MARINE ACTINOMYCETES AS SOURCE OF ANTIMICROBIAL COMPOUNDS AND AS PROBIOTICS AND SINGLE CELL PROTEIN FOR APPLICATION IN PENAEID PRAWN CULTURE SYSTEMS

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COCHIN - 682 016
2003

Dedicated to

My beloved parents,
My dear guide, Dr. Rosamma Philip
and
To the love and sacrifice of Aneesh and Anagha.

DECLARATION

I hereby do declare that the thesis entitled "Marine Actinomycetes as Source of Antimicrobial Compounds and as Probiotics and Single Cell Protein 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

Cochin- 682016
February 2003

Biji Mathew

Certificate

*This is to certify that the thesis entitled "**Marine Actinomycetes as Source of Antimicrobial Compounds and as Probiotics and Single Cell Protein for Application in Penaeid Prawn Culture Systems**" an authentic record of research work carried out by Ms. Biji Mathew 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.*

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

Ocean harbours more than 80 percent of all life on earth and remains our greatest untapped natural resource. Marine environment occupies 71 % of the earth's surface and the future of the world population depends on this environment for its food and other life-saving wonder drugs (Mary *et al.*, 1998). Hence, utilization of marine resources for developmental purpose has gained considerable attention in recent times. The biological diversity of the marine environment offers enormous scope for the discovery of novel natural products, several of which are potential targets for biomedical development (Austin, 1989; Fenical, 1997). The vast majority of antibiotics have come from terrestrial microorganisms. However these microorganisms continue to be studied extensively, the rate of discovery of novel metabolites from terrestrial microorganisms is decreasing (Bentley *et al.*, 2002). Discovery and identification of new sources of natural products, therefore, plays an important role in the unraveling of novel drug candidates and drug development processes.

Marine organisms are capable of surviving and growing in habitats of extremes. In addition to high salinity, many of them have to face high hydrostatic pressure and low or high temperature. Much of the biota in tropical seas possesses specific traits for survival in highly competitive habitats. In order to survive and grow in a highly competitive habitat, many organisms must compete for the limited resources. A variety of offensive and defensive mechanisms have evolved to allow organisms to gain selective advantage and to cope with competitors. The physiological manifestation of these defense abilities of marine organisms is in the form of bioactive metabolites. Almost every class of marine microbes produces a variety of bioactive compounds with unique structural features and often exhibits

processes distinct from those of the terrestrial environment. The bioactive metabolites are excellent candidates for a variety of applications in the pharmaceutical, agricultural and food industries.

The ocean cover contain over 2, 00,000 invertebrate and algal species (Mc Connet *et al.*, 1990). These organisms live in complex communities and in close association with other organisms both macro (algae, sponges,) and micro (non filamentous bacteria, fungi and actinomycetes) organisms (Wright, 1998). Because of the diversity of marine organisms and habitats marine natural products encompass a wide variety of chemical classes (Wright, 1998).Among the multitude of diverse organisms in the marine environment; marine microorganisms stand out as excellent source of many useful metabolites. It is well understood that marine microorganisms have been largely unexplored as a source of bioactive agents for industrial applications.

Of all the available marine forms, the actinomycetes merit special consideration in view of the proven biosynthetic capabilities of numerous isolates from soil. Marine actinomycetes however have not received the attention accorded to their terrestrial counterparts. As has already been expressed by Goodfellow and Hynes (1984), the former may comprise a selected gene pool possibly containing organisms with the potential to yield metabolic substances. The investigation held out on marine actinomycetes as sources of potential novel metabolites has prompted the suggestion that marine forms will prove to be as fruitful as those isolated from terrestrial habitats (Okami, 1986).

Actinomycetes are a group of Gram-positive bacteria that tend to form branching filaments, which in some families develop a mycelium. They produce aerial mycelia or substrate mycelia or both. But these

structures may break into rods or cocci, giving bacterial appearance. The diameter of the filaments varies from 0.5 –2μm. Filaments are not always observed because in some families as stated above, tend to fragment and leads to the formation of coccoid, elongate or diphtheroid elements. In some families true spores are formed on aerial and / or substrate hyphae. The spores vary greatly in shape and are produced at the tip of filaments. Spore formation occurs in response to nutrient depletion. Actinomycetes are widely distributed in nature and found in a variety of habitats. They are temperature tolerant and can withstand desiccation.

Actinomycetes as a source of antibiotics

The actinomycetes stand out as a unique group of prokaryotic organisms in two respects; the diversity of their morphology and their metabolic products. The prime example of the latter is the plethora of antibiotics produced principally as secondary metabolites late in the growth cycle. These antibiotic molecules, some being quite complex structures involving complicated biosynthetic pathways, number in hundreds (Ensign, 1978). More than 90% of the antibiotics are produced by actinomycetes (Baidacci *et al.*, 1966). The actinomycetes are also notable for their production of pigments, exocellular enzymes, and the terpenoid compounds that give soil its characteristic odour.

The abundance of terrestrial actinomycetes and their role in numerous biological processes is well known while their marine counterparts remain unexplored. They are the potential source of many antibiotics. Every year hundreds of antibiotics are rediscovered and many new ones are added to the list. What we know today is just the tip of an iceberg and much remains to be explored. Of all the genera of actinomycetes, streptomycetes rank first as most of the antibiotics are produced by them.

Actinomycetes as Probiotics

During the past 20 years, aquaculture industry has been growing tremendously, especially that of marine fish, shrimps and bivalves. But as with many other industries, this rapid growth has brought with it the problem of environmental pollution. Contamination of coastal waters due to aquaculture is causing serious concerns among law makers as well as scientists. Diseases caused by various etiological agents followed by mortality of cultured stock have become limiting factors in production from the aquaculture sector. Bacterial diseases are considered to be a major cause of mortality in shrimp larviculture (Wyban and Sweeny, 1991; Wilkenfeld, 1992) and fish hatcheries (Grisez and Ollevier, 1995). From the management perspective it is always better to prevent diseases rather than to make efforts to cure them.

For preventing and controlling diseases, a host of antibiotics, pesticides and chemicals were used possibly creating antibiotic resistant bacteria (Weston, 1996). Persistence of pesticides and other toxic chemicals in aquatic environment creates human health hazards. There is an increasing interest within the industry in the control or elimination of antimicrobial use. Therefore alternative methods need to be developed to maintain a healthy microbial environment in the larval rearing tanks. One such method that is gaining acceptance within the industry is the use of probiotic bacteria to control potential pathogens (Gomez-Gill *et al.*, 2000). The research on probiotics for aquatic animals is gaining importance due to the demand for environment friendly aquaculture.

Probiotics are defined as "a live microbial feed supplement, which beneficially affect the host animal by improving its intestinal microbial balance," (Fuller, 1989). As far as aquatic animals are concerned not

only the digestive tract is important but also the surrounding water. So Gram *et al.* (1999) broadened the definition by removing the restriction to the improvement to the intestine as "a live microbial supplement, which beneficially affects the host animal by improving its microbial balance". In aquaculture systems, the probiotics are used in two ways (Singh *et al.*, 2002).

1. As gut probiotics to maintain the microbial balance of the animal and thereby to reduce the number of pathogenic species inside the animal body.
2. As pond probiotics applied to the water/sediment system to degrade the organic waste in the system, which will provide a healthy environment for the animal and help in the exclusion of pathogens by producing antimicrobial substances.

Probiotics generally includes bacteria, cyanobacteria, microalgae, fungi etc. It is the "effective microbiota", which includes photosynthetic bacteria, *Lactobacillus*, Actinomycetes, Nitrobacteria, Denitrifying bacteria, Bifidobacterium, yeast etc. (Xiang-Hong, 1998).

Several bacteria have been used in the larval culture of aquatic organisms. Garriques and Arevalo (1995) reported that the use of *V. alginolyticus* as a probiotic agent might increase survival and growth in *Penaeus vannamei* post larvae by competitively inhibiting potential pathogenic bacteria.

Jiravanichpaisal and Chaauaychuwong *et al.* (1997) reported the use of *Lactobacillus* sp. as the probiotic bacteria in the giant tiger shrimp (*P.monodon* Fabricius). Maeda and Liao (1992) reported on the effect of bacterial strains obtained form soil extracts on the growth of prawn larvae of *P. monodon*. Higher survival and moult rates of prawn larvae

were observed in the experiment treated with soil extracts. Addition of the photosynthetic bacteria in the food or culture water was found to improve the growth of the prawn and the quality of water. Cui Jingjin *et al.* (1997) have reported on the application of photosynthetic bacteria in the hatchery rearing of *P.chinensis.*

Maeda and Nogami (1989) have reported the use of bacterial strains possessing vibriostatic activity to control vibriosis in prawns and thereby enhancing growth. By applying these bacteria in aquaculture, a biological equilibrium between competing beneficial and deleterious microorganisms was produced and results showed that the population of *Vibrio* spp. which frequently causes large scale damage to the larval production was decreased.

No reports could be obtained on the use of actinomycetes as probiotics in aquaculture.

Actinomycetes as Single cell protein for application in Penaeid prawn culture systems

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

The term single cell protein refers to protein in microbial cells grown, harvested, and used as animal feed and for human

consumption. Yeast, bacteria, algae etc are used in this regard. A list of reasons makes SCP advantageous-

1. Microorganisms have a very short generation time and can produce rapid biomass increase.
2. The protein content is high up to 60 %.
3. The production of SCP can be based on raw materials, which is rapidly available in large quantities.
4. SCP production can be carried out in continuous culture and thus can be independent of climatic changes.

SCP comprises of proteins, fats, carbohydrates, ash ingredients, water and other elements such as phosphorus and potassium. The use of Yeast and Bacterial SCP as feed ingredients has gained importance with the understanding of its nutritive value. Bacterial cell wall contains peptidoglycans (PG) rich in N-acetyl glucosamine and N-acetyl muramic acid (White *et al.*, 1979). The essential amino acid index of almost all bacteria was noted to be in the range of 91-94. Being above the value 90, it is of good quality for use as aquaculture feed ingredient (Penaflorida, 1989). Bacteria like *Pseudomonas* and *Methylophilus* sp. have been investigated for use as SCP in aqua feeds. They have approximately 73% crude protein, 5.7% lipid and 2.7 % NFE by weight (Kant, 1996). Brown *et al.* (1996) analyzed the composition of a few strains of marine bacteria including *Methylophilus methylophilus, Aeromonas* sp., *Werxia* sp. and *Psuedomonas* sp. to assess their nutritional value for bivalve aquaculture. Protein was the major constituent of bacteria (25-49% dry weight), with lipid a minor component (2.5 - 9.0%). Carbohydrate in bacteria ranged from 2.5 - 11%. Ash occurred in high levels 29-

40%. Glucose was the major sugar source in bacteria (12 - 73% of total sugars). Nucleic acids range from 3.3 to 8.4 % dry weight and polyunsaturated fatty acids, 20:5n-3 and 22:6n-3, were absent in the bacteria. High levels of good quality protein however, indicate their potential to provide important nutrients in a mixed diet. SCP contains more nitrogen in the form of nucleic acids (Prave *et al.*, 1987). Besides these, microorganisms are able to synthesize precursors of all macromolecules and vitamins (Prave *et al.*, 1987).

Atack and Matty (1978) evaluated the efficacy of a methanophilic bacterium, a petro-yeast, algal protein and extracted soybean as sole source of nitrogen in trout feed. Moreover, the results showed that the bacterial proteins were well accepted, digested and utilized for growth. Kussling and Askbrandt (1993) reported that the methanol bacterial SCP could replace up to 75% fishmeal in salmonid production diet. Similarly photosynthetic bacteria have also been proved to be a potential feed source for aquaculture systems (Kobayashi and Tachan, 1973; Shipmann, 1975; You *et al.*, 1992; Gebruk *et al.*, 1992). These bacteria can synthesize vitamins such as riboflavin, pyridoxine, folic acid, ascorbic acid and cholecalciferol, which may influence the growth and conversion efficiency of prawns (Kobayashi, 1970).

Actinomycetes can be used as single cell protein in aquaculture feed formulations, since they are the producers of many secondary metabolites. Nakamura *et al.* (1977) are of the opinion that some of the secondary metabolites may enhance the growth of prawns. Manju and Dhevendran (1997) have studied the effect of bacteria and actinomycetes as single cell protein feed on growth of *Macrobrachium idella*. Significant growth, better conversion efficiency and increased protein content in SCP fed prawns than those fed on control diet could

be observed. They have suggested the replacement of fishmeal with SCP to a possible extent in formulated diet.

The present work is aimed at the utilization of marine actinomycetes as source of antimicrobial compounds and as source Single cell protein and Probiotics which can be used in Penaeid prawn culture systems.

The present study was undertaken with the following objectives.

1. To isolate marine actinomycetes from the coastal waters of Cochin and offshore waters of the Arabian Sea.
2. To screen these isolates for antagonistic properties with selected Prawn pathogens and to select potential strains with best antibacterial properties.
3. To select a suitable fermentation medium for the production of antimicrobial compounds and to optimize the fermentation process and conditions.
4. To characterize the selected strains and to extract the bioactive principle using various solvents.
5. To find out the efficacy of actinomycetes as source of Single cell protein for incorporation in shrimp diets.
6. To select suitable strains of actinomycetes for utilization as Probiotics in aquaculture.

Chapter 2

ISOLATION OF ACTINOMYCETES FROM MARINE ENVIRONMENT

2.1 INTRODUCTION

2.1.1 Soil Actinomycetes

Actinomycetes constitute a significant component of the microbial population in most soils and counts of over 1 million per gram are commonly obtained (Goodfellow and Williams, 1983). The soil is the most prolific source of actinomycetes, which are found to produce antibiotics and other useful metabolites. A gram of rich agricultural soil contains 10^6 streptomycetes colony-forming units and 10^4 and 10^5 *Micromonospora*, as well as various other actinomycete genera. In soil, streptomycetes find plenty of surfaces to support their mycelial growth. The spores contribute to the survival over longer periods of drought, cold and anaerobic conditions. It appears that streptomycetes exist for extended periods as resting arthrospores that germinate in the occasional presence of exogenous nutrients. Although nutrient availability is a major factor controlling the activity of soil actinomycetes, various other environmental factors also exert an influence.

Temperature is obviously an important factor as far as soil streptomycetes are concerned. Like many soil microbes most actinomycetes behave as mesophiles in the laboratory with optimum growth rate at 25°C - 30°C. Most soil actinomycetes behave as neutrophiles growing between pH 5 and pH 9. As many soils are acidic, pH is clearly a major factor determining the distribution and activity of actinomycetes (Waksman, 1959).

2.1.2 Actinomycetes in marine environment

Actinomycetes have been isolated from marine environment largely from sediment samples from the continental shelf or from brackish water environments such as salt marshes (Hunter *et al.*, 1981).

Goodfellow and Hynes (1984) suggested that marine environment might be a valuable source for the isolation of actinomycetes with the potential to yield useful products. However, it has not yet been resolved whether actinomycetes are part of the autochthonous marine microbial community of sediments or whether actinomycetes isolated from marine sediment samples originate from terrestrial habitats and were simply carried out to sea in the form of resistant spores (Takizawa *et al.,* 1993). Jensen *et al.* (1991) reported a bimodal distribution of actinomycetes in near shore tropical marine environments with streptomycetes predominating at shallow depths and an increase in *actinoplanetes* with increasing depth. From a collection of 100 samples from five geographically different inshore locations, Grein and Meyers (1958) collected over 200 isolates. They concluded that the actinomycetes collected especially the streptomycetes do not represent an autochthonous marine flora. More likely they may be terrestrial forms that have become adapted to the salinity of seawater and sediments. On the other hand Weyland (1969) reported that there is a natural actinomycete flora in marine sediments after isolating 1348 strains from sea, away from littoral zone. Some scientists considered actinomycetes to be part of an indigenous marine microflora (Zobell, 1946; Okazaki and Okami, 1976 and Weyland, 1981) whereas, others consider them primarily as wash in components that merely survived in marine and littoral sediments as spores (Goodfellow and Haynes 1983). This view is supported by the observation that the number of actinomycetes in marine habitats decreases with increasing distance from land (Weyland, 1969; Okami and Okazaki, 1972, Attwell *et al.,* 1981 and Attwell and Colwell, 1981). Okazaki and Okami (1975 and 1976) found that actinomycetes from marine habitats were generally more salt tolerant

than terrestrial isolates but greater tolerance was found among sand dune isolates than those from sea water (Watson and Williams, 1974).

2.1.3 Selective isolation of actinomycetes

In order to isolate a wide variety of actinomycetes from soil samples, it is necessary to eliminate or greatly curtail fungal and bacterial growth in the isolation medium without producing adverse effect on actinomycetes. In general actinomycetes will grow in ordinary laboratory media, but their growth is usually slower than that of ordinary bacteria. A cell division cycle in actinomycetes may take 2 to 3 hours as compared with 20 minutes in *E. coli.* Some actinomycetes grow even more slowly

Isolation of a microorganism from its habitat depends on the nature of the microorganisms and their number relative to the other microbes within the habitat. The isolation of actinomycetes from the mixed microflora present in nature is complicated by their characteristic slow growth relative to that of other soil bacteria (Hirsch and Christensen, 1983). This has resulted in the development of selective isolation procedures based primarily on one or all of the following approaches

1. Nutritional selection in which media is formulated with nutrients which are preferentially utilized by actinomycetes (Porter *et al.*, 1960; Lingappa and Lockwood, 1961;El-Nakeeb and Lechevalier, 1963; Kuster and Williams, 1964 and Kutzner, 1981).
2. Selective inhibition in which compounds such as antibiotics are incorporated into media to inhibit non-actinomycete bacteria selectively (Dulaney *et al.*, 1955; Williams and Davis, 1965 and Kutzner, 1981).

3. Pre-treatment of the samples by which a reduction in the number of bacteria and fungi can be achieved by the careful use of heat/chemical treatment or centrifugation of the soil suspensions.

2.1.3.1 Composition of the isolation media

Manipulation of the composition of isolation medium is the most effective method for discouraging unwanted bacteria. Some of the isolation media are rather lean, since actinomycetes have the ability to survive and grow to some extent on very small amounts of nutrients that they scavenge from non-nutrient substances such as agar. Other isolation media have high carbon to nitrogen ratios, e.g. starch, casein, chitin, humic acid etc. The use of these media greatly reduces the number of bacteria in contrast with low C: N ratios and are usually unable to attack high molecular weight resistant polymers (Grey and Williams, 1971).

Benedict *et al.* (1955) has reported that Streptomycetes grow well on a glycerol medium containing L (+) arginine as the sole source of nitrogen. Starch-potassium nitrate medium was preferred for the isolation of actinomycetes by Flaig and Kutzner (1960). Starch-casein agar in which glycerol was substituted for starch was used to isolate large numbers of streptomycetes from composted soil (Küster and Williams, 1964). Okazaki and Okami (1972) recommended the use of starch-casein agar and this has been used by many workers (Pisano *et al.*, 1986).

Many media formulations have been recommended for isolation of one or more actinomycete genera, the most widely used includes, starch–casein (Kuster and Williams,1964) colloidal chitin-mineral salt agar (HSu and Lockwood, 1975), half strength nutrient agar (Rowbotham and Cross, 1977).

2.1.3.2 Inhibitory agents used in isolation media

Antibiotics have been effectively used to improve media selectivity. Antifungal antibiotics, which do not inhibit actinomycetes, have been widely used. (Williams and Davies, 1965; Cross, 1968; Cross and Johnston, 1972; Orchard *et al.*, 1972).

i) Antifungal compounds

Sl No	Compound	Concentration in the medium	Media	Reference
1.	Cycloheximide and Nystatin	50 µg/ml	Actinomycete isolation Agar	Philip and Hanel(1950),Cork and Chase, (1956) and Porter *et al.* (1960).
2	Cycloheximide & Nyslatin	50 µg/ml	-	Okazaki and Okami (1972).
3	Cycloheximide		Starch-casein agar	Pisano *et al.* 1986
4	Cycloheximide and Candiacidin	100 µg/ml	Glucose-asparagine agar	Hirsch and Christensen (1983)
5	Pimeracin	100 µg/ml	-	Porter *et al* (1960)

ii) Antibacterial antibiotics

Sl. No	Compound	Concentration in the medium	Reference
1	Nalidixic acid	10µg/ml	Takizawa *et al.* (1993).
2	Polymyxin and Penicillin	5 µg/ml 1 µg/ml	William and Davies (1965).
3	Novobiocin	50 µg/ml	Takahashi *et al.* (1991)
4	Rifampicin	25 µg/ml	Athalye *et al* (1981)
5	Tunicamycin	25 µg/ml	Wakisaka *et al.* (1982)

Successful applications of antibacterial antibiotics have been more limited, as actinomycetes are also likely to be susceptible to

these compounds. Nevertheless, they have been found to be effective for isolation of single genera or species after determination of their sensitivity patterns. Besides antibiotics Na-Propionate 4g/L (Crook *et al.*, 1950) and Rose Bengal 35 mg/L (Ottow 1972) are added in the media. Medium containing these compounds can suppress most bacteria and make even pinpoint actinomycete colonies intense pink and reduce the spreading growth of fungi.

2.1.3.3 Pre-treatment of the samples

The selectivity of the isolation procedure may be influenced by pre-treatment of the sample before plating. Most pre-treatments have been designed to reduce the number of fast growing and spreading bacteria, while still preserving the viability of actinomycetes. A variety of pre-treatments are done to enhance the isolation of specific groups of actinomycetes (Cross, 1982).

The pre-treatments included physical/chemical treatment and enrichment technique.

Heat Treatment

Heat treatment is often used as a pre-treatment of marine sediments, prior to actinomycete isolation, to reduce the number of gram-negative bacteria commonly found in sampling (Pisano *et al.*, 1986; Barcina and Egea 1987; Jensen *et al.*, 1991). This includes dry heat (Nonomura and Ohara, 1969) followed by plating on starch-casein agar (Okazaki and Okami, 1972). Nonomura and Ohara (1969) reported that treating soil with heat at 120°C drastically reduced the number of undesirable bacteria and in addition, the isolation of streptomycetes were also considerably curtailed.

Heat treatments have been used to isolate *Actinomadura, Microbiospora* (Nonomura and Ohara, 1969,1971 a, b and c and Athalye *et al.*, 1981), *Streptomyces* (Williams *et al.*, 1972) and *Gonococcus* (Rowbotham and Cross, 1977). The viability of the sporangia after desiccation has been used to isolate actinomycetes (Makkar and Cross, 1982). Simple air-drying of soil samples at room temperature could eliminate most of the unwanted, gram negative, bacteria that produce mucoid spreading colonies on soil dilution plates. A pre-treatment employing alternate drying and wetting of soil samples has been applied to eliminate unwanted competitors (Makkar and Cross, 1982).

Phenol Treatment

Phenol treatment of soil slurries (Lawrence, 1956; Panther *et al.*, 1979 and Nonomura and Hayakawa, 1988) and marine sediments (Pisano *et al.*, 1986) has been used to eliminate competing microorganisms.

A pre-treatment procedure combining heat with chemical treatment was reported by Nonomura and Hayakawa, (1988)

Enrichment

Enrichment techniques have often been used in soil bacteriology to obtain greater populations of desired organisms. It appears that some substances stimulate actinomycetes more. Both Waksman and Starkey (1924) and Porter and Wilhelm (1961) noted large increase in actinomycete numbers when blood was added to moistened soil sample. Organic materials such as Salmon Viscera meal, Peanut meal, Cotton Seed meal and dried blood flour (15mg/g soil) were used by Porter and Wilhelm (1961). Jenson (1930 and 1932) used keratin and

chitin for enrichment and observed an increase of streptomycetes in soil. Williams *et al.* (1972) have carried out enrichment with chitin for isolation of actinomycetes.

Apparently because of a change of pH favourable to actinomycetes in contrast to fungi, the addition of $CaCO_3$ to soil causes a substantial increase in the ratio of actinomycetes to fungi. Tsao *et al.* (1960) noticed that $CaCO_3$ at a rate of 100mg/g of soil was effective in selective isolation of actinomycetes. The soil was moistened and incubated at 26^{0}C for 7 to 9 days. The same observation was made by El-Nakeeb and Lechevalier (1963), who incubated three different soil samples with 1g of $CaCO_3$ per 1g of soil at high relative humidity for 10 days at 28^0 C. The highest plate count of actinomycetes was obtained by combining this technique with isolation on arginine- glycerol salts medium.

Agitation

A close association of the vegetative growth and spore chains with the mineral and organic particles of the soil or fodders necessitate a vigorous shaking of the sample. Addition of glass beads to the suspension to be treated on a shaker is a useful method.

Centrifugation

Centrifugation has been used to concentrate the propagules in marine samples (Okami and Okazaki, 1972; Goodfellow and Haynes, 1983). Centrifugation of the soil suspension brings the spores of streptomycetes to the surface whereas spores of fungi and bacteria remain in the sediment (Rehacek, 1956; Neusch, 1965).

Baiting

Baiting is a form of enrichment culture. Paraffin coated glass rods are inserted into moistened soil as "bait" for the isolation of different species (Orchard *et al.*, 1977). The motile spores of actinomycetes migrate into the glass rod and colonize on it. Various baits have been used to attract the motile spores of actinoplanetes in water or soil suspensions. Baits such as pollen (Kuznetov, 1969), human hair (Bond, 1976) and insect wings consisting of chitin have been used for this purpose by Veldkamps (1955); Jagnow (1957) and Okafor (1966) respectively.

2.1.3.4 Incubation time

Incubation at 28°C for 4 weeks was sufficient to obtain isolated colonies of actinomycetes (Pisano *et al.*, 1986). Incubation is usually at 25-30°C or 45°C for thermophiles, with most colonies developing within 14 days. Incubation period of 4-6 weeks have been recommended by Nonomura and Ohara (1969 and 1971) and Okami and Okazaki (1972).

2.1.4 Preservation of Isolates

Actinomycetes cannot be maintained by serial passage on agar slants, because this may predispose them to degeneration with consequent loss of ability to produce the desired metabolites. The cause for this loss of gene expression in actinomycetes is not fully understood, but probable causes include loss of a plasmid, breakdown of an unstable heterokaryon and insertion sequence promotion or inactivation of gene expression (Nisbet, 1980).

The most widely used method for long-term preservation of actinomycete strains is lyophilisation. Wellington and Williams (1979) developed a convenient preservation method suitable for the short-term provision of inocula to be used in fermentation and other studies. Dense vegetative cell or spore suspensions were prepared in 10 % (v/v) aqueous glycerol and dispensed into screw-cap vials, which were stored at -20°C. When inoculum is needed, the suspension is thawed and small amounts are removed with a loop or pipette.

2.2 MATERIALS AND METHODS

2.2.1 Samples

Marine water and sediment samples were used for the isolation of actinomycetes.

Collection Area

i) Sediment samples were collected during the cruise No.162 of Fisheries and Oceanographic Research Vessel (FORV) Sagar Sampada of Department of Ocean Development, Govt. of India, from the South West coast of India, up to 200 m depth in the Exclusive Economic Zone. The stations extended off Cape Comorin (8°.03'.96" N and 77°.21'.90" E) to off Dwaraka (21°. 56'.99" N and 67.57'.69" E). Samples from 38 stations were used for the study. The stations were designated as SS1 to SS38 (Fig2.1).

ii) Sediment and water samples were also collected from the coastal waters of Cochin. These stations were designated as S1 to S12 (Fig 2.2).

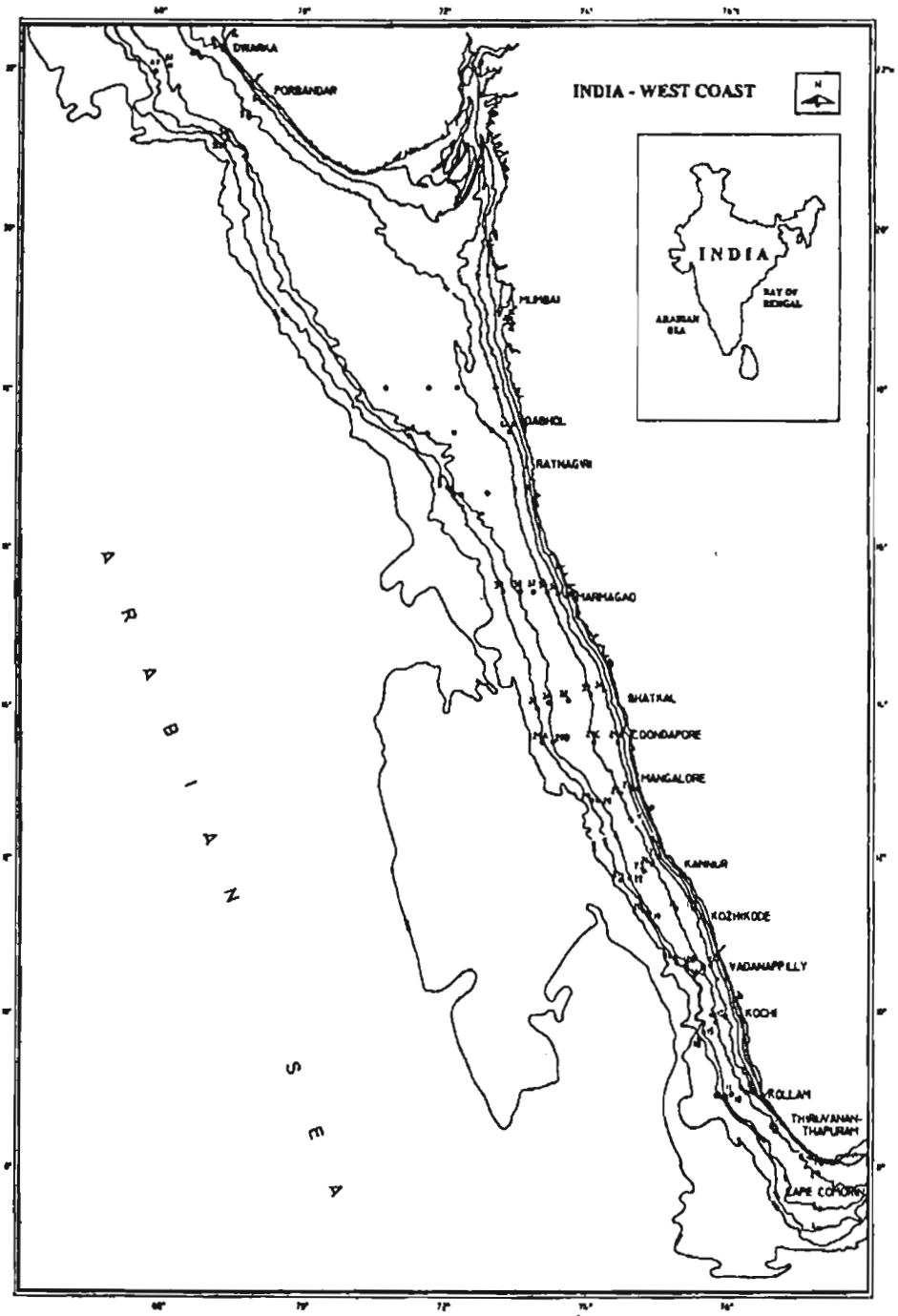


Fig. 2.1. Chart showing the location of sampling stations SS1-SS62

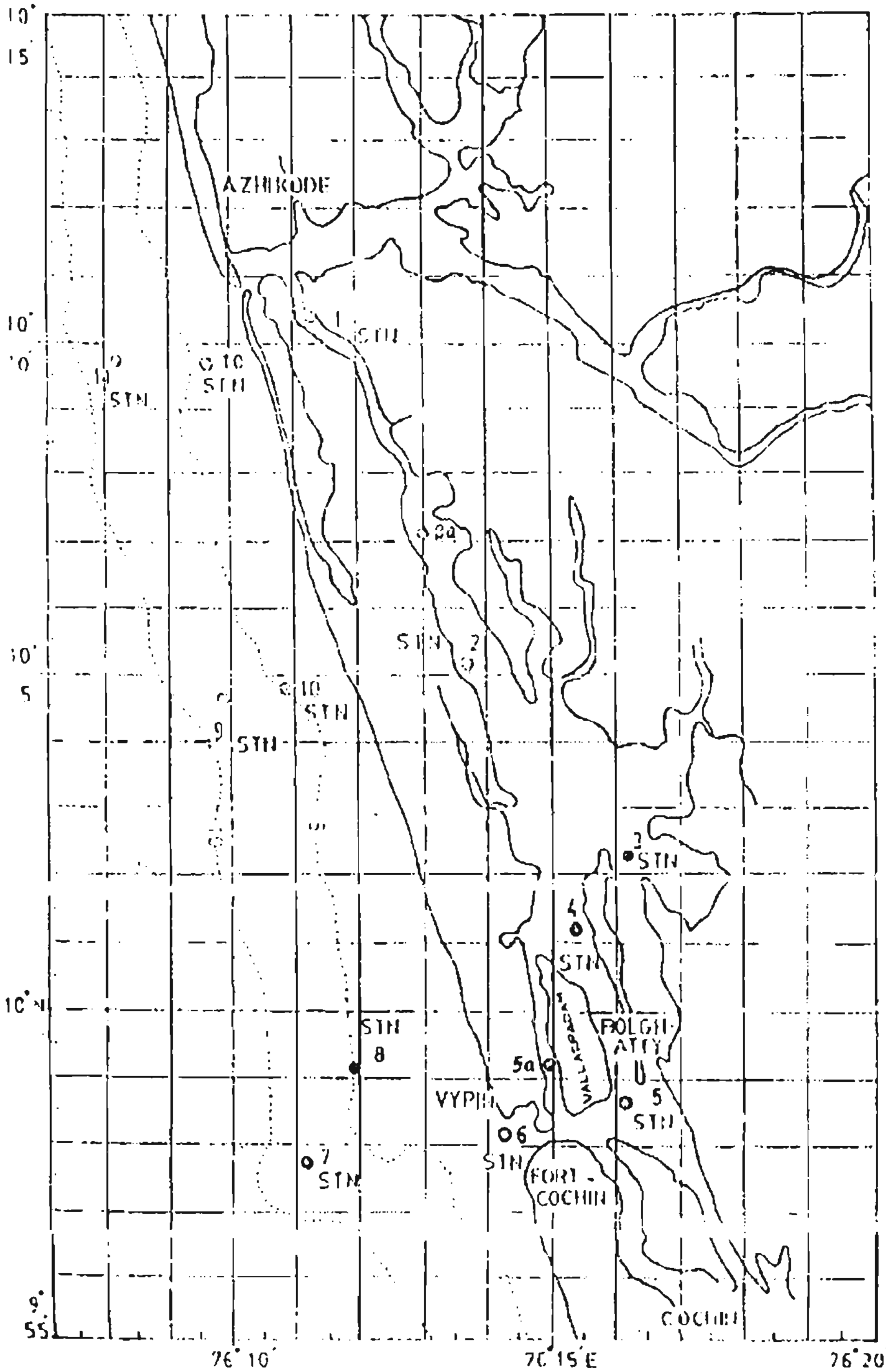


Fig 2.2 - Chart showing the location of sampling stations S1-S12

2.2.2 Pre-treatment of the samples

For the selective isolation of actinomycetes, samples were subjected to various pre-treatments as follows.

a. Heat Treatment

1) Pt 1 - Heated the air-dried soil sample at 120°C for 1 hour.
2) Pt 2 - Soil samples were heated at 40- 45°C for 2 – 16 hours.
3) Pt 3 - Soil samples were heated at 100°C for 15 minutes.
4) Pt 4 - Soil samples were heated at 50 – 60° C for one hour.
5) Pt 5 – Agitation.

   0.5 g soil was suspended in 25 ml seawater containing 0.0002% Tween 20, stirred vigorously with glass beads in a mixer. Then the suspension was placed in vacuum desiccator for 30 minutes to remove air.

6) Pt 6 – Heat and Phenol treatment.

   1 g of air-dried sediment sample after heating at 120°C for 1hour was added to 9 ml sterile seawater containing 1.5% (w/v) phenol. Mixed well and allowed to stand for 30 minutes at room temperature (28±2°C). This suspension was used as the inoculum.

7) Pt 7 – Enrichment.
   (a) Air-dried sediment (1g) samples were mixed with 0.1 g $CaCO_3$ and incubated at 26°C for a week in humidity controlled environmental chamber.
   (b) Water sample (25ml) was mixed with 1g colloidal chitin, kept for a week in a humidity controlled environmental chamber at 26°C.

2.2.3 Sample Preparation

After various pre-treatments the sediment samples except pt 6 (section 2.2.2a) were diluted up to 10^{-1} in sterile seawater, agitated well

using a vortex mixer and after settling the supernatant was used as inoculum.

2.2.4 Media used

Fourteen different media were used for the selective isolation of actinomycetes. Composition of the various media is given below.

1.Starch – Casein Agar

Starch	10.0g
Vitamin free Casamino acids	0.3g
$CaCO_3$	0.02g
$Fe_2(SO_4)_3.7H_2O$	0.01g
KNO_3	2.0g
$MgSO_4.7H_2O$	0.05g
Sea Water	1L
PH	7.2
Agar	20g

2.CSPY-ME Agar

Casein	3.0g
Maize Starch	10.0g
Peptone	1.0g
Yeast Extract	1.0g
Malt Extract	10.0g
K_2HPO_4	0.5g
Sea Water	1L
pH	7.5
Agar	20g

3.Chitin Agar

Colloidal Chitin	4.0g
KH_2PO_4	0.3g
K_2HPO_4	0.7g
$MgSO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
$ZnSO_4.7H_2O$	0.001g
$MnCl_2.4H_2O$	0.001g
Sea Water	1L
pH	8
Agar	20g

4.Glucose - Arginine Agar

L-Arginine	3g
Glucose	10g
Glycerol	10ml
K_2HPO_4	3g
$MgSO_4.7H_2O$	2g
Thiamine	5mg
Niacin	5mg
Riboflavin	5mg
Pyridoxine	5mg
Inositol	4mg
Pantothenate	5mg
PABA	5mg
$Fe_2(SO_4)_3$	100mg
$ZnSO_4.7H_2O$	500mg
$MnCl_2.4H_2O$	10mg
$CuSO_4.5H_2O$	10mg
Sea Water	1L
pH	7.5
Agar	20g

5.Nutrient Agar

Peptone	5.0g
Beef Extract	3.0g
Sea Water	1L
pH	7.2
Agar	20g

6.Glucose Casamino Acid Agar

Glucose	10.0g
Casamino Acids	2.0g
Yeast Extract	2.0g
Beef Extract	0.1g
Sea Water	1L
pH	7.2
Agar	20g

7.SS Medium

Soluble starch	25g
Glucose	10g
Yeast extract	2g
$CaCO_3$	3g
Sea Water	1L
pH	7.5
Agar	20g

8.Paraffin Agar

Liquid Paraffin	1.0ml
NH_4NO_3	4.0g
KH_2PO_4	2.0g
K_2HPO_4	6.0g
$FeSO_4.7H_2O$	0.01g
$ZnSO_4.7H_2O$	0.001g
$MnCl_2.4H_2O$	0.001g
$CuSO_4.5H_2O$	0.0025g
$Na_2B_4O_7.10H_2O$	0.00094g
$(NH_4)_6Mo_7O_{24}.42O$	0.002g
Sea Water	1L
pH	8
Agar	20g

9.Glucose Yeast Extract Agar

Glucose	10g
Yeast Extract	10g
K_2HPO_4	0.5g
Sea Water	1L
PH	7.5
Agar	20g

10.GS Medium

Glucose	10gm
Soybean Meal	10gm
$CaCO_3$	1gm
Sea Water	1L
pH	7.5
Agar	20g

11.Starch – Glycerol – Inorganic salt agar

Starch	10g
Glycerol	10ml
$(NH_4)_2SO_4$	2g
K_2HPO_4	1g
$MgSO_4.7H_2O$	1g
$CaCO_3$	2g
Sea Water	1L
PH	7.5
Agar	20g

12. Glycerol Asparginc Agar

Glycerol	10g
Aspargine	1g
K2HPO4	1g
$MgSO_4.7H_2O$	0.5g
FeSO4.6H2O	0.01g
CuSO4.H2O	0.001g
ZnSO4.7H2O	0.001g
MnSO4.H2O	0.001g
Sea Water	1L
pH	7.4
Agar	20g

14.Starch Inorganic Salt

Starch	10g
(NH4) SO4	2g
K2HPO4	1g
$MgSO_4.7H_2O$	1g
$CaCO_3$	2g
Sea Water	1L
pH	7.4
Agar	20g

14. Glycerol Arginine Agar

Glycerol	12.5g
Arginine monohydro chloride	1g
$MgSO_4.7H_2O$	0.5g
Yeast Extract	3gm
Sucrose	10.3g
Sea Water	1L
pH	7.5
Agar	20g

2.2.5 Antibiotics and Other Inhibitory Agents Used

All isolation media were supplemented with antibacterial and antifungal compounds.

i) Antibacterial Compounds

Antibiotics		***Concentration in the media***
Novobiocin (Himedia)	-	2.5mg/100ml
Vancomycin (Himedia)	-	1mg/100ml

ii) Antifungal Compounds

Compound	***Concentration in the media***
Cycloheximide (Himedia) -	2.5mg/100ml
Amphotericin B (Himedia) -	1mg/100ml
Bavistin*	13.75 mg/100ml

(BASF India Limited, Bombay)

Carbendazim 50%(2-methoxy-carbomyl benzimidazole)

2.2.6 Plating and Incubation

Medium as given in 2.2.4 were prepared and sterilized at 121°C for 15 minutes in an autoclave (except Glucose). Glucose was filter sterilized and added to the respective medium at the time of pouring into the plates. At about 45°C antibiotics and other inhibitory agents (bavistin) were added in to the media at the time of plating. Pour plate method was employed and plates were incubated in a Bacteriological incubator at 28 ±2 °C for a period of 2 to 4 weeks.

2.2.7 Isolation and Purification

Colonies with characteristic appearance of actinomycetes (tough, leathery) were isolated on to nutrient agar slants (peptone 0.5g; beef extract 0.3g; agar 2g; sea water, 100 ml; pH7). The isolates were repeatedly streaked on nutrient agar plates for purification.

Gram staining was done for all the isolates. All gram-positive forms with filamentous morphology were segregated for the study.

2.2.8 Preservation

Preservation was done in two ways.

i. All the actinomycete isolates were stocked in sterile nutrient agar vials overlaid with sterile liquid paraffin.

ii. Spore suspensions of the actinomycetes in sterile nutrient broth supplemented with 10% v/v glycerol were kept at –35°C in a deep freezer.

2.3 RESULTS

2.3.1 Isolation of Marine actinomycetes

Marine actinomycetes were found to be comparatively slow growing and only after nearly two weeks, the appearance of the colony could be observed on the isolation agar plates. Round white colonies with powdery appearance due to the presence of spores were the most prominent type. Pigmented forms like grey, orange, red and lemon yellow colonies were also present on the isolation plates (Fig 2.3). Sediment samples collected from the coastal waters of Cochin were found to be a better source of actinomycetes compared to those collected from offshore waters in the shelf region of the South - West coast of India. (Table 2.1).

Figure 2.3 Typical actinomycete colonies on starch casein agar plates

Table 2.1 Details of actinomycetes isolated from various stations

Sl No	Culture No	Station[1]	Sample[2]	Isolation media[3]	Colony characteristics
1.	B27	S12	Sediment	CSPYME	Round, white
2.	B28	S10	Sediment	CSPYME	Round, white
3.	B29	S10	Sediment	CSPYME	Round, white
4.	B30	S10	Sediment	CSPYME	Round, white powdery
5.	B39	S11	Sediment	SC	Round, white
6.	B42	S12	Sediment	SC	Round, white
7.	B44	S8	Sediment	SC	Round, white
8.	B45	S8	Sediment	SC	Large spreading white
9.	B47	S4	Sediment	CSPYME	Small convex red
10.	B72	S4	Sediment	CSPYME	Small convex red
11.	B78	S6	Sediment	CSPYME	Round, Yellow
12.	B79	S6	Sediment	SC	Small convex, white
13.	B80	S8	Sediment	CSPYME	Swarming, white
14.	B113	S8	Water	CSPYME	Spreading, yellow
15.	B181	S4	Water	CSPYME	Small convex, red
16.	B182	S5	Water	CA	Round, cream
17.	B186	S3	Water	CSPYME	Round, peach
18.	B188	S3	Water	CSPYME	Irregular, white
19.	B192	S2	Water	CSPYME	Round, yellow
20.	B194	S5	Water	CSPYME	Round, yellow
21.	B196	S5	Water	SC	Irregular, white
22.	B198	S5	Water	SC	Round, white
23.	B199	S5	Water	CSPYME	Branching, white
24.	B201	S2	Sediment	CSPYME	Branching, white
25.	B213	S5	Sediment	CSPYME	Branching, white
26.	B214	S5	Sediment	CSPYME	Branching, white
27.	B215	S5	Sediment	CSPYME	Branching, white
28.	B217	S8	Sediment	CSPYME	Hard, white
29.	B221	S8	Sediment	CSPYME	Round, white
30.	B222	S10	Sediment	CSPYME	Round, white
31.	B224	S10	Sediment	CSPYME	Round, white
32.	B226	S10	Sediment	CSPYME	Round, white

Table 2.1 Continue

33.	B227	S3	Sediment	CSPYME	Round, white
34.	B236	S7	Sediment	SA	Round, white
35.	B262	S7	Sediment	GC	White, hard
36.	B271	S7	Sediment	GC	White, hard
37.	B272	S9	Sediment	GC	White, hard
38.	B274	S9	Sediment	GC	Round, hard
39.	B278	S6	Sediment	SGA	Round, orange
40.	B280	S2	Sediment	SGA	Irregular hard
41.	B301	S2	Sediment	SGA	White powdery, black pigment
42.	B303	S2	Sediment	SGA	White powdery, black pigment
43.	B306	SS34	Sediment	SIA	Round powdery with white center
44.	B309	SS23	Sediment	SIA	Round gray
45.	B319	SS28	Sediment	SIA	Small convex powdery
46.	B337	SS28	Sediment	SIA	White, orange pigment
47.	B338	SS30	Sediment	SIA	White, orange pigment
48.	B343	S5	Sediment	SGA	White, hard
49.	B347	S2	Sediment	SIA	Opaque hard, white
50.	B350	S2	Sediment	SIA	White, hard
51.	B351	SS28	Sediment	SIA	White, hard
52.	B356	S2	Sediment	SIA	White, hard
53.	B361	S2	Sediment	SC	White powdery
54.	B365	S4	Sediment	GA	White, hard
55.	B369	S4	Sediment	GA	White, hard
56.	B373	S2	Sediment	GA	White, hard
57.	B377	SS1	Sediment	GC	White, hard
58.	B380	SS23	Sediment	GC	White, powdery
59.	B408	SS20	Sediment	SGA	White, powdery
60.	B409	SS20	Sediment	SGA	White powdery
61.	B410	SS20	Sediment	SGA	White, powdery
62.	B451	SS38	Sediment	GA	White, powdery

1 refer 2.2.1
2 S - sediment
W - water
3 refer 2.2 .4

Of the various pre-treatments, heat treatment at 50-60°C for 1 hour (Pt 4) was found to be most effective in the selective isolation of actinomycetes (Table2.2), followed by heat treatment at 100°C for 15 min (Pt3) and enrichment with calcium carbonate (Pt7a).

