyme is able to convert tyrosine to DOPA, as well as DOPA to DOPA-quinone, followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment. PO is the terminal enzyme of the so-called proPO system, a non-self recognition system present in arthropods and other invertebrates (Soderhall, 1982; Ashida, 1990; Soderhall *et al.*, 1996). The activation of this prophenol cascade is exerted by extremely low quantities (pg/l) of microbial cell wall components (lipopolysaccharides (LPS), β-1,3-glucan or peptidoglycan (PG) and results in the production of melanin pigment, which can often be seen as dark spots in the cuticle of arthropods (Soderhall, 1982; Sugumaran and Kanost, 1993). During the formation of melanin, toxic metabolites are formed which have microbicidal activity (Soderhall and Ajaxon, 1982; St. Leger *et al.*, 1988; Rowley *et al.*, 1990; Nappi and Vass, 1993).

The PO activity is measured spectrophotometrically by recording the formation of dopachrome from L-dihydrophenylalanine (l-DOPA) at 490nm (Leonard *et al.*, 1985). PO can be obtained in different ways. The proPO system is released from haemocytes by incubating them with laminarin or zymosan as elicitor in presence of Ca^{2+} (Vargas-Albores *et al.*, 1993a; Le Moullac *et al.*, 1998). PO can

also obtain from cellular lysates containing inactivated proPO system; trypsin is used to activate the proPO to PO (Smith and Soderhall, 1991). The procedure PO activity assay has been simplified, the reaction carried out completely in microtiter plates (Hernandez-Lopez *et al.*, 1996).

Phenoloxidase, the major enzyme produced during the proPO system, is necessary for the melanization process observed in response to foreign matter. Although in insects the proPO system has been detected in both cells and plasma (Ashida 1981; Saul *et al.*, 1987; Brehelin *et al.*, 1989), in shrimps, as in other crustaceans, this system is confined inside granular haemocytes (Vargas-Albores *et al.*, 1993b). The shrimp proPO system can be activated directly by laminarin (Vargas-Albores *et al.*, 1993b), marking the role of this system against microbial infections.

Biochemical studies on shrimp proPO system has been carried out in *Penaeus californiensis* (Vargas-Albores *et al.*, 1993a, 1996; Hernandez-Lopez *et al.*, 1996; Gollas-Golvan *et al.*, 1999), *P. paulensis* (Perazzolo and Barracco, 1997), *P. stylirostris* (Le Moullac *et al.*, 1997) and *P. monodon* (Sritunyalucksana *et al.*, 1999b) in the penaeid shrimp, enzymes of the proPO system re localized in the semigranular and granular cells (Vargas-Albores *et al.*, 1993a; Perazzolo and Barracco, 1997). This is in agreement with a recent study showing that *P. monodon* proPO mRNA is expressed only in the haemocytes (Sritunyalucksana *et al.*, 1999a).

In shrimp, as in all crustaceans, a dark pigmented spot appears after an animal is injured. This is due to the action of phenoloxidase (PO), which promotes hydroxylation of phenols and oxidation of *o*-

phenols to quinones, necessary for the melanization process observed in response to foreign intruder in the hemocoel and during wound healing (Johansson and Soderhall, 1989; Ashida and Yamazaki, 1990; Soderhall, 1992; Soderhall *et al.*, 1994). Quinones are subsequently transformed, by a non-enzymatic reaction, to melanin and often deposited around encapsulated objects, in haemocyte nodules and at sites of fungal infections in the cuticle. Although a direct antimicrobial activity has been described for melanin and its precursors (Nappi and Vass, 1993), the production of reactive oxygen species such as superoxide anions and hydroxyl radicals during the generation of quinoids (Song and Hsieh, 1994; Nappi *et al.*, 1995) also has an important antimicrobial role. In addition, biological reactions such as phagocytosis, encapsulation and nodulation are also activated.

In the activation of the proPO system, an LPS-binding protein has also been proposed (Ashida *et al.*, 1983; Soderhall and Hall, 1984; Soderhall *et al.*, 1990; Hughes *et al.*, 1991). In addition, a β-glucan binding protein (BGBP) has been characterized from insects (Ochiai and Ashida 1988; Soderhall *et al.*, 1988) and crustaceans (Duvic and Soderhall 1990, 1993; Yoshida *et al.*, 1996; Thornqvist *et al.*, 1994). This protein induces activation of the proPO system after its reaction with β-glucans (Duvic and Soderhall, 1990, 1993; Yoshida and Ashida, 1986; Barracco *et al.*, 1991) and can also function as opsonin for hyaline cells (Thornqvist *et al.*, 1994).

PO is present in the haemolymph as an inactive pro-enzyme called proPO. The transformation from proPO to PO involves several reactions known as the proPO activating system. This system is specifically activated by BG (Soderhall and Unestam, 1979; Ashida *et*

al., 1983; Smith and Soderhall, 1983; Vargas-Albores, 1995; Vargas-Albores *et al.*, 1996, 1997), bacterial cell walls (Ashida *et al.*, 1983; Rowley and Rahmet-Alla, 1990) and LPS (Soderhall and Hall, 1984; Hernandez-Lopez *et al.*, 1996; Gollas-Galvan *et al.*, 1997). The proPO activating system is considered a constituent of the immune system and is probably responsible, at least in part, for the non-self recognition process of the defense mechanism in crustaceans and insects (Soderhall, 1982, 1992; Ashida and Soderhall, 1984; Ratcliffe, 1985; Ratcliffe *et al.*, 1985, 1991; Johansson and Soderhall, 1989, 1992; Ashida and Yamazaki, 1990; Soderhall *et al.*, 1990, 1994; Lanz *et al.*, 1993). Furthermore, the proPO system has been proposed as invertebrate counterpart of the vertebrate complement system since it can be activated by BG (Soderhall, 1982; Smith and Soderhall, 1983; Leonard *et al.*, 1985; Vargas-Al-bores *et al.*, 1993b), has a cascade reaction, and involves proteinases (Aspan *et al.*, 1990; Soderhall, 1992; Soderhall *et al.*, 1994). However, other than these similarities, no direct lytic activity of the proPO system has been detected.

Although LPS or β-glucans can activate directly the horseshoe crab coagulation cascade (Liang *et al.*, 1985; Muta *et al.*, 1991) and the proPO activating system of arthropods (Soderhall, 1982, 1992; Soderhall *et al.*, 1990; Vargas-Albores *et al.*, 1993b; Yoshida *et al.*, 1996), the presence and participation of plasma recognition proteins can amplify this activation.