Table 2.2 Efficacy of various pre-treatments in the selective isolation of actinomycetes

Sl No	Pre treatment methods *	Actinomycete isolates (%)
1.	Pt1	Nil
2.	Pt2	Nil
3.	Pt3	24
4.	Pt4	29
5.	Pt5	13
6.	Pt6	8
7.	Pt7a	18
8.	Pt7b	8
Total number of isolates		62

* For details, refer 2.2.2

Heat treatment at 120°C for 1 hour was not found to be suitable since no actinomycete growth could be observed in the various media. Incubation of soil samples at 40-45°C for 2 to 6 hrs was not sufficient to prevent bacterial growth resulting in the over dominance of bacterial population in the isolation plates and inhibition in the development of slow growing actinomycete colonies.

CSPYME was found to be most suitable media in the isolation of actinomycetes (40.3%) followed by starch glycerol agar (12.9%), starch inorganic salt agar (12.9%), arginine glycerol agar (9.68%) and glycerol agar (9.68%) (Table 2.3).

Table 2.3 Percentage of actinomycetes isolated using various media

Sl No	Isolation media used	Actinomycetes isolated (%)
1.	Casein Starch Peptone Yeast Extract Malt Extract agar	40.3
2.	Starch Glycerol Agar	12.9
3.	Starch Inorganic-salts Agar	12.9
4.	Arginine Glycerol Agar	9.68
5.	Glycerol Casamino Acid	9.68
6.	Glycerol Agar	6.45
7.	Chitin Agar	3
8.	Nutrient Agar	3
9.	Starch Casein agar	1.6
Total number of isolates		62

Bavistin (Carbendazim 50%) at a concentration of 13.75mg/100 ml medium was found to be effective as an antifungal agent in the isolation of actinomycetes.

2.4 DISCUSSION

There is little published information describing the distribution, growth and ecological role of actinomycetes in marine habitats. This is because of the fact that actinomycetes represent a small component of the total bacterial population in marine sediments (Goodfellow and Haynes, 1984 and Goodfellow and Williams, 1985). The regular isolation of actinomycetes from littoral sediments does not indicate that these organisms are indigenous to the marine and brackish water areas (Grein and Mayers, 1958). In the present study, sixty-two actinomycetes were isolated from the Arabian Sea and the Cochin backwaters. Most of them could be isolated from sediment samples collected form coastal waters rather than offshore regions.

However, it has not yet been resolved whether actinomycetes isolated from marine sediment samples are members of true autochthonous marine microbial community or originate from terrestrial habitats and were simply carried out to sea in the form of resistant spores (Takizawa *et al.*, 1993). Jensen *et al.* (1991) reported a bimodal distribution of actinomycetes in near shore tropical marine environments with streptomycetes predominating at shallow depths and an increase in actinoplanetes with increasing depth. From a collection of 100 samples from five geographically different inshore locations, Grein and Meyers (1958) collected over 200 isolates. They concluded that the actinomycetes collected especially the streptomycetes; do not represent an autochthonous marine flora. More likely, they may be terrestrial forms that have become adapted to the salinity of seawater and sediments. On the other hand, Weyland (1969) reported that there is a natural actinomycete flora in marine sediments after isolating 1348 strains from sea away from littoral zone. Some scientists considered actinomycetes to be part of an indigenous marine micro flora (Zobell, 1946; Okazaki and Okami, 1976; Weyland, 1981). Whereas, others consider them primarily as wash in components that merely survived in marine and littoral sediments as spores (Goodfellow and Haynes, 1983). This view is supported by the observation that the numbers of actinomycetes in marine habitats decrease with increasing distance from land (Weyland, 1969; Okami and Okazaki, 1972, Attwell *et al.*, 1981; Attwell and Colwell 1981). Okazaki and Okami (1975 and 1976) found that actinomycetes from marine habitats were generally more salt tolerant than terrestrial isolates but greater tolerance was found among sand dune isolates than those from sea water (Watson and Williams, 1974).

D'souza and Vaidya (1999) isolated 45 actinomycetes from sediment samples collected from the estuarine regions of Goa of which six were potential source of antibacterial substances. Pisano *et al.* (1986, 1987 and 1989) isolated actinomycetes from marine and estuarine sediments. Takizawa *et al.* (1993) isolated actinomycetes from Chesapeake Bay and screened for bioactive compounds. The distribution of actinomycetes in near shore tropical marine sediments were studied by Jensen *et al.* (1991) and 289 actinomycetes were isolated from 15 island locations throughout Bahamas.

Very low number of actinomycetes, slow growth of these organisms and fungal contamination were the major problems encountered during the study. Long incubation period caused fungal contamination in spite of the addition of antifungal antibiotic, amphotericin B. However, with the addition of bavistin, the fungal contamination could be avoided. Concentration of bavistin below 13.75 mg/100ml medium resulted in suppressed growth of sub surface fungal colonies and a concentration higher than this value was found to be inhibitory even to actinomycetes.

Pre-treatments employed in the study could successfully eliminate contaminating microflora and isolation of actinomycetes became easier. Heat treatment is often used as a pre-treatment of samples, prior to actinomycete isolation, to reduce the numbers of gram-negative bacteria commonly found in marine samples and often dominating the isolation plates (Barcina *et al.*, 1987; Jensen *et al.*, 1991 and Pisano *et al.*, 1986)

Heat treatment at 50-60^{0}C for 1 hour was found to be most suitable for the isolation of actinomycetes. Higher temperature for long duration reduced the number of actinomycete colonies. Takizawa *et al.*

(1999) have recommended the avoidance of heat treatment at higher temperatures since heat treatment selects actinomycetes in samples allowing the out growth of actinomycetes present as heat resistant spores. Pisano *et al.* (1986) investigated on pre-treatment methods for selective isolation of actinomycetes and was of the opinion that heat treatment reduced the number of undesirable bacteria occurring on the isolation plates. Nonamura and Ohara (1969) reported that heating soil at 120°C drastically reduced the number of undesirable bacteria. In addition, the isolation of streptomycetes was also considerably curtailed. They reported heat to be selective for the isolation of *Microbiospora* and *Streptosporangium*.

In this study heat and phenol treatment, Pt_6(section 2.2.2a) was also found to be effective in eliminating unwanted microbes, especially fungi to some extent. Similar observations were reported previously by Lawrence (1956) and Pisano *et al.* (1986). It was noted that samples treated with phenol took much time to develop colonies. Most of the plates were devoid of microbial growth, bacteria or even fungi. Pisano *et al.* (1986) observed a sharp reduction in the occurence of undesirable bacteria on isolation plates on treatment with 1% phenol and found that phenol treatment was suitable for the isolation of actinomycete colonies through the suppression of not only contaminating bacteria but also of fungi.

Calcium carbonate enrichment was found to be effective in the isolation of actinomycetes. Calcium carbonate enrichment effects a change in pH, which is favorable to actinomycetes. Tsao *et al.* (1960) noticed that calcium carbonate at a rate of 100mg/g of soil was effective in selective isolation of actinomycetes. The same observation was made

by El- Nakeeb and Lechevalier (1963) who incubated soil samples with 0.1g of calcium carbonate per 1 gm of soil.

Composition of the isolation medium greatly influences the development of actinomycete colonies. Chitin agar, as a selective medium proved to be effective for the isolation of actinomycetes (Pisano *et al.*, 1986). The selectivity of chitin containing media is attributed to the near universal ability of the actinomycetes to hydrolyze chitin (Hsu and Lockwood, 1975). In this study also, in chitin agar, a few strains could be observed.

In the present study, CSPY ME agar was found to be most suitable for the isolation of actinomycetes. Although, this medium is rich in nutrients, the pretreatment procedures and addition of antibiotics and bavistin might have prevented the development of unwanted microbes promoting actinomycete growth. CSPYME agar has high carbon to nitrogen ratios. Use of these media greatly reduces the number of bacteria that are unable to attack high molecular weight resistant polymers (Grey and Williams, 1971).

Starch-glycerol agar and starch inorganic salt agar were also found to be supporting selective growth of actinomycetes. Starch as carbon source was found to be the best for minimizing bacterial growth. Supply of nitrogen in the inorganic form also might have contributed to the minimal development of many bacteria. Okazaki and Okami (1972 have demonstrated the effectiveness of starch casein–agar as an actinomycete isolation medium. Starch casein agar, starch glycerol agar, starch inorganic salts agar, chitin agar etc are lean media and actinomycetes grow well on these media since they have the ability to survive and grow to some extent on very small amounts of nutrients. Generally, for the isolation of actinomycetes,

media with minimal nutrients containing high molecular weight compounds like starch and chitin are used. Long-term incubation of these media plates enables selective growth of actinomycetes. Media with minimal nutrients can be used even without antibiotics used for preventing bacterial growth. However, the antifungal compound, bavistin was found to be essential due to the long-term incubation required for development of actinomycete colonies.

Antibacterial compounds novobiocin and vancomycin effectively reduced the number of bacterial colonies on the isolation plates. Even though the antifungal compounds like cycloheximide and amphotericin were used in the medium, fungal contamination occurred and addition of bavistin could effectively control fungal growth. Bavistin is generally used as an antifungal agent in plant tissue culture media.

Chapter 3

ACTINOMYCETES AS A SOURCE OF ANTIBACTERIAL COMPOUNDS: SCREENING FOR ANTAGONISTIC ACTIVITY AND SELECTION OF STRAINS.

3.1 INTRODUCTION

There exists a fifty-year history of screening microbial secondary metabolites for antimicrobial activity, which has led to the discovery and subsequent development of many important antibiotics. In the middle1960's, the late Umezawa of the institute of Microbial Chemistry in Tokyo began screening for molecules from microbial sources with activities as selective enzyme inhibitors. This approach, which continues today, has resulted in numerous therapeutic products

3.1.1 Antibiotics and secondary metabolites

Antibiotics are antimicrobial agents produced by microorganisms that kill or inhibit other microorganisms. A more broadened definition of an antibiotic includes any chemical of natural origin (from any type of cell), which has the effect to kill or inhibit the growth of other types of cells. They are low molecular weight (non-protein) molecules produced as secondary metabolites mainly by microorganisms. Secondary metabolites are produced from primary metabolic precursors with diverse and complex structures and are pharmaceutically active. They are usually distinctive products of particular groups of organisms, sometimes even of a single strain (Vining, 1990). Secondary metabolites owe their antibiotic activity to their ability to inhibit essential primary metabolic processes (Vining, 1990). Most antibiotics acts as antimetabolites, their functional resemblance to a normal metabolite enables them to bind at the target site and interfere with a vital activity. Microorganisms produce secondary metabolites during sporulation. Sporulation usually occurs when there is depletion in the nutrient content of the growth medium. Different classes of antibiotics and their mode of action are given in Table 3.1.

Table 3.1 Different classes of antibiotics

Chemical Class	Examples	Biological Source	Spectrum (effective against)	Mode of action
β-lactams (pencillins and cephalosporins)	PenicillinG, Cephalothin	*Penicillium notatum* and *Cephalosporium* species	Gram-positive bacteria	Inhibits cell wall synthesis
Semi synthetic penicillin	Ampicillin, Amoxycillin		Gram-positive and Gram-negative bacteria	Inhibits cell wall synthesis
Monobactams	Aztreonam	*Chromobacter violaceum*	Gram-positive and Gram-negative bacteria	Inhibits cell wall synthesis
Carboxypenems	Imipenem	*Streptomyces cattleya*	Gram-positive and Gram-negative bacteria	Inhibits cell wall synthesis
Aminoglycoside	Streptomycin	*Streptomyces griseus*	Gram-positive and Gram-negative bacteria	Inhibits translation of proteins
Glycopeptides	Vancomycin	*Streptomyces orientalis*	Gram-positive bacteria,esp.*Staphylococcus aureus*	Inhibits cell wall synthesis
Lincomycins	Clindamycin	*Streptomyces lincolnensis*	Gram-positive and Gram-negative bacteria.esp.anaerobic bacteroids	Inhibits protein Synthesis
Macrolides	Erythromycin	*Streptomyces erythreus*	Gram-positive and Gram-negative bacteria, not enterics, *Neisseria, Legionella. Mycoplasma*	Inhibits protein Synthesis
Polyenes	Amphotericin	*Streptomyces nodosus*	Fungi	Inactivate Membranes
	Nystatin	*Streptomyces noursei*	Fungi(*Candida*)	Inactivates Membranes containing sterols
Rifamycinx	Rifampicin	*Streptomyces medeterranen*	Gram-positive and Gram-negative bacteria ,*Mycobacterium tuberculosis*	Inhibits protein Synthesis
Tetracyclines	Tetracycline	*Streptomyces sp.*	Gram-positive and Gram-negative bacteria .Rickettsias	Inhibits protein Synthesis
Chloramphenicol	Chloramphenicol	*Streptomyces venuzuelae*	Gram-positive and Gram-negative bacteria .	Inhibits protein Synthesis

3.1.2 Actinomycetes as a source of antimicrobial compounds

Most of the work in the field of antibiotic production has been centered on actinomycetes, particularly on Streptomycetes as these organisms are proven source of structurally diverse secondary metabolites possessing broad range of biological activities. Actinomycetes have provided many important bioactive substances (Takizawa *et al.*, 1993). These microorganisms stand out as a unique group of prokaryotic organisms in two respects, the diversity of their morphology and metabolic products (Ensign, 1978). Approximately two thirds of the naturally occurring antibiotics have been isolated from actinomycetes (Okami and Hotta, 1988).

3.1.3 Screening actinomycetes for antimicrobial property

As the frequency of novel bioactive compounds identified from terrestrial actinomycetes decreased with time, researchers are increasingly screening actinomycetes from varied environments. Jensen and Fenical (1996) have initiated a pilot study to engineer biochemical diversity in marine streptomycete natural products, as these marine strains present a vast untapped resource of novel metabolites. Intensive screening programs carried out over the past several decades came across with rediscovery of already known bioactive compounds. One approach to address this problem is to expand the source of actinomycetes by carrying out ecological assessment of environments other than soil. The biochemical potentials of the terrestrial actinomycetes have been widely exploited, but their marine counterparts remain largely ignored (D'souza and Vaidya, 1999). There is a growing interest among researchers to screen marine actinomycetes for novel bioactive compounds. Since 1950 more than 200 macrolide antibiotics have been reported. They elicit antifungal and antibacterial

activity. Similarly the number of antibiotics isolated from these groups of organisms is numerous. It is difficult to define which genera of actinomycetes should be selected for specific types of desired activities, since a range of different genera produces antibiotics. (Table3.2)

Table 3.2 Examples for antibiotics produced by Actinomycetes

Antibiotic	Producing organism	Action	Reference
Alba flavenone	*Streptomyces abridoflavus*	Antimicrobial	Gurtler and Pedersen, 1993
Aldecalmycin	*Streptomyces* sp.	Antimicrobial	Sawa *et al.*, 1994
Nisamycin	*Streptomyces* sp.	Antimicrobial	Hayashi *et al.*, 1994
Bioxalomycins	*Streptomyces* sp.	Antimicrobial	Bernan *et al.*, 1994
Indomycinone	*Streptomyces* sp.(marine)	Antimicrobial	Biabam *et al.*, 1997
Liposidomycins	*Streptomyces* sp.	Antibacterial	Kimura *et al.*, 1998
Lactonamycin	*Streptomyces rishiriensis.*	Antimicrobial	Matsumoto *et al.*, 1998
Fattiviracin A1	*Streptomyces microflavus*	Antiviral	Yokomizo *et al.*, 1998
Diperamycin	*Streptomyces griseoaurantiacus*	Antimicrobial	Matsumoto *et al.*, 1998
Arisostatins A and B	*Micromonospora* sp.	Antimicrobial	Igarushi *et al.*, 1999
Dihydromphimycin (Macrolide)	*Streptomyces hygroscopicus*	Antifungal	Ivanova *et al.*, 1999
Wecatromicins	*Actinomadura* sp.	Antimicrobial	Momose *et al.*, 1999
Vinylamycin	*Streptomyces* sp.	Antimicrobial	Igarashi *et al.*, 1999

3.1.3.1 Methods of screening

The screening program must be done in such a way as to obtain "exotic" or highly unusual microbes. Freshly obtained wild microorganisms yield greater microbial metabolic diversity than isolates held in culture collections (Yabrough, 1993). Organisms, which are maintained in culture collections and subjected to, repeated passages (regrowth in same fermentation media) may loose some of their original capacity for gene

expression and are not as robust in their level of production of secondary metabolites (Nisbet, 1979). Screening allow the discarding of many valueless microbes and helps in the isolation of the organism of interest from a large microbial population.

Many industrial screening programs for the isolation of novel antibiotic forming actinomycetes involve initial testing of surface colonies (Bushell, 1982). Pickup *et al.*, (1993) suggested that agar cultures are suitable for examining large numbers of isolates. Screening methods for antibacterial antibiotics have been modified by the changes in target pathogens and based on the elucidation of mechanism of action of antibiotics.

There are mainly two methods of screening for antibiotics. One involves the direct screening of cultures growing on agar and the other one is the determination of activity in liquid media (Waksman, 1959 and Waksman and Lechevalier, 1962).

1. Screening on Agar

For this, cultures may be examined *in situ* on isolation plates or grown I individually and tested for activity against selected bacteria and fungi.

a) "Crowded Plate" Technique

This is one of the earliest method in which cultures were isolated when zones of inhibition were noted in crowded soil isolation plates. Since there was no control over what was being inhibited this technique was not very useful. Besides, this technique is not specific for a particular microbe of interest.

b) Four- layer plate technique (Kelner, 1948)

Kelner developed this method to overcome the objection towards crowded plate technique. This method is done in four steps;

i. A foundation layer of sterile actinomycete agar is prepared.

ii. A seed layer of 0.5 ml of soft agar containing a soil dilution designed to give 30-50 colonies per plate is poured over the agar layer.

iii. A layer composed of 5-10ml of sterile agar added after growth of the colonies on layer two.

iv. A top layer of 3 ml or more containing a suspension of test bacteria.

This has the disadvantage of making it difficult to isolate contaminant free isolates. Here only one test culture can be used on each plate.

c) Lederberg replica plate technique

This technique of antibiotic screening is done by stamping a series of plates from the isolation plates and applying a different test organism to each plate (Lechevalier and Corke,1953). This method has added advantage that more than one agar medium can be employed thereby increasing the possibility of detecting medium related zones of inhibition.

d) Cross streak plate method

Actinomycete cultures are streaked in a narrow band across the centres of agar plates, incubated for growth and preferably until sporulation. Test organisms are then streaked from the edge of the plates up to but not touching the actinomycete growth. The plates are further incubated. The inhibition by the actinomycete against each test organism is noted. The advantage is many test organisms can be tested at a time against one actinomycete. Disadvantage is both the actinomycete and test organism must be grown in the same agar medium. To overcome this handicap, cultures can be grown over the surface of agar plates and plugs or block cut outs. These plugs may be

then placed on plates seeded with desired test organism.(Ball *et al.*,1957 and Vanek *et al.*,1958).

2. Testing antibacterial activity in culture broths

a) Plate diffusion method

Agar diffusion methods using bacteria, as test organism is one of the most widely used assay methods. This method is used to find antibacterial activity in culture broth of actinomycetes. This assay is based on the technique of allowing an antibiotic to diffuse through an agar gel, which has been previously seeded with test organisms. This is the most widely used and accepted method employing the diffusion technique. This assay is done in two ways.

i. Agar well method - In which wells of definite volume are cut on agar plates seeded with test microbes. Culture filtrate is added to the wells in fixed quantity using micropipette and incubated.

ii. Kirby-baur disc method – Sterile paper discs impregnated with culture filtrates are placed over seeded agar plates with test microbes.

b. Use of supersensitive mutants

In recent screening programs to find new antibiotics active against the bacterial cell surface, an antibiotic selectively active against antibiotic resistant mutant *Staphylococcus aureus* 4R was sought. About 2000 actinomycetes strains were tested by this procedure and lead to the discovery of new dipeptide Alahopcin (Higashide *et. al.*, 1985).

c. Use of mutants supersensitive to antibiotics

To detect small amounts of antibiotic production in a culture broth by traditional agar diffusion method, a supersensitive mutant of conventional assay organism was used. A new pyrrole-amidine antibiotic TAN-868 A, which is active against bacteria, fungi and protozoa was detected by this method using sensitive strain of *Micrococus flavus* by *Streptomyces idiomorphins* was reported by Takizawa *et al.* (1987).

3.1.2 Antibiotics in aquaculture

Even though the use of antibiotics in aquaculture practice is unscientific, unwanted and harmful, antibiotics are being used for therapeutic, prophylactic and growth promoting purposes. Certain antibiotics are incorporated in shrimp feed as preservatives. The table given below is the list of antibiotics, which are clandestinely promoted for aqua cultural practice in India.

Sl.No	Antibiotics
1	Chloretetracycline
2	Oxytetracycline
3	Tetracycline
4	Doxycycline
5	Ampicillin
6	Amoxycillin
7	Furazolidone
8	Gentamycin
9	Quinoline based antibiotics
11	Chloramphenicol
12	Sulphonamides

Use of antibiotics results in the emergence of drug resistant bacteria. There are reports of the isolation of antibiotic resistant pathogens like *Salmonella, Listeria* and *Vibrio* from aquaculture farms. Besides these, chemicals may accumulate in the shrimp tissues and exoskeleton. The European Union, US FDA and Japan have notified that residues of the following antibiotics should not be present in the imported shrimp.

1. Chloramphenicol
2. Furazolidone
3. Nalidixic acid
4. Neomycin
5. Oxolinic acid
6. Oxytetraycline
7. Tetracycline
8. Sulphonamides

The Govt. of India has recently notified the maximum permitted residual level of antibiotics in fish and fishery products.

Sl.No	Sl.No.	Max.Residual level in ppm
1	Chloramphenicol	Nil
2	Furazolidone	Nil
3	Neomycin	Nil
4	Tetracycline	0.1
5	Oxolinic acid	0.1
6	Oxytetra cycline	0.3
7	Trimethoprim	0.05
8	Nalidixic acid	Nil
9	Sulphamethazole	Nil

3.2 MATERIALS AND METHODS

The actinomycete cultures isolated from marine environment were subjected to screening for antibacterial activity.

3.2.1 Microorganisms

Actinomycetes

Actinomycete isolates (41numbers) were screened for antibacterial activity against bacterial prawn pathogens.

Pathogens

Bacterial prawn pathogens isolated and maintained at the Center for Fish Disease Diagnosis and Management (CFDDM), CUSAT, Kochi were used for the study. These pathogens were isolated from sources like, larval rearing system of *Macrobrachium rosenbergii*, *Penaeus indicus* and adult *Metapenaeus dobsoni* affected with shell disease. Totally 22 pathogens were used for the study. All the pathogenic strains were *Vibrio* spp. (Table 3.3)

Culture Broth

The selected actinomycete isolates were cultured in two different fermentation media A and B (Composition given below).

Medium A

Soluble Starch	2g
Soybean mea	1.5g
KH_2PO_4	0.3 g
$MgSO_4.7H_2O$	0.05 g
$COCl_2.6H_2O$	0.0002g
$MnCl_2.4H_2O$	0.0002 g
Na_2HPO_4	0.2 g
Sea water	100ml
pH	7

Medium B

Glycerol	7ml
Glucose	3g
Beef Extract	3g
Peptone	0.8g
$NaNO_3$	0.2g
$MgSO_4.7H_2O$	0.01g
Sea water	100ml
pH	7

Table 3.3 List of pathogens used to test the antibacterial property of the marine actinomycetes

S. No	Pathogens		Source
	Culture No	Genera	
1	P1	*Vibrio* sp.	*Macrobrachium rosenbergii* larvae
2	P2	*Vibrio sp.*	*Macrobrachium rosenbergii* larvae
3	P3	*Vibrio* sp.	*Macrobrachium rosenbergii* larvae
4	P4	*Vibrio* sp.	*Macrobrachium rosenbergii* larvae
5	P5	*Vibrio* sp.	*Macrobrachium rosenbergii* larvae
6	P6	*Vibrio* sp.	*Macrobrachium rosenbergii* larvae
7	P7	*Vibrio* sp.	*Macrobrachium rosenbergii* larvae
8	P8	*Vibrio* sp.	*Penaeus monodon* larvae
9	P9	*Vibrio* sp.	*Metapenaeus dobsoni* adult
10	P10	*Vibrio* sp. *	*Penaeus monodon* larvae
11	P11	*Vibrio* sp. *	*Penaeus monodon* larvae
12	P12	*Vibrio* sp. *	*Penaeus monodon* larvae
13	P13	Vibrio *sp.*	*Penaeus monodon* adult
14	P14	*Vibrio* sp.	*Penaeus monodon* adult
15	P15	*Vibrio* sp.	*Penaeus monodon* adult
16	P16	*Vibrio* sp.	*Penaeus monodon* adult
17	P17	*Vibrio* sp.	*Penaeus monodon* adult
18	P18	*Vibrio* sp.	*Penaeus monodon* adult
19	P19	*Vibrio* sp.	*Penaeus monodon* adult
20	P20	*Vibrio* sp.	*Penaeus monodon* adult
21	P21	*Vibrio* sp.	*Penaeus monodon* adult
22	P22	*Vibrio* sp.	*Penaeus monodon* adult

* Luminescent Vibrio

100ml each of media A and B were prepared in seawater (30ppt) in a 250 ml Erlenmeyer flask and autoclaved at 15lbs pressure for 15 minutes. Glucose for medium B was filter sterilized separately and added at the time of inoculation.

A loopful of 2 week old slant culture of the actinomycete isolates were inoculated into the fermentation media and incubated at room temperature (28 ± 2 ^{0}C) for 7 days in a rotary shaker at 150 rpm. After incubation, the culture broth was centrifuged at 10,000 rpm for 15mts at 4^{0}C in a cooling centrifuge (Remi, C-30, Mumbai). The supernatant was collected and used for testing antibacterial property.

3.2.2 Testing Bioactivity

Kirby - baur disc method was employed for testing Bioactivity. Nutrient agar (peptone, 0.5g; beef extract, 0.3g; sea water (50%), 100ml; pH 7.2). Plates were prepared and swab inoculations of the pathogens (*Vibrio* spp.) were made on the surface to produce a lawn culture. Sterile filter paper discs impregnated with culture broth (30µl) were placed immediately on the agar surface. The plates were incubated at room temperature 28±2^{0}C for 24 hours and observed for halo zone formation. Presence of halo zone around the discs was recorded as positive for antibacterial property. Diameter of the zones was also measured. Based on the antibacterial property, six actinomycetes were selected for further study.

3.2.3 Testing antagonistic activity of selected strains of actinomycetes against natural flora

Natural flora Isolated from penaeid prawn culture ponds (Panangad, Kochi) and maintained in the laboratory were used for the study. Six hundred cultures were used for the sensitivity test. These included *Aeromonas* (154Nos), *Vibrio* (50), *Pseudomonas* (60), *Bacillus* (45), *Flavobacterium* (50), Enterobacteriaceae (65), *Flexibactor* (8), *Moraxella* (50), *Alcaligenes* (6), *Streptococcus* (50) and *Staphylococcus* (50), Coryneforms (10) and *Micrococcus* (2)

Assay Medium

Seed medium			Production medium		
Starch	-	1g	glycerol	-	7ml
Yeast extract	-	0.4g	glucose	-	3g
Peptone	-	0.2g	Meat extract	-	3g
Seawater	-	100ml	peptone	-	0.8g
PH	-	7	$MgSO_4$ $7H_2O$	-	0.01g
			$NaNO_3$	-	0.2g
			Seawater	-	100 ml
			PH	-	7

A loop full of actinomycete cultures of the six selected strains (B30, B272, B301, B361, B377 and B451) were inoculated into 50ml each seed medium in 250 ml Erlenmeyer flasks. Incubated for 24 hrs at room temperature (28±2°C) on a rotary shaker at 150 rpm.

10ml each of this seed medium (after incubation) was transferred to 100ml production medium. Incubated at room temperature (28±2°C) for 5 days on a rotary shaker at 150 rpm. Culture broth was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for the study.

Testing Antagonistic Activity

Kirby-baur disc method as described in (3.2.2) was adopted for study. Instead of pathogens natural flora isolated from the prawn culture systems were used for testing sensitivity.

3.2.4 Testing sensitivity of bacterial prawn pathogens to commercially available antibiotics

Antibiotics used

Fifty-two commercially available antibiotic discs (HiMedia, Mumbai) were used (Table 3.4).

Table 3.4 List of commercially available antibiotics(discs) used for the study

Sl. No.	Antibiotic	Sl .No.	Antibiotics
1	Amikacin	27	Nalidixic Acid
2	Ampicillin	28	Neomycin
3	Azytromycin	29	Norfloxacin
4	Cefamandole	30	Novobiocin
5	Cefazolin	31	Oleandomycin
6	Ceftazidime	32	Pipemidic Acid
7	Ceftizoxime	33	Polymixin-B
8	Cephadroxil	34	Roxithromycin
9	Cephaloridine	35	Sparfloxacin
10	Cephalothin	36	Spiramycin
11	Cephotaxime	37	Streptomycin
12	Cephoxitin	38	Sulphamethizole
13	Ciprofloxacin	39	Ticarcillin
14	Cloxacillin	40	Tobramicin
15	Colistin	41	Vancomycin
16	Doxycyckine hydrochloride	42	Virginamycin
17	Enrofloxacin	43	Fluconazole
18	Erythromycin	44	Trimethoprim
19	Floxitin	45	Nystatin
20	Fusidic Acid	46	Clotrimazole
21	Gentamicin	47	Sulphadiazine
22	Kanamycin	48	Rifampicin
23	Lincomycin	49	Amoxycillin
24	Lomefloxacin	50	Chloramphenicol
25	Methicillin	51	Nitrofurantoin
26	Minocyclin	52	Tetracycline

Pathogens

Twenty-two pathogens (*Vibrio* spp.) were used for the study (Table 3.3).

Sensitivity Assay

Nutrient agar plates (Peptone 0.5%, Beef extract 0.3%, Sea water 100ml, pH 7) seeded with the pathogenic vibrios (22 numbers) were prepared and the antibiotic discs (52 numbers) were kept on the surface of the plates immediately. Simultaneously filter paper discs impregnated with the fermentation broth of the six selected actinomycetes were also kept on the surface of the plates. Incubation was done at room temperature (28±2°C) for 24 hrs and the halo zone formation was noted. Diameter of the zone of inhibition was also recorded.

3.3 RESULTS

3.3.1 Screening of actinomycetes for inhibitory property

A total of 41 isolates were subjected to screening for antibacterial activity against 22 bacterial prawn pathogens (*Vibrio* spp.) and the results are given in Fig 3.1.a and 3.1.b The performance of the strains was different in the two different media in terms of its inhibitory activity against the pathogens. Generally medium B was found to be supporting better antibiotic production compared to medium A. Out of the 41 isolates tested, six inhibited more than 50% of the vibrios, when their performance in both the media were taken into account.

Medium A

Culture No. B 272 inhibited maximum number of vibrios (65%) followed by B 301 (57.8%) and B 30 (50%). All the strains exhibited inhibitory property. However, the percentage of vibrios inhibited was comparatively low for some of the actinomycetes.

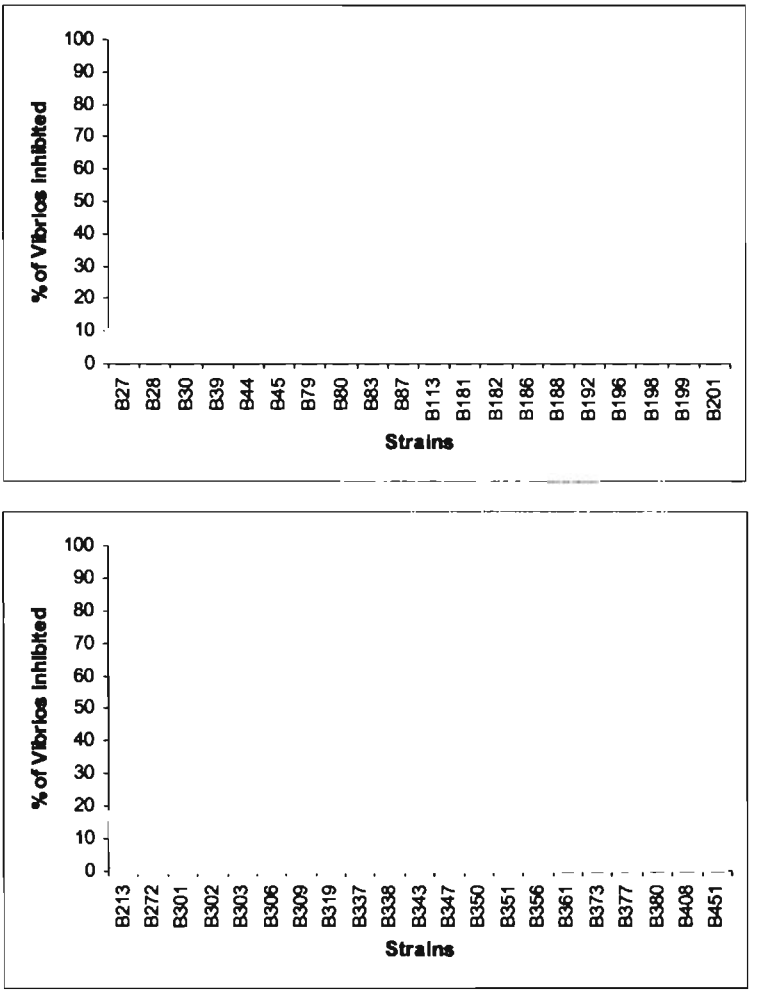


Fig 3.1(a) Percentage of vibrios inhibited by the various actinomycetes when cultured in Medium A

Medium B

Culture No. B 361 inhibited maximum number of vibrios (85%) followed by B 377 (70%) and B 451 (65%). Most of the strains inhibited more than 30% of the vibrios tested. No strain could be observed without inhibitory property.

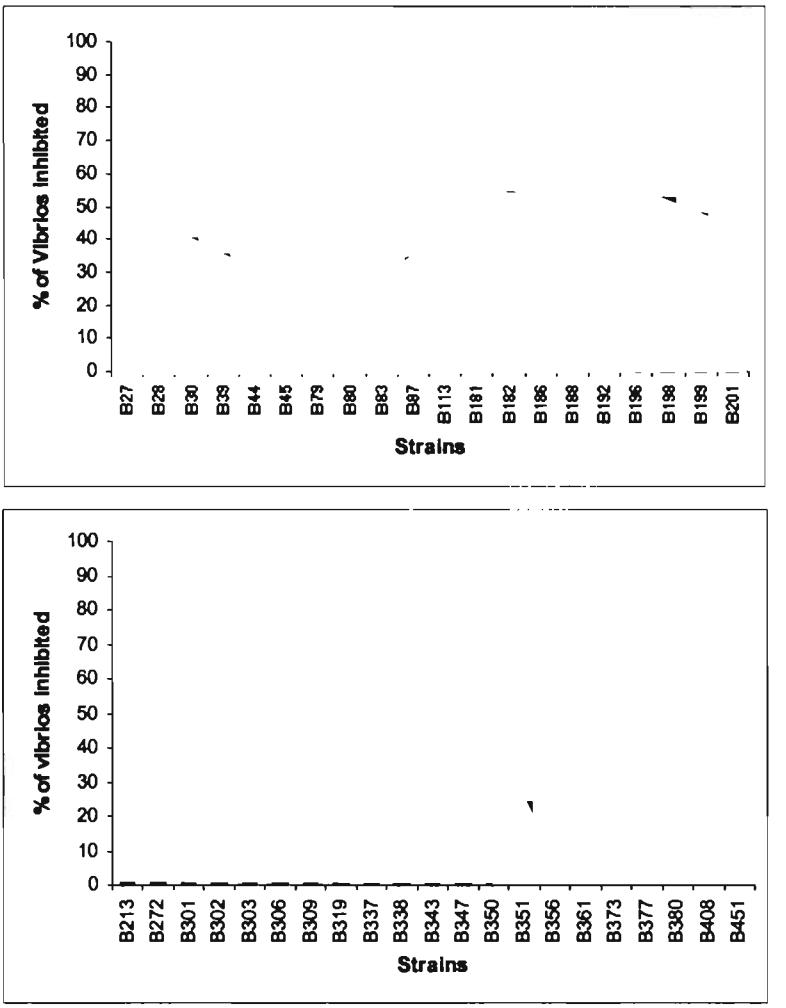


Fig 3.1(b) Percentage of vibrios inhibited by the various actinomycetes when cultured in Medium B

Based on the above results six strains were selected for further study. These strains were 1) B 30, 2) B 272, 3) B 301, 4) B 361, 5) B 377, and 6) B451.

3.3.2 Comparison of the antibiogram of commercially available antibiotics and the selected actinomycete isolates against Vibrios

Out of the 52 different antibiotics, maximum numbers of pathogens were inhibited by Neomycin (95%) (Table 3.5 and Fig3.2) Amikacin, Enrofloxacin, Streptomycin and Nitrofurantoin inhibited 90% of the pathogens.

Table 3.5 Comparison of the inhibitory activity of commercially available antibiotics and selected actinomycete strains against bacterial prawn pathogens

	Inhibition by antibiotic disc*(1-52) and actinomycete broth** (52 - 58) (halozone in cm)																										
Pathogens	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
P1	-	2	-	2	2	1	2	1	-	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
P2	1.7	-	-	2	2	2	2	3	1	2	1	2	2	-	-	2	2	2	2	3	3	1	1	2	2	-	-	1.5
P3	1	1	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2	-	1	-	2	1	-	-	-	-	-	1
P4	-	1	2	-	-	1	-	-	-	-	-	-	4	-	-	-	2	-	1	-	1	-	-	-	-	1	-	-
P5	1.5	1	1	2	2	-	1	2	1	2	2	2	3	2	-	1	2	-	2	1	2	1	-	2	2	-	-	-
P6	1	2	2	-	-	-	-	-	2	2	2	2	2	-	-	-	2	-	2	1	2	2	-	2	2	1	-	1
P7	1	-	-	2	2	-	2	2	2	-	2	-	-	-	-	-	1	-	1	1	2	1	-	1	-	-	-	0.9
P8	1.5	2	2	1	1	1	2	1	1	1	2	1	2	1	2	1	2	1	2	-	2	2	2	2	-	-	-	1
P9	1.4	1	2	2	1	2	-	2	2	1	2	2	2	2	-	2	2	1	2	1	2	1	1	1	-	-	-	1
P10	1.5	-	-	2	2	3	3	2	3	2	2	3	3	4	-	-	2	2	2	2	2	2	2	2	3	1	1	1
P11	1	2	1	-	2	2	-	2	2	1	2	-	2	1	-	1	1	-	-	-	1	2	-	-	2	-	-	1
P12	1.5	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	1	1	1	1	-	-	1	-	-	1
P13	0.9	1	2	1	-	-	-	1	-	1	1	1	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P14	1	2	1	1	1	1	-	1	-	-	-	-	-	-	-	-	2	1	2	-	2	1	-	2	-	-	-	1
P15	1	2	2	2	2	-	3	1	-	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P16	0.9	1	2	-	-	-	-	-	-	-	-	-	4	-	2	1	3	-	2	-	1	-	1	2	-	-	2	1
P17	1	-	1	1	1	1	1	1	-	1	-	-	1	1	-	-	2	-	1	1	1	1	1	-	-	-	-	1
P18	1.5	2	2	2	2	-	1	2	2	2	3	2	2	2	-	-	2	-	2	2	3	2	3	2	2	1	1	1.5
P19	1.5	2	2	3	3	2	2	3	2	2	2	2	3	3	-	3	2	1	2	3	1	2	2	2	2	2	1	1
P20	1.3	2	1	1	2	2	2	1	1	1	-	1	1	-	1	-	2	-	2	-	1	-	2	2	-	-	-	1
P21	1.5	-	-	1	1	2	2	-	-	1	2	1	2	1	-	1	1	-	1	-	2	1	-	-	-	1	-	1
P22	1.5	-	-	1	1	3	3	1	2	1	-	2	1	-	-	-	1	-	1	-	-	1	-	-	-	-	-	1
%Pathogen inhibited	90	65	60	70	70	55	55	75	50	60	55	55	75	45	20	20	90	30	80	40	80	75	35	60	40	30	20	95

Table 3.5 Continue ...

Pathogens	29	30	31	32	33	34	35	36	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
P1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	3	3	1	1.5	2	2
P2	1	3	2	-	-	1	1	1	-	2	2	2	1	-	2	-	2	-	2	2	-	2	2	-	-	-	-	1	-
P3	2	-	-	1	-	1	-	2	-	-	2	-	1	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-
P4	1	-	-	-	-	1	-	2	-	-	2	-	-	-	-	-	-	-	2	-	2	2	-	-	-	-	0.9	1	-
P5	-	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-	2	2	2	1	-	-	-	-	-	-
P6	1	-	-	-	-	-	2	1	-	1	2	2	-	-	2	-	1	-	2	-	2	2	-	2	1	-	0.8	-	-
P7	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1	-	2	2	-	-	-	1	1	1	1
P8	1	-	-	1	-	1	2	2	-	-	2	-	-	-	-	-	-	-	2	-	2	2	1	-	-	-	1	1	-
P9	-	2	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	1	1	-	1	1	-
P10	2	-	-	-	-	2	2	1	2	1	2	1	2	-	3	1	1	1	1	2	2	2	1	1	1	-	1	-	-
P11	1	-	3	-	-	-	-	1	-	-	1	-	-	-	1	-	-	-	1	-	1	1	-	1	1	-	1	-	-
P12	1	-	-	1	-	-	-	1	1	1	-	-	-	-	-	-	-	-	1	-	2	1	-	1	-	-	1	-	1
P13	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	1	-	-
P14	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	1	1	-	-
P15	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	-	-
P16	2	1	-	-	1	2	2	1	-	-	1	-	-	-	2	-	-	-	-	-	-	-	1	1	1	1	1	1	1
P17	-	1	-	-	-	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	1	1	1	-	1
P18	1	2	-	1	-	1	1	1	-	1	2	2	2	-	-	-	1	1	2	2	2	2	1	-	-	-	-	-	-
P19	1	2	1	-	-	2	2	2	-	1	2	-	1	-	-	-	2	-	2	2	2	2	2	-	-	-	1.5	1	-
P20	2	1	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	2	1	2	2	-	-	-	-	1.5	-	-
P21	1	-	-	-	-	-	-	2	1	-	2	-	-	-	1	-	-	-	1	-	2	1	1	1	1	1	0.8	1	1
P22	1	1	-	-	-	1	1	1	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	1	-	1	1	1	1
%Pathogen inhibited	75	45	15	25	5	50	50	90	15	45	65	20	25	0	30	5	25	10	65	30	75	90	50	50	50	40	90	50	35

* 1 - 52 Antibiotic discs (Himedia) refer Table 3.4

** 53 - 58 Fermentation broth supernatant of actinomycetes
53 - B30, 54 -B272, 55 - B301, 56 -B361, 57 - B377, 58 - B451

All the antibiotics except Fluconazole exhibited inhibitory property. However, nine of them showed inhibition below 10%. In terms of the extent of halo zone, Ciprofloxacin was most effective showing diameter of 3.5 cm and 3 cm for 2 pathogens each and an overall percentage of 75.

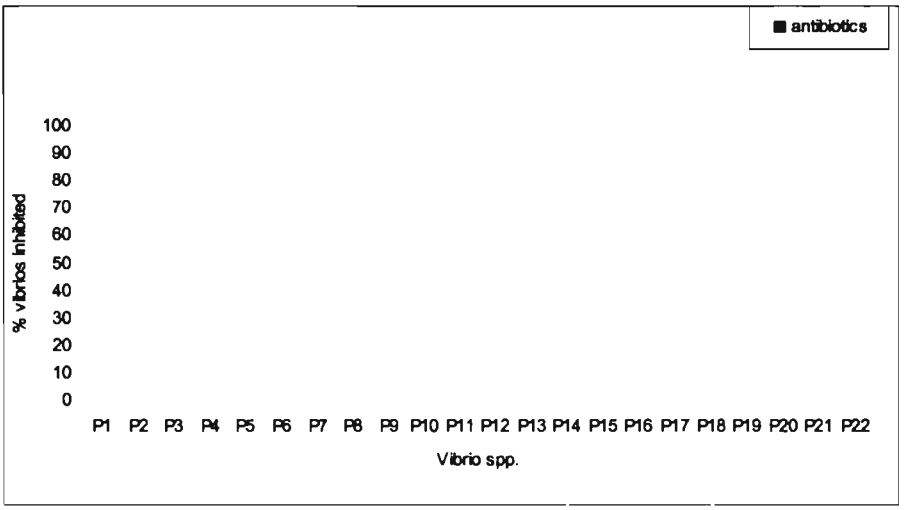
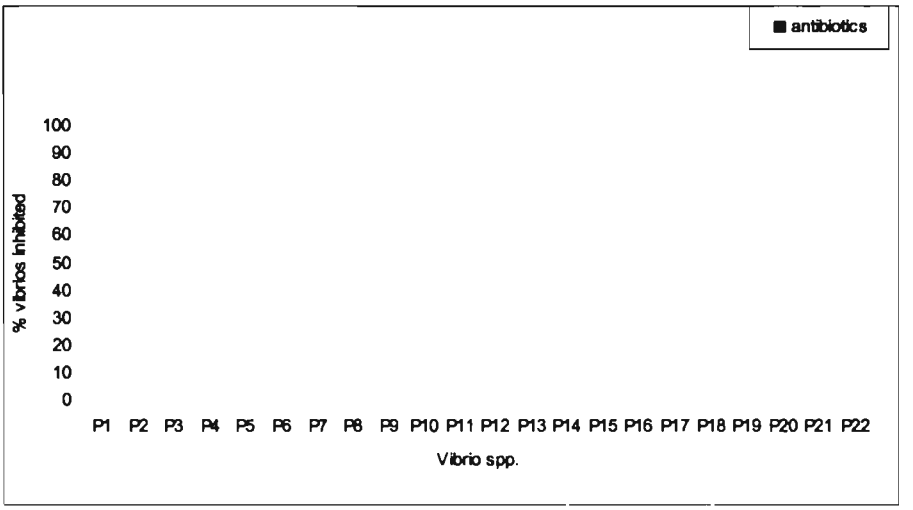


Fig.3.2 Antibiogram of commercially available antibiotics when tested against pathogenic vibrios isolated from prawns

Among the actinomycetes, B 361 inhibited 90% of the pathogens with comparatively good halo zone followed by B 30 (50%), B 272 (50%), B 377 (50%), B 301 (40%) and B 451 (35%). Of the various pathogens tested P10 and P19 was found to be most sensitive to the various antibiotics (42%) each followed by P18 (41%) and P2 (38%) (Fig 3.2). P1 was sensitive to only 17.31 % of the antibiotics tested. However, it was found to be sensitive to all the six actinomycetes tested. The area of the halo zone was also very large compared to other antibiotics, which showed inhibition. P16 and P21 were also inhibited by all the actinomycetes (Table 3.5).

3.3.3 Inhibition of selected actinomycetes to natural flora

Generally, inhibition to the natural flora by the various actinomycetes was found to be less. Maximum inhibition was exhibited by B361 followed by B 451 and B 301 (Fig 3.3.a-f and Table A.1). *Bacillus* was not inhibited by B30, B272, B301, B377 and B451. However, 20% of them were inhibited by B361.



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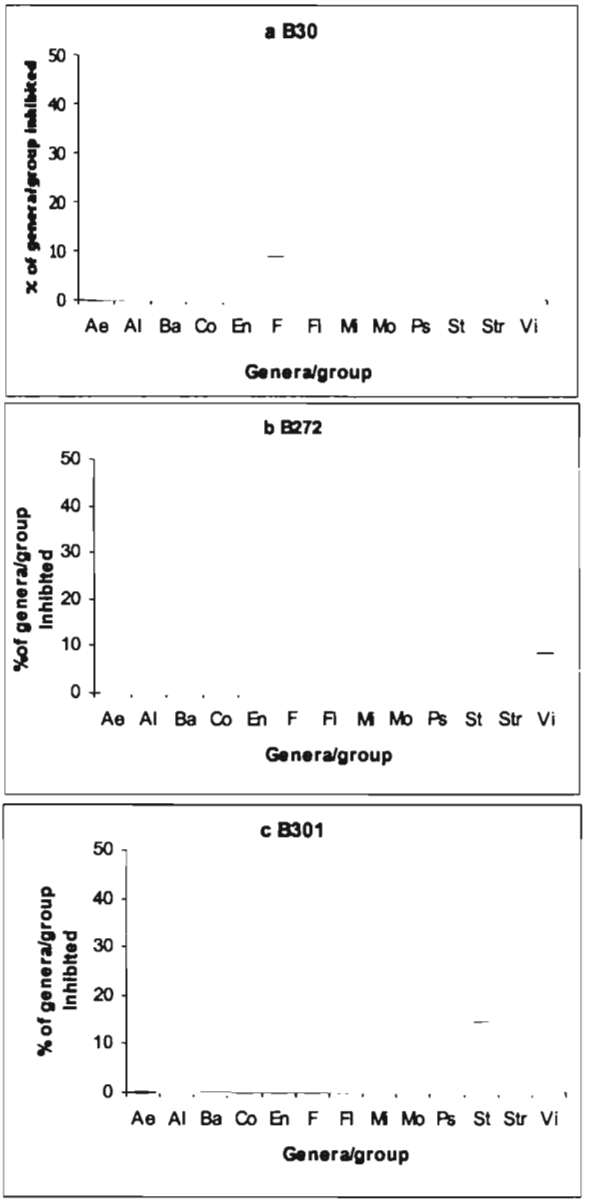
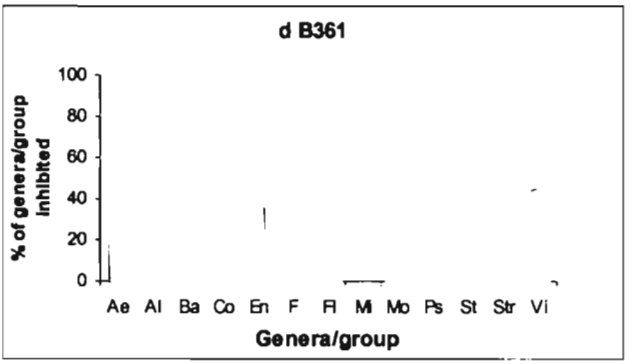
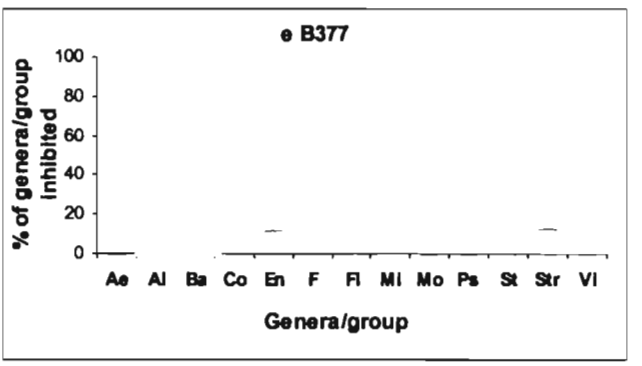


Fig 3.3 (a - c) Percentage inhibition of selected actinomycetes against natural flora

Ae – *Aeromonas*
Al – *Alacaligenes*
Ba – *Bacillus*
Co – Coryneforms
En - Enterobactiriaceae
F - *Flavobacterium*
Fl - *Flexibacter*
Mi - *Micrococcus*
Mo - *Moraxella*
Ps- *Pseudomonas*
St - *Staphylococcus*
Str - *Streptococcus*
Vi - *Vibrio*





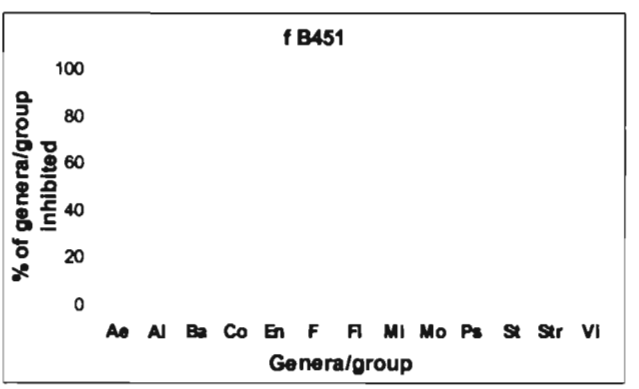


Fig 3.3 (d - f) Percentage inhibition of selected actinomycetes against natural flora

Ae - *Aeromonas*
Al - *Alacaligenes*
Ba - *Bacillus*
Co - Coryneforms
En - Enterobactiriaceae
F - *Flavobacterium*
Fl - *Flexibacter*
Mi - *Micrococcus*
Mo - *Moraxella*
Ps- *Pseudomonas*
St - *Staphylococcus*
Str - *Streptococcus*
Vi - *Vibrio*

Pseudomonas was inhibited by all the six actinomycetes. However, the percentage being inhibited was very less and was in the range 2-22%. The inhibition of *Aeromonas* was found to be in the range of 8.7% – 37.5 % by the various actinomycetes. Vibrios being inhibited varied from 2.7 to 44.4 %. Coryneforms were found to be inhibited considerably.

In general, almost all the groups except Micrococci were inhibited by B361.44.4% of the vibrios (36 No) were sensitive to this actinomycete. *Aeromonas* and Enterobecteriaceae were inhibited at 37.5 % and 35.1 % level.

3.4 DISCUSSION

Members of the family Actinomycetales have been the most widely exploited group of microorganisms in terms of biotechnological applications and as such, have been the subject of study by many scientists (Cross, 1982 and Williams and Vickers, 1986). Antibiotics, enzymes and enzyme inhibitors of commercial importance have been produced by large-scale cultivation of members of this group for the past fifty years and new compounds are discovered, patented and marketed every year. Williams and Vickers (1988) reviewed the literature and reported that out of 3477 antibiotics, Streptomyces alone produced 1984. This fact is not surprising when taking into consideration the ubiquitous nature of this genus and the prolific activity of its species in the production of secondary metabolites.

The discovery of new bioactive compounds hinges upon selective and sensitive screening methods Screening can help in the selection of the organisms of interest from a large microbial population. In the present study for the screening of marine actinomycetes for antimicrobial

compounds, a total of 41 isolates were subjected to screening against 22 bacterial pathogens (*Vibrio* spp.).

The performance of the strains was different in the two different media in terms of its inhibitory activity against the pathogens. The importance of the medium composition for the production of antibiotic activity by marine microorganisms was demonstrated by Okazaki and Okami (1972). In the present investigation, the nature of the fermentation medium directly influenced the elaboration of antimicrobial substances. This is well demonstrated by the fact that B30, B272 and B301 produced bioactive broths when grown in medium A. On the other hand, medium B elaborated the antibacterial activity of B361, B377 and B451.

Actinomycetes are proven source of antimicrobial compounds. The production of these compounds is specific to certain conditions. However, in the present study it was found that there was no strain without inhibitory property. The ability to inhibit all test organisms was not exhibited by any particular strain. By far, the best inhibitory activity was noted with B361. 85% of the total test organisms were inhibited by this strain. It has been found that many antibiotic producing organisms have the capacity of growing in the same medium and at the same time producing several different antibiotics or various chemical modifications of the same antibiotic. Many chemically related antibiotics have been found in the fermentation broth of one organism (Waksman and Lechevatier 1962)

Omura and Tanaka (1984) have reported antibiotics comprising both antifungal and antibacterial compounds produced by soil actinomycetes, majority belonging to the genus *Streptomyces*. Most of the strains inhibited more than 30% of the vibrios tested. Culture No.

B377 and B451 showed more than 60% of inhibition against the vibrios tested. Antibacterial activity of actinomycetes has been reported in the literature (Omura, and Nagakawa, 1984).

Comparison of antibiogram of commercially available antibiotics and the selected actinomycete isolates against vibrios.

In a recent study conducted by CIFT, Cochin showed that out of the 284 samples of farmed shrimp, tetracycline were detected in 122 samples and chloramphenicol in 8 samples.Even though these chemicals can inhibit pathogenic forms, the health hazards associated with them cannot be ruled out. Once the disease outbreaks happens, there is no other way than using antibiotics, one alternative to this problem is to find out novel, environment friendly antibiotics which will not leave any residue in the system.

In this study, most of the commercially available antibiotics showed inhibitory property against the vibrios tested. Neomycin inhibited 95% of the pathogens recording the highest percentage among the 52 antibiotics tested. It was reported that *V. harveyi* isolated from different shrimp farming facilities and from unfarmed waters in Asia were resistant to Ampicillin (Teo *et al.*, 2000) and several other antibacterial (Abraham *et al.*, 1997) compounds.

The use of antibiotics and especially quinolones are common in aquaculture, and new drugs have been tested that are more potent with lower resistance development and more antibacterial activity (Martinsen *et al.*, 1993). Of this group, enrofloxacin has been reported as most potent drug, within minimum inhibitory concentration (MIC) as low as 0.06mg/l for vibrios pathogenic to fish (Martinsen *et al.*, 1993) and between 1.0 and 0.05mg/l for nine vibrios isolated from

diseased shrimp (Mohney *et al.*, 1992). In this study also, enrofloxacin inhibited 90% of the vibrios. Sterptomycin and Nitrofurantoin also showed 90% inhibition to the pathogens.

The selected six actinomycetes exhibited good antagonistic activity against pathogens P1. Even the culture filtrate in very minute concentration gave clear halo zone of 2.5-3 cm compared with the pure antibiotics at 10µg /disc exhibiting only 1-2 cm. Culture No: B361 inhibited 95% of the pathogens like neomycin, enrofloxacin and other antibiotics which exhibited more than 90% inhibition of the pathogens. Neomycin is a banned drug by Govt. of India. This was found to inhibit 95% of the pathogens. P10 and P19 were sensitive to 85% of the antibiotics tested. P1, P16, P21 were sensitive to all the actinomycetes tested.

Chloramphenicol is highly effective in controlling infection caused by *Vibrio campbellii* like bacterium in the larvae and post- larvae of *P. indicus* (Hameed and Rao, 1994). Chloramphenicol inhibited 75% of the pathogens in the present study.

Inhibition of selected actinomycetes to natural flora

The aquaculture environment is a very dynamic system. The chemical/biochemical/gaseous equilibrium in the farm, i.e, water quality is maintained by the materialization process caused by the microflora mainly bacteria.

Microbes are very important and play critical roles in aquatic systems, including shrimp farming at both the hatchery and the grow out level, because water quality and disease control are directly related and influenced by microflora in the ecosystem.

So in the present study the inhibitory activity of the selected strains on natural flora in the pond ecosystem was noted. Beneficial flora such as *Bacillus* and *Pseudomonas* were not inhibited by B30, B272, B301, B377 & B451 and inhibited only at very low level by B361 i.e, *Bacillus*(20%) and16% in case of *Pseudomonas*. *Streptomyces pelveraceus* (B361) was found to inhibit the pathogenic forms (*Aeromonas*, Enterobacteriaceae, *Staphylococcus*, *Streptococcus*, Vibrios and *Flexibacter*) among the natural flora in the pond ecosystem. 44% of the vibrios were inhibited by B361.Vibriosis is a major cause of mortality both in hatcheries and grow out systems. Starin B361 is found to inhibit a good percentage of *Aeromonas* tested. This partial inhibition of only the pathogenic forms and sparing effect of other natural flora makes the Streptomyces a suitable candidate for application in penaeid prawn culture systems. There would not be significant disturbance in the microbial diversity of the pond ecosystem by the application of the antimicrobial compounds from these selected actinomycetes.

Chapter 4

SELECTION OF FERMENTATION MEDIA AND PROCESS OPTIMIZATION

4.1 INTRODUCTION

The nature of the compound produced in a fermentation process depends on the fermentation media and the fermentation conditions. The quality and quantity of the medium ingredients greatly influence antibiotic production. Antibiotics are produced under conditions, which are characteristic for the production of other secondary metabolites (Ömura and Tanaka, 1986). Antibiotic production is strain specific and unstable. The productivity tends to disappear on successive transfer of the producing organism. Active antibiotic production often occurs in association with sporulation of the producing organism, which begins with nutritional limitation in cultivation media.

The major groups of microorganisms used in fermentation are bacteria, actinomycetes, yeasts and fungi. According to Ömura (1986), among the many factors that affect screening for bioactive compounds from microorganisms, a devised fermentation, unique microorganisms and selective and sensitive detection methods for bioactivity are of utmost importance. Several studies have shown that about 30 to 40% of the total numbers of *Streptomyces* isolates have shown sufficient antibiotic activity. Obviously, the results depend on the screening methods, media used and the number and variety of the test organisms. The medium supplies nutrients for growth, energy, and building of cell substances and biosynthesis of fermentation products.

4.1.1 Fermentation media

It is generally accepted that development of fermentation media is a mixture of art and science. The scientific basis rests with those fundamental biochemical aspects of microorganism, which are general

to large group of species. The art is required when the specific biochemical details of the species of interest are unknown. The importance of the composition of the medium employed for the production of antibiotics by marine microorganisms was demonstrated by Okazaki and Okami (1972). Pisano *et al.* (1992) in his paper on chitinolysis as an indicator of potential bioactivity among marine actinomycetes reported that the nature of the fermentation medium directly influenced the elaboration of antimicrobial substances. He has demonstrated this by the fact that, 60% of the actinomycetes produced active broths when grown in medium of Tunac *et al.* (1995), as compared to 40 % in the PS medium of Okazaki and Okami (1972).

4.1.1.1 Nutritional factors in medium formulation

Nutritional ingredients in a fermentation medium are indispensable for growth of microorganisms. Selection of suitable nutrients for fermentation media is commonly related to improved antibiotic production. Fermentation media are composed of carbon sources, nitrogen sources, inorganic metallic 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 Carbonate, ammonium phosphate, phosphates of potassium etc. can be incorporated for enhanced production of antibiotics. Of all the factors used, carbon and nitrogen sources are of particular importance in the medium since microbial cells and fermentation products are composed largely of these elements.

Table 4.1 Fermentation media showing combination of carbon and nitrogen sources used for the production of following antibiotics.

Avermectins[1]	Difficidins[2]	K-252[3]	Monacolin M[4]
Cerelose 4.5%	Dextrin, 4%	Glucose 0.5%	Glycerol, 7%
Peptonized milk 2.4%	Distillers soluble, 0.7%	Soluble starch 3%	Glucose, 3%
Autolyzed yeast 0.25 %	Yeast extract, 0.5%	Soybean meal, 3%	Meat extract 3%
PolyglycolP2000, 2.5ml liter^{-1}	$CoCl_2.6H_2O$, 0.01%	Corn Steep liquor, 0.5%	Peptone 0.8 %
		Yeast extract, 0.5%	$NaNO_3$, 0.2%
		$CaCO_3$ 0.3%	$MgSO_4.7H_2O$ 0.01%
pH 7.0	pH 7.3	pH 7.2	pH 5

1 From Burg *et al.*, 1979
2 From Zimmerman *et al.*, 1987
3 From Nakanishi *et al.*, 1986
4 From Endo *et al.*,1986

In addition to nutrients, a medium also may contain various inhibitors of microbial growth and biosynthesis, which may affect antibiotic production negatively. Antibiotic production is either not initiated or proceeds at a very low rate when glucose, amino acids and other carbon and nitrogen sources required for microbial growth are present at levels exceeding certain thresholds. For example .the production of β-lactam antibiotics (Cephalosporin C and Penicillin N) by *Acremonium chrysogenum* is inhibited by glucose, ammonium ions and inorganic phosphate when one or more of these nutrients are present at high concentration in a medium (Zanca and Martin, 1983). The result obtained by Tanaka *et al.* (1986) contradicts this phenomenon. He reported that the production of the macrolide antibiotic, tylosin by *Streptomyces fradiae* does not appear to be affected by 4% glucose added to the production medium. The production of tylosin was not at all affected by high concentration of glucose. In general, inhibiting nutrients can be glucose or other easily assimilable carbon sources,

ammonia and other easily assimilable nitrogen sources, inorganic phosphate and other phosphate generating ingredients.

4.1.1.2 Seed media and Production Medium

Seed media (inoculum) usually differ in composition from production media. Seed media are compounded for quick yield at large numbers of microbial cells in their proper morphological states, without sacrificing genetic stability of the cells. Seed medium helps in enzymatic adaptation of cells to substrates and eliminates lag period during initial stage of production. This is usually with fewer nutrients compared to production media.

Streptomycetes in contrast to most other bacteria grow well on a glycerol medium containing L (+) Arginine as the sole source of nitrogen (Benedict *et al.*, 1955)

4.1.2 Fermentation process optimization

Many studies on the optimization of antibiotic production in batch and fed-batch cultures have been carried out (Bajpai and Reuss., 1981; Mou and Cooney., 1983). The biosynthesis of antibiotics is normally characterized by a phase of rapid growth (trophophase) followed by the phase of antibiotic production (idiophase). The production of antibiotics is strongly influenced by the nature and concentration of the carbon source, nitrogen source, phosphorous source and trace elements (Yegneswaran *et al.*, 1988). The production of secondary metabolites by different actinomycete strains is regulated by various factors. The production of actinomycin was found to be regulated by Nitrogen and Carbon sources (Williams and Katz, 1977; Aharonowitz, 1980; Foster and Katz, 1981)

4.1.2.1 Effect of nitrogen sources

While optimizing a medium for the production of 1, 2-epoxytetradecane by *Nocardia carollina* B-276 Furuhashi *et al.* (1984) found that the production was enhanced as the concentration of urea had been increased. Same result was obtained when sodium nitrate and various ammonium salts were used as the nitrogen sources.

Streptomyces Venezuelae produce chloramphenicol in a well-buffered medium with a variety of carbon, energy and nitrogen sources (Chatterjee *et al.*, 1983). However, the form in which nitrogen is supplied has a marked influence on the yield as well as on the relationship between biomass and product accumulation (Shapiro and Vining, 1984). For example, ammonium, a readily assimilated nitrogen source, supports lower titre of antibiotics than slowly consumed amino acid nitrogen sources such as proline (Shapiro and Vining, 1985). In general, higher production is obtained in media where the availability of nitrogen limits the growth rate (Shapiro and Vining, 1984).

Cultures growing in a medium containing glucose and ammonium sulphate, produce antibiotics in the stationary phase, after the nitrogen source has been depleted whereas cultures in glucose - proline medium produce chloramphenicol and biomass concurrently while proline is being used (Shapiro and Vining, 1985).

Ammonium is known to repress the synthesis of enzymes required to assimilate alternative nitrogen sources. Shapiro and Vining (1984) reported that *S. Venezuelae* preferentially assimilates ammonium during growth in a medium containing ammonium nitrate and amino acids but uses ammonium and amino acids concurrently.

4.1.2.2 Effect of glucose concentration

Furuhashi and Takagi (1984) while optimizing a medium found that increasing the glucose concentration to 40 gl^{-1} caused an increase in Dry Cell Weight (DCW) and decrease in pH. From these concentrations onwards, DCW did not increase, indicating that glucose was no longer a growth-limiting factor for final biomass. The spiramycin production was maximum at 20 gl^{-1} glucose. Above and beyond this concentration production decreased rapidly.

4.1.2.3 Effect of phosphate concentration

Several mechanisms have been proposed to explain the effect of phosphate on the production of secondary metabolites (Martin, 1977).

a. Phosphate favours primary metabolism; a shift down in primary metabolism depresses secondary metabolism.

b. Phosphate limits synthesis of inducers of the antibiotic pathway.

c. Phosphate shifts carbohydrate catabolic pathways.

d. Phosphates inhibit formation of antibiotic precursors.

e. Phosphate inhibits or represses phosphatases necessary for antibiotic synthesis.

Vegetative growth increased with the initial phosphate concentration up to 5mM, a further increase of phosphate supply showed no significant effect on cell yield (Lounes *et al.,* 1996). Further, it has been shown that there is a sharp decline in the spiramycin production above concentrations of 10 mM.

Several studies showed that antibiotic production in *Streptomyces* was sensitive to phosphate concentration. (Martin, 1977 and Vu-Trong *et al.,* 1981). However, the formation of many secondary metabolites is inhibited by inorganic phosphate concentration (Weinberg, 1978). Among the elements added to fermentation media used in secondary metabolite production, phosphate is one of the most critical factors (Weinberg., 1974). Many antibiotics are produced at concentrations of inorganic phosphate sub optimal for growth. Phosphate in the range of 0.3 - 5.0 mM permits excellent cell growth whereas 10 mM phosphate often suppresses biosynthesis of antibiotics.

Despite the widely accepted notion that low phosphate media are favourable, high phosphate medium containing 1- 2% inorganic phosphate was successfully employed in the fermentation media yielding pyrrolnitrin, an antidermatophytic antibiotic (Arima *et al,* 1964).

High phosphate favours antibiotic production in three ways; as a constituent atom of an antibiotic molecule, as a pH control factor and as an agent to suppress production of other known bioactive compounds. Hall and Hassal (1970) demonstrated that a single microorganism in low and high phosphate concentrations produced different antibacterial compounds.

In contrast to high ammonium salt and high phosphate, Tanaka and Ömura (1988) developed ammonium ion depressed and phosphate ion depressed fermentation for antibiotic screening. They used trapping agents for ammonium ion and phosphate. This led to the discovery of eight new bioactive compounds listed in table.

Table 4.2 Trapping agents used in fermentation media

Sl.No.	Trapping agents	Amount Added (%)	Antibiotics
1.	(NH_4^+) Magnesium Phosphate	1.0	Leucomycin
2.	Sodiumphosphotungstate	0.5	Spiramycin
3.	Natural zeolite	1.0	Tylosin
4.	Magnesium Phosphate	1.0	Cephalosporin
5.	NH_4^+-saturated zeolite (PO_4^{3-})	1.0	Cerulenin
6.	Allophane	0.5	Tylosin
7.	Allophane	0.5	Nanaomycin
8.	Allophane	0.5	Candicidin

4.1.2.4 Fatty acids and amino acids

The fatty acid spectrum of a mutant strain of *S. fradiae* was changed by the supplement of certain fatty acids and high concentration of sodium ions to the medium (Arima *et al.*, 1973)., Okazaki *et al.* (1974) reported that this change of fatty acid spectrum induces the formation of neomycin. D-amino acids, often present as constituents of peptide antibiotics, can inhibit antibiotic production when provided in the culture medium (Katz and Brown, 1989). D - valine inhibits synthesis of actinomycin (Katz.,1960; Katz *et al.*, 1961; Yajima *et al.*,1972) and penicillin (Demain.,1956). D-leucine blocks formation of etamycin (Kamal and Katz., 1976).

4.1.2.5 Effect of Sodium, Potassium and other metal ions

Metal ions affect antibiotic production. These include both promotion and inhibition of antibiotic biosynthesis. For example Furuhashi and Takagi (1984) observed marked difference on the epoxide production and cell growth between sodium and potassium

salts of phosphate. In the presence of sodium salts, the growth was poor, but the activity of the cells was higher than that of the cells cultivated in potassium phosphate buffer.

Trace metals such, as Fe, Mn, Mg, Zn and Cu are known to be essential not only for primary metabolism, but also for production of secondary metabolites. CO_2 stimulated the production of Sagamicin by *Micromonospora Sagamicinsis* and Bialaphos by *S. hygroscopicous*. Similarly, Zn^{2+}, Fe^{2+} and Mg^{2+} are involved in candicin production by *S. griseus*.

4.1.2.6 Effect of aeration

Oxygen, necessary for aerobic growth of microorganisms is dissolved into culture fluids by shaking culture flasks on a reciprocal shaker or by aeration and agitation in a jar fermentor. According to Arai *et al.* (1976), when a strain of Streptomyces sp. was grown in a jar fermentor with agitation at 500rpm,the minimum oxygen tension was sufficiently high to allow production of mimosamycin and chlorocarcins A, B and C. When the agitation was reduced to 250 rpm no antibiotic production occurred. Yegneswaran *et al.*, (1998) studied the effects of reduced oxygen on growth and antibiotic production in *S. clavuligerus* and found that at reduced oxygen levels no production had occurred after stationary phase.

4.1.2.7 Effect of NaCl concentration

Okazaki and Okami (1975) conducted a study on actinomycetes tolerant to increased NaCl concentration and their metabolites. They used actinomycetes isolated from sea mud. Results showed that the medium with 3% NaCl supported the growth of 70% of the marine isolates but only 41% of terrestrial isolates. Also. observed that strains

with increased tolerance to NaCl (3.5%) exhibited a tendency to form novel metabolites. Marine microorganisms, such as *S. tenjimariensis*, producer of an amino glycoside, istamycin tolerated 5 -7% NaCl in culture media (Hotta *et al.*, 1980). Some cultures required 3- 5% NaCl for growth. For example, a marine streptomycete isolated from the surface of Jellyfish, required salt water for optimal growth and Salinamide production.

4.1.2.8 Effect of medium with alkaline pH

The pH of the culture medium is an important measure to monitor antibiotic production as well as mycelial growth. It is usually controlled in flasks (fermentation) by adding mineral acids, $CaCO_3$, phosphate salts, ammonia and Na_2CO_3 to fermentation media. Actinomycetes and fungi are alkalophilic microorganisms. They are surveyed for their ability to produce new antibiotics under alkaline conditions. This trial led to the discovery of a new antibacterial and antifungal peptide antibiotic No.1907, from *Paecilomyces* sp. growing at pH 11 (Sato *et al.*, 1980).

4.1.2.9 Effect of temperature

Antibiotic production varied when growth temperatures were varied. A cryophilic strain of *Streptomyces* produced an antibacterial antibiotic A-60 at 15°C, but did not do so at 28°C, a permissible temperature, because an enzyme that inactivates the antibiotic was produced at 28°C (Ogata *et al.*, 1977). Yoshida *et al.* (1973) in their studies on antibiotic complex SP-35 produced by a psychrophilic actinomycete showed that maximum cell yield was obtained at 12°C while most rapid growth occurred at 25°C. Production of antibiotic occurred between 15°C and 28°C.

Padma *et al.* (2002) evaluated the optimum process conditions, pH, temperature, inoculum size, agitation and aeration for vancomycin production and found that a pH of 7.6, a temperature of 29°C and inoculum size of 4.5 %, agitation of 55 rpm and an aeration of less than 1:10 medium-to-air ratio favored increased production of vancomycin.

In a study conducted by Higashide *et al.,* (1961) for optimizing culture conditions for production of zygomycinA by *S. pulveraceus*, a medium consisting of glucose 2%, lactose 1%, Soybean flour, 2%, peptone 0.5%, NaCl 3% and $CaCO_3$ 0.5% gave a broth with high potency.

Vinogradova *et al.* (2002) investigated on the culture conditions optimization aimed to maximize the secretion of extra cellular L-glutamate oxide by *Streptomyces cremeus* and showed that calcium ions at the concentration of 5-20 mM and 0.1% ammonium sulphate enhanced activity of enzyme to four folds.

4.2 MATERIALS AND METHODS

4.2.1 Selection of Fermentation Media

a) Microorganisms used

The six potential actinomycete strains were selected based on their performance in the preliminary screening test for production of antimicrobial compounds and were used for the fermentation study.

The six selected strains were B30, B272, B301, B361, B377and B451.

These strains were then identified by Institute of Microbial Technology (IMTECH), Chandigarh, India as follows

B30	*Streptomyces californicus*
B272	*Streptomyces* sp.
B301	*Streptomyces griseoflavus*
B361	*Streptomyces pulveraceus*
B377	*Streptomyces* sp.
B451	*Streptomyces fradiae*

b) Inoculum Preparation

The six strains were streaked on to nutrient agar (peptone, 0, 5g; beef extract, 0.3g, agar; 2g; pH, 7.2) slants prepared in seawater and incubated at room temperature (28±2°C) until sporulation. A loop full of this actinomycete culture with spores was inoculated into 50 ml seed medium in 250ml Erlenmeyer flask and incubated for 48 hrs at room temperature (28 ± 2°C) on a rotary shaker at 150rpm.

Composition of seed medium

Starch	-	1g
Yeast extract	-	0.4g
Peptone	-	0.2g
Sea water	-	100ml
pH	-	7

A loop full of actinomycete spores were transferred to 50ml seed medium in 250 ml Erlenmeyer flask.

c) Fermentation Media

Fourteen different fermentation media were selected for the study based on the literature survey and they were designated as M1 to M14.

M1

Glycerol	- 3g
Cotton seed meal	- 2g
Dried yeast cells	- 2g
KH_2PO_4,	- 2.18g
$Na_2HPO_4.12H_2O$	- 1.43g
$MgCl2.6H_2O$	- 0.5g
Sea water	- 100ml
pH	- 7.2

M2

Soluble starch	- 2g
Cotton seed meal	- 0.5g
Wheat germ	- 0.5g
Dried yeast cells	- 0.25g
Corn steep liquor	- 0.25g
$KH2PO_4$	- 0.5%
$Na2HPO_4.12H_2O$	- 1.25g
Sea water	- 100ml
pH	- 7

M3

Dextrin	- 4g
Distillers soluble	- 0.7g
Yeast extract	- 0.5g
$CoCl_2.6H_2O$	- 0.01g
pH	- 7.3

M4

Glucose	- 0.5g
Corn steep liquor	- 1g
Oat meal	- 1g
Pharmamedia	- 1g
K_2HPO_4	.5g
$MgSO_4$, $7H_2O$	.5g
Sea water	- 100ml
PH	- 7

M5

Soluble starch	- 2.5g
Glucose	- 0.1g
Yeast extract	- 0. 2g
$CaCO_3$	- 0. 3g
Sea Water	- 100ml
pH	- 7.5

M6

Glucose	- 1g
Soluble starch	- 1g
Cornsteep liquor	- 0.3g
Oat meal	- 1g
Pharmamedia	- 1g
Basic Magnesium Carbonate	- 0.5g
Sea water	- 100ml
PH	- 7

M7

Glycerol	- 1.5g
Oat meal	- 3g
Dried yeast cells	- 0.5g
KH_2PO_4	- 0.5g
$Na_2HPO_4.12H_2O$	- 0.5g
$MgCl_2.6H_2O$	- 0.1g
Sea water	- 100ml
pH	- 7

M8

Glycerol	- 2g
Starch	- 2g
Corn steep liquor	- 0.2g
Soybean meal	- 1g
Meat extract	- 0.5g
Yeast extract	- 0.3g
$CaCO_3$	- 0.3g
$Mg_3 (PO_4) 2.8H_2O$	- 1g
Sea water	- 100ml
pH	- 7

M9

Glucose	- 0.5g
Soluble starch	- 3g
Soybean meal	- 3g
Corn steep liquor	- 0.5g
Yeast extract	- 0.5g
$CaCO_3$	- 0.3g
Seawater	- 100ml
pH	- 7.2

M10

Glycerol	- 1.25g
Arginine monohydro chloride	- 0.1g
$MgSO_4.7H_2O$	- 0.05g
Yeast Extract	- 0.3gm
Sucrose	- 1.0.3g
Sea Water	- 100ml
pH	- 7.5

M11

Glucose	- 1gm
Soybean Meal	- 1gm
$CaCO_3$	- 0.1gm
Sea Water	- 100ml
pH	- 7.5

M12

Glycerol	- 2ml
Soybean meal	- 0.2g
Citrulline	- 0.01g
$CoCl_2.6H_2O$	- 0.002g
Allophane	- 0.5g
Sea water	- 100ml
pH	- 7

M13

Soluble Starch	- 2g
Soybean meal	- 1.5g
KH_2PO_4	- 0.3 g
$MgSO_4.7H_2O$	- 0.05 g
$COCl_2.6H_2O$	- 0.0002g
$MnCl_2.4H_2O$	- 0.0002g
Na_2HPO_4	- 0.2g
Sea water	- 100ml
pH	- 7

M14

Glycerol	- 7ml
Glucose	- 3g
Meat extract	- 3g
Peptone	- 0.8g
$NaNO_3$	- 0.2g
$MgSO_4.7H_2O$	- 0.01g
pH	- 5.0

All the 14 media (100ml) were prepared in 250 ml Erlenmeyer conical flasks and autoclaved at 121°C for 15 minutes.

5 ml aliquots of 48-hour seed culture were inoculated into 100ml fermentation media. Incubated at room temperature (28±2 °C) on a rotary shaker for 5 days at 150 rpm and antibiotic production was assessed.

d) Antibacterial assay

Twenty-two bacterial prawn pathogens (*Vibrio* spp.) as given in Table 3.4 were used for the sensitivity test. Culture broths were centrifuged at 10000 rpm in a cooling centrifuge (Remi, C-30, Bombay)

at 4°C for 15 minutes. The supernatant was tested for antibacterial property. Disc agar diffusion assay as given in section 3.2.2 was employed for the antibacterial assay.

Based on antibiotic assay results two media were selected for further studies. Medium M13 for B30, B272, B301 and medium M14 for B361, B377 and B451.

4.2.2 Process Optimization

Optimum physico-chemical conditions for maximum growth and antibiotic production for the selected actinomycetes were tested using the two selected fermentation media.

4.2.2.1 Parameters

Salinity, pH, Phosphate concentration and Calcium carbonate concentration in the media were the parameters subjected to the study.

4.2.2.2 Media preparation (Fermentation media)

Medium M13 was used for B30, B272 and B301 since it was found to be the best medium for the antibiotic production by these three cultures. Similarly, medium M14 was used for B361, B377 and B451.

Optimum salinity

Medium M13 and M14 were prepared using seawater of different salinity (10, 20, 25, 30 and 35 ppt).

Optimum pH

Medium (M13 and M14) was prepared at different pH 5, 6, 7 and 8 using various buffers as given below.

Buffer	***pH***
Sodium acetate-acetic acid	5.0
Tris-maleic acid buffer	6.0 and 7.0
Tris-HCl Buffer	8.0

Optimum phosphate concentration

Media (M13 and M14) with different phosphate concentration (0.1, 0.25, 0.5, 0.75 and 1 %) were prepared. KH_2PO_4 and Na_2HPO_4 were added in equal quantity to get the final concentration to the required level. Glucose was sterilized separately in case of medium M14 since glucose and phosphate may react when heated.

Optimum calcium carbonate concentration

Media (M13 and 14 with five different concentrations of $CaCO_3$ (0.1, 0.2, 0.3, 0.4 and 0.5 %) were prepared.

4.2.2.3 Inoculum

Spore suspensions of the six actinomycetes were prepared in seawater using two week old slant cultures. Triton (0.0001 %) was added to the seawater before sterilization to make a homogeneous spore suspension. Spore density was adjusted to 10^6 /ml and the suspensions were used as inoculum.

4.2.2.4 Inoculation and incubation.

1 ml of the spore suspension was inoculated into 50 ml medium in a 250ml Erlenmeyer flask. Incubation was done at room temperature (28±2^{0}C) for a period of 10 days.

4.2.2.5 Determination of growth

The culture broth after 10 days incubation was centrifuged at 10000 rpm for 15 minutes in a refrigerated centrifuge at 4^{0}C .The supernatant was carefully decanted and used for bioactivity tests. Then

the biomass was separated, washed with saline and dried at 80°C to constant weight and the dry weight was calculated to estimate growth.

4.2.2.6 Estimation of antibiotic production

Antibiotic production was noted by testing the inhibitory activity of the culture supernatant against five bacterial prawn pathogens (P1, P2, P15, P17) and one Luminescent Bacteria (LB). The disc agar diffusion assay was employed as given in section 3.2.2.

35 µl each of the culture supernatants was impregnated on to filter paper discs and kept on nutrient agar plate inoculated with the pathogens. Incubation was done at room temperature (28±2°C) for 24 hours and the observation was made for halo zone formation. The diameter of the halo was also measured.

4.3 RESULTS

4.3.1 Comparison of various media for antibiotic production

The selected six strains of actinomycetes showed varied performance in the 14 different fermentation media (Fig 4.1 a-f). Of the 14 media, M13 and M14 were found to be the best in terms of its support for antibiotic production. Culture No: B30, B272, and B301 preferred M13 whereas B361, B377 and B451 preferred M14 (Fig.4.2).

***Streptomyces californicus* B 30**

The activity of B30 in 14 media are presented in Fig 4.1 a. The maximum number of pathogens was inhibited by the culture filtrate of B30 when cultured in Medium M13 (55%) followed by M3 (30%), M8 (20%) and M5 (20%).

***Streptomyces* sp. B 272**

Medium M13 supported better antibiotic production. 60% of the vibrios were inhibited when cultured in M13 followed by 30% in M12.

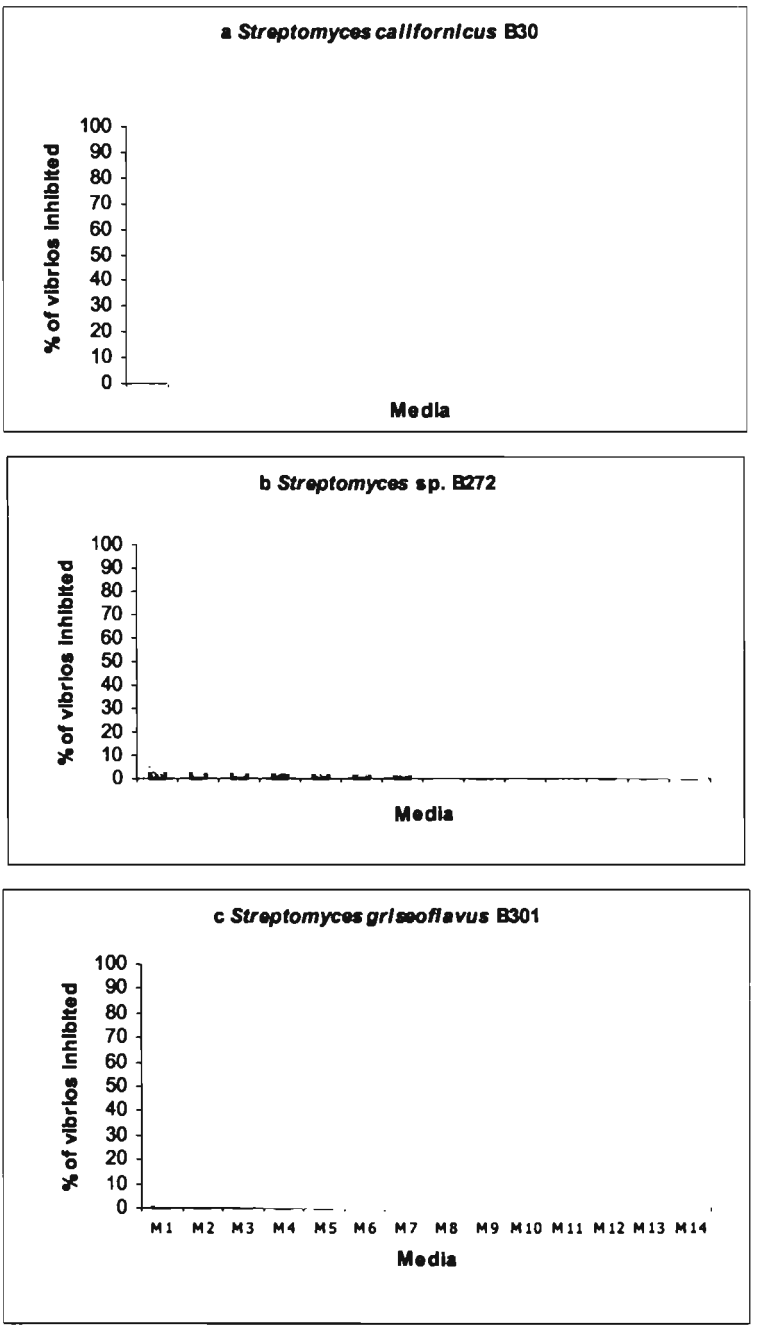


Fig 4.1.a-c percentage of vibrios inhibited by selected strains of Streptomyces spp. when cultured in 14 different fermentation media

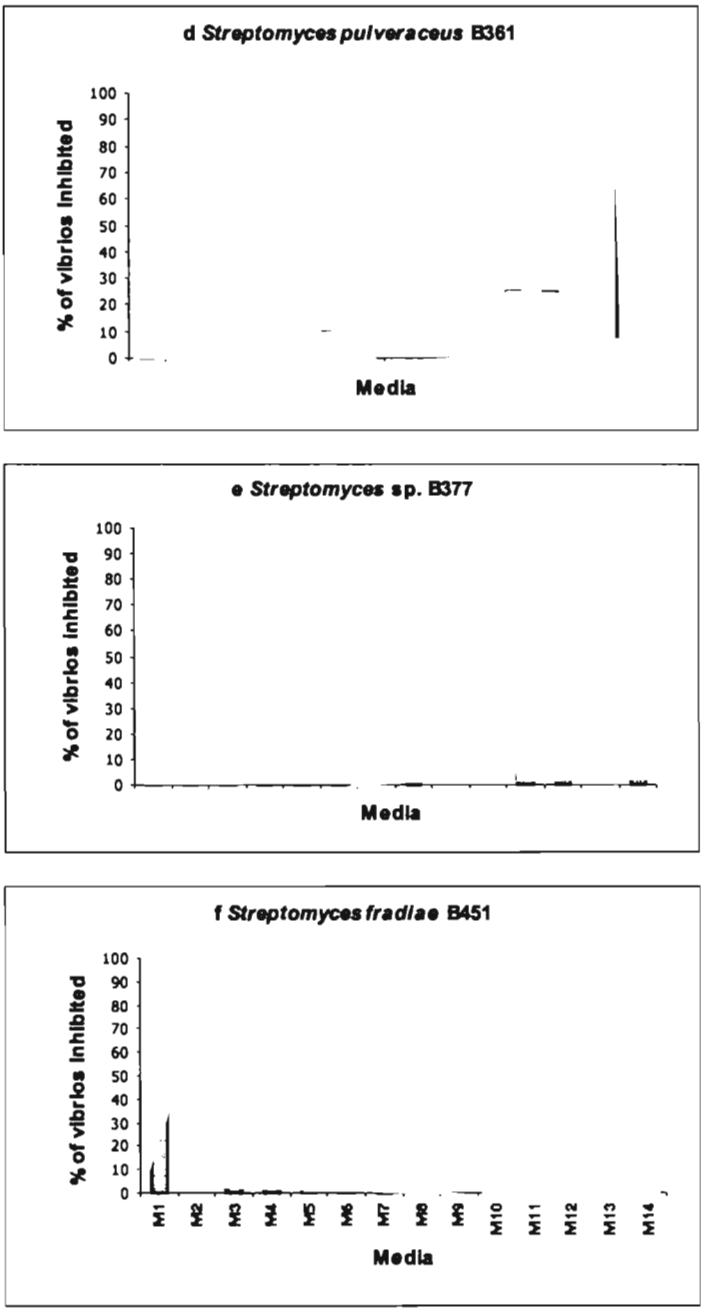


Fig 4.1.d-f percentage of vibrios inhibited by selected strains of Streptomyces spp. when cultured in 14 different fermentation media

Streptomyces griseoflavus B 301

B301 also gave maximum activity in Medium M13.Vibrios were inhibited when grown in this medium. 60 % of the vibrios were inhibited when grown in this medium. When cultured in M1 and M14 35% of the pathogens were found to be inhibited. The rest of the media were not found to support significant production of the bioactive compound.