The first immune process is the recognition of invading microorganisms, which is mediated by the haemocytes and by plasmatic proteins (Vargas-Albores and Yepis-Plascencia, 2000; Marques and Barracco, 2000). There is little information about the

molecular mechanisms that mediate recognition; however, in crustaceans, several types of modulator proteins have been described that recognize cell wall components of microorganisms. In freshwater crayfish *P. leniusculus*, a β-1,3 glucan-binding protein has been characterized and cloned, and a haemocyte receptor has been partially characterized that binds the plasmatic glucan-binding protein after the latter has reacted with β-1,3-glucan (Duvic and Soderhall, 1992). In the shrimp, *P. californiensis*, a protein that responds to β-1,3 glucan has been purified and characterized (Vargas-Albores *et al.*, 1996).

Measurement of plasma protein concentration

Crustaceans have an open circulatory system in which the haemolymph carries out several physiological functions. One of these functions is the transport of molecules such as the respiratory protein (haemocyanin), which is the most abundant molecule of the haemolymph (60% to 95% of total protein) (Djangmah, 1970) followed by the clotting protein and other humoral components. The measurement of plasma protein is based on Lowry's method (Lowry *et al.*, 1951).

Chisholm and Smith (1994) found a relation between the protein concentration and water temperature, showing low plasma protein concentrations when temperatures are at their lowest and highest in the year. The concentrations of total proteins are also related to the moult cycle of the shrimp. In *P. japonicus*, Chen and Cheng (1993) have reported lower levels of protein concentration during postmoult stage (41.37 mg/ml) as opposed to higher levels (74.90 mg/ml) found in early premoult (D0). In apparently healthy *P.*

vannamei juveniles reared under laboratory conditions the plasma protein concentration is around 120 mg/ml (unpublished data), but this concentration changes under different environmental conditions. Engel *et al.* (1993) reported a negative effect of low levels of dissolved oxygen on haemocyanin concentration in serum of the blue crab, *C. sapidus*.

To undertake health monitoring on farms, it is necessary to monitor immune status of animals to know the relationships between environmental conditions and normal or abnormal values of immune responses of shrimp. The use of resistance criteria opens many research possibilities: survivors of experimental infections and shrimp with high level of expression of resistance markers could be used in the selection of broodstock.

6.2. MATERIALS AND METHODS

Microorganisms used

Based on the results obtained through the feeding experiments, two marine yeasts *Debaryomyces hansenii* S8 and *Candida tropicalis* S186 were selected for this study. *Saccharomyces cerevisiae* (MTCC 36) was also included as a reference strain.

Preparation of yeast biomass

Yeast biomass was prepared as given in 5.2.1

Preparation of experimental feeds

Experimental feed preparation was done as described in 5.2.4. Four different feeds were prepared incorporating the two different

marine yeast biomass, Baker's yeast *S. cerevisiae* and the control feed (without the yeast biomass). These feeds were air-dried over night and stored at -20^0 C in a freezer. (Fig. 6.1)

Feeding experiment

Adults of Indian white prawn (*Fenneropenaeus indicus*) were brought from a farm in Kannamali, Cochin, acclimatized to laboratory conditions and maintained on the control diet for a period of one week (Fig.6.2). They were distributed in aquarium tanks. Feeding experiment was carried out for 14 days. Physico-chemical parameters of the rearing water were monitored daily (Table.6.1). Salinity, NH_3N, NO_2-N and dissolved O_2 were estimated as per APHA (1995).

Fig. 6.2 Fenneropenaeus indicus adults used for the experiment

Challenge of the experimental animals with white spot virus

After the termination of the feeding experiment (14 days) all the treatment groups including the control were maintained under the same rearing conditions mentioned earlier. Challenge with White spot

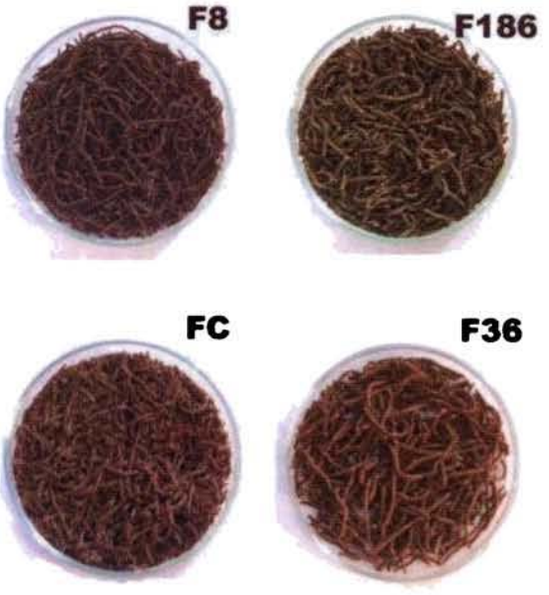


Fig. 6.1 Experimental diets prepared by incorporating the yeast biomass

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 (*Fenneropenaeus indicus* adult). 24 hours after challenge, they were maintained on the test feeds and survival was noted everyday till the 10th day post challenge.

Table .6.1. Rearing conditions and Water quality parameters of the experimental system

Rearing and feeding conditions	
Initial body weight (average)	: 40-60g
Number of prawns stalked	: 50
Tank capacity	: 1000L
Feeding level	: 10-15% body weight
Feeding frequency	: Twice daily
Feeding period	: 14 days
Water Quality Parameters	
Water temperature	: 28 - 30°C
pH	: 7 - 7.5
Salinity (ppt)	: 26 - 28ppt
NH_3 (mg/L)	: 0.01 - 0.02mg/L
NO_3 (mg/L)	: n.d
NO_2 (mg/L)	: <0.01mg/L
Dissolved O_2	: 7-8mg/L

n.d = not detectable

Haematological parameters

Haemolymph was taken from *Fenneropenaeus 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), 24 hr post challenge, 48 hr post challenge, 72 hr post challenge, 5 days post challenge and 7 days post challenge.

Haemolymph was drawn from 4 animals maintained on a particular test diet (n=4) and the various assays were done.

Preparation of the anticoagulant buffer solution.

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

Haemolymphcollection

Haemolymph was drawn from the rostral sinus of the animals. The spot for haemolymph withdrawal was wiped with sterile cotton swabs and using specially designed glass capillary tubes (rinsed thoroughly with anticoagulant) haemolymph was withdrawn and transferred to an eppendorf rinsed with anticoagulant and maintained at 4^{0}C till further analysis. Haemolymph of four animals from each treatment were taken for the analysis (n=4).

The following parameters were then analysed.

1. Total haemocyte count
2. Phenol oxidase
3. Nitroblue tetrazolium reduction
4. Alkaline phosphatase
5. Acid phospahatase

1. Total haemocyte count

An aliquot of fresh haemolymph soon after withdrawal was placed on Neubauer haemocytometer for enumeration of total circulating haemocytes. The result was expressed as number of cells per ml haemolymph.

2. Phenoloxidase activity

Phenoloxidase activity was estimated using L-dihydroxyphenylalanine (L-DOPA, Sigma) as substrate. 100µl haemolymph was taken in an eppendorf and 100µl 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, pH 7) solution. SDS was used to activate the pro-enzyme (Prophenoloxidase). The absorbance at 420nm was recorded every 30 seconds, 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).

Haemolymphprotein

To 100µl haemolymph 1.9 ml ethanol was added and centrifuged. The supernatant was discarded and the residual pellet consisting of precipitated haemolymph protein was dissolved in 1 N NaOH and used for protein estimation by Bradford method (1976).

3. Intracellular super oxide anion (NBT) assay

This test allows to indirectly assess the intracellular superoxide anion 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. 100µ NBT (2mg/ml NBT in Tris-HCl buffer, pH. 7.6) solution was added to the samples and incubated at room temperature for one hour. After incubation, the samples were centrifuged at 7000 rpm for 10 minutes. 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 minutes. The supernatant was again discarded after centrifugation and the tubes were dried in vacuum desiccators 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 60µ KOH. 70µ DMSO was added and mixed well. 2 ml of distilled water was added to this coloured solution to make it upto a readable volume. The optical density was read at 620nm in a Hitachi 200UV-Visible spectrophotometer against a blank. The blank was prepared using all reagents in the same volume with equal volume of distilled water instead of haemolymph. The optical density was expressed per mg haemocyte protein.

Haemocyte protein

100µl haemolymph was centrifuged at 2000 rpm at 4°Cin a refrigerated centrifuge (Remi, C-30, Mumbai) and washed with phosphate buffered saline twice and the supernatant was decanted. 0.1 ml of 1N NaOH was added to the residual pellet to dissolve and the estimation was done by Bradford method.

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 haemolymph solution was added to 2ml of *ρ*-

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

5. Acid phosphatase assay

Acid phosphatases were assayed according to Reichardt *et al.* (1967) using *ρ*-nitrophenyl phosphate a colourless substrate that produces a colorimetric end-product p-nitrophenol. The buffer-substrate mixture for the assay was prepared by dissolving 0.5% in citrate buffer. The mixture was incubated at 37°C for 30 minutes. At the end of the incubation period the enzyme reaction was terminated by adding 2.9ml of 0.1 N NaOH. The yellow coloured solution was read against a blank at 405nm. The blank is prepared by incubating a mixture of 2.9ml of 0.1N NaOH and 2ml of substrate solution to which finally 100µl of haemolymph was added.

6.3. RESULTS

Generally the performance was best with the two marine yeast incorporated feeds, F8 and F186. The performance of Baker's yeast was almost the same as that of the control feed.

6.3.1 Post challenge survival

Post challenge survival was found to be significantly high with *F.indicus* maintained on feed F8 compared to other feeds (Fig. 6.3). 3rd

day post challenge data was almost similar for feeds F186 and FC. However, on the 7th day the survival percentage of *Fenneropenaeus indicus* reduced considerably for all the feeds. 9th day post challenge survival was found to be significantly high for feed F8. Death by WSSV infection was confirmed by the presence of white spots on the carapace of the infected prawns.

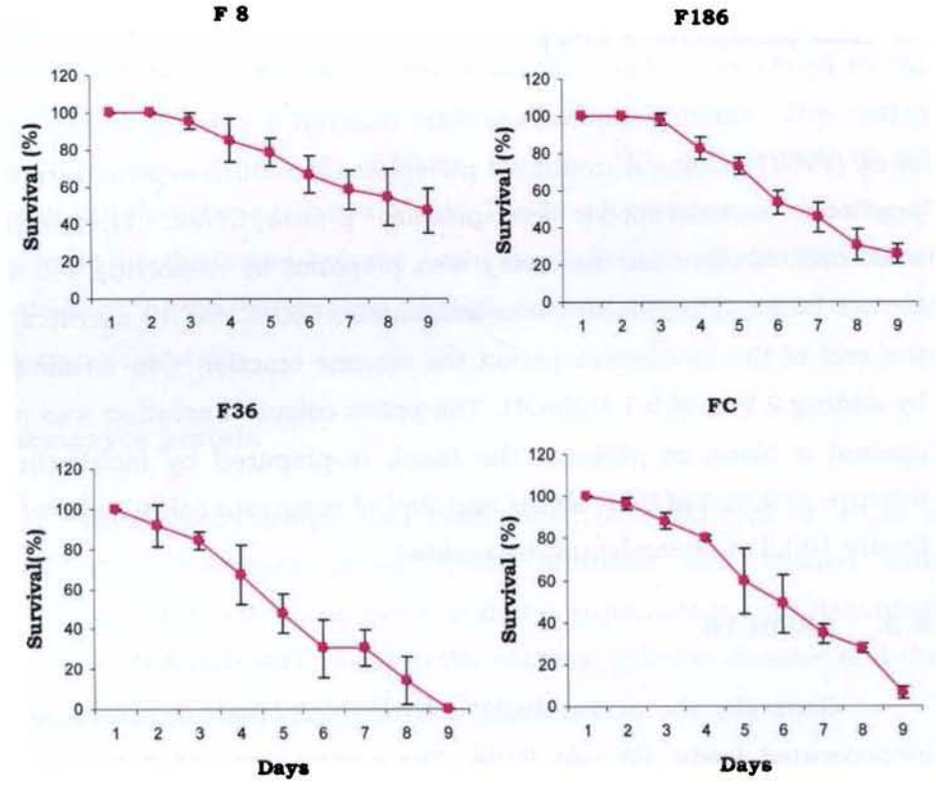
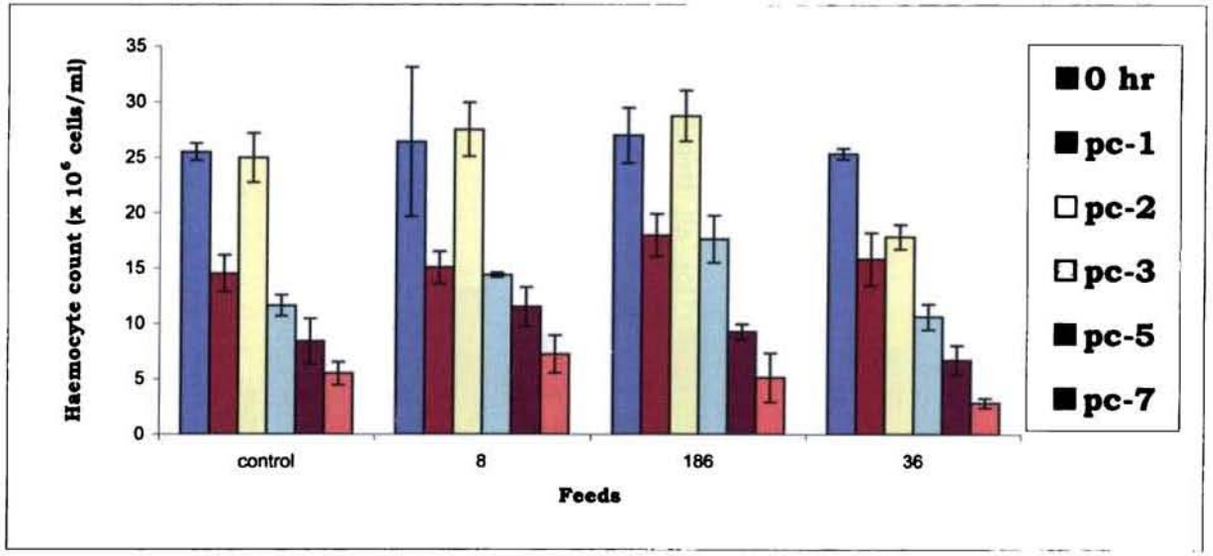