Streptomyces pulveraceus B361

85% of the vibrios were found to be inhibited by B361 when cultured in M14. The inhibition was found to be below 30% when cultured in all other media.

Streptomyces sp. B377

M14 was found to be the best supporting medium for antibiotic production against the prawn pathogens. 70% of the pathogens were inhibited when the isolate was cultured in this medium followed by M4 (55%).

Streptomyces fradiae B 451

Streptomyces fradiae B451 also preferred Medium M14 for maximum bioactivity (70%) followed by M6 (60%) and M5 (50%).

Fig. 4.2Actinomycetes in medium M13 and M14

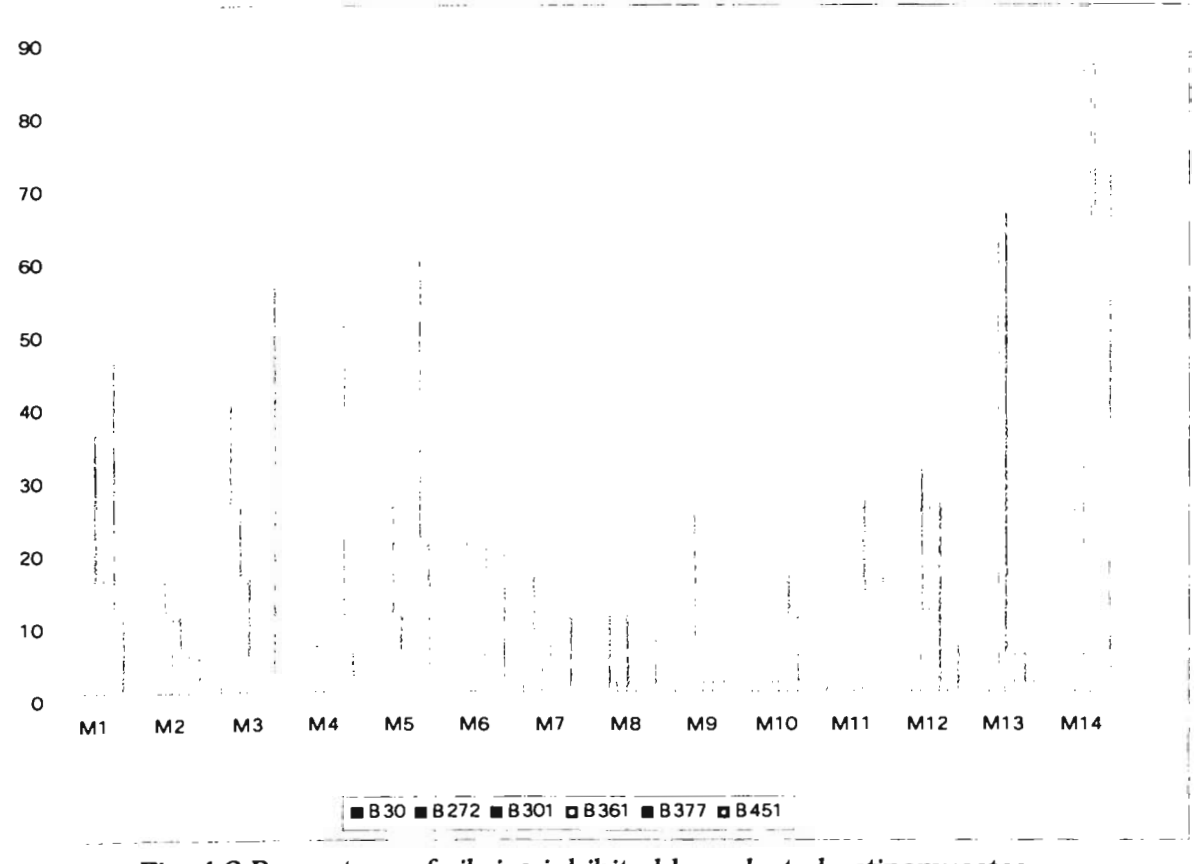


Fig. 4.3 Percentage of vibrios inhibited by selected actinomycetes when cultured in 14 different fermentation media

4.3.2 Optimal conditions for growth and antibiotic production by the selected actinomycetes.

a. Effect of salinity on growth and antibiotic production.

Generally, a salinity of 35 ppt was preferred by the various strains for growth and antibiotic production (Fig. 4.3 a-f).

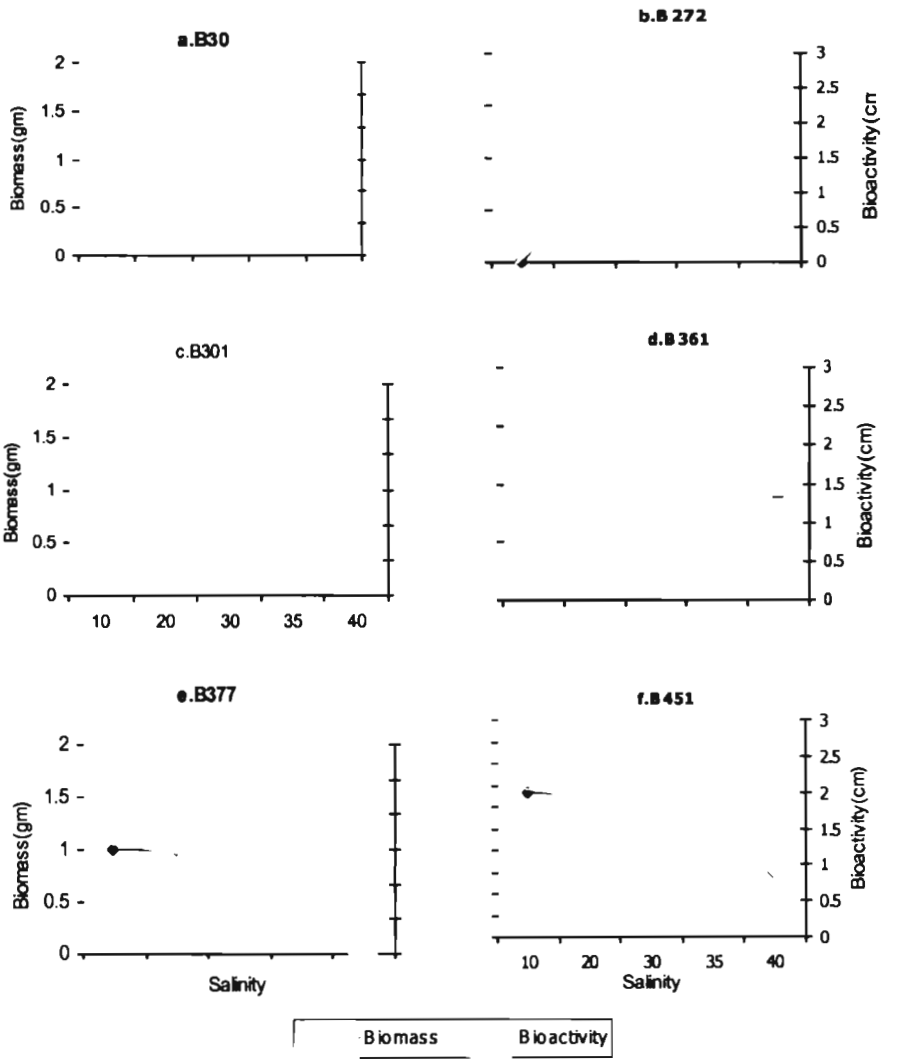


Fig 4.3.a-fGrowth (biomass) and antibiotic production (bioactivity) by selected streptomyces spp. at various salinity in the fermentation medium

All the six strains showed maximum growth at 35 ppt and the antibiotic production was maximum at 30 ppt for B30, B272 and at 35 ppt for B 301, B361 and B451.

B30, B361 and B451 did not show much variation in growth and antibiotic production with respect to salinity whereas the other three strains showed considerable decrease in growth and bioactivity with increase or decrease of salinity.

b. Effect of pH on growth and antibiotic production.

The effect of different pH on growth and antibiotic production are presented in figure 4.4.a-f. Generally, growth and antibiotic production was found to be optimum at pH 7 for the various strains. Antibiotic production was found to be negligible for *Streptomyces californicus* (B30) and *Streptomyces* sp. (B272) at pH 5 and a sharp increase could be observed at pH 6. No significant difference in the antibiotic production could be observed between pH 6, 7 and 8 by most of the strains except B30 for which production was significantly low at pH 7 and 8.

c. Effect of phosphate concentration on growth and antibiotic production.

The optimum phosphate concentration for growth and antibiotic production varied for the different strains (Fig 4.4a-f). Generally 0.3% $CaCO_3$ Concentration was fond to be optimal for the growth and bioactivity. Except for the growth of B30 *(S.californicus)* and B301 *(S.griseoflavus)* (0.1%) and bioactivity of B451 (0.4%) (Fig 4.5a-f).

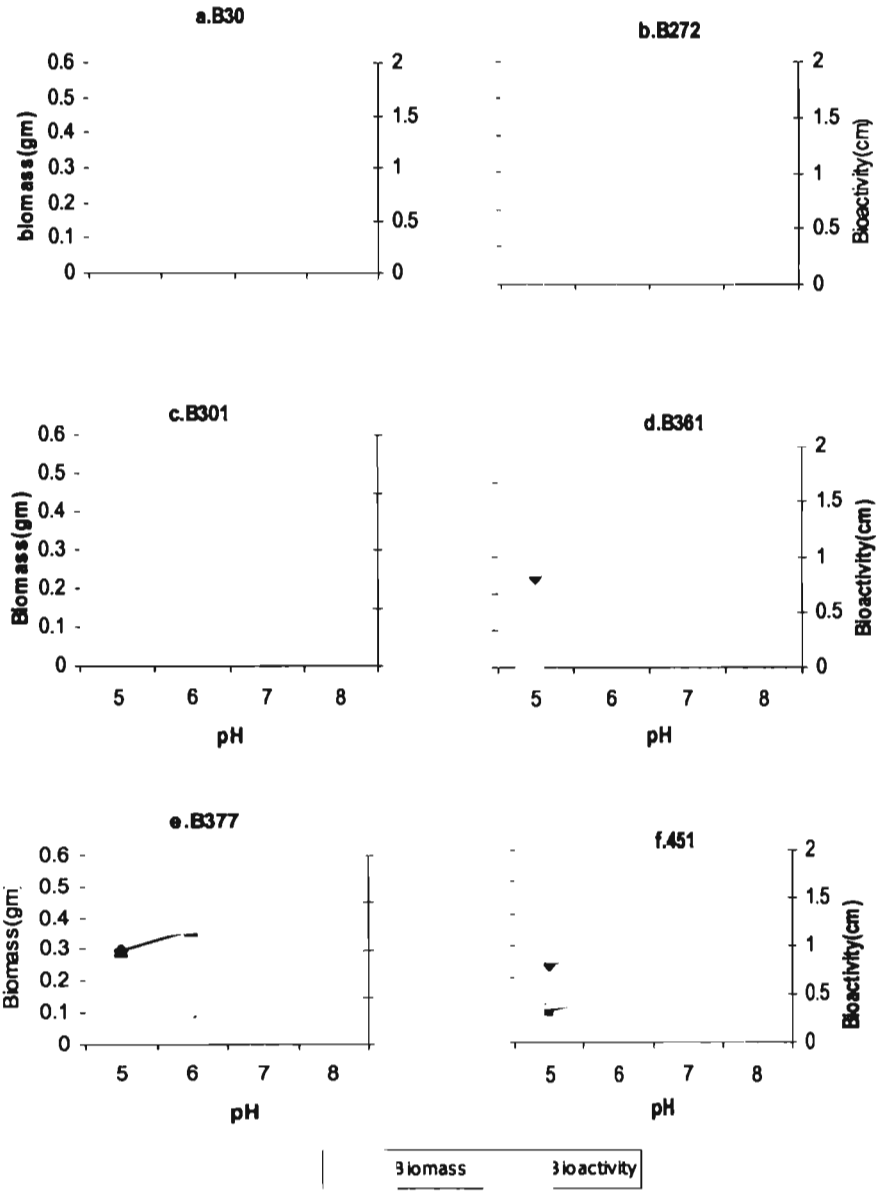


Fig 4.4 a - f Growth (biomass) and antibiotic production (bioactivity) by selected Streptomyces spp. at various pH in the fermentation medium.

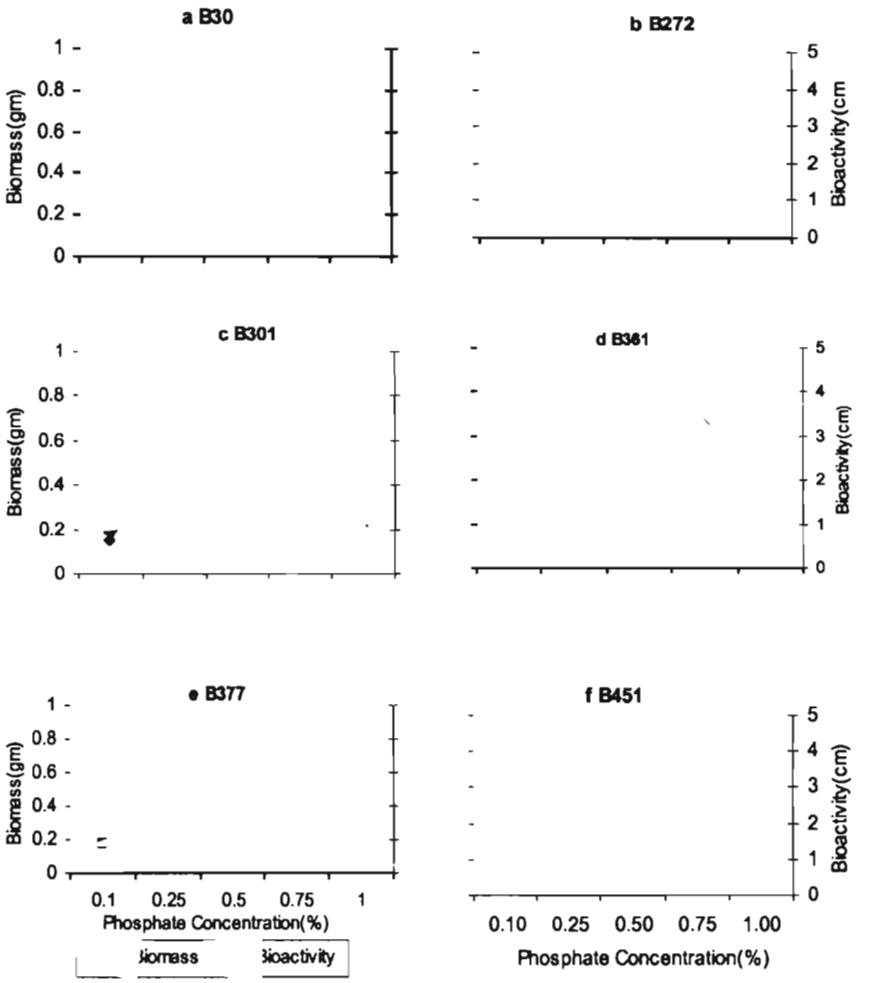


Fig 4.5 a - f (biomass) and antibiotic production(bioactivity) by selected streptomyces spp. at various phosphate concentration in the fermentation medium.

Phosphate in the range of 0.25 to 0.75% was found to be optimal for growth by the various strains. Phosphate concentration of 0.75% was found to be optimal for B272 (*Streptomyces* sp.) and B301 (*S.griseoflavus)* for growth and 0.5% for B30 *(S.californicus)* and B377 (*Streptomyces* sp.). Bioactivity was maximum at 0.25% for B30, B301, 0.5% for B451 (*S.fradiae*) and at 0.75% for B272 and B361.

d. Effect of Calcium Carbonate concentration

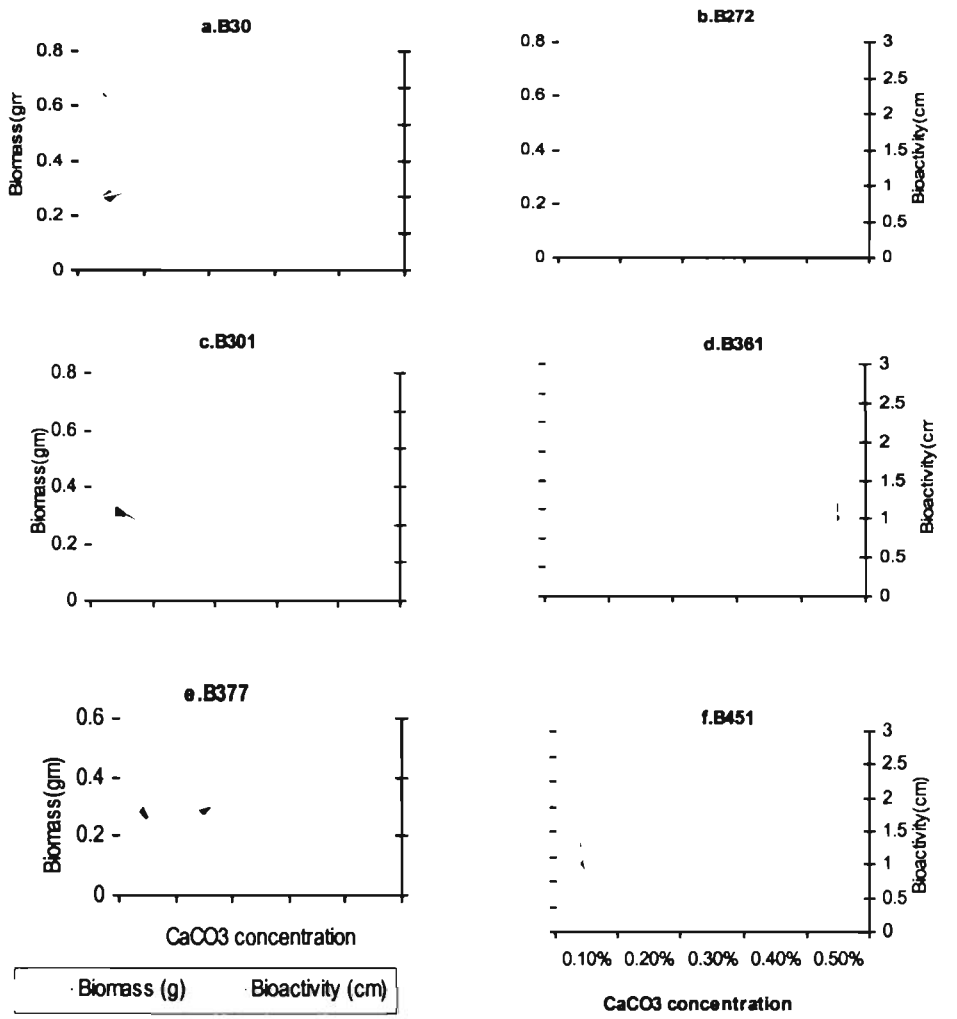


Fig 4.6a-f (biomass) and antibiotic production(bioactivity) by Streptomyces spp. at various calcium carbonate concentration in the fermentation medium.

Since the optimal values of the various physico-chemical parameters varied for growth and bioactivity (Table 4.3 & 4.4) for the selected strains, the optimal values for bioactivity were given weightage and selected for further fermentation study.

Table 4.3 Optimal conditions for growth by the selected actinomycetes

Culture No.	pH	Salinity	PO_4 (%)	$CaCO_3$ (%)
B30	8	35	0.5	0.1
B272	5	35	0.75	0.3
B301	5	35	0.25	0.1
B361	6	35	0.75	0.1
B377	7	35	0.1	0.4
B451	7	35	0.5	0.3

Table 4.4 Optimal conditions for antibiotic production by the selected actinomycetes

Culture No.	pH	Salinity	PO_4 (%)	$CaCO_3$ (%)
B30	7	30	0.25	0.3
B272	8	30	0.75	0.4
B301	6	35	0.75	0.2
B361	7	35	0.25	0.3
B377	7	30	0.5	0.3
B451	7	35	0.25	0.3

4.4 DISCUSSION

4.4.1 Selection of medium

The importance of the composition of the fermentation medium employed for the production of antibiotics by microorganisms was demonstrated by Okazaki and Okami (1972). In the present investigation also the composition of the fermentation medium was found to influence the elaboration of antimicrobial substances. This was demonstrated by the fact that strains B30, B272 and B301 exhibited higher bioactivity when grown in the medium M13 and the other three strains (B361, B377and B 451) showed a broad spectrum of activity when grown in medium M14.

The medium supplies nutrients for growth, energy, building of cell substances, and biosynthesis of fermentation products. Good yields of fermentation products occur only if relatively large amounts of carbon and nitrogen are chanelled through the metabolic pathways of the microorganisms. Moreover, the antibiotic production is strain specific and is unstable. Active antibiotic production as suggested by Omura and Tanaka (1986) occur in association with sporulation of the producing organism, which begins with nutritional limitation of carbon or nitrogen sources and inorganic phosphate. Culture no B30, B272 and B301 exhibited better bioactivity when cultured in medium M13. Medium M13 contains starch (2%) as carbon source and soybean meal (1.5%) as nitrogen source besides 0.5% phosphate salt 0.05% magnesium salt and trace amounts of iron and cobalt. Some of these nutrients might have induced the production of antimicrobial compounds by the three *streptomyces* species (B30, B272 and B301). It has been reported that salts added in higher concentration can enhance bioactive compound production (Shimada et al., 1986). Magnesium salt at concentrations 0.5-1.0 can produce octaprin D, Gilvocarcins, Ancovenin and Monacolins.

Actinomycetes B361 (*S.pulveraceus*), B377 (*Streptomyces* sp) and B451 (*S.fradiae*) preferred medium M14 for better production of bioactive compound. This medium contained a high percentage of glycerol (7%). Benedict *et al.* (1955) observed that streptomycetes, in contrast to most other bacteria grew well on a glycerol medium containing L (+) arginine as the sole source of nitrogen. Similar observations were made by Kuster &Williams (1964). They were of the opinion that the starch –casein agar, in which glycerol may be

substituted for starch can be used to grow streptomycetes from a compost soil.

Glucose (3%) was another major ingredient in the medium. It has been reported that glucose very often but not always suppresses antibiotic production (Tanaka *et al.*, 1986). Nevertheless, the production of macrolide antibiotic tylosin by *Streptomyces fradiae* was not found to be affected by 4% glucose added to the medium. Parekh *et al.*, (1999) evaluated the effectiveness of glucose on solvent production and found an optimum of 6% glucose for the production of butanol.

4.2.2 Process Optimization

Results obtained in the present study suggest that the optimum concentrations of nutrients for growth and antibiotic production do not coincide. Fermentation process is generally influenced by physiochemical conditions like pH, salinity, phosphate and calcium carbonate concentration. Optimization of these parameters was essential to achieve maximum yield as growth and antibiotic production.

Salinity

All the six strains (*Streptomyces* spp.) used in the study showed 35ppt as their optimal salinity for growth in both M13 and M14 media. Antibiotic production by the *Streptomyces griseoflavus* (B301), *S. pulvaraceus* (B361), *Streptomyces* sp. (B377), *S. fradiae* (B451) were optimum at 35ppt whereas, the other two strains B30 and B272 preferred 30ppt. All these strains were isolated from marine sediment samples and their preference for 30 to 35 ppt seawater shows their indigenous marine nature.

pH

An initial pH of 7.0 was found to be very much favourable for growth and antibiotic production by majority of the strains. During the course of fermentation, normally the pH changes. Literature stress the importance of alkaline pH for growth and antibiotic production of streptomycetes. Actinomycetes were surveyed for the ability to produce new antibiotic under alkaline conditions, which led to the discovery of a new antibacterial and anti fungal peptide, number 1907, from *Paecilomyces* sp. No 1907 grown at pH 11 (Sato *et al.*, 1980). Jamaka (1986) reported that the inhibitory effect of glucose on production of bacitracin resulted from a pH effect. Okazaki and Okami (1975) showed that a medium with 3% NaCl supported growth of 70% of actinomycetes isolated form sea muds. Hotta *et al.* (1980) observed that *Streptomyces tenjimariensis* produced an amino glycoside antibiotic at 3.5 % NaCl concentration at maximal level. A marine streptomycete isolated form the surface of jellyfish, required salt water (3.5%) for optimal growth and salinamide production

Phosphate Concentration

Phosphate can enhance or suppress the production of desired metabolites at different concentrations. In the present study the optimum phosphate concentrations was found to be in the range of 0.25 to 0.75 %. Several studies showed that antibiotic production in *Streptomyces* was sensitive to phosphate concentration. (Martin, 1977; Vu-Trong *et al.*, 1981 and Lebriti *et al.*,1987). However, the formation of many secondary metabolites is inhibited by inorganic phosphate concentration (Weinberg, 1978). Among the elements added to fermentation media used in secondary metabolite production, phosphate is one of the most critical factors (Weinberg, 1974). Many

antibiotics are only produced at concentrations of inorganic phosphate sub optimal for growth. Phosphate in the range of 0.3 – 5.0 mM permits excellent cell growth whereas 10 mM phosphate often suppresses biosynthesis of antibiotics.

Calcium Carbonate concentration

Calcium carbonate can control pH of the fermentation media and has its own effect on antibiotic production. Tanaka (1986) reported calcium carbonate as the most common buffering agent used to avoid acidic conditions in the fermentation medium. Adverse effect of calcium carbonate has been reported by Iwai *et al.* (1973) who pointed out that calcium carbonate added to the production medium inhibited cerulin production. In the present investigation, presence of 0.3% calcium carbonate in the medium was most favourable for growth and antibiotic production. Use of calcium carbonate in the antibiotic production media has been reported by many workers. In most of the cases, 0.3% of $CaCO_3$ was added to the medium. Furumai *et al* (2000) used 0.35% $CaCo_3$ in the production medium of Aristostatins A and B from a *Micromonospora* sp. Similarly Luminacins by *Streptomyces* sp. (Naruse et al., 2000), Dihydroniphimycin (Ivanova *et al.*, 2000) by *S.hygroscopicus* and Vinylamycin (Igarashi *et al.*, 1999) from a *Streptomyces* sp. was produced when 0.3% $CaCO_3$ was added to the medium.

Chapter 5

CHARACTERIZATION OF ACTINOMYCETES AND EXTRACTION OF BIOACTIVE COMPOUNDS

5.1 INTRODUCTION

5.1.1 Characterization of actinomycetes

Pridham et al. (1958) grouped streptomycetes in sections based on spore color en masse. Using only the spore bearing hyphae and spore chains Nishimura et al. (1960) had placed them in fourteen series comprising five sections. Cross and Mac Iver (1966) devised a key which employed melanin reaction on peptone – iron agar, nature of spore surface, morphology of the aerial mycelium, color of the substrate mycelium and carbon utilization. Spore morphology is a valuable taxonomic criterion in Streptomyces speciation. An international Co-operative effort to define acceptable criteria and methods of studying streptomycetes for taxonomic purposes has been carried out and the results and recommendations are published by Shirling and Gottlieb (1966).

In summary, the following are considered today to be the most reliable criteria for classifying streptomycetes.

1 Spore chain morphology

2 Spore morphology

3 Spore color *en masse*

4 Chromogenecity (melanin production)

5 Carbon source utilization patterns

6 Colour of the non-sporulated aerial mycelium

7 Colour of the substrate mycelium

8 Level of NaCl concentration

9 Soluble pigments

5.1.2 Extraction of Bioactive compounds

A fermentation broth is a complex mixture of components that often contains only trace amounts of a desired product. Microorganisms typically produce a desired product at low concentration (<3% w/v) and may be present at few parts per million concentrations in fermentation broth (Gailliot, 1998). It can be seem a formidable task, knowing that there is one group of molecules that has to be separated from the dark viscous fermentation broth. These molecules possibly be represent only about 0.0001% or 1 ppm of the total biomass and are dispersed throughout the broth, intimately bound up with other molecules (Cannel, 1998).

The primary goals of the initial capture stage are to isolate an unknown compound responsible for a particular biological activity and to purify sufficient amount of compound from dissimilar impurities. This includes solids removal, solvent extraction, solid phase adsorption and expanded adsorption. The first one, solid removal includes filtration or centrifugation of the fermentation broth. The second step is the solvent extraction procedures with water miscible or immiscible solvents. The third phase is solid phase extraction using adsorbents. The fourth section uses techniques, which enables resin-column recovery of product directly from unclarified fermentation broth.

To begin with, the isolation of a compound, the nature of the compound needs to be known. This can be determined by evaluating the properties like solubility, acid/base properties, charge, heat stability and size of the material. An indication of the polarity of the compound can be determined by carrying out a range of solvent partitioning experiments between the culture filtrate and a number of solvents differing in polarity.

Acid/Base properties can be obtained by partitioning experiments with a range of pH values. The supernatant is adjusted with acid or alkali, and then the solvent is added, mixed and the assay of the two phases are done (Cannel, 1998). Charge can be obtained by adding portions of different ion exchangers to the culture supernatant. A typical heat stability test would involve incubation of the sample at 80/90°C for 10 minutes in a water bath and the assay for unaffected compound. Large molecules like misleading proteins can be eliminated by passing through ultra filtration membrane by pressure, vacuum or centrifugal force.

The first and most important factor in the extraction procedure is to determine, whether the compound of interest is in the supernatant or in the cells.

Clarification of fermentation broth can be accomplished by either centrifugation or filtration. Although many broth properties influence the choice between filtration and centrifugation, the dominating factors are the solid's particle size and solid-liquid density difference.

5.1.2.1 Solvent Extraction

Crude culture filtrate containing antibiotics are usually mixtures, and the presence of dissimilar components may prevent a clear interpretation of antimicrobial spectrum data. Solvent extraction provides the ease of liquid handling, the potential for high throughput operation and the potential for adaptation to continuous operation (Gailliot, 1998). Both water-miscible and immiscible solvents are used for extracting compounds from the biomass. Multiple approaches can be employed to purify a fermentation-derived product. A number of literature reviews of liquid-liquid extraction of antibiotics (Hatton, 1985) small molecules (Schugerl, 1993) and biopolymers (Kulla, 1985)

have been published. Ethyl acetate was used by number of workers for initial extraction of compounds from culture supernatant (Fernandez-Chimeno *et al.*, 2000; Enomoto *et al.*, 2001; Kurosawa *et al.*, 2001).

Selection of Solvent

Table 5.1 Properties of common solvents used in Fermentation Broth Extraction *(From Natural Products isolation, 1998 Cannel, R.J.P(ed.).*

Solvent	Polarity index	Solubility in water % w/w
Acetic acid	6.2	100
Acetone	5.1	100
Acetonitrile	5.8	100
Benzene	2.7	0.18
Butyl acetate	4.0	0.43
n-Butanol	3.9	7.81
Carbon tetrachloride	1.6	0.08
Chloroform	4.1	0.815
Cyclohexane	0.2	0.01
1,2-Dichloroethane	3.5	0.81
Dichloromethane	3.1	1.6
Dimethylformide	6.4	100
Dimethyl sulfoxide	7.2	100
Dioxane	4.8	100
Ethanol	5.2	100
Ethyl acetate	4.4	8.7
di-ethyl ether	2.8	6.89
Heptane	0.0	0.0003
Hexane	0.0	0.001
Methanol	5.1	100
Methyl-t-butyl-ether	2.5	4.8
Methyl ethyl ketone	4.7	24
Pentane	0.0	0.004
n-Propanol	4.0	100
Iso-Prpanol	3.9	100
di-iso-Propyl ether	2.2	-
Tetrahydrofuran	4.0	100
Toluene	2.4	0.51
Trichloroethylene	1.0	0.11
Water	9.0	100
Xylene	2.5	0.018

Selection of the most appropriate solvent is an important step in extraction process. Commonly used water immiscible solvents include alcohols (n-butanol, isobutanol), ketones, acetates (butyl, ethyl, isopropyl), hydrocarbons (toluene, hexanes) and methylene chloride. Common water-miscible solvents are the alcohols particularly methanol. Table 5.1 shows different solvents used in fermentation broth extraction with their polarity index and solubility in water. Solvent extraction ends up with an extract that can easily be vacuum concentrated and used in final purification steps.

5.1.2.2. Solid Phase Extraction

Solid phase extraction utilizes adsorbents for sample cleanup, trace enrichment and fractionation of desired products in crude solvent extracts from fermentation broth (Gaillot, 1998). Usually there are three general classes of adsorbents - polar, ion exchange and non-polar.

5.1.2.3. Thin Layer Chromatography (TLC)

Analytical TLC has found application in the detection and monitoring of compounds through a separation process. Qualitative and quantitative information regarding the compounds can be gathered concerning the presence or absence of a metabolite. Natural products may be "tracked" by running analytical TLC of fractions from other separation processes, such as column chromatography or HPLC (Gibbons and Gray, 1998). TLC on silica gel is the most commonly adopted method (Gibbons and Gray, 1998). Table 5.2 shows common solvent systems used for detection of compounds in TLC.

Table 5.2 Solvent Systems for TLC *(From Natural Products isolation, 1998 Cannel, R.J.P(ed.).*

Solvent system	Sorbent	Compounds
Hexane: Ethyl acetate	Silica gel	Universal system
Petrol: Diethyl ether	Silica gel	Terpenes and fatty acids
Petrol: chloroform	Silica gel	Cinnamic acid derivatives
Toluene: Ethyl acetate: Acetic acid	Silica gel	Acidic metabolites
Chloroform: Acetone	Silica gel	Medium polarity products
Benzene: Acetone	Silica gel	Aromatic products
Butanol: Aceticacid: Water	Silica gel	Flavonoid and glycosides
Butanol: Water: Pyridin: Toluene	Silica gel	Sugar
Methanol: Water	C18	-
Acetonitrile: Water	C18/C2	Universal
Methanol: Water	Polyamide	Universal
Methanol: Water	Cellulose	Sugars and glycosides

Preliminary identification can be carried out on crude culture broths (Porter *et al.*, 1964). Snell *et al.* (1956) used ascending one-directional paper chromatography. Useful solvent system was butanol: acetic acid: water (74:3:25). Yajima (1955); Balan *et al.* (1962) and Batina (1964) have analyzed and identified different antibiotics from crude culture filtrates by using different solvent systems including (1) distilled water (2) Butanol saturated with water (3) Ethyl acetate saturated with water and (4) Benzene saturated with water.

i) Detection of compounds in TLC

Effective visualization or detection is crucial to obtain pure compounds. Exposure to ultraviolet, spray detection with various reagents and Iodine vapor exposure are the three common methods for detection of bioactive compound on TLC plate.

a) UV detection

This involves the use of UV active compounds (indicators) that are incorporated in to the sorbent of TLC plates. Compounds absorb light and appear as dark spots against a light background. The advantage of this method is that, it is a non-destructive detection of compounds.

b) Spray detection

It relies on a color reaction between the compound on the TLC plate and spray reagent. Some of the most common spray reagents are listed in Table 5.3.

Table 5.3 Spray reagents for natural products in TLC visualization *(From Natural Products isolation, 1998 Cannel, R.J.P(ed.).*

Detection spray	Compounds
Vanillin/Sulfuric acid	A universal spray
Phosphomolybdic acid (PMA)	Terpenes
Ammonium molybdate(IV)	A universal spray
Antimony (III)chloride	di-and triterpenes
Tin(IV)chloride	Flavonoids and terpenes
Dragendorff's Reagent	Alkaloids
2,4 Dinitro-phenyl hydrazine	Aldehydes and ketones
Perchloric acid	Steroids and triterpenes
Borntrager Reagent	Coumarins and antraquinones
Ninhydrin	Aminoacids, amines, alkaloids
Ehrlich Reagent	Amines,indoles, ergot alkaloids
Anisaldehyde/sulfuric	Terpenes, phenols and steroids
Bial's Reagent (Orcinol-ferric chloride)	Sugars
Triphenyl-tetrazolium chloride	Reducing sugars, corticostroids
Fluorescein	Lipids
Ferric chloride	Phenolics

ii) Preparative TLC (PTLC)

PTLC helps to separate partially purified mixture in large quantities without running many plates. PTLC is nearly always used as a final purification step in an isolation procedure.

iii) TLC Bioassays

Two types of Bioassays are performed on TLC plates.

a) TLC Direct Bioautography

This technique is used with spore forming fungi and bacteria. It is a very sensitive assay and gives accurate localization of active compounds (Rahalison *et al.*, 1991). In this technique TLC, plates are run and then the microorganism is introduced to the plate as a spray in the form of suspension in a growth medium and incubated. Inhibitory zones give indication of active compounds. Dellar *et al.* (1994) have isolated antifungal sequiterepenes and prostantherol by this method.

b) TLC overlay assay

In this type of assay, the extract is run on a TLC plate, which is then covered by a medium seeded with appropriate microorganism. Rahalison *et al.* (1991) have applied this technique for the evaluation of antimicrobial extracts against *Bacillus subtilis*.. Batistia *et al.* (1995) used this technique to isolate an antibacterial diterpene by using *S. aureus* as test organism. Hamburger and Cordell (1987) used this to investigate some plant sterols and phenolic compounds. Nocolans *et al.* (1961) carried out bioautography with sensitive test organism plus triphenyl tetrazolium salts. Antibiotic areas became light yellow, while the remainder of the plate was brown.

5.1.4 Characterization of bioactive compounds

Waksman and Lechevalier (1962) list the following properties and techniques as useful in characterizing antibiotics. Properties such as solubility, stability, color reactions, fluorescence and light absorption (UV, Visible, infrared) are helpful in characterization. Paper chromatography of whole broth antibiotic and decomposition products, electrophoresis, counter current distribution, elementary analysis, physical constants and mass spectrometry are the techniques generally employed for characterization.

An antibiotic identification can be achieved based on its antimicrobial spectrum, ionic character and behavior on chromatographic plates. Kimura *et al.* (1998) extracted Liposidomycins from *Streptomyces* sp. culture filtrate using n-butanol. This butanol extract was passed through silica gel column and eluted with chloroform: Methanol (1:2) as preliminary steps of extraction prior to purification by HPLC. Cyclosporin A was extracted by Kuhnt *et al.* (1996) using ethyl acetate from the culture broth. This ethyl acetate extract was dried and redissolved in methanol and was subjected to HPLC. Ethyl acetate was used as solvent for the extraction of antibiotic from the culture broth of Actinomycetes by various workers (Seki-Asano *et al.*, 1994; Kunigami *et al.*, 1998; Matsumoto, 1999; Igarashi *et al.*, 1999; Sato *et al.*, 2000). TLC on silica gel plate was performed for initial separation of the compound and to find out the suitable solvent system for further purification of the compound on column and HPLC. BuOH: AcOH: H_2O (4:1:2) was used as the solvent system for the separation of Liposidomycin from a *Streptomyces* sp. Liposidomysins were distinguished by Rf values of 0.35 and 0.41 respectively. Further analysis was done on HPLC. Igarashi et al. (1999) performed TLC of

Vinylaycin with Kiesel gel 60 using $CHCl_3$-MeOH (4:1) as the solvent system. Spot detection on TLC was done using molybdophosphoric acid – sulphuric acid and UV quenting (Rf value 0.5).

Decatromicins gave a positive color reaction with molibdophosphoric acid - sulphuric acid, 2,4-dinitrophenyl hydrazine, Rydon - Smith and anisaldehyde - sulphuric acid reagents, but a negative one with ninhydrin reagent (Momose et al., 1999). Similarly, macrolide antibiotics FD-891 and FD-892 gave positive reaction to iodine, H_2SO_4 and Vanillin-H_2SO_4, but negative to ninhydrin (Seki- Asano et al., 1994). Nisamysin an antibiotic from *Streptomyces* sp. K 106 was purified with preparative thin layer chromatography. The crude powder was obtained by passing ethyl acetate extract through a silica gel column and stepwise elution using $CHCl_3$-MeOH. Albaflavenone, a sequiterepene ketone produced by a streptomycete was reported by Gurtler *et al.* 1994. The majority of terpenoids are produced by eukaryotic organisms. However, a variety of actinomycetes, most notably members of the genus *Streptomyces*, produce sequiterepenes which have been implicated in the production of earthy odors in water Gerber, 1979; Weet *et al., 1979)* and in soil (Buttery and Garibaldi, 1976).

A neutral absorber resin, Amberlite XAD-16 when added to fermentation broth absorbs practically all of the excreted metabolites and at the same time concentrates and stabilizes them. This adsorbed metabolite can be eluted using a suitable solvent and then concentrated. HPLC with diode array and light scattering detectors are used to characterize the extracts according to peak patterns and UV spectra. This has repeatedly led to the discovery of new compound for which biological effects were later discovered. (Reichenbach *et al.*, 1996).

5.2 MATERIALS AND METHODS

Microorganisms

Marine actinomycete strains B30, B272, B301, B361, B377 and B451 selected based on antibacterial property as described in chapter III were used in this study.

5.2.1 Taxonomic studies

Carbon source requirements, fatty acid profile, whole cell sugar pattern and cell wall analysis were done by the Institute of Microbial Technology (IMTECH), Chandigarh, India for identification of the strains and the characteristics are presented in Table 5.4.

5.2.2 Morphological studies

Morphological characteristics were studied by cultivating these strains on eight different media described by Shirling and Gottlieb (1966).

Media used

1) Tryptone - Yeast extract broth (Pridham and Gottlielb, 1948).
   - Tryptone - 5.0g
   - Yeast extract - 3.0g
   - Agar - 20.0g
   - Sea water - 1L
   - pH - 7.2

2). Yeast extract - Malt extract agar (Pridham *et al.*, 1956-57).
   - Yeast extract - 4.0g
   - Malt extract - 10.0g
   - Dextrose - 4.0g
   - Agar - 20.0g
   - Sea water - 1L
   - pH - 7.3

Table 5.4 Taxonomical Characteristics of the selected strains (as obtained from IMTECH Chandigarh, India)

Name of the Test	B30	B272	B301	B361	B377	B451
Gram's staining	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Cell wall amino acids – Diagnostic	LL-DAP	LL-DAP	LL-DAP	LL-DAP	LL-DAP	LL-DAP
Cell wall sugars – Diaganostic	Nil	Nil	Nil	Nil	Nil	Nil
Utilization of sugars						
Carbon free	-	-	-	-	+w	-
Arabinose	+	-	+	+w	+	+
Fructose	+	-	-	+	+	-
Galactose	+w	-	+w	+w	-	+w
Glucose	-	-	+w	+w	+	+
Meso-inositol	+	-	+	-	-	-
Mannitol	+	-	+	+	+	-
Raffinose	-	-	-	-	+	-
Rhamnose	+	-	+	+	-	+
Salicin	+	-	+	+w	-	-
Sucrose	-	-	-	-	+	-
Xylose	+w	+	+	+	+	-
Degradation of						
Elastin	-	-	-	-	+	+
Chitin	-	-	-	-	-	-
Guanine	-	+	-	-	+	+
Adenine	-	+	-	-	+	+
Testosterone	-	+	-	-	+	+
Pectin	-	-	-	-	-	-
Hypoxanthin	-	+	-	-	+	+
Esculin	-	+	-	-	-	-
Casein hydrolysis	+	+	-	-	+	+
Starch hydrolysis	-	-	+	+	+	+
Gelatin hydrolysis	-	+	+	+	+	+
Tween 80 hydrolysis	-	-	-	-	-	-
Growth on nitrogen sources						
Methionine	+	+	-	-	+	+
Valine	+	+	-	-	+	+
Proline	+	+	-	-	+	+
Arginine	+	+	-	-	+	+
Phenylalanine	+	+	-	-	+	+
Serine	+	-	-	-	+	+
Tyrosine	-	-	-	-	-	-
Threonine	+	-	-	-	+	+
Histidine	+	+	-	-	+	+
Growth on Potassium nitrate	+	+	-	-	+	+
Spore chain morphology	Recti.	Recti.	Retina.	Spiral.	Recti.	Retina.

3) Oat meal agar (Kuster, 1959).

Oatmeal	-	20.0g
Agar	-	20.0g
Seawater	-	1L
PH	-	7

Cooked 20 g oatmeal in 1000ml seawater and filtered through cheesecloth. Seawater was added to restore volume to 1000ml. pH adjusted to 7.2 with 0.1 N NaOH and 20 g of agar was added.

4) Inorganic salts- starch agar (Kuster, 1959).

Starch	-	10.0g
K_2HPO_4	-	1.0g
$MgSO_4.7H_2O$	-	1.0g
$(NH_4)_2SO_4$	-	2.0g
$CaCO_3$	-	2.0g
Agar	-	20.0g
Seawater	-	1L
pH	-	7.0 - 7.4

5) Glycerol- asparagine agar (Pridham and Lyons, 1961).

L- Asparagine	-	1.0g
Glycerol	-	10.0g
K_2HPO_4	-	1.0g
Agar	-	20.0g
Seawater	-	1L
pH	-	7.0-7.4

6) Peptone - Yeast extract Iron agar (Tresner and Danga, 1958)

Peptone	-	15.0g
Proteose peptone	-	5.0g
Ferric ammonium citrate	-	0.5g
Dipotassium phosphate	-	1.0g
Sodium thiosulfate	-	0.08g
Agar	-	20.0g
Seawater	-	1L
pH	-	7.2

7) Tyrosine Agar (Shinobu, 1958).