Fig.6.3 Post challenge survival of F.indicus adults maintained on yeast incorporated diets while infected with White Spot Virus (via diet)



Days	FC	F8	F186	F36
0 hr	25.49^{a}	26.41^{a}	27.01^{a}	25.34^{a}
pc-1	14.525^{a}	15.0725^{ab}	17.99^{b}	15.8225^{ab}
pc-2	24.9725^{b}	27.54^{bc}	28.755^{c}	17.85^{a}
pc-3	11.6275^{a}	14.4175^{b}	17.64^{c}	10.615^{a}
pc-5	8.4025^{ab}	11.5225^{c}	9.265^{bc}	6.7175^{a}
pc-7	5.5^{b}	7.235^{b}	5.1175^{ab}	2.835^{a}

Fig.6.4.Haemocyte count of Fenneropenaeus indicus fed on different experimental diets and challenged with White Spot Virus (via diet)

0hr : Just before challenge, PC-1 : Post challenge 1 day, PC-2 : Post challenge 2 day, PC-3 : Post challenge 3 day, PC-5 : Post challenge 5 day, PC-7 : Post challenge 7 day.

6.3.2 Haematological parameters

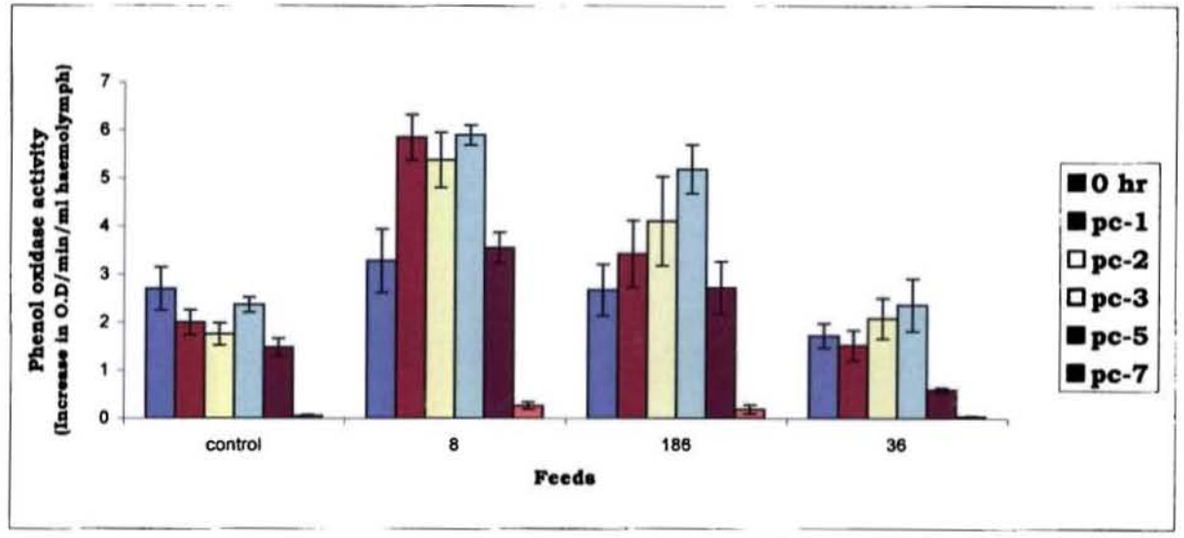
Feed F8 exhibited significant immunostimulatory property being evidenced by better post challenge survival and haematological profile supporting an immune boost up. The other feeds F36 and FC did not show a significant increase in the performance compared to F8.

6.3.2.1Haemocyte count

No remarkable change could be observed in the haemocyte count between the various treatment groups (Fig.6.4). However, the prawns fed on marine yeast incorporated feeds showed a marginal increases on haemocyte count. Uniformly for all treatment groups reduction in the haemocyte could be observed after 24 hours followed by an increase at 48 hours and subsequent lowering of the haemocyte count resulting in very low count on 7th day.