Glycerol	-	15.0g
L- tyrosine	-	0.5g
Asparagine	-	1.0g
K_2HPO_4	-	0.5g
$MgSO_4.7H_2O$	-	0.5g
$FeSO_4.7H_2$	-	0.01
Agar	-	20.0g
Seawater	-	1L
pH	-	7.2 - 7.4

8) Marine Actinomycete Growth medium

Starch	-	10.0g
Yeast extract	-	4.0g
Peptone	-	2.0g
Agar	-	20.0g
Seawater	-	1L
pH	-	7.0

Media were prepared and sterilized at 121°C for 15 minutes. In the case of medium 2 dextrose was filter sterilized separately and added to the medium at the time of pouring in to the plates. 20 ml each sterile media were poured in to petridishes, surface drying was done at 35°C for 24 hours in an oven and checked for contamination prior to inoculation.

Preparation of inoculum

With the help of a sterile wire loop spores were harvested from a two-week-old culture slants, in to 5 ml sterile seawater. This was used as the inoculum.

Inoculation of plates for morphological studies

One loop full of inoculum suspension was spread near one edge of the perti plate and streaked on to the agar plates across the plate. Incubated the plates at room temperature (28±2°C) and morphological observations were made after 5-7 days incubation.

5.2.3 Fermentation

A loop full of mature slant cultures of all the six strains (Nutrient agar slants prepared in sea water) were inoculated in to a 50 ml seed medium (starch 1%, yeast extract 0.4%, peptone 0.2% sea water 100ml, and pH 7) taken in a 250 ml Erlenmeyer conical flask. The flasks were incubated at room temperature (28±2°C) for 2 days on a rotary shaker (200 rpm). 10 ml aliquots of the seed culture were transferred into 500 ml conical flasks containing 100 ml of the production media (modified M13 and M14). For actinomycete culture No. B30, B272, and B301 modified M13 was used and for culture No. B361, B377 and B 451 modified M14 was used as per the Table 5.5 a & b obtained after optimization of physico-chemical parameters for

growth and antibiotic production. Fermentation was carried out for 7 days at room temperature (28±2°C) on a rotary shaker at 200 rpm.

Table 5.5a Composition of medium M13 (modified)

Ingredients	Quantity		
	B30	**B272**	**B301**
Soluble Starch	2g	2g	2g
Soybean meal	1.5g	1.5g	1.5g
$MgSO_4.7H_2O$	0.05 g	0.05 g	0.05 g
$COCl_2.6H_2O$	0.0002g	0.0002g	0.0002g
$CaCO_3$	0.3g	0.4g	0.2g
Phosphate*	0.25g	0.75g	0.75g
Sea water	100ml	100ml	100ml
Salinity	30	30	35
PH	7	8	6

Table 5.5 b Composition of medium M14 (modified)

Ingredients	Quantity		
	B361	**B377**	**B451**
Glycerol	7ml	7ml	7ml
Glucose	3g	3g	3g
Beef Extract	3g	3g	3g
Peptone	0.8g	0.8g	0.8g
$NaNO_3$	0.2g	0.2g	0.2g
$MgSO_4.7H_2O$	0.01g	0.01g	0.01g
$CaCO_3$	0.3g	0.3g	0.3g
Phosphate*	0.25g	0.5g	0.25g
Sea water	100ml	100ml	100ml
Salinity	35	30	35
PH	7	7	7

*K_2HPO_4 and KH_2PO_4 in equal amounts.

5.2.4 Chemical Screening for bioactive compounds

Fermentation Broth

Fermentation broth of the actinomycete isolates was prepared as given in b section 5.2.3 and the supernatant was collected after centrifugation at 10,000 rpm for 15 min in a refrigerated centrifuge. Reagents giving specific colour reactions were used for the detection of each type of compounds.

Test for Alkaloids

Dragendorff Reagent

10 mL of 40 % aqueous solution of KI to 10 mL solution of 0.85 g of basic bismuth sub nitrate with acetic acid and water in the ratio 1:2:10 (Gibbons and Gray, 1998).

Primary screening – Culture filtrate was divided into two parts. pH was adjusted to 4.0 with 0.1 N HCl. Few drops of reagent were added to acidified culture filtrate. Presence of yellow participate was noted as positive.

Secondary screening: – If the primary screening test was positive, a secondary screening test was conducted on the reserved culture filtrate. 0.5ml of the filtrate was made alkaline with NH_4OH and extracted with one volume of butyl acetate. The butyl acetate layer was extracted with 1ml of 0.1 N HCl and tested with Dragendorff Reagent.

Test for sequiterepenes, Lactones and Glycosides

Baljeet reagent

Solution – I – 1g Picric acid in 100 ml Ethanol

Solution – II – 10g NaOH in 100ml Water

Both solutions were mixed (1:1) before use and two to three drops were added to the culture filtrate. Presence of orange to deep red coloration was recorded as positive.

Test for Flavonoids

To the culture filtrate, concentrated sulphuric acid is added. The presences of flavonoids are indicated by change of color from deep yellow to orange red.

5.2.5 Antibacterial assay

Antibacterial activity of the whole fermentation broths as well as the solvent extracts (concentrated) of fermentation broth were evaluated by conventional Kirby-bauer disc agar diffusion assay using five selected prawn pathogenic vibrios. The assay was performed as given in section 3.2.2. The diameter of the inhibition zone was also noted.

5.2.6 Solvent extraction

Solvents of differing polarity were screened for extraction of the bioactive compound to find out the suitable solvent. The fermentation broth was centrifuged at 10000rpm for 15 minutes in a cooling centrifuge to separate the mycelia from the supernatant. The supernatant was extracted with three solvents differing in polarity.1.Hexane, 2. Ethyl acetate and 3.Chloroform – Methanol.

Culture supernatants were mixed with equal volume of the solvents sealed using Parafilm and kept over night on a magnetic stirrer for thorough agitation. The solvent layer was separated using a separating funnel and bio -assayed.

5.2.7 Detection of the bioactive compounds using Thin Layer Chromatography (TLC)

Analytical TLC was done on silica gel 60 (0.2mm thickness) plates (20x20 cm) for the detection of the bioactive compounds.

5.2.7.1 Preparation of TLC plates

Five glass backing plates of 20x20 cm size were cleaned with acetone and dried. These plates were placed on the TLC plates coater with spacer plates at either end. The applicator was adjusted to 0.2mm thickness and placed at one end on the spacer plate. Silica gel (30g) was taken in a stoppered conical flask, 60ml distilled water was added and mixed well for 30 seconds to prepare homogenous slurry. This slurry was poured immediately in to the applicator and in a one steady movement; the applicator was pulled across the plates. Plates were air dried for one hour and then activated in an oven at 115°C for 4 hours prior to use.

5.2.7.2 TLC of whole broth

The fermentation broths were centrifuged at 10,000 rpm for 15 min.at 40°C in a refrigerated centrifuge (Remi,C-30, Mumbai. The supernatants of the culture broths were used as the samples.

The samples were spotted near the bottom of the plate 1.5 cm above from bottom with the help of a capillary (5-10µl). The spot was air dried to dryness.

Four solvent systems were used to develop the sample

Butanol: Acetic acid	: Water	(4:1:2)
Methanol	: Water	(8:2)
Pyridine: Acetic acid	: Water	(50:35:15)
Ethyl acetate	: Methanol	(5:1)

Detection reagents

Dragendorff reagent, Conc. H_2SO_4, H_2SO_4: Vanillin, Ferric Chloride, Wood's and Ehrlich. (1 g 4 – dimethylmino benzaldehyde in 100ml 36% HCl/MeOH (3:1) and dimethylamino benzaldehyde in 100ml ethanol).

The solvent tanks were saturated with solvent systems and then kept for developing. Development was achieved by allowing the solvent front reach the plate top. The plates were then taken out and the solvents were evaporated. Detection was done by using spray reagents. Rf values were calculated by using the formula,

$$Rf = \frac{\text{Compound distance from origin}}{\text{The solvent front distance from origin}}$$

5.2.7.3 TLC of solvent extracts

Ethyl acetate extracts of culture No. B 361 and B451 was found to exhibit better inhibitory activity against the pathogenic vibrios tested. Therefore TLC was performed for these extracts.

Solvent systems used.

1 Ethyl acetate - 100%

2 Ethyl acetate : hexane (80:20)

3 Ethyl acetate : Hexane (60:40)

4 Ethyl acetate : Hexane (40:60)

5 Ethyl acetate : Hexane (20:80)

Detection was done by placing the plates in an iodine vapour saturated tank.

5.2.8 Preparative TLC – was performed on silica gel plates (0.5mm thickness 20x 20cm). Plate preparation was done as described in

section **5.2.7.1** Ethyl acetate extract concentrate of *S.fradiae* (B451) was applied in a line with the help of a capillary tube.

Sample

Ethyl acetate extract of *S.fradiae*(B451) was used since it showed distinct spots with iodine vapour in solvent system.

Solvent system - Ethyl acetate: Hexane (60:40), which gave good separation of compounds for ethyl acetate extract of B451.

Detection - Detection of spots was done by using iodine vapour.

Bioassay - Antibacterial property of separated compounds were done by disc agar diffusion assay on nutrients agar plates swabbed with prawn pathogenic vibrios, P1 (*Vibrio* sp.) and LB (Luminescent bacteria).

5.2.9 Compound Isolation

Ethyl acetate extract on PTLC plates showed one distinct band when exposed to iodine vapour. Plates after developing with solvent system was air-dried to remove solvents and band was scraped off from the plates with the help of a reference plate detected with iodine vapour. The band was extracted with minimum quantity of ethyl acetate, centrifuged with 5000rpm for 10 min. in a cooling centrifuge and supernatant was separated. Sterile paper discs impregnated with this isolate (crude compound) were left overnight to remove the solvent from the disc, then placed over nutrient agar plates swabbed with pathogen P1 (*Vibrio* sp.). A control disc impregnated with ethyl acetate alone and left overnight for removal of the solvent was also kept. Incubated at room temperature for 24 hours and halo zone of inhibition was recorded.

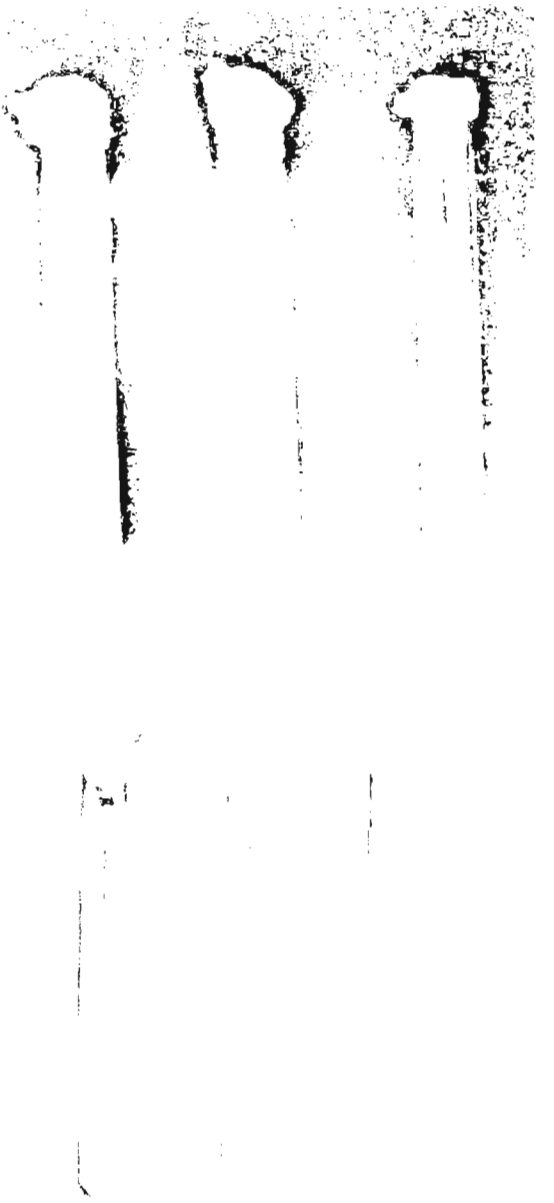


Fig. 5.1 Slant culture of the selected actinomycetes

Fig. 5.2 a-f Slide culture of the selected strains

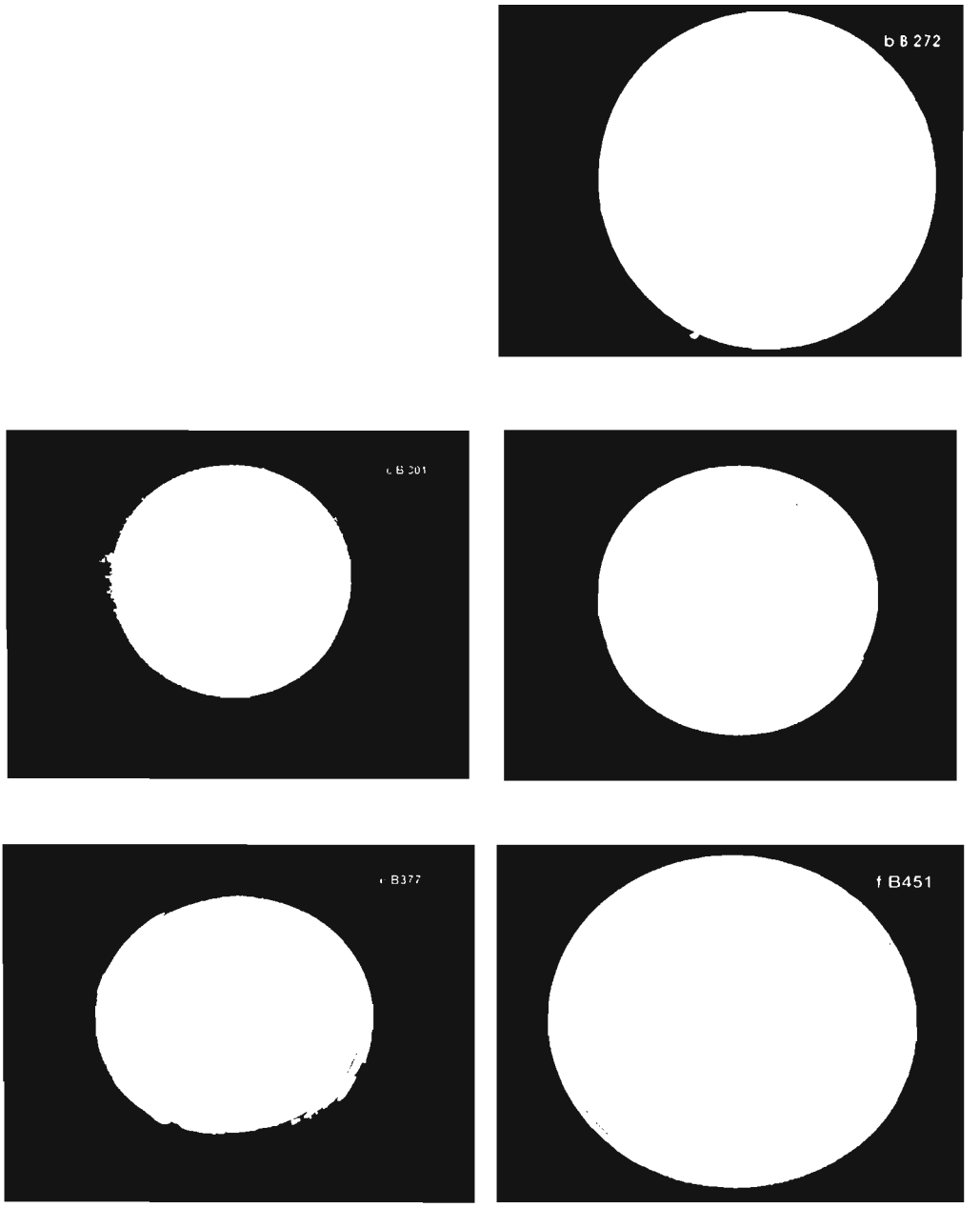


Fig. 5.3 a-f morphological characteristics of the selected strains

5.3 RESULTS

5.3.1 Morphological characteristics

The morphological characteristics obtained on various media are presented in Table 5.6 a-f.

Table 5.6.a Cultural characteristics of B30

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble Pigment
Tryptone – yeast extract agar	Moderate	White powdery	Yellowish white	-
Yeast extract – Malt extract agar	Scanty	White powdery	Yellowish	Golden yellow
Oat meal agar	Scanty	Light grey	Creamy	-
Inorganic salts – Starch agar	Faint	White creamy	Cream whitish	-
Glycerol – Asparagine agar	Scanty	White powdery	Yellowish	Faint yellow
Peptone – yeast extract agar	Scanty	White powdery	Colorless	-
Tyrosine Agar	Moderate	White powdery	Light gray	Yellowish
Marine Actinomycete growth agar	Good, abundant	Black with white powder	Yellowish black	Dark brown

Table 5.6.b Cultural characteristics of B272

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble Pigment
Tryptone – yeast extract agar	Good, spreading	Pale White powdery	Pale white	-
Yeast extract – Malt extract agar	Good	Golden yellowish with white powder	Golden Yellowish	Yellow
Oat meal agar	Moderate	White powdery	White	-
Inorganic salts – Starch agar	Faint, dot like	Pale creamy	Cream whitish	-
Glycerol – Asparagine agar	Good	White powdery	Pale white	-
Peptone – yeast extract agar	Good	White powdery	Yellowish creamy	-
Tyrosine Agar	Good	White powdery	Light gray	Yellowish
Marine Actinomycete growth agar	Abundant	White powdery	Yellowish	-

Table 5.6 c Cultural characteristics of B301

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble Pigment
Tryptone – yeast extract agar	Good, dot like	White powdery	Light cream	-
Yeast extract – Malt extract agar	Good	Light yellowish powdery	Yellowish brown	Brown
Oat meal agar	Faint	Faint White powdery	White	-
Inorganic salts – Starch agar	Faint, dot like	White powdery	White	-
Glycerol – Asparagine agar	Abundant, large, elevated	Pale yellowish White powdery	Light yellow	Yellow
Peptone – yeast extract agar	Good, large, elevated.	Light yellowish powdery	Yellow	Light yellow
Tyrosine Agar	Good, dot like	Yellowish white powdery	Yellowish orange	Yellow
Marine Actinomycete growth agar	Good	White powdery	Cream	-

Table 5.6 d Cultural characteristics of B361

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble Pigment
Tryptone – yeast extract agar	Faint	White powdery	Light cream	-
Yeast extract – Malt extract agar	Good	White powdery	Pale gray	Light yellowish
Oat meal agar	Faint	Cream	White	-
Inorganic salts – Starch agar	Poor	-	White	-
Glycerol – Asparagine agar	Good	White powdery, ring formation	Cream	-
Peptone – yeast extract agar	Faint	White powdery	Cream	Light yellow
Tyrosine Agar	Good	Pale White powdery	Light yellow	Yellow
Marine Actinomycete growth agar	Good	White powdery	Cream	-

Table 5.6 e Cultural characteristics of B377

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble Pigment
Tryptone – yeast extract agar	Good	White powdery ring formation	Pale white	-
Yeast extract – Malt extract agar	Good	White powdery elevated	Brownish yellow with dark brown center	Brown
Oat meal agar	Faint		White	
Inorganic salts – Starch agar	Faint	Pin point white powdery	White	-
Glycerol – Asparagine agar	Good	White powdery center with a baby margin	Pale white	-
Peptone – yeast extract agar	Good star like colonies	White powdery	White with dark brownish center	Light yellow
Tyrosine Agar	Faint	White powdery	Yellowish	Light brown
Marine Actinomycete growth agar	Good, elevated center	White powdery with elevated center	Light brown	-

Table 5.6 f Cultural characteristics of B451

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Soluble Pigment
Tryptone– yeast extract agar	Faint, pin point	White powdery	Yellowish, cream	-
Yeast extract – Malt extract agar	Good	White powdery	Yellowish	Yellowish
Oat meal agar	Faint	White powdery	Cream	-
Inorganic salts – Starch agar	Good	White powdery	White	-
Glycerol – Asparagine agar	Good	White powdery	Yellowish cream	Yellow
Peptone– yeast extract agar	Good	White powdery	Bright yellow	Crimson
Tyrosine Agar	Good	White powdery	Yellow	-
Marine Actinomycete growth agar	Good	White powdery	Cream	-

5.3.2 Chemical Screening

Chemical screening to detect the nature of the compound gave different reactions (Table5.7). Strains B301 (*Streptomyces griseoflavus*) and B451 (*S.fradiae*) gave positive reaction with dragendorff reagent forming deep orange precipitate, indicating presence of alkaloid compounds. B30, B272 and B377 were positive for the tests of flavonoids, sequiterepenes and glycosides. B451 was also positive for Flavonoids.

Table 5.7 Chemical screening using different reagents for six selected actinomycete strains

Strains	Alkaloids	Sequiterepenes, Lactones and Cardiac glycosides	Flavonoids
B30	-	++	+
B272	-	+++	++
B301	++	-	-
B361	-	-	-
B377	-	++	+
B451	+	-	+

5.3.3 Bioactivity of whole fermentation broth and the various solvent extracts of the fermentation broth.

The bioactivity of the selected actinomycetes, cultured under optimal conditions was tested against five *Vibrio* spp. Whole broth and various solvent extracts were tested for bioactivity against these vibrios. Of the three solvents tested for the extraction of the bioactive compound, ethyl acetate was found to be most suitable for B361 (*S.Pulveraceus*), B377 (*Streptomyces* sp.), and B451 (*S.fradiae*). For the other three strains, none of the tested solvents was found to be effective enough for the separation of the antimicrobial principle. Extract of culture no. B30 (*S.californicus*), B272 (*Streptomyces* sp.) and B301 (*S.griseoflavus)* showed low-level inhibition against the vibrios.

However, the extracts of the other three strains did not show any inhibition at all. Chloroform, methanol extract of culture No. B30 exhibited a low level inhibition against two of the pathogenic vibrios and no inhibition could be observed with the fermentation broth extract of other strains. Ethyl acetate extracts of all the strains showed inhibitory activity against the pathogenic vibrios. B451 and B361 showed better performance compared to other strains. B30 and B272 exhibited only very low inhibitory activity.

5.3.4 Thin Layer chromatography for bioactive compounds

5.3.4.1 TLC of fermentation broth

Thin layer chromatography of fermentation broth was performed with different solvent systems and the results are presented in Table-5.8(a-c).

Butanol: Acetic acid: Water

With Dragendorff reagent distinct spots could be obtained for culture B30 (*S.californicus*), B301 (*S.griseoflavus*), B361 (*S.pulveraceus*) and B451 (*S.fradiae*) when butanol: acetic acid: water was used as the solvent system.

Table 5.8 (a - c) Detection of bioactive compounds in the fermentation broth of selected Actinomycetes using TLC

Solvent System a: Butanol: acetic acid: water

Spray reagent	Rf value of the compounds in various fermentation broth					
	B30	B272	B301	B361	B377	B451
Dragendorff	0.29	-	0.26	0.2	-	0.17
H_2SO_4	-	-	-	-	-	-
H_2SO_4: Vanillin	-	-	-	-	-	-
Woods	-	-	-	-	-	-
Ferric Chloride	-	-	-	-	-	-

Solvent system b: Methanol: Water

Spray reagent	Rf value of the compounds in various fermentation broth					
	B30	B272	B301	B361	B377	B451
Dragendorff	0.15	0.32	0.5	0.9	0.7	-
H_2SO_4	0.2	0.8	0.7	-	-	-
	0.4	-	0.8	0.8	0.9	-
H_2SO_4:Vanillin	0.6	0.7	0.8	0.8	0.8	0.8
	0.8	0.8	0.6	-	0.7	-
Woods	-	-	-	-	-	-
Ferric Chloride	-	-	-	-	-	-

Solvent system c: Pyridine: acetic acid: water

Spray reagent	Rf value of the compounds in various fermentation broth					
	B30	B272	B301	B361	B377	B451
Dragendorff	0.7	0.8	0.7	0.7	0.8	0.8
H_2SO_4	-	-	-	-	-	-
H_2SO_4:Vanillin	-	0.9	-	1	0.9	0.6
Woods	0.8	0.9	0.9	0.8	0.7	0.7
Ferric Chloride	0.6	-	-	-	-	-

Methanol: Water

More number of distinct spots could be obtained when methanol: water was used for development. All the strains except B451 (*S.fradiae*) showed spots with various Rf values ranging from 0.15 to 0.7. With Dragendorff, reagent distinct spot could be obtained for B 272(*Streptomyces* sp.) at Rf value 0.32. H_2SO_4: Vanillin spray showed spots of comparatively high Rf value ranging from 0.6 to 0.8.

Sulphuric acid

Sulphuric acid spray showed two distinct spots in the case of B30 (*S.californicus*). (Rf value 0.2 and 0.4). However, all the strains showed spots at 0.7 to 0.9.

Pyridine: Acetic acid: Water.

Positive reaction could be obtained with various reagents like Dragendorff, H_2SO_4: Vanillin, Woods and Ferric chloride reagent, for all the strains. The spots were mostly in the range of 0.6 to 1. Well-separated spots could be obtained for *S.fradiae* (B451) (H_2SO_4: Vanillin) and *S.californicus* (B30) (Ferric chloride). With Dragendorff and Woods reagent spots of more or less same Rf value could be obtained with the various strains.

5.3.4.2 The TLC of solvent (ethyl acetate) extract of B361and B451

Ethyl acetate extract of B 451 and B 361 exhibited good bioactivity when tested for inhibitory property against vibrios Therefore these two extracts were subjected to TLC. With *S.fradiae* considerable separation could be obtained. Out of the four different ratio of the solvent system used Ethyl acetate: Hexane (60:40) was found to be suitable for effective separation. Distinct spots could be observed with iodine vapour. There were two spots E1 and E2 (Rf values 0.18 and 0.25) (Fig.5.4). However, the ethyl acetate extract of *S.pulveraceus*, B361, did not show distinct separation of the compound.

Fig. 5.4 Thin layer chromatogram of the ethyl acetate extract of Streptomyces fradiae

Fig. 5.5 Bioactivity of fraction RB 451E

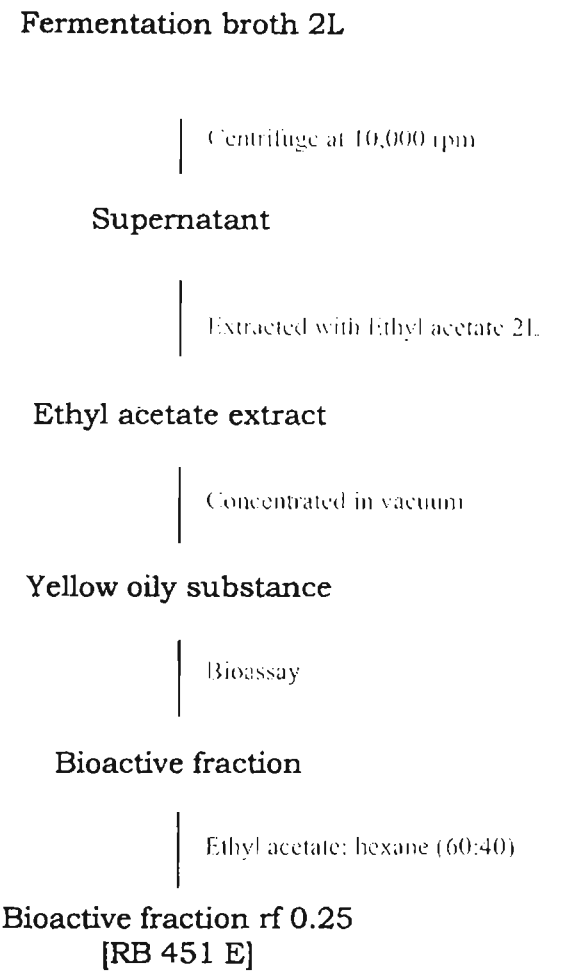


Fig 5.5 Protocol developed for the isolation of antimicrobioal compound, RB451, from B451, streptomyces fradiae

5.3.5 PTLC of Ethyl acetate extract of B451E

The ethyl acetate extract of B451 was applied on PTLC plates in a line. Two bands of same Rf value could be observed when one reference plate was developed in iodine vapour. The ethyl acetate extract eluants of the bands corresponding to Rf 0.25 showed inhibitory activity against pathogens (*Vibrio* spp.). The ethyl acetate

concentrate of this band was a reddish orange substance when it was scrapped off, redissolved and concentrated.

5.4 DISCUSSION

The strains selected with potential antivibrio property were all belonging to Streptomycetes. Streptomycetes are known producers of many valuable antibiotics and other bioactive compounds. Moreover, the six strains used in the present investigation were isolated from marine sediments, and therefore expected to be potential producers of novel bioactive compounds.

The extraction and purification of active principle from the fermentation broth is cumbersome. The most important fact in this regard is an understanding of the nature of the compound. Since the compound is unknown, the methods followed are to be hypothetical. As the proverbal saying, "needle in a haystack", lots of hay have to be removed just to find out the needle. Microorganisms typically produce a desired product at low concentration (<3%w/v) and may be present at the few parts per million concentrations in fermentation broth (Gailliot, 1998). These molecules possibly may represent only about 0.0001% or 1 ppm of the total biomass and are dispersed throughout the broth, intimately bound up with other molecules (Cannel, 1998). The antibacterial property exhibited by the culture filtrate stresses that, the dark viscous fermentation broth contained molecules responsible for their activity against bacteria.

Solvent extraction of the culture supernatants made extraction more specific. The hexane extract of the supernatants did not show any antibacterial property. This indicates that the compounds of interest are not non-polar in nature. Extraction with the polar solvents

chloroform-methanol was also not satisfactory. The result obtained in ethyl acetate suggests that compound of interest is with medium polarity.

The bacteria free culture filtrate extracted by organic solvents contained an antibacterial principle similar to the one present in the broth. The results show that, antibiotic principles extracted in solvent or present in the culture broth are active against prawn pathogenic vibrios and are soluble and extractable in ethyl acetate. Ethyl acetate was used by number of workers for initial extraction of compounds from culture supernatant Furumai *et al.*(2000) isolated two new antibiotics Aristostatins A and B from *Micromonospora* sp.isolated from sea water. For the initial extraction purpose the culture supernatant was extracted with ethyl acetate at pH 8.0.This extract was vacuum concentrated to a brown oily substance. Further purification was done with these extract. Another work conducted by Bibiani *et al.*(1997) whole broth was extracted with ethyl acetate which resulted in the discovery of Indomycinone.Similarly Dihydroniphimycin, a macrolide antibiotic produced by *S.hygroscopicus* was isolated with Butanol (Ivanova *et al.*, 2000) Hayashi *et al.*(1994)isolated Nisamycin from *Streptomyces* sp.K 106 by ethyl acetate extraction. Other examples for ethyl acetate extraction are Lactonamycin (Matsumoto *et al.*, 1999) *S.rishiriensis* and Vinylamycin (Igarashi *et al.*, 1999) by *Streptomyces* sp. Ethyl acetate is used in the present study since it is water immiscible and therefore seperation from fermentation broth was easy. The culture supernatant contains the active fraction dispensed in large amount of water. Removal of water from the culture broth is a tedious process.

5.4.1 Thin layer chromatography

Fermentation broth

TLC of whole fermentation broth gave spots mostly at Rf of 0.7 to 0.8 with the various spray reagents like, Dragendorff, H_2SO_4, H_2SO_4: Vanillin, Wood's reagent and ferric chloride. All these may be due to the nutrients present in the fermentation broth. Dragendorff reagent gave spots for the various fermentation broth at an Rf of about 0.2 for four strains, which may also be due to the nutrients. Dityromycin (AM-2504) an antimicrobial compound was isolated by using Dragendorff by Ömura *et al.* (1977) and Theshima *et al.* (1988). Ferric chloride was used by Inouye *et al.* (1975) to detect an antibacterial compound L-β-(3-hydroxy ureido)-alanine from *Streptomyces chibaensis* SF-1346. Antimicrobial antibiotics like Dienomycin (Umezawa et al., 1970a, 1970b) and Uredamycin (Drautz *et al.*, 1986) were detected by using Wood's and Ehrlich colour reagents respectively from *Streptomyces* spp. Ehrlich reagent was used by many workers for the detection of number of antibiotics, for example Keller-Schierlein *et al.* (1983 and 1985) isolated two compounds from *S. fimbriatus* and *S. parvulus.*

S. griseoflavus (B301) developed a clear spot at Rf 0.5 with Dragendorff reagent and *S.californicus* (B30) at 0.4 and 0.6 while H_2SO_4, and H_2SO_4: vanillin was used as spray reagents.

Dragendorff reagent is positive for alkaloids. Omura *et al.* (1974) found several new alkaloids from the culture broths of streptomycetes. An alkaloid AM-6201, identified as reductiomycin (shimizu *et al.*, 1981a and 1981b) exhibits anti tumour activity. Another alkaloid antibiotic AM-2504 (dityromycin) was isolated from streptomycetes which posses activity against gram-positive bacteria. Further, Omura

et al. (1979) and Hirano *et al.* (1979) have discovered two alkaloids, herquline and neoxaline, from streptomycetes in screening using Dragendorff's reagents. Kunze *et al.* (1989) isolated a new iron-chelating compound myxochelin A, from the culture broth by using TLC. The compound showed positive reaction to $FeCl_3$.

Chemical screening of the culture filtrate for alkaloid antibiotics with *S.griseoflavus* (B301) also gave positive reaction by forming a precipitate with Dragendorff reagent. Therefore, the active principle present in this strain against pathogenic vibrios of prawns may be an alkaloid secondary metabolite. Preliminary detection of compounds in crude culture broths using colour reagents has been reported by Porter *et al.* (1964).

5.4.2 Solvent extract of fermentation broth

Ethyl acetate extract of *S.Pulveraceus* (B361) and *S.fradiae* (B451) exhibited good bioactivity in terms of its inhibition to pathogenic vibrios. Therefore, these two extracts were subjected to TLC and only 451 E developed spots when exposed to iodine vapour. A spot at Rf of 0.25 was very distinct and other one at 0.18 was found with a tailing. Spot at Rf 0.25 exhibited good inhibitory activity and it was designated as RB 451 E. The separation could be effected with a solvent system, Ethyl acetate: Hexane (60:40). Strain B451 is *Streptomyces fradiae*, which has been already reported to produce tylosin (Tanaka *et al., 1986).*

Further work is to, be carried out for characterization, structural elucidation and identification of the compound.

Chapter 6

MARINE ACTINOMYCETES AS SINGLE CELL PROTEIN FOR APPLICATION IN PENAEID PRAWN CULTURE SYSTEMS

6.1 INTRODUCTION

During the past few decades, there has been an increasing interest to use alternative plant and animal protein, as low cost substitutes of fishmeal (Moore and Stanley, 1982; Kohler and Kruegger, 1986; Koshio *et al.*, 1992; Tidwell *et al.*, 1992, 1993 a & b and 1994) and many of the results obtained so far have proved encouraging. In aquaculture operations, essential and expensive components of the feed are proteins. Among unconventional protein sources, single cell proteins (SCP) of microbial origin appear to be a promising candidate. Many workers have reported partial replacement of fishmeal with yeast, bacteria and soybean protein (Cho *et al.*, 1974; Bergstrom, 1978; Spinelli *et al.*, 1978). The very high protein content of SCP's make them suitable for inclusion at high levels in pelleted fish rations (Nose, 1975).

6.1.1 Single Cell Protein

The term single cell protein refers to protein in microbial cells grown, harvested, and used as animal feed and for human consumption. Yeast, bacteria, algae etc are used in this regard. A list of reasons makes SCP advantageous-

1. Microorganisms have a very short generation time and can produce rapid biomass increase.
2. The protein content is high up to 60 %.
3. The production of SCP can be based on raw materials, which is readily available in large quantities.
4. SCP production can be carried out in continuous culture and thus can be independent of climatic changes.

SCP comprises of proteins, fats, carbohydrates, ash ingredients, water and other elements such as phosphorus and potassium. The use of Yeast and Bacterial SCP as feed ingredients has gained importance with the understanding of its nutritive value. Bacterial cell wall contain peptidoglycans (PG) rich in N-acetyl glucosamine and N-acetyl muramic acid (White *et al.*, 1979). The essential amino acid index of almost all bacteria was noted to be in the range of 91-94. Being above the value 90, it is of good quality for use as aquaculture feed ingredient (Penaflorida, 1989). Bacteria like *Pseudomonas* and *Methylophilus* sp. have been investigated for use as SCP in aqua feeds. They have approximately 73% crude protein, 5.7% lipid and 2.7 % NFE by weight (Kant, 1996). Brown *et al.* (1996) analyzed the composition of a few strains of marine bacteria including *Methylophilus methylophilus, Aeromonas* sp., *Werxia* sp. and *Psuedomonas* sp. to assess their nutritional value for bivalve aquaculture. Protein was the major constituent of bacteria (25-49% dry weight), with lipid a minor component (2.5 - 9.0%). Carbohydrate in bacteria ranged from 2.5 - 11%. Ash occurred in high levels 29-40%. Glucose was the major sugar source in bacteria (12 - 73% of total sugars). Nucleic acids range from 3.3 to 8.4 % dry weight and polyunsaturated fatty acids, 20:5n-3 and 22:6n-3, were absent in the bacteria. High levels of good quality protein however, indicate their potential to provide important nutrients in a mixed diet. SCP contains more nitrogen in the form of nucleic acids (Prave *et al.*, 1987). Besides these, microorganisms are able to synthesize precursors of all macromolecules and vitamins (Prave *et al.*, 1987).

Atack and Matty (1978) evaluated the efficacy of a methanophilic bacterium, a petro-yeast, algal protein and extracted soybean as sole

source of nitrogen in trout feed. Moreover, the results showed that the bacterial proteins were well accepted, digested and utilized for growth. Kussling and Askbrandt (1993) reported that the methanol bacterial SCP could replace up to 75% fishmeal in Salmonid production diet. Similarly, photosynthetic bacteria have also proved to be a potential feed source for aquaculture systems (Kobayashi and Tachan, 1973; Shipmann, 1975; You *et al.*, 1992; Gebruk *et al.*, 1992). These bacteria can synthesize vitamins such as riboflavin, pyridoxine, folic acid, ascorbic acid and cholecalciferol, which may influence the growth and conversion efficiency of prawns (Kobayashi, 1970).

Actinomycetes can be used as single cell protein in aquaculture feed formulations, since they are the producers of many secondary metabolites. Nakamura et al. (1977) are of the opinion that some of the secondary metabolites may enhance the growth of prawns. Manju and Dhevendran (1997) studied the effect of bacteria and actinomycetes as single cell protein in aquaculture diet by observing the growth, conversion efficiency and protein increment of the juvenile prawn, *Macrobrachium idella*. The feeding experiment included diet containing bacterial mixture, actinomycetes, bacterial mixture + actinomycetes and photosynthetic bacteria.

The results showed a higher growth percentage with bacterial mixture and Actinomycetes.

6.1.2 Diseases in Aquaculture and Prevention

Aquaculture is a fast growing industry in the world and a major threat to this industry is the frequent outbreak of diseases, especially of viral origin. White spot disease (WSD) is a devastating problem in aquaculture affecting most of the commercially cultured shrimps globally. Diseased shrimps show white spots on the carapace and reddish discolouration of the body.

The prevention and control of diseases are now considered priorities for shrimp aquaculture in the vast majority of the shrimp producing countries. The maintenance and development of this industry are at stake as shrimp aquaculture faces increasingly significant ecological and pathological problems on a global scale. The use of antibiotics and other chemotherapeutics have several shortcomings including the risk of generating resistant pathogens, the problem of drug residues in the treated animal, and the impact of environmental pollution. The need for alternate methods for regulating the number of pathogenic bacteria and also detrimental effects of viruses in aquaculture, have led researchers to turn to different methods of treatment such as probiotics and immunostimulants.

Immunostimulants aim at enhancing the nonspecific defense mechanisms in animals. A number of different biological and synthetic compounds have been found to enhance the nonspecific defense system in animals, including shrimp (Song and Sung, 1990; Sung *et al.*, 1991). They have been shown to increase the barriers of infection against a series of pathogens simultaneously, both specific and opportunistic ones. (Raa *et al.*, 1992). The use of immunostimulants may increase the resistance level sufficiently to abolish infections by opportunistic

pathogens and therefore, lead to improved performance, enhanced growth and reduced mortality throughout the production period.

Most evidences indicated that shrimp pathogens were 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 non - specific immunity of shrimp to provide them with broad-spectrum defensive ability should effectively protect shrimp against infections from pathogens.

Bacterial derivatives like muramyl peptide (Olivier *et al.*, 1985; Kodama *et al.*, 1993), low molecular weight peptides (Kitao and Yoshida, 1986;Bogwald *et al.*, 1995; Gildberg *et al.*, 1996;) and Freund's (complete) Adjuvant (Patterson and Fryer, 1974; Olivier *et al.*, 1985, 1986) have proved to confer immunostimulation.

There are several evidences to suggest that bacterial cell walls have the ability to enhance the non-specific resistance of fish and shrimp against bacterial infections. In another experiment using peptidoglycans, shrimp fed with peptidoglycan-incorporated diet showed hemocytic activity, disease resistance and higher tolerance to variations in dissolved oxygen, salinity and stress (Booyratnapalin, 1990).

The oral administration of peptidoglycans from *Bifidobacterium thermophilum* protected juvenile rainbow trout challenged with *Vibrio anguillarum* (Mastsuao and Miyazano, 1993). Peptidoglycan derived from the cell walls of Gram-positive bacteria, *Brevibacterium lactofermentum* was continuously fed to black tiger shrimp (*Penaeus*

monodon) larvae (aged 15 days) for 8 weeks. Shrimp fed with pre-supplemented feed showed better survival and higher disease resistance against Yellow Head Baculovirus (YHBV) than those fed with a normal diet.

Lipopolysaccharides (LPS), the outer cell envelope of the gram-negative bacteria is also known for its immunostimulatory properties in activating both macrophages and lymphocytes (Burrel, 1990). LPS is less toxic to fish and shrimp and has been shown to be active as an immunostimulatory complex that increases disease resistance in fish (Jorgensen, 1994) and shrimp (Song and Sung, 1990).

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, 1997). Improvements in the health status of aquatic organisms can certainly be achieved by balancing the diet with regard to nutritional factors, in particular lipids and anti-oxidative vitamins, which is primarily an input of substrates and co-factors in a complex metabolic system.

Some nutritional factors are so intimately interwoven with the biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors (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).

The antibacterial properties of culture filtrates of the selected actinomycete strains were demonstrated in the previous chapters 3, 4 and 5.After separating the bioactive compounds actinomycete biomass

is generally discarded. Since bacteria are proven source of single cell protein, an attempt was made in this chapter to study the efficacy of Actinomycete biomass as single cell protein for the application in penaeid prawn culture systems for supporting growth as well as increased protection against microbial infections.

6.2 MATERIALS AND METHODS

6.2.1 Microorganisms used

The six selected actinomycetes (*Streptomyces californicus* (B30), *Streptomyces* sp. (B272), *S.griseoflavus* (B301), *S.pulveraceus* (B361), *Streptomyces* sp(B377) and *Streptomyces fradiae*(B451) were used for the study.

6.2.2 Medium used

Modified medium M13 and M14 obtained after process optimization in chapter 4 was used for making actinomycete biomass. Medium was prepared in 1000 ml Erlenmeyer flasks and sterilized in an autoclave at 121^{0}C for 15 minutes.

6.2.3 Inoculation and Incubation

One loopfull each of spores from one-week-old slant culture of actinomycete were inoculated into 10ml sterile marine actinomycete growth medium. Incubation was done at room temperature (28±2 ^{0}C) for 48 hours in a rotary shaker at 150 rpm. Then the culture broth (10ml) was transferred as such into 500 ml medium and kept on a magnetic stirrer (Remi, Mumbai) with teflon pellets for agitation at room temperature (28±2^0 C).

6.2.4 Separation of Actinomycete Biomass

After one-week incubation the culture, broths were centrifuged at 10,000rpm for 15 minutes in a refrigerated centrifuge (Remi,C-30, Bombay) at 4°C and the residual biomass was collected after decanting the supernatant .The biomass of the six actinomycetes generated in this way were used for feed preparation. The biomass was kept at –20°C in a freezer (Labline, Kochi) until used for feed preparation.

6.2.5 Proximate composition of the experimental diets, actinomycete biomass and prawn flesh

Protein, lipid and carbohydrate content of the actinomycetes biomass, experimental diets and prawn flesh were estimated. Protein estimation was done as per Lowry *et al.* (1951), lipid by phospho-vanillin method (Folch et al., 1959) and carbohydrate as per Mendel *et al.* (1954)

6.2.6 Feeding experiment with penaeid prawns using actinomycete biomass as SCP

Two feeding experiments were conducted on penaeid prawns using actinomycete biomass incorporated feeds.

Expt .1 *Penaeus monodon* post larvae were used

Expt .2 *Penaeus monodon* juveniles were used

6.2.6.1 Feeding Experiment I with Penaeus monodon post larvae

Experimental Animals

Post larvae (PL25) of *Penaeus monodon* of size range 30 – 50 mg were brought to the laboratory from Matsyafed Prawn hatchery, Ponnani.

Preparation of Experimental Feeds

Powdered ingredients (except actinomycete biomass) as given in table 6.1 were mixed well into dough with 100 ml water.

Table 6.1 Composition of diets used for feeding experiment

Feed composition – Experiment I

Ingredients	Control Feed (g)	Experimental Feed (g)
Prawn shell powder	10	10
Fish meal	30	30
Ground nut oil cake	8	8
Soya flour	10	10
Carboxy Methyl Cellulose	10	-
Rice bran	20	20
Refined wheat flour	10	10
Actinomycete biomass (wet wt)	0	10
Vitamin mineral supplement*	2	2

Feed Composition - Experiment II

Ingredients	Control Feed (g)	Experimental feed (g)
Prawn shell powder	10	10
Fish meal	30	30
Ground nut oil cake	8	8
Soya flour	10	10
Carboxy Methyl Cellulose	10	5
Rice bran	20	20
Refined wheat flour	10	10
Actinomycete biomass (dry.wt)	0	5
Vitamin mineral supplement*	2	2

*** vitamin mineral supplement**
vitamin mg kg^{-1} diet

p-amino benzoic acid,150.8; biotin, 6.3; inositol, 6320.0; niacin, 632.0; Ca pantotenate, 948.0; pyridoxin HCl, 189.6; riboflavin, 126.4; thiamin HCl, 63.2; menadione, 63.4; α-tocopherol, 316.0;β-kerotene' 151.7; calciferol, 19.0; cyanocobalamin, folic acid ,12.6; choline chloride ,9480.0.

mineral mix mg kg^{-1} diet

K2HPO4 ,11.69; Ca3 (PO4)2 , 15.91;MgSO4.7H2O, 17.78; and NaH2PO4. 2H2O, 4.62.