6.3.2.2 Phenoloxidase activity

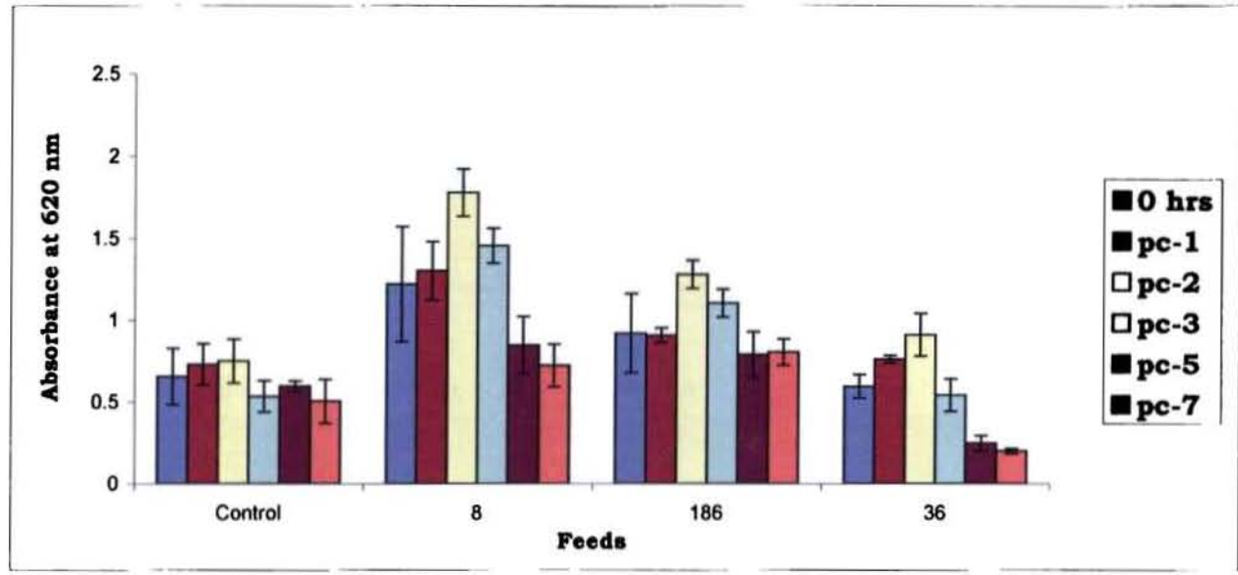
The performance of prawns fed on feed F8 was best followed by F186 (Fig.6.5). No significant difference could be observed in the performance of F 36 with that of the control. An increase in phenol oxidase activity could be observed at post challenge for the prawns fed on marine yeast incorporated feeds (F8, F186) reaching a maximum on 3rd day followed by a decrease culminating in very low values on 7th day.



Days	FC	F8	F186	F36
0 hr	0.80925[b]	0.98375[b]	0.80175[b]	0.5175[a]
pc-1	0.59875[a]	1.755[c]	1.02875[b]	0.45625[a]
pc-2	0.53175[a]	1.615[c]	1.23325[b]	0.6255[a]
pc-3	0.70875[a]	1.771[c]	1.557[b]	0.71125[a]
pc-5	0.44175[b]	1.06625[d]	0.81625[c]	0.1775[a]
pc-7	0.015[a]	0.07625[b]	0.0555[b]	0.0125[a]

Fig.6.5 Phenoloxidase activity of Fenneropenaeus indicus fed on different experimental diets and challenged with White Spot Virus (via diet)

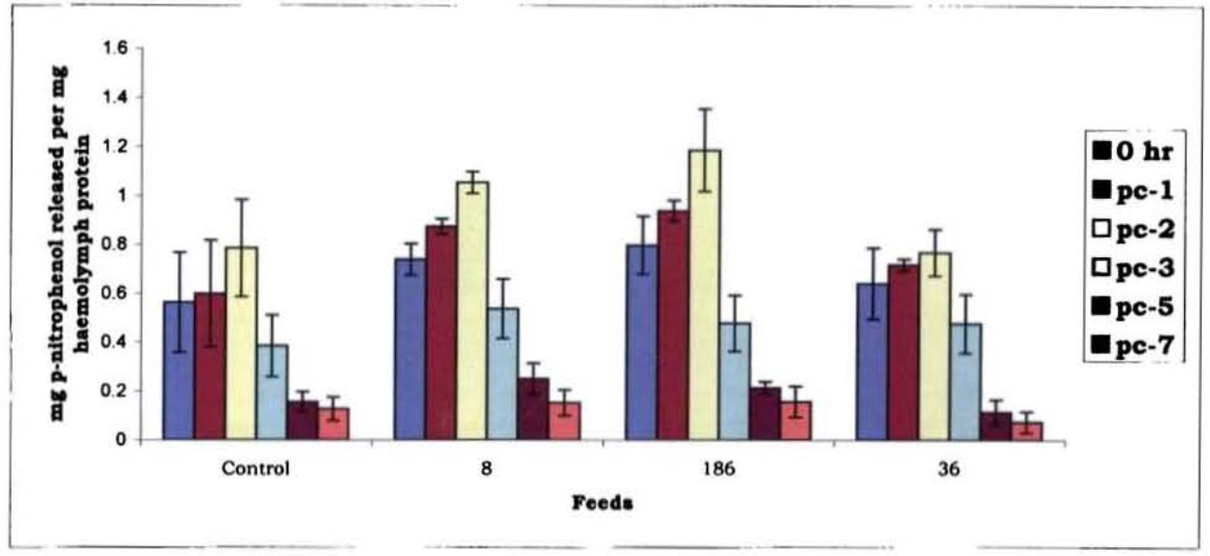
0hr : Just before challenge, PC-1 : Post challenge 1 day, PC-2 : Post challenge 2 day, PC-3 : Post challenge 3 day, PC-5 : Post challenge 5 day, PC-7 : Post challenge 7 day.



Days	FC	F8	F186	F36
0 hrs	0.65625[a]	1.2225[b]	0.92175[ab]	0.59625[a]
pc-1	0.73125[a]	1.305[b]	0.909[b]	0.76125[a]
pc-2	0.75125[a]	1.78175[c]	1.2825[b]	0.91125[a]
pc-3	0.535[a]	1.4575[c]	1.1075[b]	0.54225[a]
pc-5	0.5975[b]	0.85[c]	0.7925[c]	0.2475[a]
pc-7	0.505[b]	0.725[c]	0.8075[c]	0.19975[a]

Fig.6.6 NBT activity of Fenneropenaeus indicus fed on different experimental diets and challenged with White Spot Virus (via diet)

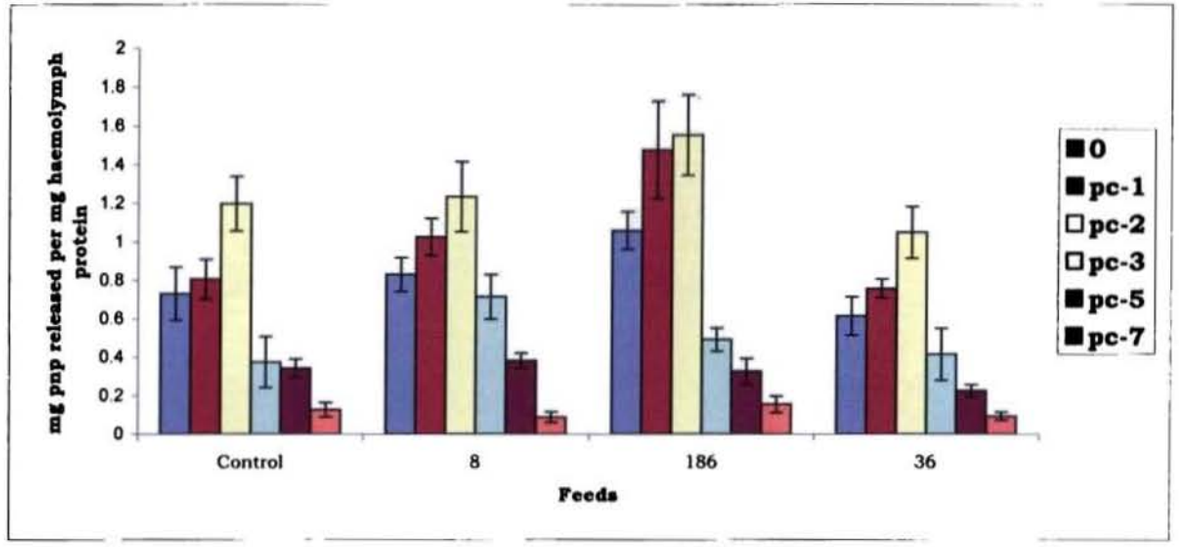
0hr : Just before challenge, PC-1 : Post challenge 1 day, PC-2 : Post challenge 2 day, PC-3 : Post challenge 3 day, PC-5 : Post challenge 5 day, PC-7 : Post challenge 7 day.



Days	FC	F8	F186	F36
0 hr	0.562^{a}	0.739^{a}	0.798^{a}	0.64125^{a}
pc-1	0.59775^{a}	0.87425^{bc}	0.939^{c}	0.7185^{ab}
pc-2	0.78425^{a}	1.05415^{ab}	1.18645^{b}	0.7685^{a}
pc-3	0.3845^{a}	0.53725^{a}	0.47825^{a}	0.47755^{a}
pc-5	0.1555^{ab}	0.25^{c}	0.21325^{bc}	0.1145^{a}
pc-7	0.1265^{a}	0.15175^{a}	0.15575^{a}	0.07375^{a}

Fig.6.7. Alkaline Phosphatase activity of Fenneropenaeus indicus fed on different experimental diets and challenged with White Spot Virus (via diet)

0hr : Just before challenge, PC-1 : Post challenge 1 day, PC-2 : Post challenge 2 day, PC-3 : Post challenge 3 day, PC-5 : Post challenge 5 day, PC-7 : Post challenge 7 day.



Days	FC	F8	F186	F36
0	0.72975^{ab}	0.83^{b}	1.05975^{c}	0.6145^{a}
pc-1	0.8055^{ab}	1.02575^{b}	1.47725^{c}	0.756^{a}
pc-2	1.19725^{a}	1.2345^{a}	1.55325^{b}	1.04725^{a}
pc-3	0.37425^{a}	0.71475^{a}	0.49275^{a}	0.415^{a}
pc-5	0.3425^{a}	0.38275^{b}	0.32725^{b}	0.22475^{a}
pc-7	0.12675^{ab}	0.08775^{a}	0.15525^{b}	0.093^{a}

Fig.6.8 Acid Phosphatase activity of Fennerpenaeus indicus fed on different experimental diets and challenged with White Spot Virus (via diet)

0hr : Just before challenge, PC-1 : Post challenge 1 day, PC-2 : Post challenge 2 day, PC-3 : Post challenge 3 day, PC-5 : Post challenge 5 day, PC-7 : Post challenge 7 day.

6.3.2.3 NBT Activity

NBT reduction was found to be considerably better for prawns fed on marine yeast incorporated feeds compared to Baker's yeast and the control feed (Fig.6.6). Unlike the phenol oxidase activity the maximum performance could be observed at 48 hours followed by a gradual decrease over the 7 day observation period.

6.3.2.4 Alkaline phosphatase Activity

Considerable increase in alkaline phosphatase activity could be observed in prawns fed on marine yeast incorporated feeds F8 and F186 % (Fig 6.7). Generally in all treatment groups maximum activity could be observed on 2nd day followed by lower activity during the ensuing days resulting in very low values in 7th day. Performance of Baker's yeast incorporated feed F36 was slightly better compared to the control feed.

6.3.2.5 Acid phosphatase Activity

Maximum acid phosphatase activity was exhibited by F186 (*Candida tropicalis*) incorporated feed followed by F8 (*Debaryomyces hansenii*) (Fig.6.8). In this case also the activity was maximum at 48 hours post challenge followed by a sharp decrease resulting in very low values on 7th day.

6.4 DISCUSSION

Aquaculture production is directly related to growth and the survival of animals in the culture ponds and losses attributed to bacterial, viral and fungal diseases remain an important concern to

the industry. Immunostimulants have been reported to increase resistance to these infectious diseases in teleost fish and shellfish by enhancing the non-specific immune system.

In this study the potency of two marine yeasts as immunostimulant to *Fenneropenaeus indicus* was studied. The immunological assays employed were haemocyte count, phenol oxidase activity, NBT reduction, alkaline and acid phosphatase assay. When the experimental animals were challenged with white spot virus, maximum survival could be obtained in *Fenneropenaeus indicus* fed with marine yeast incorporated diet compared to that of Baker's yeast diet and the control diet. Yeasts are rich nutritionally with proteins, vitamins and carbohydrate and this might have contributed to better health and resistance to the pathogens. Better performance by both the marine yeasts compared to *S. cerevisiae* may be due to the presence of desired nutritional components including minerals in the rate and proportion required for penaeid prawns. Cell wall component, β-1,3 glucan also might have contributed to the improved immune response exhibited by the yeast fed animals compared to the controls. Burgents *et al.*, 2004 have reported the disease resistance in *Litopenaeus vannamei* following the dietary administration of yeasts. It was found that when the animals were challenged with *Vibrio* after the administration of yeast incorporated diets for 3 weeks, the survival was significantly high compared to the control. In the present study a significant increase in the disease resistance of marine yeast fed animals could be observed compared to the control and *S. cerevisiae* fed yeasts. Both the marine yeasts used in this study had higher β-1,3 glucan content (*D. hansenii* S8-16.73% and *C. tropicalis* – 16.23%) as per Vrinda (2002). This higher glucan content can be one of the

reasons for the better immunostimulation effected in prawns by these marine yeasts. Andlid *et al.* (1995) showed that yeast may improve fish health as antagonists to pathogens and by immunostimulation. A detailed analysis of the biochemical composition of these yeasts is essential to elucidate the reason for this differential efficacy in immunostimulation. Marine yeasts are rich in minerals and these minerals might be imparting immunostimulation Pipe *et al.* (1995) reported that cadmium at 400 ug /l resulted in significantly enhanced number of circulating haemocytes and increased uptake of neutral red in marine mussel, *Mytilus edulis*.