This was steamed for 10 minutes in an autoclave. Biomass was added and pelletized using a laboratory model hand pelletizer having a

1mm die. Pellets were dried in an oven at 50°C for 18 hours. The pellets were broken into pieces of 4-5 mm size. Six different feeds were prepared incorporating the biomass of six actinomycetes and a control diet without biomass. Immersed the pellets in seawater for 8 hours and examined the stability by visual observation. Feeds were stored in airtight polythene bags at –20°C in a freezer.

Feeding Schedule

Prawns were fed twice daily at 10 a.m and 5 p.m with seven 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 (Fig.6.1). Water quality was monitored daily and was maintained as per table 6.2.

Table 6.2 Rearing conditions and water quality

Parameters	Experiment I	Experiment II
Initial body wt (average)	30 – 50 mg	0.52 - 0.73g
Stocking Density (per tank)	25 PL/tank	5 juveniles/tank
Tank Capacity	30 L	30 L
Feeding level	15 -20 % body wt.	15 -20 % body wt.
Feeding frequency	Twice daily	Twice daily
Feeding period	21 days	28 days
Water temperature	24-27°C	24-27°C
pH	7.5 – 8	7.5 – 8
Salinity (ppt)	15 – 18 ppt	15 – 18 ppt
NH_3 (mg/ml)	0.01 - 0.02 mg/L	0.01 - 0.02S mg/L
NO_3^- (mg/ml)	Below detectable	Below detectable
NO_2^- (mg/ml)	0.00 - 0.01mg/L	0.00 - 0.01mg/L
Dissolved oxygen	6 -7 mg/L	6 -7 mg/L

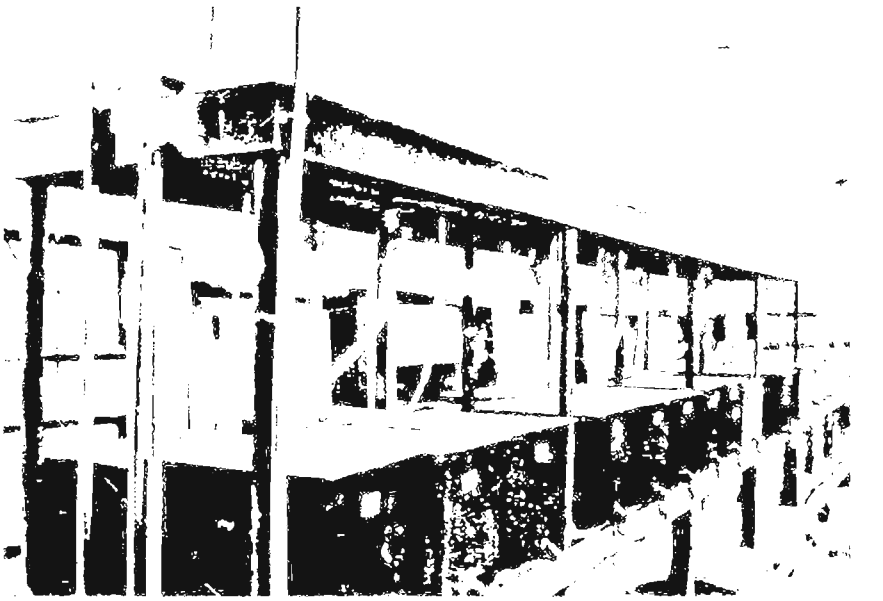


Fig. 6.1 Experimental setup

On alternative days after removing the faeces and left over feed, 50% of the water was exchanged from all the experimental tanks. Aeration was provided from a 1HP compressor through air stones. Physiochemical parameters like salinity, ammonia-N, nitrite-N, nitrate-N and dissolved oxygen of the rearing water were estimated on alternate days by following standard procedures (APHA, 1995).

Design of Experiment

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

Measurements

The initial wet body weight of all the prawns in each rearing tank was recorded. They were weighed on a precision balance after they were blotted free of water by tissue paper. The mean weights of all the prawns in a tank were calculated (mean ± 0.01 mg). After 28 days final weights of all the prawns were measured and mean weight was found. Parameters including individual increase in weight (production), Specific Growth Rate (SGR) and Relative Growth Rate (RGR)) were determined based on the data collected during the experimental period.

The formula used for calculating the growth parameters were given below: -

Production = final weight (W2) – initial weight (W1).

SGR = [(ln final weight – ln initial weight) * 100]/Days of feeding experiment.

RGR = (W2 - W1) / mean weight / no of days

Challenge Experiment

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

Survival rate was recorded everyday for a period of seven days by which time almost complete mortality was recorded in some groups.

Morality by WSSV infection was confirmed by checking the characteristic white spots on the carapace and other regions of the shell of infected animal.

6.2.6.2 *Feeding Experiment II with Penaeus monodon juveniles*

Experimental Animals

Penaeus monodon juveniles of size range of 0.52 to 0.73g were used for the study.

Rearing facility

As described in 6.2.6.1

Experimental design

After one week of acclimatization, the prawns were distributed in fiberglass tanks, 5 each in tank containing 15 L water. Triplicate tanks were maintained for each treatment. Feeding was done for 28 days.

Preparation of Experimental Feeds

Powdered ingredients as given in table 6.2 were mixed well into dough with 100 ml water. This was steamed for 10 minutes in an autoclave and pelletized using laboratory model hand pelletizer having 1mm die. Pellets were dried in an oven at 50^0 C for 18 hours. The pellets were broken into pieces of 4-5 mm size. Six different feeds were prepared incorporating six-actinomycete biomass plus the control diet with out biomass. Stability of the feeds was checked by immersing the feeds in seawater for 8 hours followed by visual observation. Feeds were stored in airtight polythene bags at -20^0C in a freezer.

Feeding Schedule

Feeding was done as given in 6.2.6.1.Uneaten feed was collected twice daily by siphoning and was washed gently with distilled water to

remove salt and filtered through a pre-weighed filter paper and dried to constant weight in an electric oven at 80°c for 24 hours.

Measurements

The initial wet body weight of all the prawns in each rearing tank was recorded. They were weighed on a precision balance after they were blotted free of water by tissue paper. The mean weights of all the prawns in a tank were calculated (mean ± 0.01 mg). After 28 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), Consumption per Unit weight per Day(CUD) 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 (W2) – initial weight (W1).

FCR = Food consumed/live weight gain

SGR = [(ln final weight – ln initial weight) x 100]/Days of feeding experiment.

RGR = (W2 - W1) / mean weight / no of days

GGE = [(W2-W1) / Food Consumed] x100

CUD = Food consumed / (mean weight x no. of days)

Data analysis

Data obtained in the feeding experiments were subjected to Duncan's multiple range tests to bring out the difference between the

treatment means. The statistical analysis was performed using the SPSS 10.0 package for windows.

Challenge Experiment

After a period of 28 days feeding experiment all treatment groups including control were challenged as given in 6.2.6.1.Survival rate was recorded everyday for a period of seven days. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of the animals.

6.3 RESULTS

In both the feeding experiments, the actinomycete incorporated feed F361 exhibited better performance in terms of production, FCR, SGR and RGR etc. The growth parameters exhibited by this feed (F361) was found to be significantly different compared to the other actinomycete incorporated feeds and control feed.

6.3.1 Feeding experiment I

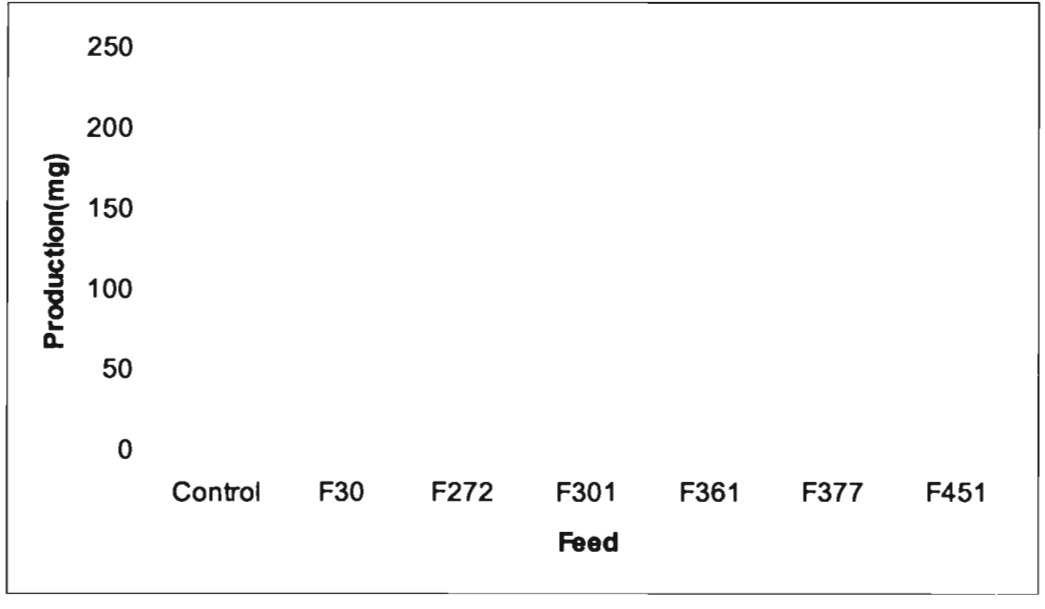
Two feeding experiments were conducted incorporating actinomycete biomass in the feed of giant tiger prawn, *Penaeus monodon* (Post larvae and juveniles). In both the experiments, the experimental feed (F361) incorporated with the biomass of B361 (*Streptomyces pulveraceus)* gave the best performance in terms of various biogrowth parameters (production, Food Conversion Ratio, Relative Growth Rate and Gross Growth Efficiency) and post challenge survival with white spot virus.

6.3.1.1 Biogrowth parameters

Significant weight gain (production) was noted with *Penaeus monodon* larvae maintained on experimental feeds, F361, F451 and F377 incorporated with the biomass of *S.pulveraceus, S.fradiae, and Streptomyces* sp.respectively (Fig. 6.2 a). The best production was recorded for feed F361 (181mg) followed by F451 (170mg) and F377 (160mg). SGR was maxim um with feed F361 (8.0) followed by the control (6.38) (Fig.6.2b). However, Relative Growth Rate (RGR) was found to be maximum for F272 (0.246) closely followed by F361 (0.239) and F451 (0.215) (Fig 6.2c). Survival after the feeding experiment was above 80% for all the treatment groups including the control feed. The post challenge survival after the feeding experiment was 80% for all the treatment groups and the maximum was observed with feed F 361(Fig 3a).

6.3.1.2 Post challenge survival

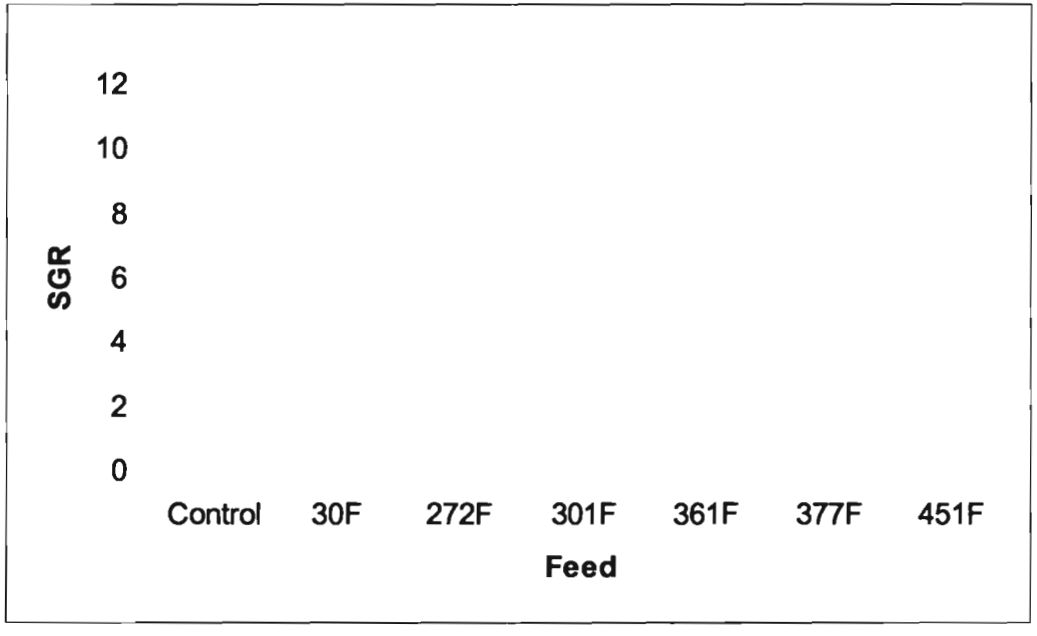
The percentage survival of the post larvae fed on experimental diets after challenge with white spot virus (WSV) is given in Fig.6.3b. The percentage survival for treatment diets varied from 20% to 79%. The survival was best with feed F361 (79%) followed by F30 (70%), F272 (57%) and F451 (50%).



Feed	*Production (mg)
Control	73.9±18[a]
F30	123±11[abc]
F272	116±27[abc]
F301	90±23[ab]
F361	181±20[c]
F377	160±20b[c]
F451	170±18b[c]

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

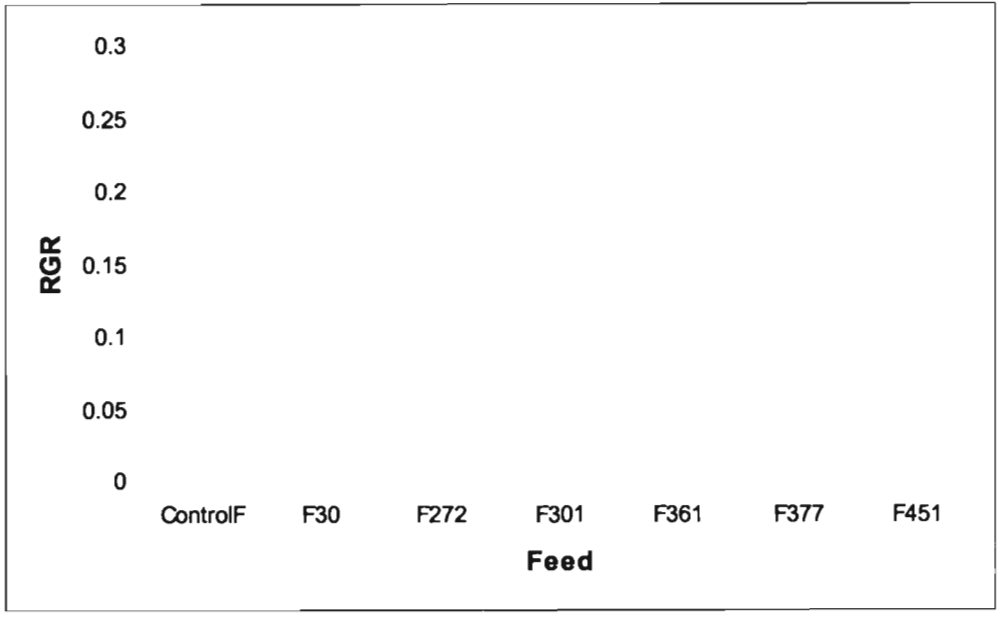
Fig 6.2.a. Weight gain (production) obtained in P. monodon post larvae when fed with various experimental feeds



Feed	*SGR
Control	6.38±2.81[a]
F30	5.62±2.5[a]
F272	4.85±2.4[a]
F301	4.84±2.6[a]
F361	8.00±2.9[a]
F377	5.89±2.1[a]
F451	3.65±2.8[a]

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

Fig.6.2b. Specific Growth Rate (SGR) obtained in P. monodon post larvae when fed with various experimental feeds



Feed	*RGR
Control	0.212±0.029[a]
F30	0.150±0.019[a]
F272	0.246±0.017[a]
F301	0.09±0.017[a]
F361	0.239±0.023[a]
F377	0.182±0.019[a]
F451	0.215±0.017[a]

* (mean ±s.d.)

* Values with the same superscript does not vary significantly

Fig 6.2c. Relative growth Rate (RGR) obtained in P.monodon post larvae when fed with various experimental feeds

Fig 6.3 a Percentage survival of P.monodon post larvae after feeding experiment

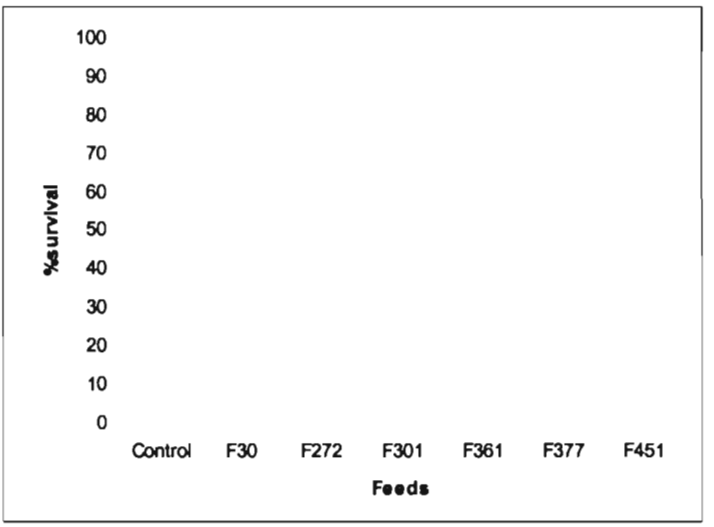


Fig 6.3 b Percentage survival of P.monodon post larvae on seventh day post challenge with white spot virus

6.3.1.3 Statistical analysis

Duncan's multiple range test showed that the performance of the feeds in terms of biogrowth parameters differed significantly and is presented in the supporting data for the Fig. 6.2.a to 6.2c. Production with all the experimental diets differed significantly from the control diet and the performance of F361 in terms of production was found to be significantly different from all other feeds. However, for SGR and RGR no significant difference could be obtained between the various feeds including the control feed.

6.3.2. Feeding Experiment II

6.3.2.1. Proximate composition of actinomycete biomass (SCP), feed and prawn flesh.

a. Proximate composition of actinomycete biomass

Protein content of the actinomycete biomass ranged from 41.6 to 56.1% with the maximum in B361 (56.1). Lipid content was also maximum in B361 (10.46%) biomass and there was no much variation between the rests of the strains (1.1 to 2.6%). Carbohydrate content was comparatively high for *S.californicus* (B30), *Streptomyces* sp. (B272) and *S.griseoflavus* (B301) and lower values could be observed for the other *Streptomyces* spp.B361 and B377 and B451(Fig 6.4).

b. Proximate composition of feeds

Protein content of the feeds ranged from 39.1 to 52.3% with the maximum in feed F361 (Fig 6.5). Lipid was also maximum in feed F361 (14%) and there was no much variation between other feeds. No significant variation could be obtained in the carbohydrate of the biomass content of various strains and the value ranged from 24.4 to 30.1%.

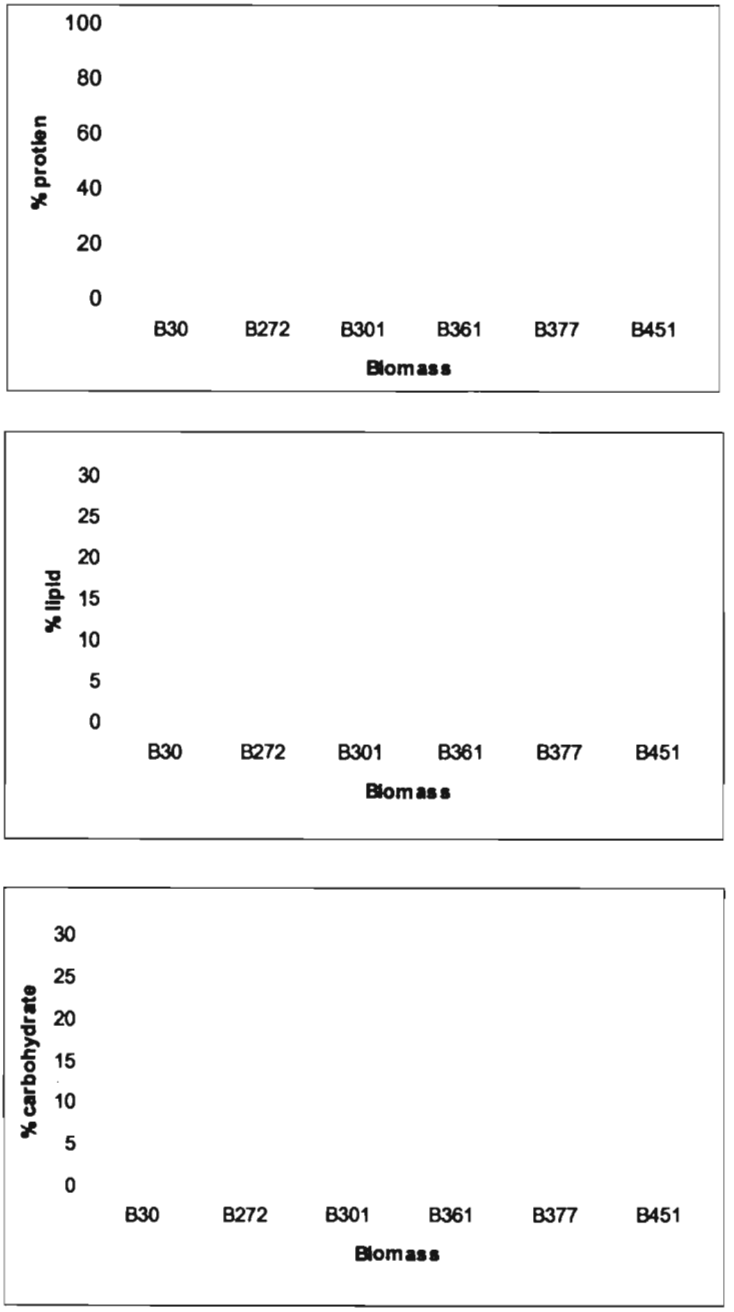


Fig 6.4. Proximate composition of actinomycete biomass

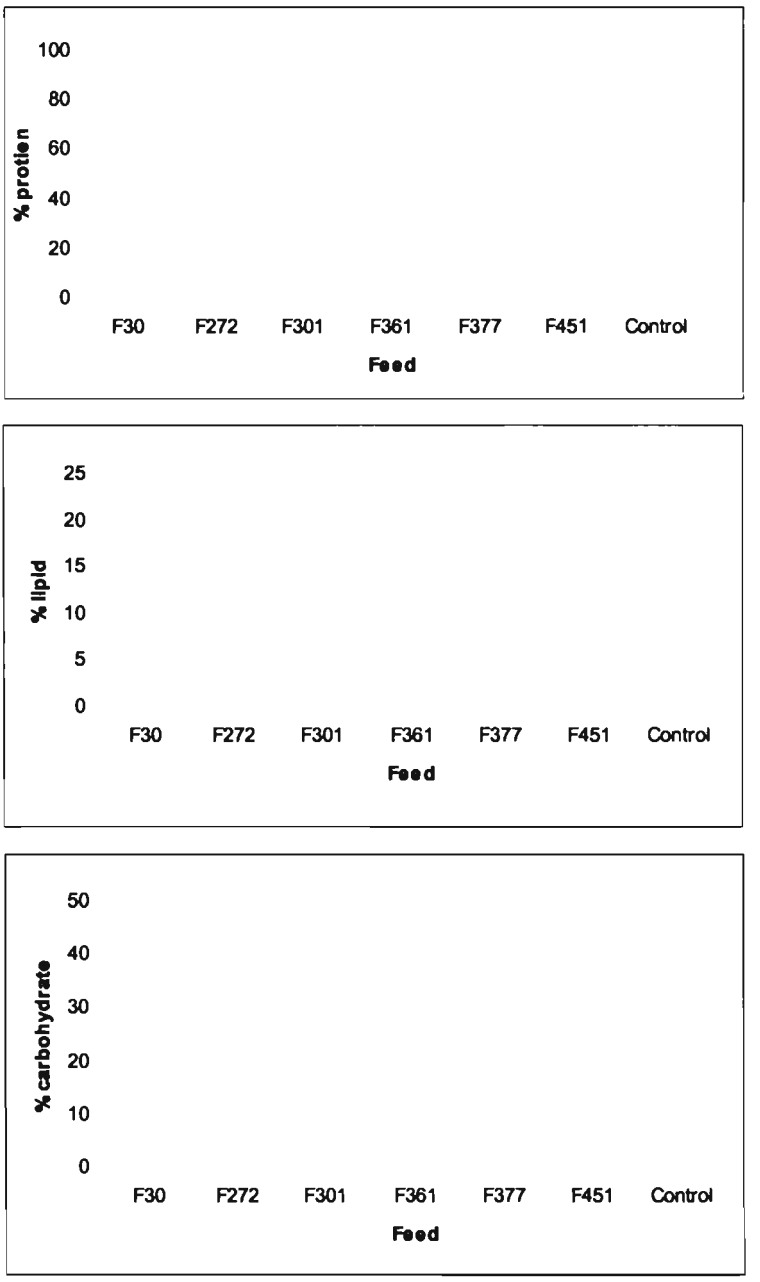


Fig 6.5. Proximate composition of experimental feeds

c. Proximate composition of prawn flesh

Prawns fed on various diets exhibited almost the same protein content and the percentage of protein at the beginning of the experiment was maintained without much change (Fig 6.6). The percentage of lipid was found to be maximum in prawns fed with feed F361 (7.85%) followed by F301 (7.75%), F451 (6.3%) and F30 (6%). Carbohydrate content varied considerably in the prawns fed varied diets. The maximum was observed in prawns fed with feed F272 and control feed whereas lower values could be obtained for F361, F377 and F30 similar to the initial protein content of the prawns. S

6.3.2.1. Biogrowth parameters

The data collected from the experiments were analyzed and the bio-growth parameters like production, food conversion ratio (FCR), specific growth rate (SGR), relative growth rate (RGR), gross growth efficiency (GGE) consumption per unit weight per day (CUD) were determined and are presented in figure 6.7a to f. An overall analysis of bio-growth parameters showed that the performance of F361 was the best followed by F451 when compared to other feeds. Production and FCR of the shrimps fed feeds F361 and F451 were found to be significantly different from the other feeds. Feed F30 was next in the order of performance followed by F377.Feeds F272 and F301 were found to be poor in terms of growth parameters when compared to the control.

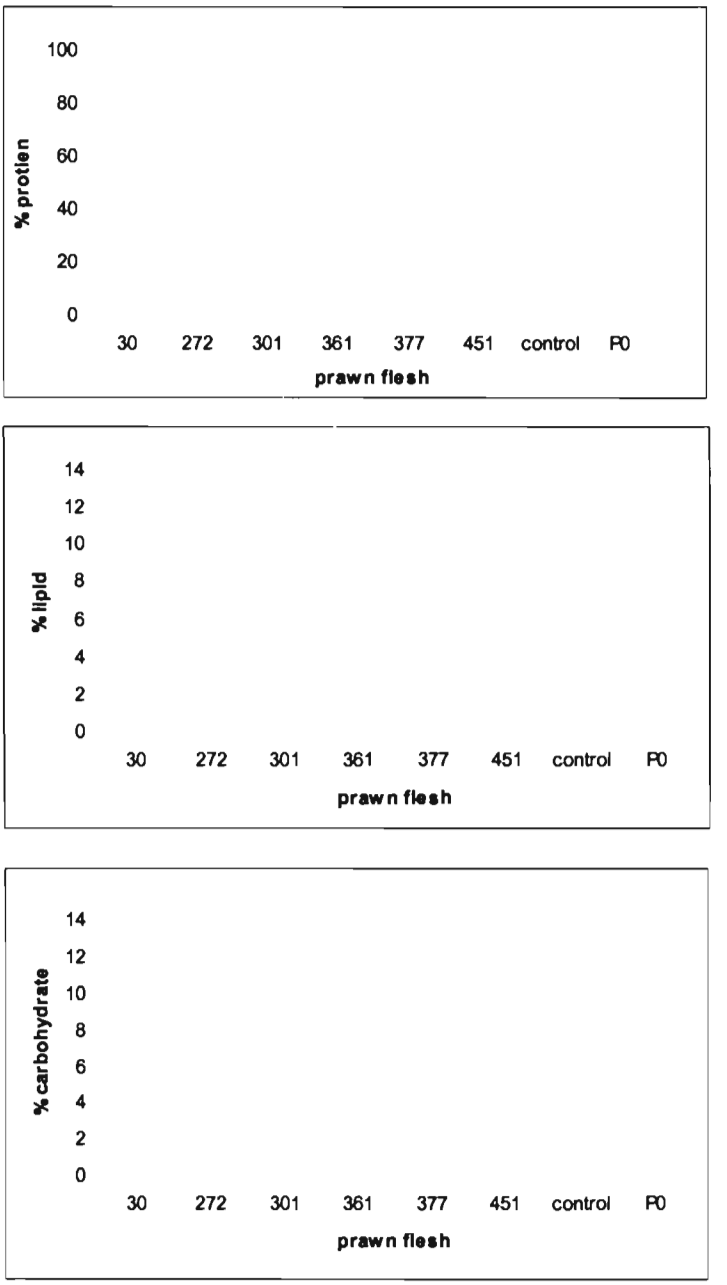


Fig 6.6. Proximate composition of prawns when fed with various actinomycete incorporated feeds

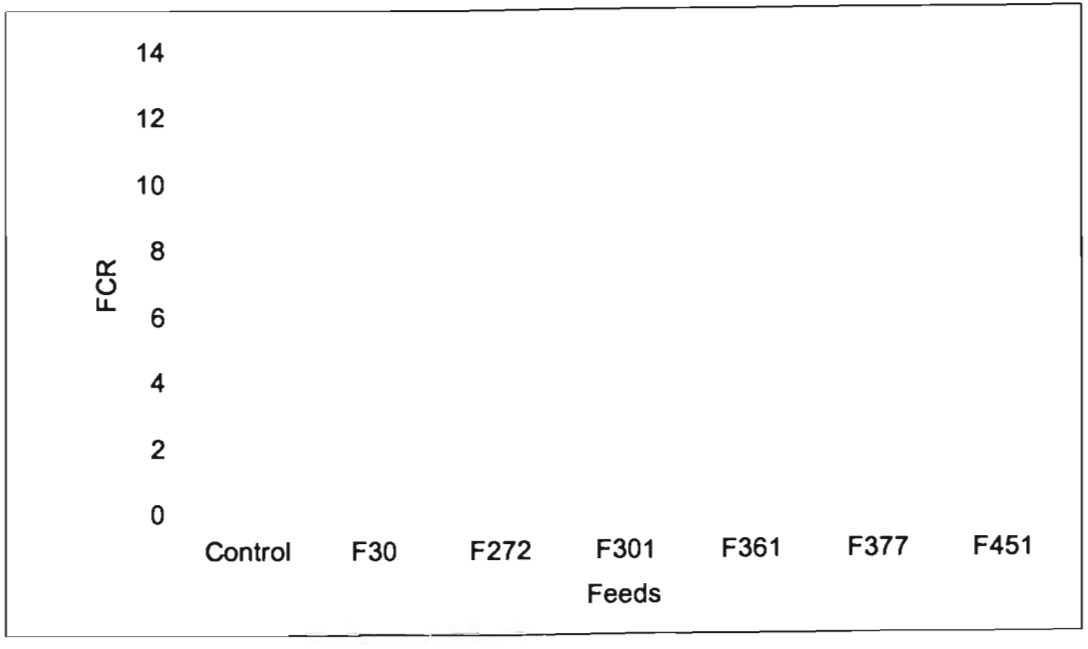
Po - initial prawn content (beginning of feeding experiment)

Feed	* PRO (mg)
Control	382.10±49.2^{abc}
F30	489.27± 64.98^{bc}
F272	249.20±40.96^{a}
F301	310±49.8^{ab}
F361	848.14±65^{d}
F377	422.27±47.46^{abc}
F451	518.88±47.6^{c}

* (mean ± s.d.)

* Values with the same superscript does not vary significantly

Fig.6.7a. Weight gain (Production) obtained in P. monodon juveniles when fed with various experimental feeds



Feed	*FCR
Control	6.43±0.38[a]
F30	5.88±0.63[b]
F272	11.54±0.82[d]
F301	8.93±0.74[c]
F361	2.93±0.18[a]
F377	4.71±0.52[ab]
F451	2.89±0.81[a]

* (mean ± s.d.)

* Values with the same superscript does not vary significantly

Fig.6.7 b Food Conversion Ratio (FCR) obtained in P. monodon juveniles when fed with various experimental feeds

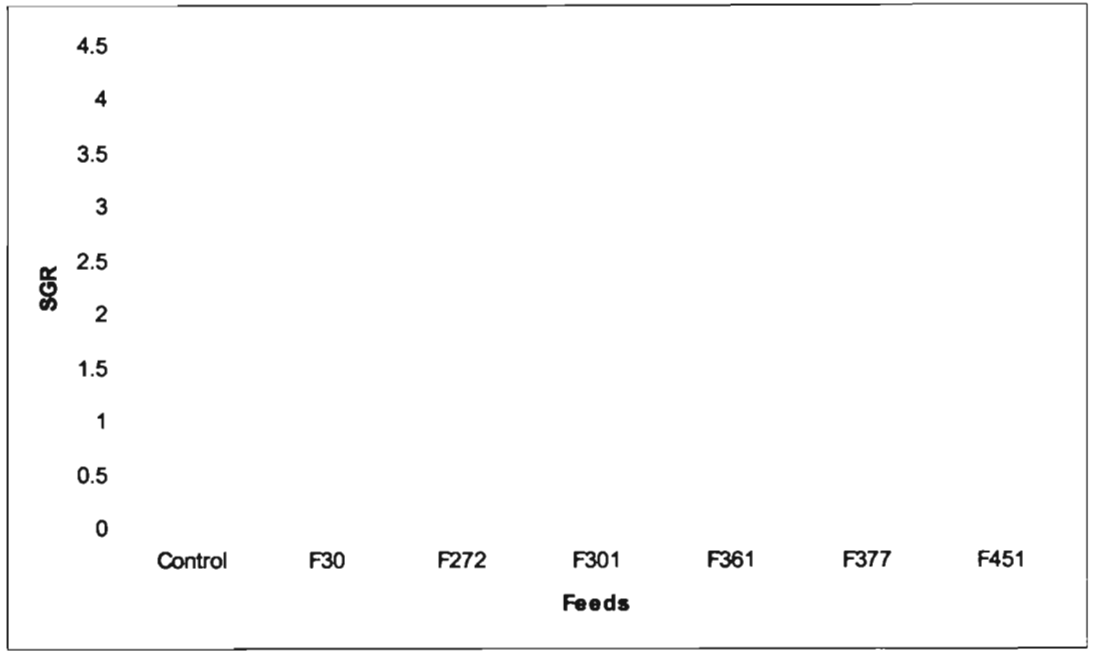
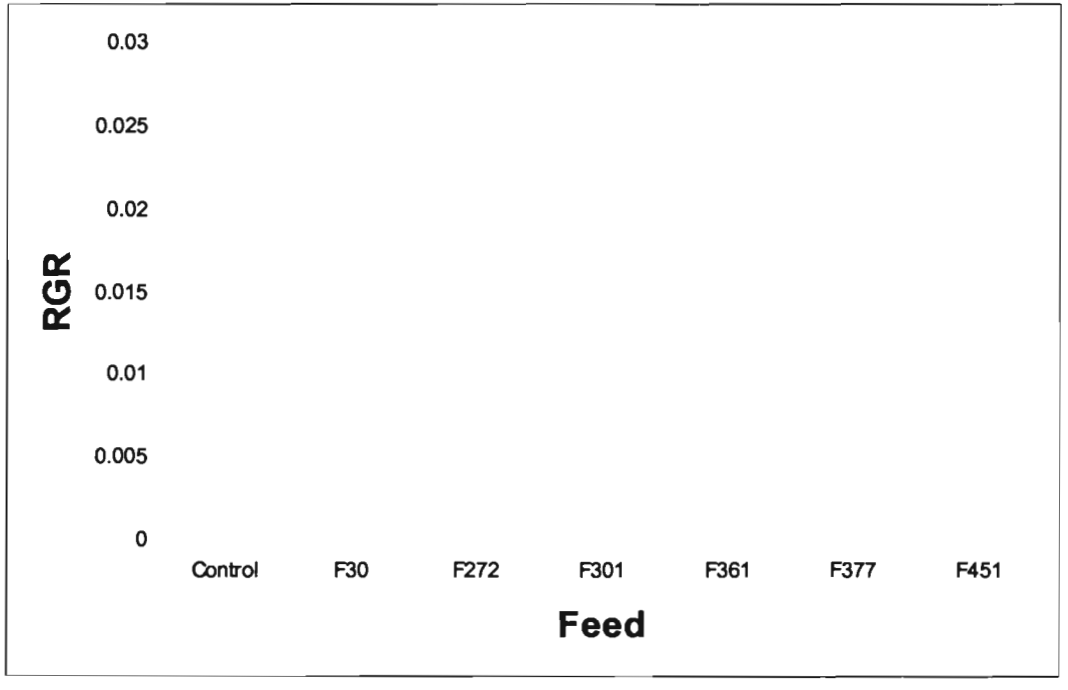


Fig.6.7 c. Specific Growth Rate (SGR) obtained in P. monodon juveniles when fed with various experimental feeds

Feed	*FCR
Control	6.43±0.38[a]
F30	5.88±0.63[b]
F272	11.54±0.82[d]
F301	8.93±0.74[c]
F361	2.93±0.18[a]
F377	4.71±0.52[ab]
F451	2.89±0.81[a]

* (mean ± s.d.)

* Values with the same superscript does not vary significantly



Feed	*RGR
Control	0.013±0.002[a]
F30	0.015±0.003[a]
F272	0.012±0.002[a]
F301	0.012±0.001[a]
F361	0.024±0.001[b]
F377	0.013±0.003[a]
F451	0.012±0.002[a]

* (mean ± s.d.)

* Values with the same superscript does not vary significantly

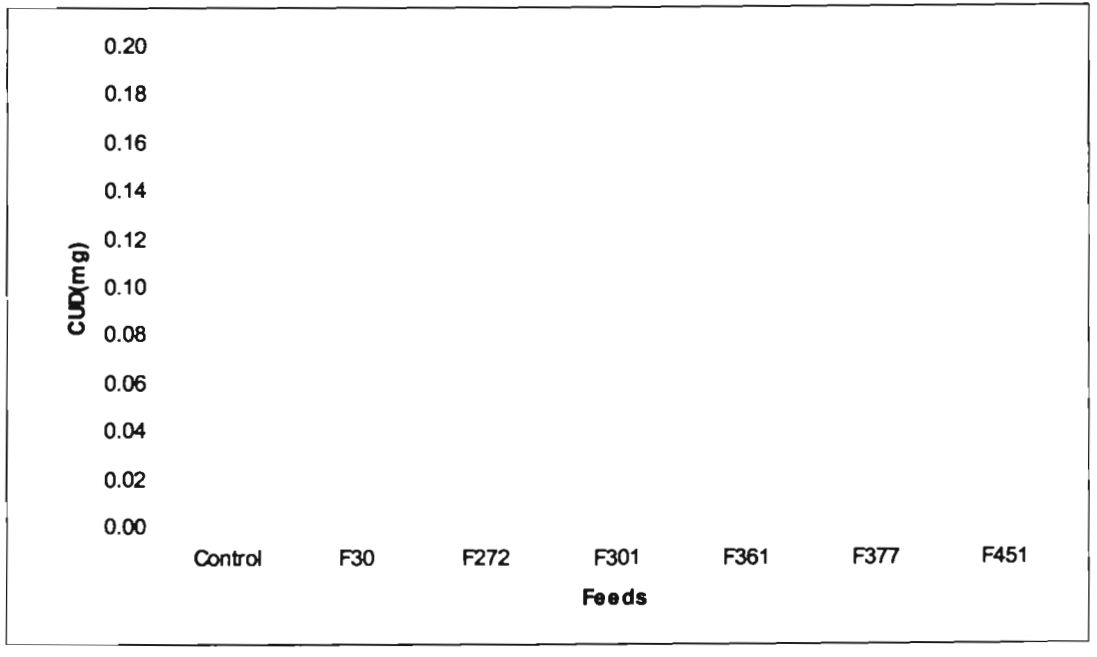
Fig.6.7 d. Relative Growth Rate (RGR) obtained in P. monodon juveniles when fed with various experimental feeds

Feed	*GGE
Control	16.5±1.05^{ab}
F30	17.1±1.85^{ab}
F272	8.8±1.35^{a}
F301	12.1±1.25^{a}
F361	34.1±1.05^{c}
F377	22.5±1.22^{b}
F451	36.1±1.54^{c}

* (mean ± s.d.)

* Values with the same superscript does not vary significantly

Fig.6.7e. Gross Growth Efficiency (GGE) obtained in P. monodon juveniles when fed with various experimental feeds



Feed	*CUD
Control	0.08 ± 0.01^{ab}
F30	0.09 ± 0.2^{ab}
F272	0.15 ± 0.02^{c}
F301	0.11 ± 0.01^{bc}
F361	0.06 ± 0.01^{ab}
F377	0.08 ± 0.2^{ab}
F451	0.03 ± 010^{a}

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

Fig.6.7 f. Consumption Per Unit weight per day (CUD) obtained in P. monodon juveniles when fed with various experimental feeds

The highest production was recorded in prawns fed with feed F361 (848mg) followed by F451 (518mg) and the lowest was recorded for F272 (249mg). Food conversion ratio was found to be the best with feed F361 (2.93) followed by F451 (2.89) (Fig. 6.7 b). FCR obtained with feeds F30 (5.8) was found to be comparable to that of the control feed (6.43) and were significantly different from the values of F361 and F451. Specific growth rate (SGR), relative growth rate (RGR) and gross growth efficiency (GGE) are parameters which expressed different facets of growth efficiency and a similar trend of best performance was exhibited by diet F361 for all these parameters (Fig 6.7 c & d). Specific growth rate varied from 1.55 to 3.37 in the prawns when fed with the various experimental diets.) . Relative growth rate ranged from 0.011 to 0.024 (Fig.6.7.d). The highest value was recorded for F361 (0.024) it was significantly different, from all other feeds statistically. The highest gross growth efficiency (GGE) values were for feed F451 (36.18) followed by F361 (34.17) and F377 (22.55) (Fig 6.7.e). The GGE of feeds F361 and F451 was significantly different from the rest of the feeds.

Consumption was found to be maximum for feed F451 (0.03) and significantly different from all other feeds. Survival was found to be above 70% for all the feeds after the one-month feeding experiment (Fig 6.8 a).

6.3.2.2. Post Challenge Survival

Post challenge survival was found to be significantly high with *P.monodon* maintained on feed F361 (91.5) compared to the other feeds (fig 6.8 b). All other feeds except F30 recorded better survival compared to the control.

Death by WSSV infection was confirmed by the presence of white spot on the carapace of the infected prawns.

Fig 6.8 a Percentage survival of P.monodon juveniles after 28 days feeding Experiment

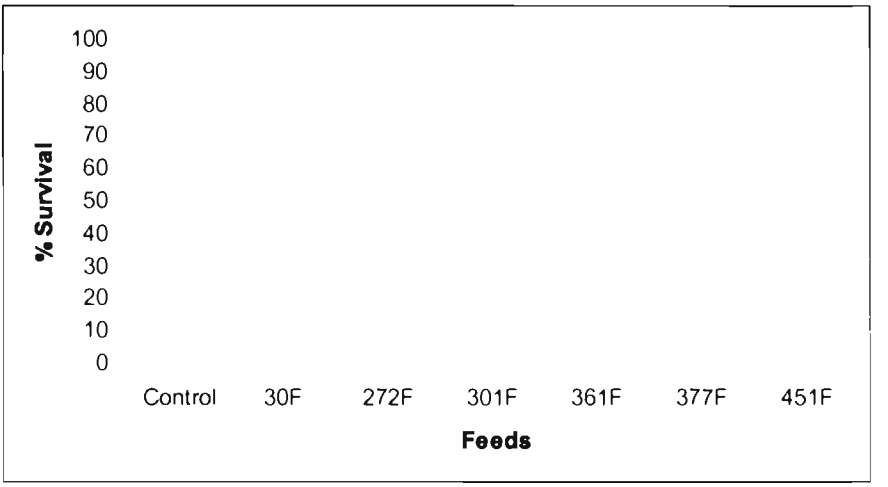


Fig 6.8 b Percentage survival of P.monodon juveniles on seventh day post challenge with white spot virus

6.3.2.3. Statistical analysis

Duncan's multiple range analysis of the various growth parameters effected by the different feeds showed that the performance of the feed F361 and F451 were significantly different from other feeds including the control feed.

6.4 DISCUSSION

The protein content of the feeds ranged from39-52 %. This range was found acceptable for optimum growth in penaeid prawns as shown by various earlier feeding experiments. Dietary protein has been reported as the most essential nutrient for the growth of prawns (Andrews *et al.*, 1972; 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 amino acids which are also crucial to the biological value of the protein.

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

contributed by microbial protein. The nutritional value of the microorganisms used in aquaculture depends on their digestibility and assimilation characteristics and the target animal. Very few reports are available on the effects of feeding bacterial and actinomycete biomass directly to prawns.