Presence of higher amounts of β-1,3 glucan in the cell wall of both the marine yeasts could be another reason for better immunostimulation. β-1,3 glucan of fungi and yeasts have already been proved as an effective immunostimulant in fin fishes and shell fishes against microbial infection. Sung *et al.* (1994) found that immersion of *P. monodon* in high concentration of glucan (>1mg/ml) had adverse effects on shrimp, causing tissue damage and lower resistance to infection. Song *et al.* (1997) have reported an enhanced resistance of *P. monodon* to Vibriosis and WSSV infection by the application of β-1,3 glucan. Oral administration of Beta glucan at 2g kg^{-1} diets for 10-20 days significantly induced the resistance of post larvae, juveniles and adult shrimp, *P. monodon* to *Vibrio damsela*, *Vibrio harveyi* and WSV infection (Su *et al.*, 1995; Liao *et al.*, 1996; Chang *et al.*, 1999; Chang *et al.*, 2000 and Chang *et al.*, 2003). Dose and frequency of administration of immunostimulants are very much important since overdose can cause immunosuppression and mortality succumbing to infection and challenge by pathogens. In this study yeast biomass was incorporated at 10% level in the diet and was

administered *ad libitum* on all the days. Glucan at a dose of 0.1% in the diet was found to be optimal for immunostimulation in *P.monodon*. Scholz *et al.* (1999) studied the efficacy of 5 different yeast supplemented diets in prawns and found that *Phaffia rhodozyma* supported better performance in terms of bacterial clearance in prawns. Burgents *et al.* (2004) found that 1% yeast (dried) in the diet imparted maximum resistance in *Litopenaeus vannamei* against white spot virus infection.

In the present study, the immunological parameters *viz*, haemocyte count, NBT reduction and the alkaline and acid phosphatase activity was uniformly found to be maximum at day 2 post challenge whereas phenol oxidase activity was maximum at day 3. This pattern of enhancement in immune parameters has not been reported earlier in shrimp on challenge with pathogens. However, a sudden depression in the haemocyte count, NBT activity and phenol oxidase post challenge could be observed by Chang *et al.* (2003). Immunological parameters were found to be at a low level even on day 6 post challenge followed by a gradual increase showing higher values at day 9, 12 and 24. These observations are not in agreement with our findings when we have witnessed a depression immediately after challenge (day 1) only in haemocyte count and all the other immunological parameters showed an increase. This continued up to day 2 and gradually decreased further recording very low values on day 7 uniformly for all the parameters except for phenol oxidase for which the maximum was observed on day 3.

The present study shows that acid and alkaline phosphatase activity also can be used as a reliable index in the assessment of

immune status in penaeid prawns. Lysosomal enzymes play an important role in the lysis of phagocytosed particles and therefore elevated the acid and alkaline phospahatse in haemolymph indicate higher defense potential of the infected animals.

On challenge with white spot virus, about 50% survival could be obtained on 7th day for prawns fed on marine yeast incorporated diet. In a similar study Burgent *et al.* (2004) could obtain about 50% survival on 6th day in *P. vannamei* fed on yeast-incorporated diet. They found that the percentage of survival remained the same after 9th day post challenge without any mortality up to 24 days whereas the 100% mortality could be observed in the case of controls on the 9th day itself.

The present study shows that the marine yeasts are a good source of immunostimulants with potential application in aquaculture. Utilization of whole cell yeast instead of the cell wall component β-1,3 glucan warrants much importance due to the nutritional quality of the yeast biomass. While extracting glucan from the cell wall all other nutritional components are lost, besides time, energy and production cost. However, a study on the comparative efficacy of yeast (whole cell) and the cell wall component β-1,3 glucan as immunostimulant to prawns should be made before making any conclusion in this matter. Landolt (1989) has reported that nutritional factors especially vitamins and minerals play an important role in enhancing immunity in fishes.

White spot virus is a highly virulent pathogen causing 100% mortality within 4-7 days of infection. Application of these immunostimulants helps to enhance the disease resistance through stimulation of the non-specific immune system of the prawns.

Enhanced disease resistance resulting in reduced mortality in prawns itself would be a big relief to the farmers since they get sufficient time to plan for a harvest and thereby avoid crop loss to a considerable extent.

Summary and Conclusion

Feed is a major concern for the shrimp farmers, representing upto 60% of the total variable production costs. The main ingredient of the feed as in many cases is protein. In aquaculture, the primary dietary animal protein source is fishmeal, but its availability is limited and supply varies due to various reasons. This can seriously affect sustainability and profitability. Yeasts are a rich source of proteins and B-complex vitamins and have been used as a supplement in animal feed and are recommended as a substitute for other ingredients in fishmeal

In addition, they are considered as a cheap dietary supplement as they are easily produced on an industrial level from a number of carbon rich substrate by products such as citrus pulp, molasses, paper industry waste, and fruit waste as well as from hydrocarbons. Yeasts can be produced much more efficiently and economically than the presently used photosynthetic algae because of shorter generation time and the use of inexpensive culture.

The present work was undertaken to study the potential of marine yeasts as a source of single cell protein and immunostimulant for application in aquaculture. The results of the present study are summarized as follows:

- Totally about 260 marine yeasts belonging to 22 genera could be isolated out of which *Candida* was the most predominant followed by *Filobasidium, Leucosporidium, Mastigomyces, Lodderomyces* etc.

- *Candida, Leucosporidium, Mastigomyces, Rhodotorula, Schizosaccharomyces, Kluyveromyces, Torulaspora, Oosporidium and Dipodascus* were mainly isolated from the offshore waters. *Lodderomyces, Hormoascus, Cryptococcus, Williopsis, Saccharomycopsis, Lipomyces, Geotrichum* and *Arxioxyma* were isolated only from the coastal waters.

- Yeast isolates were found to be with good hydrolytic potential especially with protease (63.1%) and lipase (61.5%) production. *Mastigomyces, Candida* and *Filobasidium* were the potent genera in enzyme production.

- Protein content of yeast biomass of various genera was found to be in the range of 22-30% and the maximum was encountered in S169 (30.45%) belonging to *Debaryomyces* sp. Lipid content varied between 2 to 8.25% the maximum being in S28 (*Kluveromyces* sp.). Carbohydrate content of yeast biomass was in the range 22.36 tto29.68%.

- Protein, lipid and nitrogen free extract (NFE) of the feeds were in the range 40.2 to 55.4%, 7 to 11.2% and 20.9 to 36.8% respectively.

- All the yeast biomass incorporated feeds supported better biogrowth parameters (production, FCR, SGR, GGE) compared to the control feed. F8 and F186 gave the best performance in terms of biogrowth parameters followed by F165 and F100.

- Statistical analysis (Duncan's multiple range test) showed that the performance of yeast incorporated experimental feeds varied significantly from that of control feed ($P<.05$).