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

In the present study, lipid was found to be highest in feed F361 (14.07%) followed by F377 (9.03%). Higher lipid values could be due to bacterial biomass. Yongmanitchai and Ward (1989) have reported marine bacteria that produced EPA. Microorganisms contain a diverse range of fatty acid composition and are rich sources of useful unsaturated fatty acids like PUFA (Brown et al., 1996). Other studies have shown that some bacteria contain 20: 5n-3(Yazawa et al. 1988). Recommended lipid levels for commercial shrimp feeds range from 6% to 7.5% and a maximum level of 10% was suggested by Akiyama and Dominy (1989). Among the lipid compounds in the 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.

Various studies with *Penaeus japonicus* have demonstrated that dietary phospholipids enhance growth and survival of larvae (Teshima *et al.*, 1982; Kanazawa *et al.*, 1985; Camara *et al.*, 1987) and growth and stress resistance in post larval/ juvenile stages (Sandifer and Joseph, 1976; Levin and Sulkai, 1984; Kanazawa *et al.*, 1979a, b; Camara *et al.*, 1997; Kontara *et al.*, 1977). Watanabe *et al.* (1994, 1995) 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 Maifa *et al.* (1988).

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*. In the present study, significant negative correlation is obtained between the percentage survival and the carbohydrate concentration in the various diets.

The over all results showed superior growth performance by feed F361. The performance of feed 361 supporting good survival during post challenge with WSSV in both the experiments was noteworthy. Growth performance coupled with high post challenge survival in *P.monodon* was obtained when fed with *S.pulveraceus* incorporated feed F361. This strain

B361 (*S.pulveraceus*) was found to inhibit 85% of pathogenic vibrios in the earlier experiments *in vitro*.

There are so many evidences to suggest that bacterial cell walls have the ability to enhance the non specific resistance of fish and shrimp against bacterial infections. Shrimp fed with peptidoglycans incorporated diet have showed increased disease resistance (Booyratnapalin, 1990). The immunostimulatory effect of peptidoglycans was also reported by Matsau and Mazano(1993).

Feed, F361 supported remarkable survival (91.5%) of *P.monodon* post challenge with white spot virus on 7th day showing a protective effect. The whole picture showed a general trend in which F 361, F 377 and F 451 were capable of supporting greater survival compared to control feed apart from growth recorded earlier. Feed F 361 proved its dual capacity to support growth and survival remarkably well. On the other hand, F 301, which showed better protection by registering a post challenge survival of 70%, failed to exhibit comparable performance in growth enhancing properties registering poor growth, high food conversion ratio and other biogrowth parameters. Laramore (1992) reported an increase of 17% in total yield in post larvae *Pennaeus vannamei* exposed to formalin-killed suspension of *Vibrio*. Another similar experiment recorded 40% increase in yield of vaccine (*Vibrio*) treated animals (Laramore, 1992). Feed F361 holds promise as a valuable addition to the aquaculture industry by virtue of its growth promoting and immunostimulating properties. Certain components of the actinomycete biomass (B361) may be acting as immunostimulants or the antimicrobial compounds, which are produced intracellularly by the actinomycetes and may be conferring protection to the animal. A detailed investigation is necessary to elucidate the reason.

There are two types of bacterial components of interest in the present experiment that are believed to confer immunostimulant properties. They are the peptidoglycan of Gram-positive microorganisms and the antimicrobial compounds possibly present in the mycelia of actinomycetes.

Since prawns do not have specific memory as in fishes, their non-specific immune system requires the regular use or at least intermittent use of immunostimulants. The phenomenon of immunoprotection and growth enhancing property exhibited by actinomycete biomass incorporated feed reveal an interesting aspect, which warrants further study.

Chapter 7

ACTINOMYCETES AS PROBIOTIC FOR PENAEID PRAWNS CULTURE SYSTEM

7.1 INTRODUCTION

Penaeid shrimps are a very important commercial fishery and aquaculture species worldwide. Shrimp farming provides roughly 30% of the shrimp supplied to the world market. Disease out breaks are being increasingly recognized as a significant constraint on aquaculture production and trade (Verschuere et al., 2000). Bacterial diseases are considered as a major cause of mortality in shrimp larviculture (Wyban and Sweeney, 1991; Wilkenfeld, 1992).

Mortalities in larvae of shrimp, *Penaeus monodon* due to infection by luminous bacteria, *Vibrio harveyi* have been reported from a number of countries (Lightner 1983; Sunaryanto and Mariam, 1986; Tansutapanit and Ruangpan, 1987; Lightner, 1988; Lavilla – Pitozo *et al.*, 1990; Baticadog *et al.*, 1991, Karunasagar *et al.*, 1994). Karunasagar *et al.*, (1996) found that even after the addition of antibiotics to the larval tanks, *V. harveyi* survived in the tanks.

Conventional approaches such as the use of disinfectants and antimicrobial drugs, have had limited success in the prevention or cure of aquatic diseases (Subasinghe, 1997). The massive use of antimicrobials for disease control and growth promotion in animals increase the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance (Weston, 1996). Such resistance can be readily transferred to other strains following alterations either to the existing genomes or by transfer of genetic material between cells (Towner, 1995). Several alternative strategies for the use of antimicrobials in disease control have been proposed and applied successfully in aquaculture. One such method that is gaining acceptance within the industry is the use of probiotic

bacteria to the control of potential pathogens. Yasuda and Taga (1980) anticipated that bacteria would be useful as food and biological control agents of fish disease and activators of the rate of nutrient recirculation in aquaculture.

Intensive shrimp culture practice for higher shrimp yield cause stressful environmental changes, which can cause problems for shrimp (Lightner and Redman, 1998). The major problem due to intensification of the culture system is the occurrence of disease (Primavera, 1994). Therefore, the disease management strategies include the maintenance of good water quality. Water quality in pond is improved by enhancing the mineralization process and reducing the accumulation of organic loads (Shariff *et al.*, 2001).

King (1982) observed increased removal of ammonia using a biofilter containing chemo-autotrophic bacteria. Another study reported accelerated nitrification and rapid decomposition of organic solids using bacterial consortia in aquaculture ponds (Ehrlich *et al.*, 1988). Shariiff *et al.* (2001) have studied the effectiveness of commercial microbial products in tiger shrimp *P. monodon.*

Eli Metchnikoff's work at the beginning of 20th century is regarded as the first research conducted on probiotics (Fuller, 1992). He describes the term as "microbes ingested with the aim of promoting good health". This same definition was modified to "organisms and substances, which contribute to intestinal microbial balance" (Parker 1974). Fuller (1989) gave a precise definition of probiotics, which is still widely referred as "a live microbial feed supplement, which beneficially affect the host animals by improving its intestinal balance". This definition originally was applied to farm animals. In aquatic animals, not only the digestive tract is important but also the

surrounding water. The bacteria present in the aquatic environment influences composition of the gut microbiota and *vice versa.* Surrounding bacteria are continuously ingested either with the feed or with water intake. Aquatic larvae depend on their primary microbiota partly on the water in which they are reared (Cahill, 1990; Hagiwara *et al.*, 1994; Ringu and Birkbeck, 1999). Therefore, the property of the bacteria in the ambient water is of utmost importance (Skjeromo *et al.*, 1997). Therefore, the definition for 'probiotics' by Fuller is modified in aquaculture. Probiotic in aquaculture is defined as a live microbial feed adjunct which has a beneficial effect on the host by modifying the host associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host's response towards disease or by improving the quality of its ambient environment (Verschuere, 2000).

7.1.1 Selection of probionts

Selection of probiotic bacteria has usually been an empirical process based on limited scientific evidence (Gomez-Gill *et al.*, 2000). Selection steps have been defined for getting appropriate microorganisms. It is essential to understand the mechanisms of probiotic action and to define a selection criterion for potential probiotics . General selection criteria are mainly determined by biosafety considerations, methods of production and processing, the method of administration of probiotics and the location in the body where the microorganisms are expected to be active (Veld *et al.*, 1994). Methods to select probiotic bacteria for use in the larviculture of aquatic animals might include the following steps

1. Collection of background information
2. Acquisition of putative probiotics

3. Evaluation of the ability of putative probiotics to compete pathogenic strains
4. Assessment of the pathogenicity of the putative probiotics
5. Evaluation of the effect of the putative probiotics in larvae
6. Economic cost benefit analysis (Gomes - Gill *et al.*, 2000)

7.1.2 Mode of application of probiotics

1. Addition to the artificial diet
2. Addition to the culture water
3. Bathing
4. Via live feed

7.1.3 Mechanism of action of probiotics

The exact modes of action of probiotics are rarely completely elucidated. The possible modes of action according to Verschuere (2000) are as follows.

1. Production of inhibitory compounds

Microbial populations may release chemical substances that have bactericidal or bacteriostatic effect on other microbial populations, which can alter inter population relationship by influencing the out come of competition for chemicals or available energy (Fredrickson and Stephanopoulos, 1981; Lemos *et al.*,1991). The antibacterial effect is due to the following factors i.e. production of antibiotics (Westerdahl *et al.*, 1991), bacteriocins (Bruno and Montville, 1993; Vandenbergh, 1993), siderophores, lysozymes, proteases and / or hydrogen peroxides and the alteration of pH values by the production of organic acids (Sugita *et al.*, 1997).

Lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms (Vandenbergh 1993). The possible involvement of lactic acid bacteria as probiotics in aquaculture is discussed by Ringu and Gatesoupe (1998). The mode of inhibition of lactic acid bacteria towards other microorganisms is attributed to their adhesive and antagonistic properties. Jiravanichpaisal and Chuaychuwong *et al.* (1997) reported the use of *Lactobacillus* sp. as the probiotic bacteria in the giant tiger shrimp, *P.monodon.* They have designed to investigate an effective treatment of *Lactobacillus* sp.against vibriosis and white spot diseases in *P.monodon.* Nair *et al.*, (1985) showed that a large proportion of marine bacteria produced bacteriolytic enzymes against *V. parahaemolyticus.* Imada *et al.*, (1985) isolated and characterized *Alteromonas* sp. from near shore seawaters of Japan, producing an alkaline protease inhibitor called monastatin. This monastatin showed activity against *Aeromonas hydrophila* and *V. anguillarum* in an *in vitro* assay. Many studies have demonstrated the occurence of bacterial strains showing *in vitro* inhibition towards pathogens known in prawn culture systems. (Rengpipat *et al.*, 1998; Ruiz *et al.*, 1996; Sugita *et al.*,1996 and 1997; Tanasomwang *et al.*,1998). However, it has not been demonstrated that the production of such inhibitory compounds occurs under *in vivo* conditions

2. Competition for chemicals or available energy

Competition for chemicals or available energy may determine how different microbial populations coexist in the same ecosystem (Fredrickson and Stephanopoulos, 1981). The microbial ecosystem in aquaculture environments is generally dominated by heterotrophs, competing for organic substrate both as a carbon and energy source

(Verschuere, 2000). Rico-Mora *et al.*, (1998) selected a bacterial strain for its active growth in organic poor substrate and inoculated into a diatom culture, where it prevented the establishment of an introduced *V. alginolyticus*. Since the strain had no *in vitro* inhibitory effect on *V. alginolyticus*, it was suggested that the strain was able to compete *V. alginolyticus* due to its ability to utilize the exudates of the diatoms. Similar studies were conducted by Verschuere *et al.*, (1997) on several selected bacteria and suggested that the selected strain exhibited their protective action by competing with pathogens for chemicals and available energy.

3. Competition for iron.

Virtually all microorganisms require iron for growth. Siderophores are low molecular weight, ferric ion-specific chelating agents and can dissolve precipitated iron there by making it available for microbial growth (Neilands,1981). Harmless bacteria which can produce siderophores could be applied as probiotics to compete with pathogens from which is known for their pathogenicity due to siderophore production or competition for iron or to out compete all kinds of organisms requiring ferric iron from solution.

4. Competition for adhesion sites

A mechanism for preventing colonization by pathogens could be competition for adhesion sites on gut or other tissue surfaces. It is recognized that the ability to adhere to enteric mucous and wall surfaces is necessary for bacteria to become established in fish intestine (Olsson *et al.*,1992 and Westerdahl *et al.*,1991). As bacterial adhesion to tissue surface is of importance during the initial stages of pathogenic infection (Krovacek *et al.*,1987), competition for adhesion

receptors with pathogens might be the first probiotic effect (Montes and Pugh,1993). Adhesion can be non-specific, based on physico-chemical factors, or specific involving adhesion molecules on the surface of adherent bacteria and receptor molecules on the epithelial cells. The adhesion capacity and growth on or in intestinal or external mucus has been demonstrated *in vitro* for fish pathogens like *V.anguillarum* and *Aeromonas hydrophila* (Krovacek *et al.*, 1987 and Garcia *et al.*, 1997) and for candidate probiotics such as *Carnobacterium* K1 and several isolates inhibitory to *V.anguillarum*. Adhesion of probiotics to the gut wall or other tissues does not necessarily imply competition for adhesion sites as(only) mode of action for the probiotic effect. It is conceivable that bacteria are able to colonize for example the intestinal gut wall of a fish and exert their protective action against a pathogen by excreting inhibitory compounds.

5. Enhancement of the immunity response

Immunostimulants are chemical compounds that activate the immune system of animals and render them more resistance against infections by viruses, bacteria, fungi and parasites (Raa,1996).Shrimps have immune system that are less well developed than adult fish and are primarily dependent on non-specific immune responses for their resistance to infection (Sohell and Cerenius, 1998).There are a lot of reports that bacterial compounds act as immunostimulants in fish and shrimp (Sakai, 1999), but only specific cell components or non living cells have been applied in these studies.

6. Improvement of water quality

Gram positive *Bacillus* sp.are generally more efficient in converting organic matter back to CO_2, as compared to Gram negative bacteria, which would convert a greater percentage of organic carbon to bacterial biomass or slime (Stanier et al., 1963). By maintaining higher levels of these Gram positive bacteria farmers can minimize the build-up of dissolved and particulate organic carbon during the culture cycle, while promoting more stable phytoplankton blooms through the increased production of CO_2 (Scura, 1995). Nitrifiers are responsible for the oxidation of ammonia to nitrite and subsequently to nitrate. Seeding of biological filters with nitrifying bacteria is effective in reducing the activation time of new biofilter (Carmignani and Bennet, 1997) Perfettini and Beanchi(1990) used inocula consisting of frozen cells to accelerate the conditioning of new closed sea water culture systems and the time to establish nitrification was shortened by about 30%.

7.1.4 Screening and pre-selection of the putative probiotics

In vitro antagonism tests

The common way to screen the candidate probiotics is the performance of *in vitro* antagonism tests in which pathogens are exposed in liquid (Gildberg et al., 1995 ;Gildberg *et al.*,1997 and Gram *et al* 1999,48) or solid (Austin et al 1995;Nogami and Maeda,1992;Olsson et al.,1992;Sugitha et al.,1996) medium to the candidate probiotics or their extra cellular products. Furthermore, growth inhibition may not always be a consequence of the production of inhibitory substances like antibiotics, but growth inhibition caused by primary metabolites or changes in pH are also possible (Ten Brink and Minnekus, 1987).

7.1.5 Probiotic studies in the larval cultures of shrimp

Several bacteria have been used in the larval culture of shrimps. *Vibrio alginolyticus* has been employed as a probiotic in many Ecuadorian shrimp hatcheries since 1992 (Gibson *et al.*, 1998). The addition of probiotics is a common practice in commercial shrimp hatcheries in Mexico (Rico-Mora *et al.*, 1998). Griffith (1995) reported that, following the introduction of probiotics in Ecuador in 1992, hatchery down-time between batches was reduced from 7 days per month to 21 days annually, production volumes increased by 35%, and overall antimicrobial use decreased by 94%.

In Asia, there are reports of several species of bacteria being used in the larviculture of *Penaeus monodon* and *Penaeus penicillatus* with promising results. Maeda (1992) tested the addition of sterile soil extract plus diatoms to larval rearing tanks of *Penaeus monodon.* Garriques and Arevalo (1995) tested the pathogenicity of *Vibrio alginolyticus* isolated from the seawater next to their hatchery on *Litopenaeus vannamei* larvae. No mortalities were observed in a bath challenge pathogenicity test, whereas, 100% mortality was obtained

with *Vibrio parahaemolyticus* strain after 96 hours. In a trial, repeated three times on different dates, the probiotic strain was introduced into the larval rearing tanks and resulted in an average survival of 90.1% and a wet weight of 7.8 mg. Tanks that were treated with antimicrobials had 83.8 % survival and a weight of 6.0 mg and the controls averaged 74.5% and 7.1 mg. *Vibrio* counts on TCBS agar showed non-fermenting colonies (green colonies) in the probiotic tanks, with some appearing in the control and antimicrobial treatments. Garriques and Wyban (1993) observed that *Litopenaeus vannamei* larvae grown with probiotics were larger and more active, and no luminous bacteria were observed. Zherdinant *et al.* (1997) suggested that the guts of protozoea larvae were already colonized with bacteria from surrounding water, which might have interfered with probiotic experiment. The inoculation of a probiotic bacterial strain in a tank with nauplii stage V of *Litopenaeus vannamei* at a density of 10^3 cells ml $^{-1}$ prevented colonization by a pathogenic strain, even when challenged at a density of 10^7 cells ml^{-1}.

V. alginolyticus is a frequently tested bacterium with promising results. The work by Austin *et al.* (1995) and by Garriques and Arevalo (1995) suggested that *V. alginolyticus* might have characteristics capable of conferring some degree of protection against disease. Gatesoupe (1990) also detected *V. alginolyticus* in healthy rotifer and he established a positive correlation between the survival of turbot larvae and the proportion of *V. alginolyticus* in the rearing environment.

Appropriate probiotic applications were shown to improve intestinal microbial balance, thus leading to improved food absorption (Parker, 1974; Fuller, 1989) and reduced pathogenic problems in

gastro-intestinal tract (Lloyd *et al.*, 1977; Snoeyenbos *et al.*, 1978; Pivnick *et al.*, 1981; Cole and Fuller, 1984).

7.2 MATERIALS AND METHODS

7.2.1 Actinomycete Strains

Actinomycete cultures isolated and maintained in the laboratory were used for the study. Twenty actinomycetes were screened for utilization as pond probiotics.

7.2.2 Testing Hydrolytic enzyme production

The ability of actinomycetes to produce amylase, lipase and protease were tested.

Amylase

Amylase production was tested using the agar medium of Harrigan and McCance (1972) supplemented with starch as the substrate.

Composition of the medium

Peptone	:	10g
Beef extract	:	10g
Starch (Soluble)	:	5g
Agar	:	20g
Seawater	:	1L
pH	:	7.2

Starch agar plates were prepared, inoculated the test isolates by spot inoculation and incubated at room temperature (28 ± 2 ^{o}C) for 5 – 7 days. The production of amylase was tested by flooding the plates with Gram's iodine solution (Potassium iodide, 2g; Iodine, 1g and

Distilled water, 300ml). Unhydrolysed starch formed a blue colour and amylolytic colonies developed a clear zone around them.

Lipase

Production of lipase was tested on agar medium of Harrigan and McCance (1972) supplemented with tributyrin.

Composition of medium

Peptone	:	10 g
Calcium Chloride	:	0.1 g
Tributyrin	:	10 ml
Agar	:	20 g
Seawater	:	1 L
pH	:	7.2

The prepared medium was autoclaved and poured into sterilized plates. Isolates were spot inoculated and the plates were incubated at room temperature (28 ± 2 0C) for 5 – 7 days. Lipase production was detected by the appearance of halo zone around the colony.

Gelatinase

Frazier's gelatin agar medium (modified medium of Harrigan and McCance (1972) was used for the detection of gelatinase production

Composition of the medium

Peptone	:	10 g
Beef extract	:	10 g
Gelatin	:	10 g
Agar	:	20 g
Seawater	:	1 L
pH	:	7.2

Test isolates were inoculated by spot inoculation on the prepared gelatin agar plates and incubated at room temperature (28±2°C) for 5 – 7 days. The plates were flooded with Mercuric chloride (Mercuric Chloride 15g, Conc. HCl, 20ml, Distilled water 80 ml) and the colonies with the halo zones were noted as gelatinolytic bacteria.

7.2.3 Testing antagonistic property against pathogenic vibrios

Antagonistic property against pathogenic vibrios isolated from diseased prawns was tested. The selected four actinomycete strains were cultured in Marine actinomycete growth (MAG) medium. After 7 days incubation (150 rpm in a rotary shaker) at room temperature 28±2°C.The cells were separated by centrifugation at 10000 rpm in a refrigerated centrifuge (Remi, C-30, Bombay). The inhibitory property of the culture supernatants were tested by agar diffusion assay using pathogenic vibrios isolated from prawns.

7.2.4 Testing antagonistic activity against Natural flora

Natural flora isolated from penaeid prawns culture ponds and maintained in the microbiology laboratory of School of Marine Sciences was used for the study. Six hundred cultures were tested for its sensitivity to the selected actinomycetes. This included *Aeromonas, Vibrio, Pseudomonas, Bacillus, Flavobacterium, Flexibacter, Moraxella, Alcaligenes, Streptococcus, Staphylococcus* etc. Kirby-bauer disc diffusion assay was adopted for the study. Based on the above three screening, four actinomycete strains were selected for the study. The selected strains, B301, B361, B451 and L5 were inoculated into the marine actinomycete growth medium and incubated for one week at room temperature, 28±2°C on a rotary shaker at 150rpm. The culture broth was centrifuged at 10000 rpm for 15 minutes in a refrigerated

centrifuge and the supernatant was used for testing antagonistic activity. Sterile filter paper discs impregnated with the culture filtrates (35µl) were placed over nutrient agar plates already swabbed with the natural flora. These plates were incubated for 24 hours at room temperature (28±2°C), the zone of inhibition was noted and the diameter of the halo was measured.

7.2.5 Screening for pathogenicity

7.2.5.1 Testing hemolytic property

Based on the results obtained in the above screening procedures, strains were selected and they were tested for hemolytic property on blood agar plates. Blood agar base medium (peptone, 0.5g; Beef extract, 0.3g;seawter, 100ml;pH, 7.2) was prepared and autoclaved at 121°C for 15 minutes and the medium was supplemented with 10% human blood just before plating. Surface dried plates were spot inoculated with the test strains. The plates were then incubated at room temperature 28 ±2°C for 48 hours and the clearing zone was noted.

7.2.5.2 Testing pathogenicity to penaeid prawns

Strains B301, B361, B451 and L5 were screened for pathogenicity to *Penaeus monodon*, if any.

i) Preparation of experimental animals and experimental design

Experimental animals

Healthy black tiger shrimp (*Penaeus monodon*) juveniles of the size range 0.32 to 0.45 g were used for the study.

ii) Rearing facility

Fibre reinforced rectangular plastic (FRP) tanks of 30L capacity were used for the study (Fig 6.2). Water quality was monitored daily

and was maintained as per Table (6.2). 50% of water was exchanged on alternate days for all the experimental tanks. Aeration was provided from a 1HP compressor. Physiochemical parameters like salinity, ammonia –nitrogen, nitrate – nitrogen, nitrite-nitrogen and dissolved oxygen of the rearing water were estimated daily by following standard procedures (APHA, 1995).

iii) Design of Experiment

The *P. monodon* juveniles were acclimatized for one week in laboratory conditions. The larvae were then stocked in 30L rectangular fiberglass tank containing 20L seawater with 25 individuals per tank. The experiment was conducted in triplicate. Shrimps were fed twice daily with Higashimaru feed. Similarly, one control tank in triplicate was also maintained

7.2.5.4 Testing pathogenicity of Actinomycetes, if any

iv) Challenge through rearing water

5 ml of 48 hr culture broth of the selected actinomycetes (B301, B361, B451 and L5) in Marine actinomycete growth (MAG) medium were introduced into the culture tanks containing 20L seawater. These actinomycetes were introduced in to the tanks at a frequency of 5 days for a period of 30 days. Simultaneously a pathogenic luminescent bacteria grown in nutrient agar (peptone, 0.5g; beef extract, 0.3g; agar, 2g; seawater, 100ml and pH 7.2) and harvested using sterile seawater (30ppt) was also introduced into a set of tanks and a control set was also maintained. All the treatments were uniformly fed on a commercially available feed (Higashimaru, Kochi). Survival of the prawns was noted after 30 days.

V) Challenge via diet

Since the expression of pathogenicity could not be observed even with luminescent bacteria treated prawns (positive control), all the treatment groups except control were challenged with the corresponding actinomycetes and luminescent bacteria via diet (pellet feeds were coated with live microorganisms using a binder and immediately fed to prawns). Post challenge survival was noted for a period of seven days.

7.2.6 Testing the efficacy of selected actinomycetes as probiotics.

Based on the various screening assays (Enzyme production, production of antibiotics, inhibition to natural flora, non haemolytic property and non pathogenicity) two strains were selected and used for efficacy test.

7.2.6.1 Animals used

Penaeus monodon juveniles of the size range 0.43 to 0.56 g were used for the study.

7.2.6.2 Design of experiment

P.monodon juveniles were acclimatized for one week in laboratory conditions. The experiment was conducted in two ways.

Treatment I

The *P.monodon* juveniles after acclimatization were stocked in 30L rectangular flbreglass tank containing 20L seawater (15ppt) with 25 individuals per tank.

Treatment II

The P.*monodon* juveniles were stocked in 30L rectangular fibreglass tanks with a sediment bed of 5cm height and containing 15L sea water (15ppt).

The experiment was done in triplicate. Shrimps were fed twice daily with commercial shrimp feeds (Higashimaru). 50% water was exchanged once in 5 days. However the left over feed and faecal matter was not removed. Aeration was provided from a 1HP compressor.

7.2.6.3 Analysis of water quality.

a. physico chemical parameters.

Salinity, pH, ammonia, nitrite, nitrate, phosphate, alkalinity and hardness of the rearing water was estimated as per APHA (1995). Analyses of the parameters were made once in 5 days and that too just before the water exchange was made.

b. Microbiological quality

Total Plate Count and vibrio count was taken once in 5 days i.e, just before the water exchange in the tank.

Total plate count

Water and sediment samples were collected from representative tanks of each treatment group. Serial dilutions were made and plating (pour plate) was done using Zobell's 2216 e agar media (peptone 0.5g, yeast extract 0.1g,Ferric phosphate 0.002g, agar 2g, seawater 100ml, and pH 7). The plates were incubated at room temperature (28±2°C) for 3-5 days. The colonies were counted and the population was calculated as colony forming units cfu/ml or /g for water and sediment respectively.

Vibrio count

Both water and sediment was analysed for vibrio population. After making serial dilution of the samples, spread plate inoculation was done on Thiosulphate Citrate Bile Salt sucrose(TCBS) agar media. Incubation of the plates were done at room temperature (28±2°C) for a period of 3-5 days. Sucrose fermenting (yellow) and non sucrose fermenting (green) colonies were counted and the total population of

vibrios were calculated and expressed as colony forming units (cfu) per ml water or per gm dry wt of the sediment.

7.3 RESULTS

7.3.1 Hydrolytic enzyme production by the various actinomycetes

Table 7.1 Hydrolytic enzyme production by the various actinomycetes

Sl No	Actinomycetes Culture No	Amylase	Lipase	Protease
1.	L5	+++	+++	++
2.	L6	+	++	-
3.	L18	+++	-	-
4.	L27	-	+++	++
5.	L28	-	+++	++
6.	L46	-	+	-
7.	L85	-	+++	+++
8.	L87	+	++	+++
9.	L90	+++	++	+++
10.	L100	++	+++	+++
11.	L107	-	++	-
12.	L125	-	++	-
13.	L130	-	++	-
14.	BA5	-	+++	-
15.	B30	-	+++	-
16.	B272	-	+++	+++
17.	B301	+	+++	+++
18.	B361	++	+++	+++
19.	B377	+	+++	+++
20.	B451	++	++	++

\- Negative

\+ Halo zone up to 1 cm

++ Halo zone up to 2 cm

+++ Halo zone more than 2 cm

Twenty actinomycete isolates were tested for their hydrolytic property. The hydrolysis of complex substances like proteins, lipids

and carbohydrates by various strains can be considered as an indication of their efficiency as probiotics. Almost all the isolates were found to produce lipase enzyme showing halo zone of more than 2cm diameter. Protease was produced by 60% of the tested isolates, whereas amylase production was restricted to 50% of them. (Table 7.1) Culture No. L5, L90, L100, B301, B361, B377 and B451 were capable of producing all the three enzymes.

7.3.2 Inhibition of pathogenic vibrios (non luminescent and luminescent) by actinomycetes

Based on hydrolytic potential and non-haemolytic nature four strains were selected for studying their potential as a probiotic for aquaculture applications

Culture no. B361 and B451 inhibited pathogenic viobrios significantly (85% and 65% respectively. Whereas B301 and L5 exhibited less than 50% inhibition (33.5 And 40% respectively) Culture No. B361 (*Streptomyces pulveraceus*) and B451 (*S. fradiae*) were found to exhibit broad spectrum of inhibition against luminescent bacteria. Culture no. L5 could inhibit only three of the 17 LB tested. However, none was inhibited by B301 (Fig 7.1 and Table 7.2).

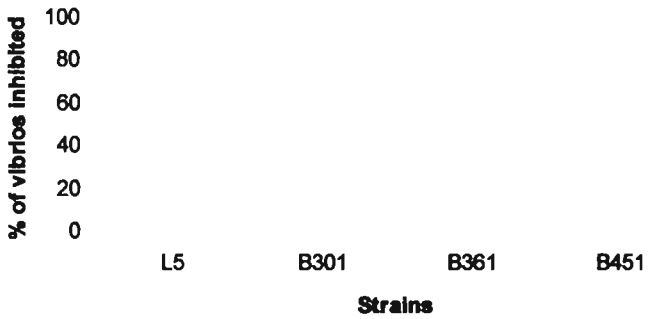


Fig7.1 Inhibition of Pathogenic vibrios by Actinomycetes

Table 7.2 Inhibitory activities of actinomycetes against luminescent bacteria isolated from penaeid hatchery

Luminescent bacteria	Actinomycetes			
Pathogen	L5	301	361	451
LB1	+	-	++	++
LB2	-	-	++	++
LB3	-	-	-	+
LB4	-	-	++	-
LB5	-	-	++	++
LB6	+	-	++	++
LB7	-	-	++	++
LB8	+	-	++	++
LB9	-	-	++	++
LB10	-	-	++	++
LB11	-	-	++	++
LB12	-	-	-	++
LB13	-	-	++	-
LB14	-	-	++	++
LB15	-	-	+	++
LB16	-	-	++	++
LB17	-	-	+	++

\+ Halo zone up to 1 cm

++ Halo zone up to 2 cm

7.3.3 Inhibition of Natural Flora

The inhibitory activity of selected actinomycetes against natural flora isolated from penaeid prawn culture systems is given in table 7.3. Generally, inhibition to the natural flora by the various actinomycetes was found to be less. Maximum inhibition was exhibited by B361 followed by B 451 and B 301. *Bacillus* was not inhibited by B301, and B451. However, 20% of them were inhibited by B361. *Pseudomonas* was inhibited by all the four actinomycetes. However, the percentage being inhibited was very less and was in the range 2 – 22%. The

inhibition of *Aeromonas* was found to be in the range of 8.7% – 37.5 % by the various actinomycetes. Vibrios being inhibited varied from 2.7 to 44.4 %. Coryneforms were found to be inhibited considerably.

Table 7.3 Inhibitory activity of selected actinomycetes against natural flora isolated from Penaeid prawn culture system

Genera	Total No tested	L5	B301	B361	B451
Aeromonas	149	23	20.1	37.5	15.4
Alcaligenes	3	0	0	100	0
Bacillus	15	0	0	20	0
Coryneforms	10	40	40	40	40
Enterobacteriaceae	54	0	7.4	35.1	0
Flavobacterium	47	8.5	8.5	8.5	8.5
Flexibacter	4	75	75	75	75
Micrococcus	2	0	0	0	0
Moraxella	41	0	0	37.7	7.3
Pseudomonas	50	4	22	16	2
Staphylococcus	69	12	14.4	21.7	14.4
Streptococcus	26	23	11.5	19.2	0
Vibrio	36	35	16.6	44.4	11.1
Total	600	8.67	13.17	29.83	9.17

Almost all the genera/ groups except micrococci were inhibited by B361. 44.4% of the vibrios (36 No) were sensitive to this actinomycete. *Aeromonas* and Enterobecteriaceae were inhibited at 37.5 % and 35.1 % level.

7.3.4 Haemolytic property on blood agar.

All the 20 isolates were tested for the haemolysis on blood agar. Except L90, L100, B30, B272 & B377, all the isolates were non haemolytic (Table7.4)

Table 7.4 Haemolytic property of the actinomycetes on blood agar

Serial No	Actinomycetes culture No	Haemolysis
1.	L5	-
2.	L6	-
3.	L18	-
4.	L27	-
5.	L28	-
6.	L46	-
7.	L85	-
8.	L87	-
9.	L90	+
10.	L100	+
11.	L107	-
12.	L125	-
13.	L130	-
14.	BA5	-
15.	B30	+
16.	B272	+
17.	B301	-
18.	B361	-
19.	B377	+
20.	B451	-

7.3.5 Pathogenicity test *in vivo.*

The pathogenicity of the selected actinomycetes to *Penaeus monodon* were tested in a bioassay system using luminescent bacteria (LB) as a positive control. All the four actinomycetes (L5, B301, B361 and B451) were found to be nonpathogenic to the *Penaeus monodon* post larvae in a bioassay system.

Challenge via rearing water

No significant mortality of prawns could be observed by challenge via water. All the treatments showed above 90% survival and the luminescent bacteria treated tanks gave an average of 83% survival (Fig 7.2).

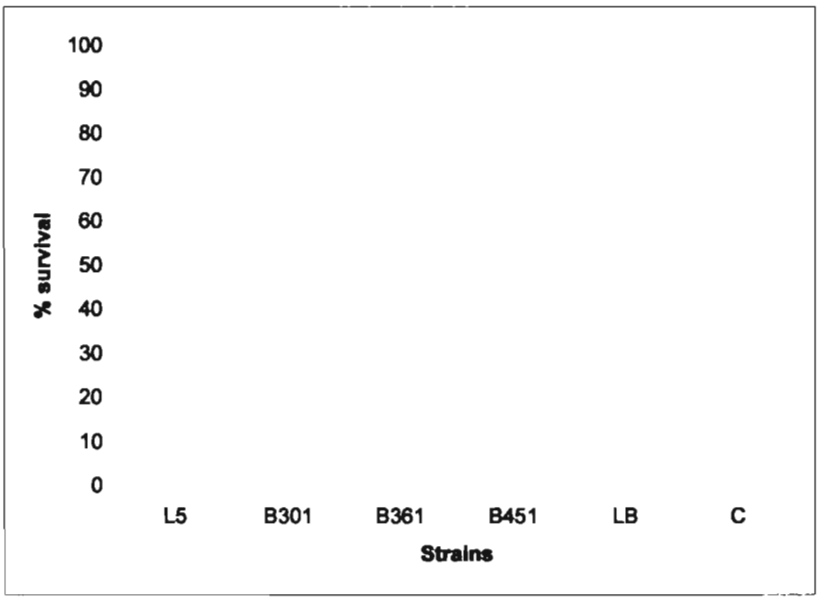
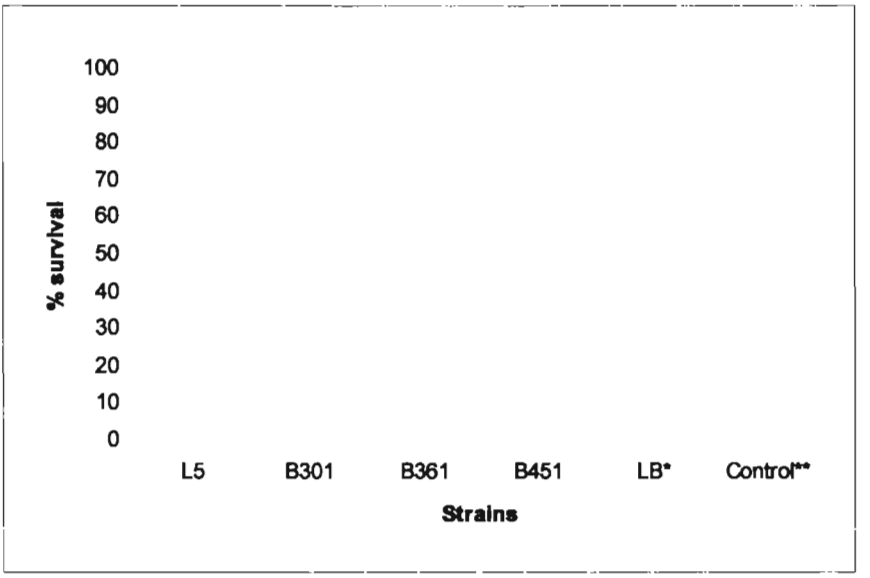


Fig 7.2 Percentage survival of penaeus monodon juveniles after 30 days exposure to the actinomycete strains and luminiscent bacteria (LB)

Challenge via diet

Since even the pathogenic LB did not cause mortality, the challenge was done via diet. Significant mortality (24. 5%) could be observed for prawns in the tanks challenged with luminescent bacteria and all other treatments showed good survival (above 87%). Maximum survival could be obtained with B301 (*S. griseoflavus*) followed by B361 (*S. pulveraceus*) and B451 (*S. fradiae*). The results confirmed the non-pathogenicity of the selected actinomycetes in the current assay conditions. Death of prawns due to LB was confirmed by streaking nutrient agar plates with the flesh of moribund prawns and observing the appearance of the luminescent Bacterial colonies after 24 hrs (Fig 7.3).



V – vibrio(Luminiscent)

Fig 7.3 Percentage survival of Penaeus monodon juveniles on challnge with the various actinomycetes and luminiscent bacteria (orally via feed)

7.3.6 Testing the Efficacy of the selected strains as a probiotic in a bioassay system.

Hydrolytic (degradative) potential of the actinomycetes was found to be better in tanks with a sediment bed, which was evidenced by the increase in ammonia level in the water. B451 (*S. fradiae*) was found to be better in its performance in terms of water quality parameters of the rearing tanks and the control of vibrios in the system.

7.3.6.1 Treatment 1. (Rearing tanks with water.)

Water quality

a. Physico chemical factors

Ammonia level was found to increase upto 10th day after which it reduced in various tanks including control. Considerable increase in

nitrate could be observed on 15th day onwards and became lesser after 25 days in the tanks with the actinomycetes B301 (*S.griseoflavus*) and B451 (*S.fradiae*). However, in the control tank it continued to increase. Nitrate and phosphate was considerably high in tank inoculated with B301 (*S.griseoflavus*) compared to B451 (*S. fradiae*) and control. In spite of the variations in other parameters a stable pH within the range, 7-8 could be observed in various tanks. Alkalinity and hardness also remained stable without much alteration in the 30 day rearing period.

b Microbiological quality

Uniformly the total plate count was found to be lesser on the 5th day compared to the initial value and the same trend continued till 20th day after which an increase in population could be encountered upto the 30th day in both the test groups (B301 and B 451). However, a sharp decrease in the count could be seen in the case of control.

Even though the vibrio population remained more or less the same upto the 15th day of rearing, slight increase was observed for control and for B301 on the 20th day onwards. However, for B451 even though there was initial increase in the vibrio count (on 5th day), the number reduced considerably afterwards and remained at very low level throughout the rearing period.

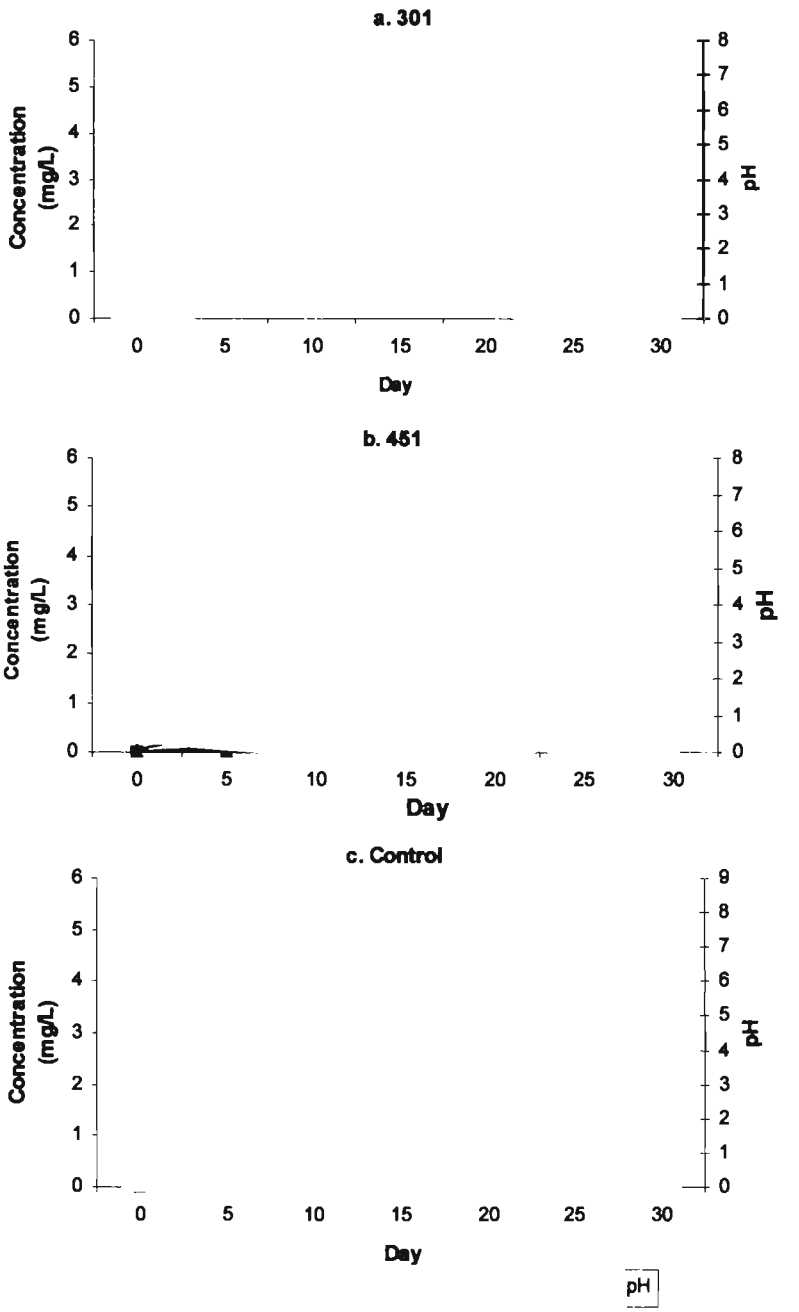


Fig.7.4 a - c water quality parameters in the penaied prawn bioassay system in the presence of actinomycetes(B301 and B451) as probiotics(Treatment I)

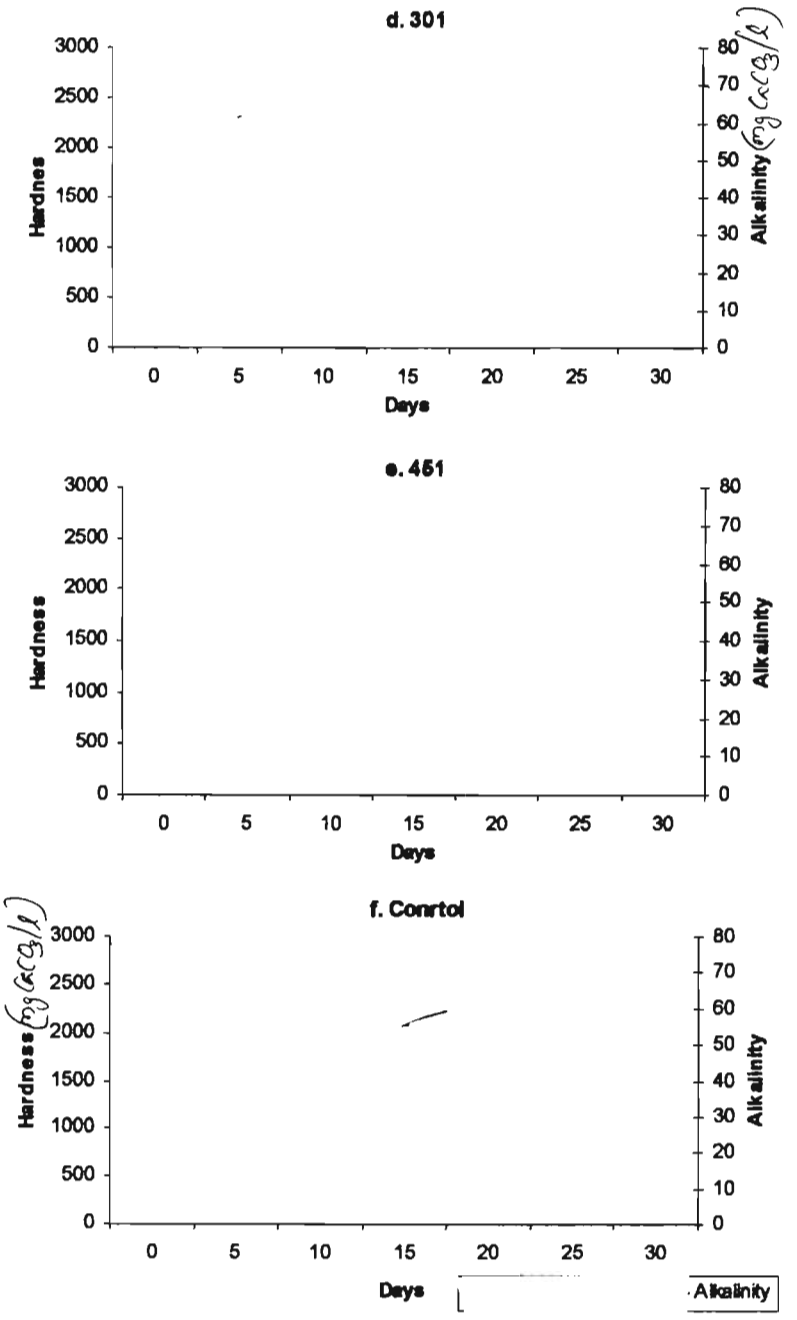


Fig. 7.4 d - f water quality parameters in the penaied prawn bioassay system in the presence of actinomycetes (B301 and B451) as probiotics (Treatment I)

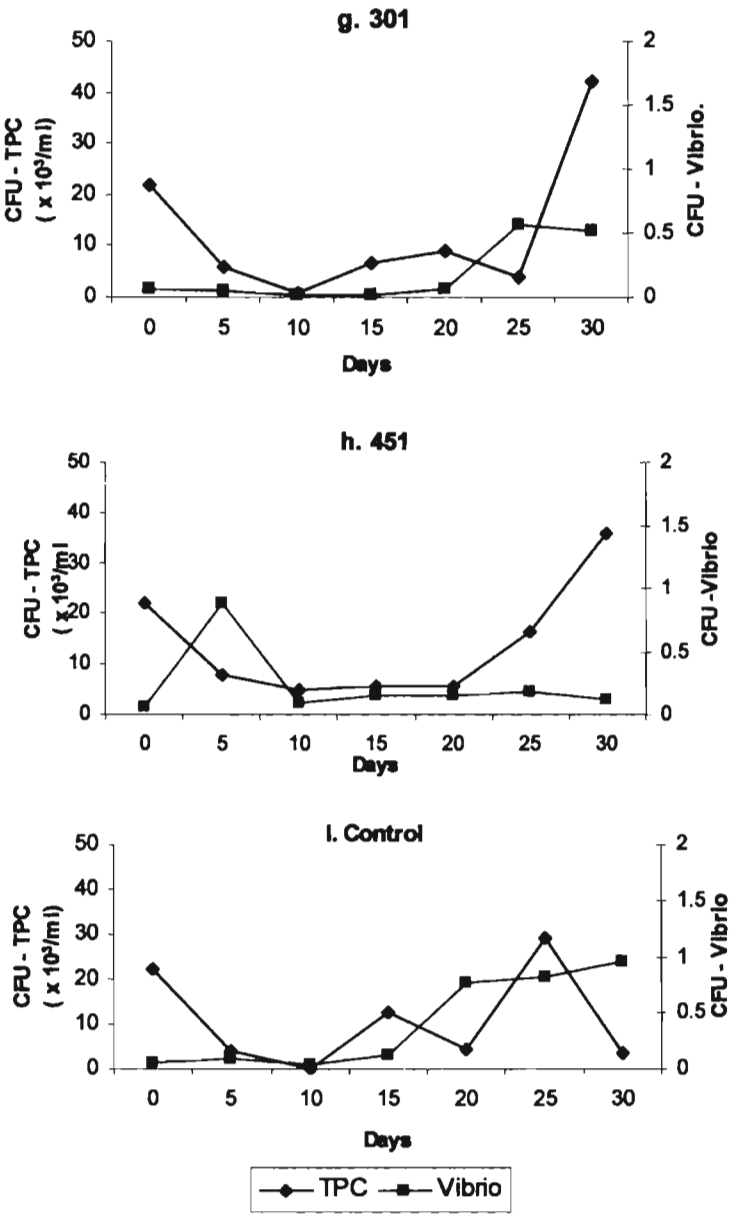


Fig 7.4. g -i Total plate count(TPC) and vibrio count in the rearing water of penaeid prawn bioassay system in the presence of actinomycetes (B301 and B 451) as probiotics (Treatment I)

7.3.6.2 Treatment 2

Water quality

a. Physico-chemical factors.