- Generally a salinity of 30ppt was preferred by various strains for growth. Growth was favoured by a salinity of 25ppt for S100 and 20ppt for S165.

- Molasses at a concentration of 9mg/ml (total sugars) was found to be optimum for growth of the selected strains.

- Generally a pH of 6 was preferred by various strains for growth. A pH of 7 favoured growth of S 186.

- The optimum peptone concentration for growth was found to be 0.75% for all four strains.

- The optimum yeast extract concentration for growth was found to be 0.5% for all four strains.

- Generally the optimum magnesium sulphate concentration for growth was found to be 0.25% for S8, S100 and S186. Growth was also favoured at 0.5% for S165.

- The optimum potassium dihydrogen phosphate concentration for growth was found to be 0.3% for S8, S100 and S165. For S186, 0.2% was found to be favourable for maximum for growth.

- The optimal calcium chloride concentration for growth was found to be 0.15% for all the strains.

- Based on the preliminary feeding experiment on *P. indicus,* S8, S100, S165 & S186 were selected for further study. In addition, Baker's yeast *S.cerevisiae* was also included in study for comparison.

- No significant variation in proximate composition could be observed in prawns fed yeast diets, commercial diet and control diet. Protein and lipid content was found to be maximum (70.21% and 12.41% respectively) in prawns fed F8 and carbohydrate with feed F100 (1.07%).

- All the three marine yeast incorporated feeds were superior in performance compared to the commercial feed and control feed. Feed F8 incorporated with the biomass of *Debaryomyces hansenii* S8 supported the best performance followed by F186, F100 and commercial feed. FCR of 1.35 to 2.00 could be obtained with the marine yeast incorporated feeds where as for the commercial feed it was 2.6 and *S. cerevisiae* incorporated feed recorded 2.96.

- Production (weight gain) in marine yeast fed prawns were almost double that of other feeds used in the study. Specific growth rate (SGR) was about 7 with the marine yeast diets compared to 6 that of Baker's yeast diet and commercial diet.

- Gross Growth Efficiency (GGE) was approximately in the range 60-75 for marine yeast feeds whereas for F36, CF and control the values were almost half of it.

- Relative Growth Rate (RGR) did not vary much among the various feeds (0.37 to 0.5).

- Considerable variation could be observed in the Protein Efficiency Ratio (PER) of the various feeds, the maximum being observed for F8 (1.7) followed by F186 (1.23), F100 (0.97), CF (0.81), F36 (0.75) and control feed (0.73).

- Consumption per unit weight per day (CUD) was found to be comparatively low for marine yeast incorporated feeds F8 (0.07), F100 (0.09) and F186 (0.08) whereas for the other three feeds (CF, F36 and control feed) the value ranged from 0.11 to 0.13.

- Histological analysis of the hepatopancreas did not show any structural or functional abnormalities with feed F8, F100, F186, F36, commercial feed and control feed.

- Based on the results obtained from the growth studies and post challenge survival, two marine yeasts were selected for the immunostimulant aspects of the study.

- No remarkable change in the haemocyte count could be observed between the various treatment groups. A remarkable reduction in the haemocyte count could be observed at 24 hours after infection followed by a remarkable increase at 48 hours and subsequent lowering resulting in a very low count on 7^{th} day.

- An increase in phenol oxidase activity could be observed at post challenge for prawns fed on marine yeast incorporated feeds.

PO activity was found to be maximum at 3rd day post challenge followed by significant decrease ensuring days.

- An increase in NBT reduction could be observed in prawns fed on marine yeast incorporated feeds on post challenge with white spot virus. Maximum NBT reduction could be observed on 2nd day post challenge with a gradual decrease during the following days.

- Acid phosphatase activity was maximum in prawns fed on S186 (*Candida tropicalis*) incorporated feeds followed by S8 (*Debaryomyces hansenii*). The activity was maximum on 2nd day. Post challenge with white spot virus followed by a sharp decrease resulting in very low values on 7th day.

The study revealed the potential of marine yeasts as a source of single cell protein and immunostimulant for prawns. Prawns fed with the selected marine yeasts were showing more growth compared to the control feed and commercial feed. Yeasts being rich with proteins, vitamins and carbohydrates serve as a growth promoter for prawns as being evidenced in this study. The better performance of marine yeasts, *D. hansenii* S8 and S100 and *C. tropicalis* S186 compared to *S. cerevisiae* S36 as a feed supplement is worth investigating. Besides being a rich nutritional source, yeasts act as immunostimulants by virtue of its high carbohydrate (β,1-3 glucan) and RNA content. β, 1-3 glucan, a cell wall component of yeasts/fungi is the most commonly used immunostimulant in aquaculture. The present study shows that even the whole cell yeast could serve as a good immunostimulant when supplied through diet. Extraction of β-1,3 glucan results in the removal of nutrients like proteins, vitamins etc. from the cell biomass.

Utilization of the yeast biomass as such in the diet would help perform a dual role as nutritional component and immunostimulant for aquaculture applications.

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Appendix 1

Table. A.1. Proximate composition of yeast biomass

Culture No.	Genera	Proximate composition (%)		
		Protein	Lipid	Carbohydrate
S 3	*Williopsis*	22.13	2.15	22.45
S 8	*Debaryomyces*	23.45	3.16	23.56
S 13	*Lodderomyces*	24.56	4.25	26.48
S 28	*Kluveromyces*	23.35	8.25	25.68
S 30	*Aciculoconidia*	22.56	2.31	22.93
S 42	*Rhodotorula*	25.23	3.14	26.53
S 48	*Lodderomyces*	26.55	3.62	22.36
S 50	*Cryptococcus*	24.35	4.25	26.45
S 56	*Pichia*	26.22	4.22	23.47
S 69	*Lipomyces*	27.33	6.35	24.86
S 70	*Hormoascus*	27.85	2.85	29.68
S 81	*Arxioxyma*	25.36	3.26	25.89
S 87	*Debaryomyces*	25.42	4.22	24.63
S 100	*Debaryomyces*	28.65	6.78	27.89
S 165	*Candida*	27.89	3.65	24.69
S 169	*Debaryomyces*	30.45	3.25	25.68
S 170	*Dekkera*	23.45	6.54	24.36
S 186	*Candida*	24.95	2.53	26.27
S 297	*Leucosporidium*	24.22	3.65	23.45
S 303	*Torulaspora*	27.89	4.58	26.89
S 382	*Candida*	25.36	2.69	28.96
S 394	*Leucosporidium*	23.48	3.96	25.63
S 425	*Dipodascus*	26.45	5.86	22.56
S 434	*Filobasidium*	27.89	3.65	23.54
S 437	*Mastigomyces*	25.65	4.31	25.65