Considerable increase in ammonia nitrogen during the first 10-15 days could be observed in both the test groups compared to the control and afterwards the level remained more or less same in various test groups and control.

A decrease in ammonia was accompanied by the increase in nitrite levels during the 10^{th} –12^{th} day of rearing. Uniformly nitrate and phosphate levels were at negligible level in the various tanks. A slow and steady increase in the pH could be observed in both test groups and control. However, the range remained within desirable limit. Alkalinity and hardness also remained the same without much variation.

b Microbiological quality

A drastic reduction in total plate count (TPC) could be observed in the rearing water as well as sediment on the 5^{th} day and the TPC remained very low in the water of both the test groups B301 and B451. The same trend was observed for the microbial population in the sediment. Vibrio population remained more or less same through out the culture period. However, in the control group a considerable increase could be observed after 20^{th} day, in both water and sediment. In both the test groups even though there was an increase on 25^{th} day, the vibrio count decreased considerably on the 30^{th} day.

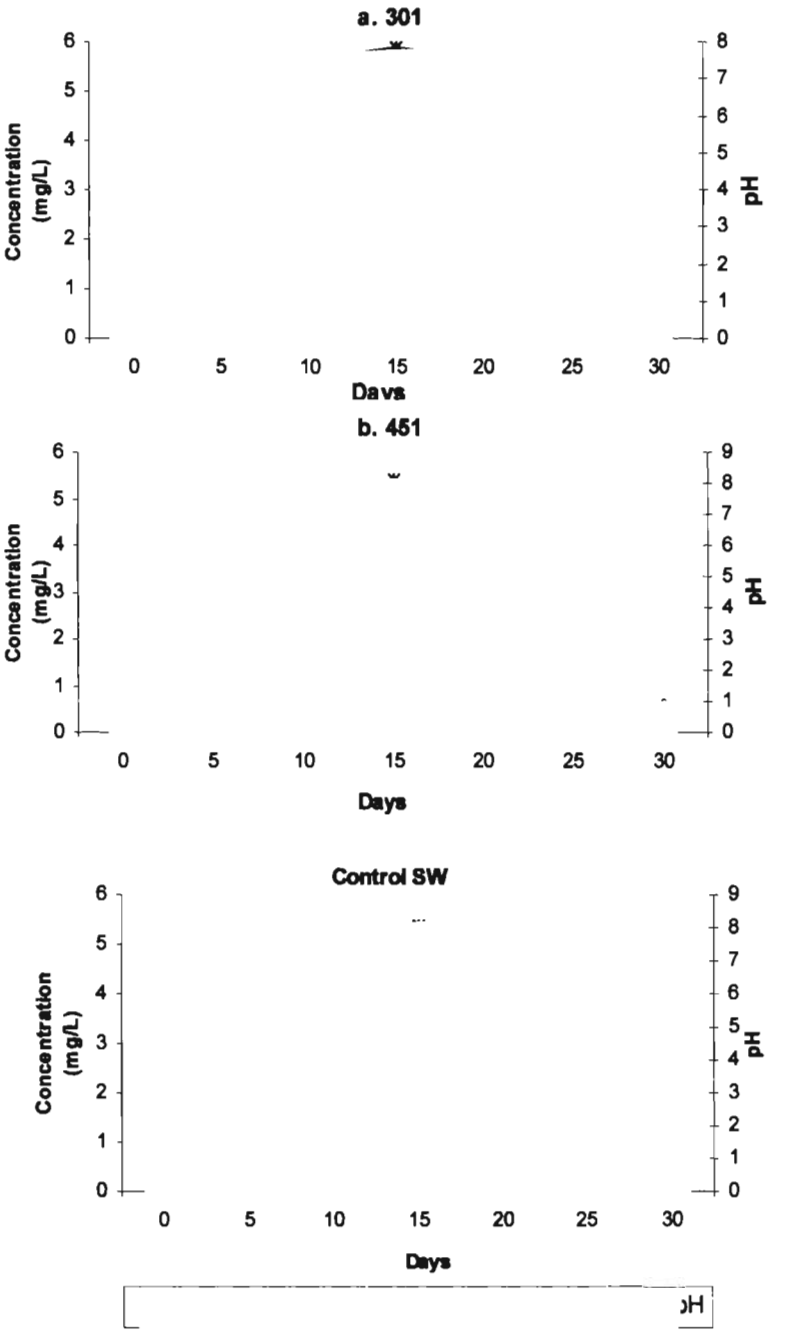


Fig.7.5 a - c water quality parameters in the penaied prawn bioassay system (with sediment bed) in the presence of actinomycetes as probiotics(Treatment II)

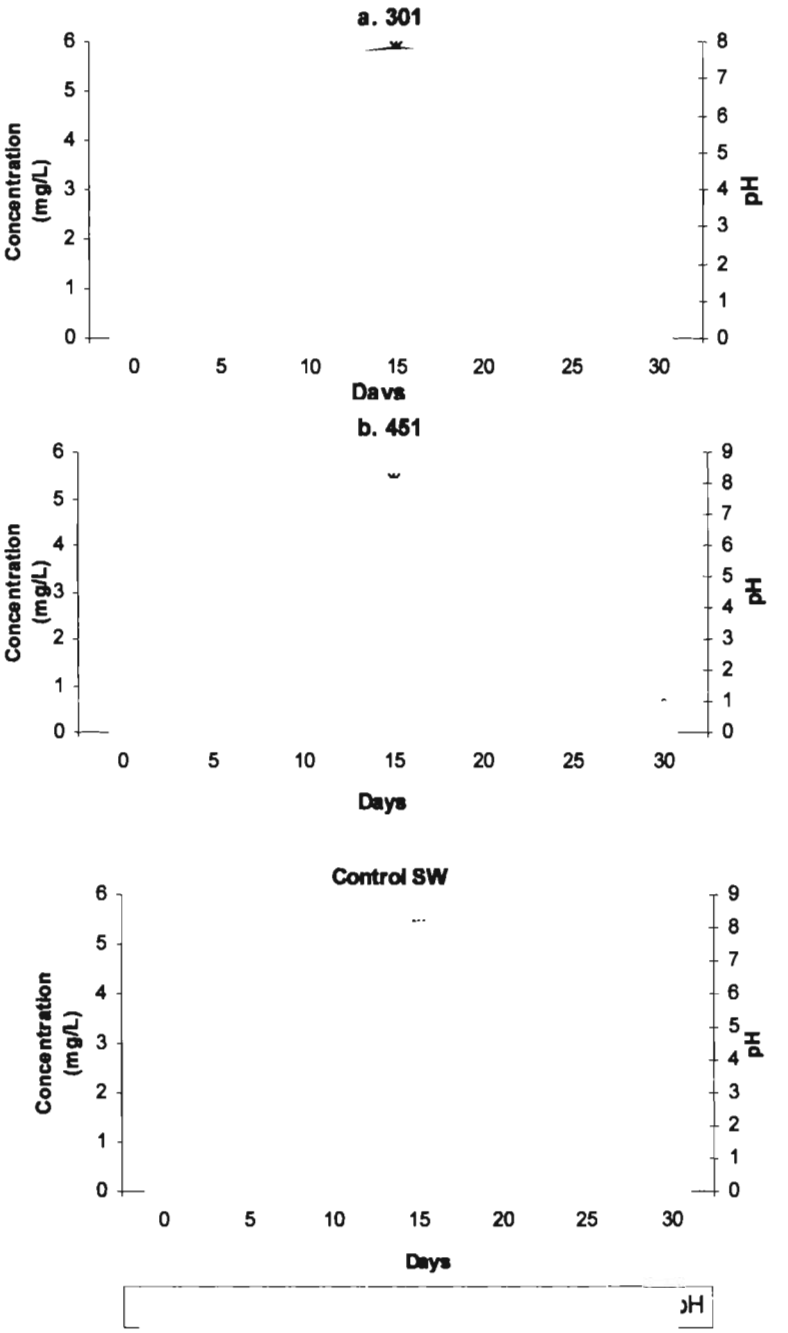


Fig.7.5 a - c water quality parameters in the penaied prawn bioassay system (with sediment bed) in the presence of actinomycetes as probiotics(Treatment II)

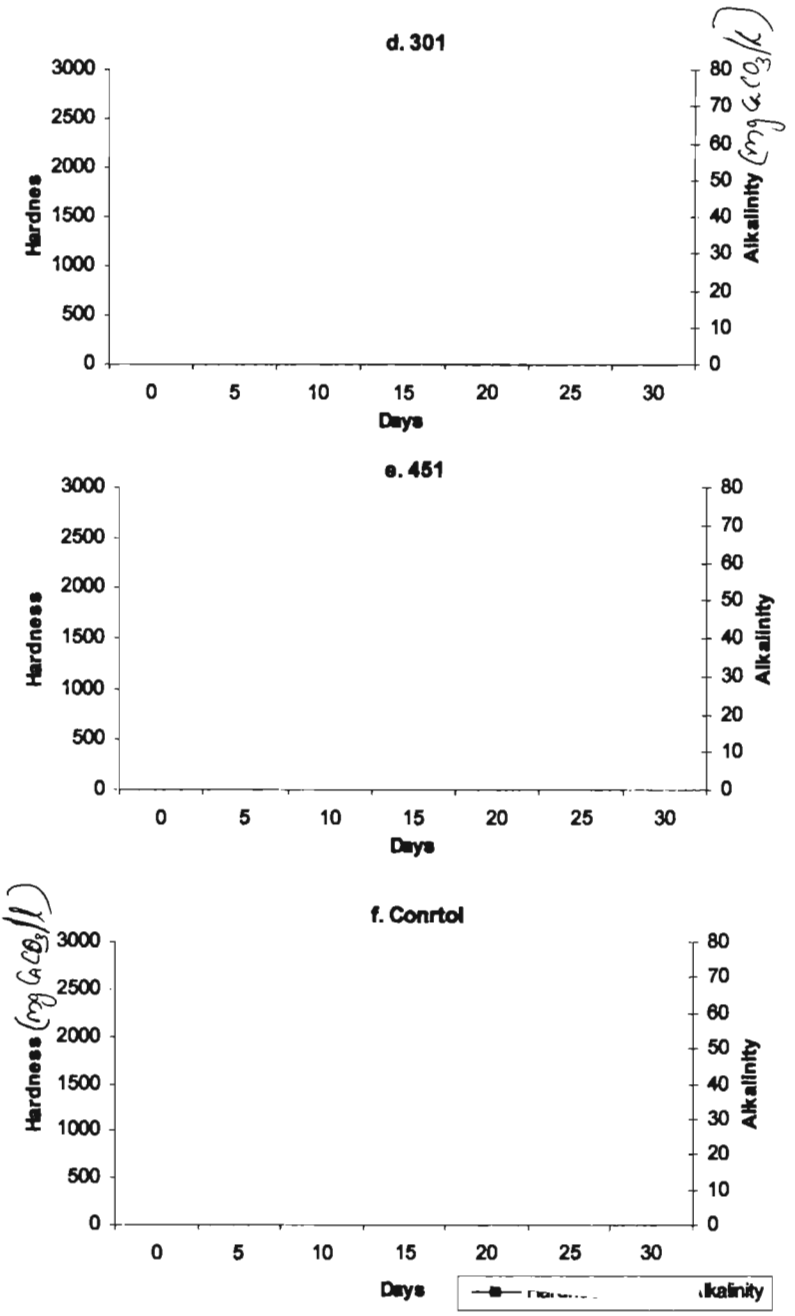


Fig.7.5 d - f water quality parameters in the penaied prawn bioassay system in the presence of actinomycetes as probiotics (Treatment II)

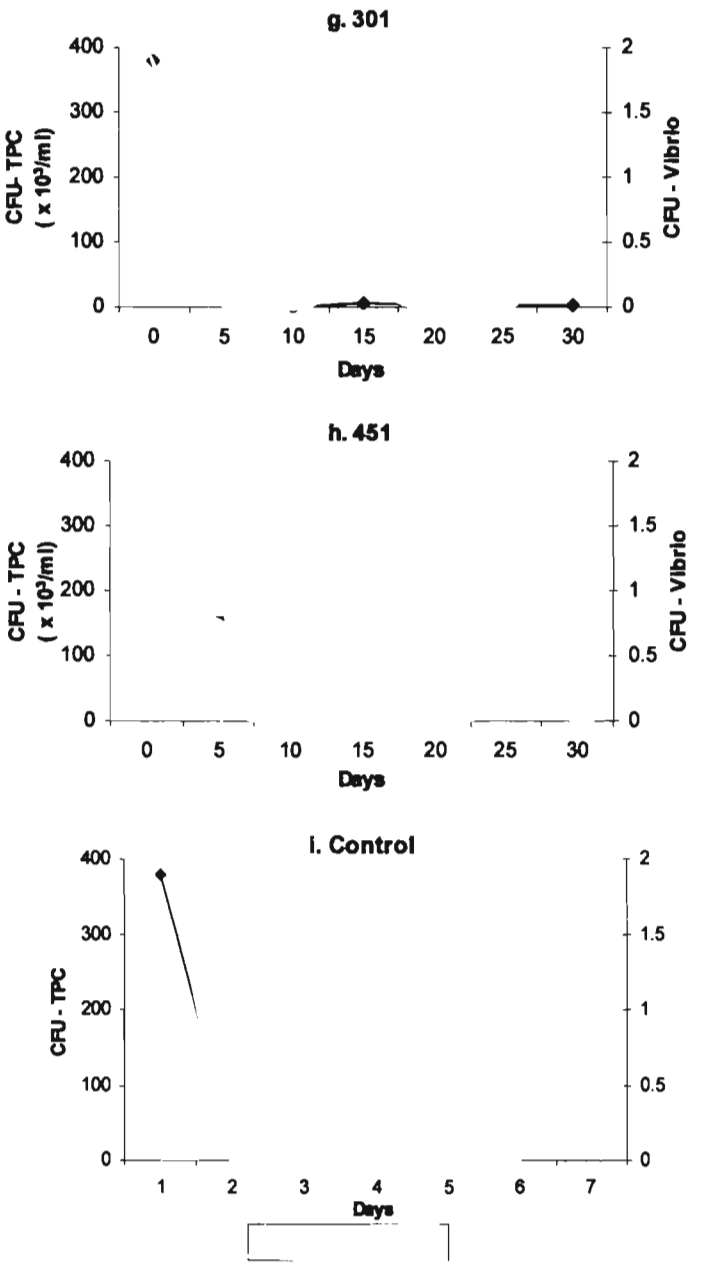


Fig 7.5.g -i Total plate count (TPC) and vibrio count in the rearing water of penaeid prawn bioassay system in the presence of actinomycetes as probiotics (Treatment II)

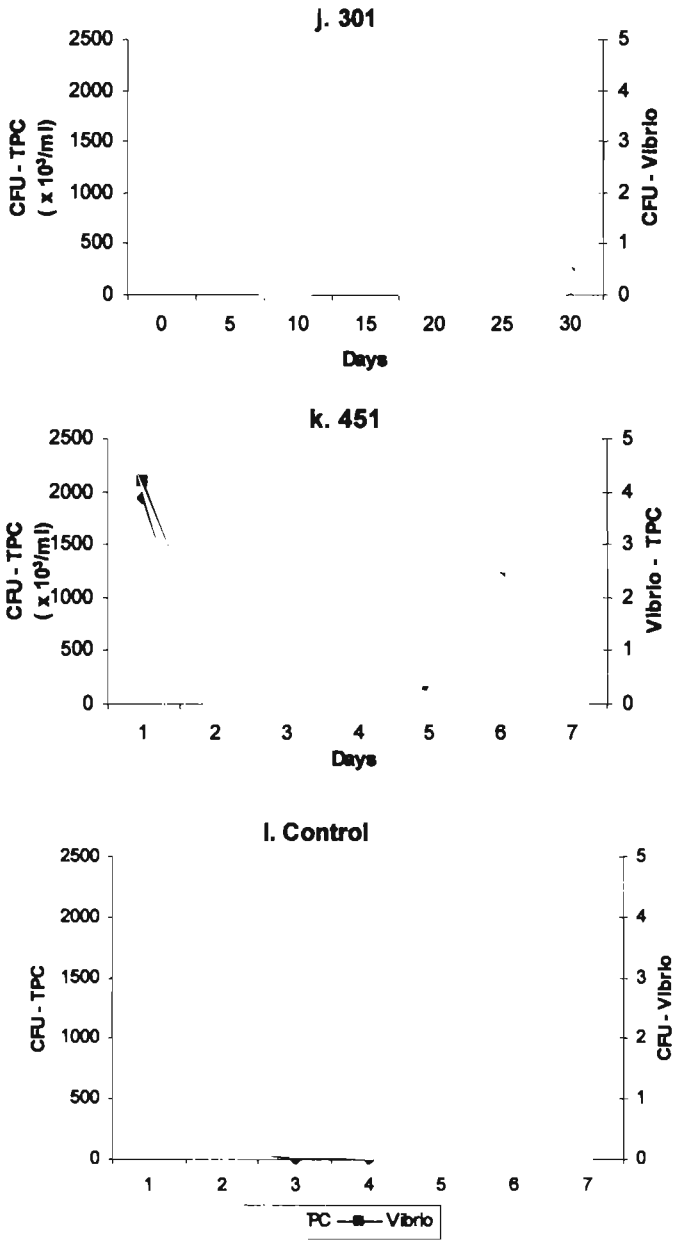


Fig 7.5. j -l Total plate count (TPC) and vibrio count in the sediment of penaeid prawn bioassay system in the presence of actinomycetes as probiotics (Treatment II)

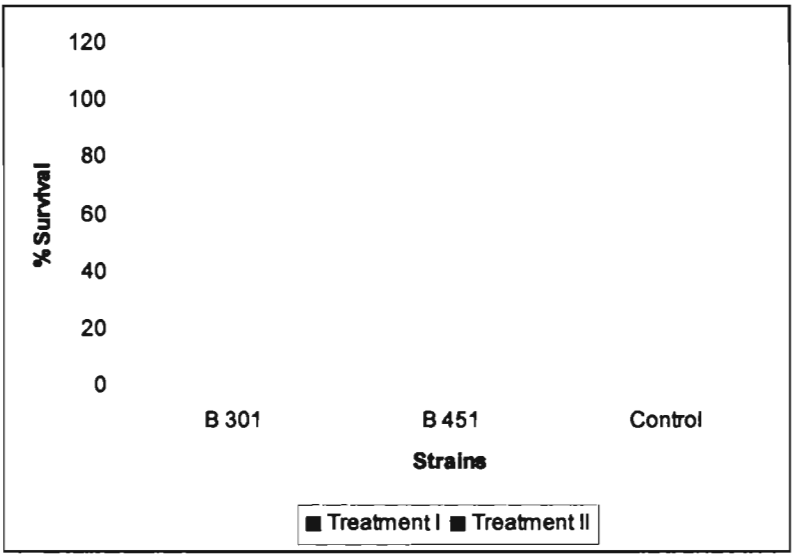


Fig. 7.6 Percentage survival of P.monodon post larvae treated with probiotic actinomycetes after challenging with Luminescent vibrio

7.4 DISCUSSION

The application of probiotics in aquaculture is relatively recent and the interest in such environment friendly treatments is increasing rapidly. Aquatic animals are quite different from the land animals for which the probiotic concept was developed and the term 'probiotic' inevitably referred to gram positive bacteria associated with the genus *Lactobacillus*: Much more than terrestrial animals, aquatic farmed animals are surrounded by an environment that supports their pathogen independently of the host animals in aquaculture. The fact that both environmental conditions and chance influence of microbial communities opens opportunities for the concept of probiotics as biological conditioning and control agents. Instead of allowing spontaneous primary colonization of the rearing water by bacteria accidentally present, the water could be pre-emptively colonized by the

addition of probiotic bacteria/ if the probiotics are indigenous, a single addition may suffice to achieve colonization and persistence in the host/ or its ambient environment. Otherwise, the probiotic will have to be supplied on a regular basis to achieve and maintain artificial dominance. The present work was carried out to develop an indigenous probiotic for application in shrimp farms.

In the current study, actinomycetes with degradative capacity were selected and they were screened for pathogenicity by testing the haemolysis on blood agar and ability to cause infection in penaeid prawns in a bioassay system. Most of the actinomycetes were found to be potential producers of hydrolytic enzymes. Grey and Williams (1971) reported that actinomycetes could efficiently degrade high molecular weight polymers. This criterion is even been exploited in the selective isolation of actinomycetes where chitin, paraffin, starch, casein etc are used as substrates in the isolation media. In aquaculture the degradation of excess feed, faecal matter and other organic substances in the system is very much important for the maintenance of water quality and thereby the health of the ecosystem. Smitha (2002) has reported the dominance of actinomycetes in penaeid prawn culture system during the end of the culture period. The animals were healthy giving good production and the culture environment was found to be healthy.

Actinomycetes are potential source of antibiotics and of the entirely known antibiotics 75% are of actinomycete origin. Among these *Streptomyces* are the major group producing most of the antibiotics. In the present study, also the potential producers of antimicrobial substances were found to be *Streptomyces* spp. Production of inhibitory substances help in the exclusion of pathogens

from the culture system. Maeda and Nogami (1989) reported some aspects of biocontrolling method in aquaculture. In their study, bacterial strains possessing vibriostatic activity, which improved the growth of prawn and crab larvae, were observed. By applying these bacteria in aquaculture, an equilibrium between beneficial and deleterious microorganisms can be produced and the results show that the population of *Vibrio* spp., which frequently causes large scale mortality in hatcheries was decreased. In this study the preliminary screening of the actinomycetes were done based on their inhibition towards pathogenic vibrios in culture system and the potential strains with bioactivity were selected. The possibility of inhibition of the natural flora is also important since the probiotics are applied as live microbial preparations. The percentage inhibition of the beneficial flora such as *Bacillus* and *Pseudomonas* were found to be very less compared to vibrios, *Enterobecteriaceae,* Staphylococci and *Aeromonas*. Vibrios and *Aeromonas* are mostly opportunistic pathogens, which invade and proliferate under stressful conditions when the host is weakened and the defense system fails in elimination of the pathogen. *Enteobacteriaceae* and *Staphylococcus* are human pathogens which may get entry into the culture system while manuring the ponds and through various human interventions. Inhibition of this flora is considered beneficial since the microbial quality of the post harvest products will be good.

In the current scenario with the ban of most of the antibiotics in aquaculture prophylaxis is the most preferred option i.e., probiotics, immunostimulants and vaccines. Manipulation of the microbial balance of the ambient environment with desirable and beneficial microflora is

considered promising in the health maintenance of the aquatic animal as well as the culture environment.

Probiotic selected should be nonpathogenic. In order to test the non-pathogenicity of the selected actinomycetes *P.monodon* post larvae were maintained in a bioassay system where these actinomycetes were inoculated in to the water. As a positive control, Luminescent bacteria were also inoculated in to another set of tanks. No infection could be observed even after 30 days of exposure to these actinomycetes and the Luminescent Bacteria. However, the oral challenge resulted in heavy mortality in the case of Luminescent Bacteria treated tanks showing its pathogenicity. Prawns in both the actinomycetes (B301 and B451) treated tanks exhibited good survival indicating the non pahtogenicity of the strains under the experimental set up. However, with field trials only, the confirmation of the non pathogenicity of the strains can be emphasized.

7.4.1 Efficacy as a probiotic in aquaculture

Ability of the strains in the water quality maintenance and the exclusion of vibrios in the culture system were estimated. Two experimental designs were executed.

I. Tanks with seawater, 2. Tanks with a sediment bed and overlying seawater. The degradative potential was found to be better in tanks with sediment bed. Water quality of the tanks with the actinomycetes gave a better performance in terms of mineralization compared to the control. However, a significant difference in the water quality could not be obtained with the tanks containing the probiotics compared to the control.

However, both these actinomycetes were found to be effective in the control of vibrios especially towards the later stages of the culture period (30 days). This delay may be due to the time taken for the adaptation and colonization of the actinomycetes in the system.

Most probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria (*Lactobacillus, Carnobacterium* etc), to the genus *Vibrio* (*Vibrio alginolyticus*), to the genus *Bacillus* and to the genus *Pseudomonas*. The inhibitory effect of Lactobacillus is attributed to their adhesive and antagonistic properties (competition for attachment sites, promotion of mucus products and competition for nutrients). Moriarty (1998) has studied the effective displacement of luminescent bacteria from shrimp culture systems using *Bacillus* sp.

Gram *et al* (1999) used *Pseudomonas fluorescens* against fish pathogenic bacterium Vibrio anguillarum where the inhibition was considered to be due to the production of siderophores, which deprive the fish pathogen of iron. Chythenya *et al* (2002) have reported the potential of marine *Pseudomonas* 1-2 strain a probiotic for shrimp culture. This strain was found to produce inhibitory compounds against shrimp pathogenic vibrios including *Vibrio harveyi, V. flavialis, V. parahaemolyticus, V. damsela and V. vulnificus*. The inhibitory substance was found to be low molecular weight compound, heat stable and soluble in chloroform and resistant to proteolytic enzymes. Enhancing the mineralization process and reducing the accumulation of organic loads improve water quality in a pond. Bioremediation or bioaugmentation is a concept of reducing organic waste to environmentally safe levels through the use of microorganisms. Rengpipat et al (1998) also noticed that a reduction in total bacterial

count and *Virbio* count in the rearing water when *Bacillus* S11 was applied as a probiotic on black tiger shrimp *Penaeus monodon*. Austin *et al.* (1995) found that probiont, *Vibrio alginolyticus* applied to salmon could reduce disease caused by *Aeromonas salmonicida*, *V. anguillarum* and *V. ordalii*. Shariff *et al.* (2001) have tested the efficacy of a commercial microbial product as a probiotic and noted considerable increase in the Total heterotrophic Bacterial population especially in sediment. In the present study also same observation was made where an increase in TPC could be noticed in the sediment. A decrease in the vibrio count as reported by Shariff *et al* (2001) was noticed in this study also which may be due to the antagonistic activity of the actinomycetes introduced in to the culture tanks.

Summary and Conclusion

Ocean harbours more than 80% of all life on earth and remains our greatest untapped natural resource. This biological diversity of the marine environment offers enormous scope for the discovery of novel natural products, several of which are potential targets for biomedical development. A variety of offensive and defensive mechanisms have evolved to allow organisms to gain selective advantage and to cope with competitors. The physiological manifestation of these defense abilities of marine organisms is in the form of bioactive metabolites. Almost every class of marine microbes produces a variety of bioactive compounds with unique structural features and often exhibits processes distinct from those of the terrestrial environment. The bioactive metabolites are excellent candidates for a variety of applications in the pharmaceutical, agricultural and food industries. Among the multitude of diverse organisms in the marine environment, marine microorganisms stand out as excellent source of many useful metabolites. Of all the marine microbes, the actinomycetes merit special consideration in view of the proven biosynthetic capabilities of numerous isolates from soil. Marine actinomycetes however have not received the attention accorded to their terrestrial counterparts. The actinomycetes stand out as a unique group of prokaryotic organisms in two respects; the diversity of their morphology ands their metabolic products. The prime example of the latter is the plethora of antibiotics produced principally as secondary metabolites late in the growth cycle More than 90% of the antibiotics are produced by actinomycetes.

The present work was undertaken to study the potential of marine actinomycetes as a source of antimicrobial compounds against prawn pathogens, their potential as a probiotic and as a source of single cell protein for application in aquaculture. The results of the present study are summarized as follows.

- Totally sixty-two actinomycetes could be isolated during the study from the various stations. More isolates could be obtained from the sediment of near shore waters compared to the offshore waters. CSPY-ME medium was found to be most effective in the isolation of actinomycetes.
- Bavistin (Carbendazim 50%) at a concentration of 13.75% in the medium was found to be very effective as an antifungal agent.
- Pre-treatment methods were found to be very effective in the selective isolation of actinomycetes and the heat treatment at 50-60°C for 1 hr was found to be most suitable followed by heat treatment at 100°C for 15min.
- All the cultures exhibited antibacterial property against vibrios. Six of them inhibited more than 50% of the 22 vibrios tested. These strains were B30 *(Streptomyces californicus)*, B272 (*Streptomyces* sp.), B301 (*Streptomyces griseoflavus*), B361, (*Streptomyces pulveraceus*), B377 *(Streptomyces* sp.) and B451 *(Streptomyces fradiae).*
- Out of the 52 different commercially available antibiotics tested, neomycin inhibited maximum number of vibrios (95%) followed by enrofloxacin, streptomycin and nitrofurantion all inhibiting 90% each. In terms of the extent of inhibition, neomycin was found to be most effective.
- *Streptomyces pulveraceus* (B361) inhibited 90% of the pathogens (*Vibrio* spp. with comparatively better halo zone. Of the various

pathogens tested P10 and P19 (*Vibrio* sp) were found to be most sensitive to the various antibiotics followed by P18 and P2. P1 was sensitive to only 17.31% of the antibiotics tested. However it was found to be sensitive to all the six actinomycetes showing halo zone in the range of 1-3 cm diameter.

- Generally growth and antibiotic production was found to be optimum at pH 7 for the various strains. No significant difference in the antibiotic production could be observed between pH 6, 7 and 8 by the strains.
- Generally a salinity of 35ppt was preferred by the various strains for growth and antibiotic production.
- A phosphate concentration of 0.25% was found to be generally the optimum for growth and antibiotic production by the selected strains.
- Calcium carbonate concentration of 0.3% was found to be optimal for antibiotic production by the various strains. Growth was also favoured at 0.3% $CaCO_3$ for B30, B301 and B361 and for others at 0.1%.
- Of the three solvents tested for the extraction of the bioactive compounds ethyl acetate was found to be most suitable for B361, (*Streptomyces pulveraceus*), B377 *(Streptomyces* sp.) and B451 *(Streptomyces fradiae).* For the other three strains B30 *(Streptomyces californicus),* B272 (*Streptomyces* sp.) and B301 (*Streptomyces griseoflavus*) none of the tested solvents were found to be effective enough for the separation of the antimicrobial compounds.
- Solvent system (Ethyl acetate: Hexane, 60:40) for the effective separation of the bioactive fraction on TLC from the ethyl acetate extract of the fermentation broth of *Streptomyces fradiae* B451 was developed.

- Fraction of ethyl acetate extract of *S.fradiae* at Rf 0.25 exhibited bioactivity (inhibition against pathogenic vibrios).
- In the feeding experiments with *P. monodon*, the performance of B361 (*Streptomyces pulveraceus)* as a source of single cell protein was significant showing its potential as an aquaculture feed ingredient.
- Biogrowth parameters like production (weight gain), food conversion ratio (FCR), relative growth rate (RGR) and Gross growth efficiency (GGE) were found to be significantly high for F361 compared to other feeds and the control feed.
- Post challenge survival with white spot virus was also significant with feed *Streptomyces pulveraceus* (F361) showing its immunostimulant effect. Possibility of having an anti viral compound associated with the strain B361 is worth probing.
- Of the 20 actinomycetes screened for hydrolytic enzyme production all were found to produce lipase, protease and amylase production was exhibited by 60% and 50% respectively.
- Actinomycete cultures *S.pulveraceus* (B361) and *S. fradiae* (B451) were found to be inhibitory to most of the luminescent bacteria isolated from diseased prawn larvae.
- Of the actinomycetes selected for probiotic application maximum inhibition to the natural flora was exhibited by *S.pulveraceus*, B361 (29.83%) followed by *S.griseoflavus*, B301 (13.17%), *S. fradiae*, B451 (9.17%) and the unidentified strain L5 (8.67%).
- Test on haemolytic property showed the presence of both haemolytic and non-haemolytic strains. Only the non-haemolytic strains were selected as Probiotics.

- All the tested actinomycetes were found to be non-pathogenic to penaeid prawns (luminescent bacteria) and therefore safe for application as Probiotics.
- Of the various actinomycetes tested, *Streptomyces fradiae*, B451, was found to be best in terms of its performance in bioremediation of the rearing water and the control of vibrios in the system.

The study revealed the potential of marine actinomycetes as a source of antimicrobial compounds. The selected streptomycetes were found to be capable of inhibiting most of the pathogenic vibrios, which is a major problem both in hatcheries and grow out systems. The bioactive principle can be incorporated with commercial feeds and applied as medicated diet for the control of vibrios in culture systems. The hydrolytic potential inhibitory property against pathogens and non-pathogenicity to penaeid prawns make the selected *Streptomyces* spp.an effective probioic in aquaculture. Since there is considerably less inhibition to the natural in pond ecosystem the microbial diversity is being maintained and thereby the water quality. Actinomycetes was found to be a good source of single cell protein as an ingredient in aquaculture feed formulations. Large amount of mycelial waste (actinomycete biomass0 is produced from antibiotic industries and this nutrient rich waste can be effectively used as a protein source in aquaculture feeds.

This study reveals the importance of marine actinomycetes as a source of antimicrobial compounds and as a probiotic and single cell protein for aquaculture applications.

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Appendix

Table A1 : Antagonistic activity of selected strains of Actinomycetes against Natural flora

Genera	**Total no. Tested**	**Inhibition by the varicus actinomycetes (%)**					
		30	272	301	361	377	451
Aeromonas	154	8.7	9.3	20.1	37.5	16.1	15.4
Alcaligenes	6	0	0	0	100	0	0
Bacillus	45	0	0	0	20	0	0
Coryneforms	10	40	40	40	40	40	40
Enterobacteriaceae	65	7.4	7.4	7.4	35.1	11.1	0
Flavobacterium	50	8.58.5	8.5	8.5	8.5	8.5	8.5
Flexibacter	8	0	0	75	75	0	75
Micrococci	2	0	0	0	0	0	0
Moraxilla	50	0	4.8	0	37.7	2.4	7.3
Pseudomonas	60	2	6	22	16	2	2
Staphylococcus	50	1.4	1.4	14.4	21.7	4.3	14.4
Streptocccus	50	0	23	11.5	19.2	11.5	0
Vibrio	50	2.7	8.3	16.6	44.4	2.7	11.1
Grand Total	600	5.0	8.7	13.17	29.83	8.8	9.17

Table A2 a-f : Comparison of various media for antibiotic production by selected Actinomycetes

a. Streptomyces californicus

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
P1	-	-	-	-		-	-	-	-	-	-	-	++	++
P2									-	-	-	-	++	-
P3			++	+	++	+	+		-	-	-	-	-	-
P4									-	-	-	-	+	-
P5	-	-	+	+	-	-	-	-	-	-	-	-	+	-
P6	-	-	+++	-	+	++	+	-	-	-	-	-	-	-
P7	-	++	++	-	-	-	-	+	-	-	-	-	-	-
P8	-	-	++	-	-	-	-	-	-	-	-	-	+	-
P9	-	-	++	-	-	-	-	-	-	-	-	-	-	-
P10	-	-	++	-	-	-	-	-	-	-	-	-	-	-
P11	-	-	++	-	+	-	+	-	-	-	-	-	-	-
P12	-	-		-	++	-		+	-	-	-	-	-	-
P13	-	-	++	+	-	-	-	-	-	-	-	-	-	-
P14	-	-	++	-	-	-	-	-	-	-	-	-	-	-
P15	-	-	-	-	-	-	-	-	-	-	-	+	+	-
P16	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P17	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P18	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P19	-	-	++	-	-	-	-	-	-	-	-	-	-	-
P20	-	-		-	-	-	-	-	-	-	-	-	-	-
Total	-	-	11	3	4	2	3	2	-	-	-	1	8	2
%	-	5	40	-	20	10	15	10	-	-	-	5	55	10

b. Streptomyces sp.

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
P1	-	-	-	-	-	-	-	-	-	-	-	-	+	+
P2	-	-	-	-	-	-	-	-	-	-	-	-	+	-
P3	-	-	+	+		+	-	-	-	-	-	-	+	-
P4	-	-	-	-	-	-	-	-	-	-	-	-	+	-
P5		++	+	-	-	-	-		+		-	+	-	-
P6	+	-	-	-	+	++	-	-	-	-	-	-	+	-
P7	-	++	+	-		++	-	-	-	-	-	-	-	-
P8	-	-	-	-	+	-	-	-	-	-	-	-	+	-
P9	-	-	-	-	+	-	+	-	++	-	-	+	+	-
P10	-	-	-	-		-	-	-	+	-	-	+	+	+
P11	-	-	-	-	+	-	-	-	-	-	-	-	-	-
P12	-	-	+	+	-	-	-	-	-	-	-	++	-	-
P13	-	-	-	-		+	-	-	-	-	-	-	+	+
P14	-	-	-	-	+	-	-		-	-	-	+++		++
P15	-	-	-	-	-	-	-	-	-	-	-	-	+	-
P16	-	-	-	-	-	-	-		++	-	-	+	-	-
P17	-	-	-	-	-	-	-		++	-	-		+	+++
P18	-	-	-	-	-	-	-	-	-	-	-	-	+	-
P19	+	++	+	-	-	-	-			-	-	-	-	-
P20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	1	2	4	2	5	4	1		5	-	-	6	12	5
%	5	10	20	10	25	20	5		25	-	-	30	60	25

c. Streptomyces greseoflavus

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
P1	-	-	-	-	-	-	-	-	-	-	-	-	+	-
P2												-	+	-
P3	++		+	-	-	+	-		-	-	-	-	+	-
P4	-	-	-	-	++	-	-	+	-	-	-	-	++	-
P5	+	+	+	-	-	-	-	-	-	-	-	-	-	-
P6	+	-		-	-	+	++	-	-	-	-	-	+	-
P7		-	+	-	-	+	-	-	-	-	-	-	++	-
P8	+	-	-	-	-	-	-	-	-	-	-	-	+	+
P9	+	-	-	-	-	-	-	-	-	-	-	-	-	
P10	-	-	-	-	-	-	-	-	-	-	-	-	+	++
P11	-	-	-	-	-	-	-	-	-	-	-	-	-	++
P12	-	-	-	-	-	-	-	-	-	-	-	-	-	
P13	-	-	-	-	-	-	-	-	-	-	-	-	+	+
P14	-	-	-	-	-	-	-	-	-	-	-	-	-	++
P15	-	-	-	++	+++	-	-	-	-	-	-	-	+	
P16	-	-	-	-	-	-	-		-	-	-	-	+	+
P17	+	-	-	-	-	-	-	-	-	-	-	-	+	+++
P18		-	-	-	-	-	-	++	-	-	-	-	+	-
P19	+	+	-	-	-	+++	-	-	-	-	-	-	-	-
P20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	7	2	3	1	2	4	1	2	-	-	-	-	11	7
%	35	10	15	5	10	20	5	10	-	-	-	10	55	35

d. Streptomyces pulveraceus

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
P1	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P2	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P3		+++				+	-	-	-	-	-	-	-	
P4	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P5	-	-	+	-	-	-	-	-	-	-	++	-	-	-
P6	+	-	-	-	-	+	-	-	-	-	-	-	-	+
P7	-	-	-	-	-	-	+	-	-	-	-	-	-	+
P8	-	-	-	+	-	-	-	-	-	-	+	-	-	+
P9	-	-	-	-	-	-	+	-	-	+++	+++	++	-	+
P10	-	-	-	-	-	-	-	-	-	++	+	+	-	+
P11	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P12	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P13	++	-	-	-	-	-	-	-	-	-	-	-	-	+
P14	-	-	-	++	-	-	-	-		-	-	++	-	+
P15	-	-	-	++	++	-		-	-	-	-	++	-	+
P16	-	-	-	+	-	-	+++	-	-	-	-	++	-	+
P17	-	-	-	-	-	-	-	-	-	++	-	-	-	+
P18	-	-	-	-	-	-	-	-	-	-	++	-	-	+
P19	++	++		+	-	-	+++	-	-	-			+	
P20	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Total	3	2	1	5	1	2	4	-	-	3	5	5	1	17
%	15	10	5	25	5	20	20	-	-	15	25	25	5	85

e. Streptomyces sp.

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
P1	-	-	+	-		-	-	-	-	-	-	-	-	+
P2	-	-	-	-	-	-	-	++	-	-	-	-	-	+
P3	-	-	+	-		+	-	-	-	-	-	-	-	+
P4	+	-	-	-	++	-	-	-	-	-	-	-		+
P5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P6	-	-	+	-	-	++	-	+	-	-	-	-	-	+
P7	-	-	+	+	+	-	-	-	-	-	-	-	-	+
P8	-	-	+	-	-	-	-	-	-	-	-	-	-	+
P9	-	-	+	-	-	+	-	-	-	-	+	-	-	+
P10	-	-	+	-	-	+	-	-	-	-	+	-	-	+
P11	-	-	+	-	-	-	-	-	-	-	-	-	-	-
P12	-	-	-	-	-	-	-	-	-	-	+	++	-	-
P13	-	-	+	-	-	-	-	-	-	-	-	-	-	-
P14	+++	-	-	-	-	-	-	-	-		-	++	-	++
P15	-	-	+	-	++	-	-	-	-	-	-	-	-	+
P16	-	-	+	-	-	-	-	-	-	-	-	-	-	+
P17	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P18	-	+	-	-	+	-	-	-	-	-	-	-		+
P19	-	-	-	-	-	-	-	+	-	-	-	-	-	
P20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	2	1	11	1	4	4	-	3	-	-	-	2		14
%	10	5	55	5	20	20	-	15	-	-	15	10		70

f. Streptomyces fradiae

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14
P1	-	-	-	-	-	-	-	-	-	-	-	-	-	+
P2	-	-	-	-	+	-	-	-	-	-	-	-	-	+
P3	-	-	+			+	++	-	-	-	-	-	-	-
P4	++	-			+		-	-	-	-	-	-	-	++
P5	++	-	+		++		-	-	-	-	-	-	-	-
P6	++	-	-	+	+	+	-	-		-	-	-	-	-
P7	++	-	-			+	-	-	-	-	-	-	-	-
P8	-	-	-	+	+	-	-	-	-	-	-	-	-	-
P9	++	-	-	+	+	-	-	-	-	++	++	-	-	-
P10	-	-	-	+	++	-	-	-	-	++	-	-	-	
P11	++	-	-	+	+	-	-	-	-	-	-	-	-	++
P12	-	-	-	-		-	-	-	-	-	-	-	-	+
P13	++	-	-	-		-	-	-	-	-	-	-	-	
P14	-	-	-	++	+	-	-	-	-	-	++	-	-	++
P15		-	-	+++	+	-	-	-	-	-	-	-	-	+
P16	-	-	-	+	+	-	-	-	-	-	-	-	-	++
P17	-	-	-	-	-	-	-	-	-	-	-	-		+
P18	++	+	-	-	-	-	-	-	-	-	-	-		+
P19	++	-	-	+	+	-	-	-	-	-	-	-	+	+
P20	-	-	-	+	-	-	-	-	-	-	-	-		+
Total	9	1	2	10	12	3	2	-	2	2	2	-	1	13
% inhibition	45	5	10	50	60	15	10	-	-	10	10	-	5	65