ROLE OF *BACILLUS* AND *LACTOBACILLUS* FROM MARINE ENVIRONMENT FOR SUSTAINABLE AQUACULTURE PRACTICES

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By

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CERTIFICATE

Certified that the thesis entitled **"Role of *Bacillus and Lactobacillus* from marine environment for sustainable aquaculture practices"** is a record of independent bonafide research work carried out by **Smt. Ajitha. S** (Reg. No. 2117) during the period of study from June 1999 to June 2004 under my supervision and guidance for the degree of **Doctor of Philosophy** and that the thesis has not previously found the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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Declaration

I hereby declare that the thesis entitled **"Role of Bacillus and Lactobacillus from Marine Environment for Sustainable Aquaculture Practices"** is an authentic record of the work done by me and that no part there of has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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1. Introduction

The worldwide demand for marine shrimps continues to attract interest and investment for the development of shrimp culture in India. During the past 20 years, aquaculture industry has been growing tremendously, especially that of marine fish, shrimp and bivalves in addition to fresh water fish and prawn. Penaeid shrimps are among the most important and extensively cultured crustaceans in the world (about 60 countries). In 2001, world's shrimp farmers produced and estimated more than 1.0 million metric tons of whole shrimp while marine shrimp production continues to increase on a global scale.

Uncontrolled and haphazard development of shrimp farming with poor farm design and management have also lead to the water quality degradation while lead to frequent attack by viral and bacterial disease inflicting huge losses to about US $ 5 billion. Pathogenic microorganisms implicated in these outbreaks were viruses, bacteria, algae, fungi and protozone parasites. *Vibrio harveyi*, the causative agent of luminous bacterial disease is considered a serious pathogen when it exceeds the permitted limits in larval shrimp hatcheries. (Lavelia-pitogo et. al., 1990, Karunasagar et. al., 1994). Application of antibiotics, vaccines was widely followed to control the pathogen and to improve the production of larvae (Baticados et. al., 1990). But the unscrupulous use of antibiotics and other

chemicals have negative effects on biological filters in controlled recirculating systems particularly on nitrifying bacteria and may adversely affect algae in the culture system besides leaving undesirable or hramfull residues in cultured animals (Sindermann, 1986). Chemical treatment with antibiotics and drugs increase the virulence of pathogen. The presence of residual antibiotics in sediments will promote bacterial resistance and may infact have human health implications. Thus environmental degradation results in a substantial reduction of water quality of shrimp culture ponds themselves causing disease outbreak and loss of production. Microbes purify water, cycle oxygen and CO_2 , maintain fertility of water (30% productivity) yield food and medicine and provide the genetic richness to improve fish and shellfish.

For preventing and controlling diseases particularly in aquaculture, the best method is the improving health of culture organisms and elimination of pathogen by improving aquatic environment. Improvement in the ecological environment of aquaculture has become the focus of attention of international aquaculture. The researchers have proved the use of probiotic bacteria in aquaculture to improve water quality and improve the immune potential of the animal by balancing bacterial flora in water and reducing pathogenic bacterial load.

Probiotics can be defined as cultures (single or mixed) of selected strains of bacteria that are used in culture and production system to modify or manipulate the microbial communities in water and sediment, reduce or eliminate pathogen i.e., species of micro-organisms, and generally improve growth and survival of targeted species. Microbes are very important and has critical role in aquaculture including shrimp farming at both the hatchery and the grow out level, because water quality and disease control are directly related to microbial activity. Solution to this problem lies in microbial ecology and environmental microbiology by competitive exclusion principles and not in the field of pharmacology.

There are various ways through which probiotics may act in aquaculture system, including by competitive exclusion of pathogen by enhancing digestion through the supply of essential enzymes by moderating and promoting their direct uptake of dissolved organic materials, by active production of pathogen inhibiting substances and other possible mechanisms. In general, it is most accepted that probiotics in ponds probably act through competitive exclusion. According to Moriarty (1996a), the species composition of a microbial community such as that in a pond will be determined partly by chance and partly by physiological factors that allow a species to grow and divide more rapidly than others and dominate numerically.

Currently the most common used probiotic in animal nutrition are (LAB) lactic acid bacteria and some strains of spore forming *Bacillus* species. Aqua farm (1996), has hinted the general characteristics of *Bacillus* species which favour their use to improve the pond condition and productivity.

(1) Bacillus can easily move around (mobile) because they have a whip like 'tail' (flagella).

(2) Bacillus from endospores which are useful under stress conditions, (or when nutrient are limited). Endospores allow Bacillus to produce when conditions are favourable.

(3) Bacillus produce antibiotics (Bacitracin polymupein, tyrocodin, gramicidin and circulans) or special compounds (enzymes) that can break down polysaccharides, nucleic acids and lipids.

(4) They produce H_2O_2 and prevent the adhesion of pathogenic bacteria.

(5) Produce metabolites which are able to neutralise bacterial toxins.

(6) They stimulate the non-specific immune system of the host, proliferate in the digestive tract and compete with pathogenic bacteria.

(7) Bacillus can be easily isolated from soil or air.

Pond environmental condition must be efficiently managed so that addition of probiotic such as bacillus cultures can have significant beneficial effects. According to Moriarty, (1996a) vendors of commercial bacterial amendments are not aware of the ecological and physiological requirements of aquatic microbes. Their bacterial products have and do not pass this information down to their client. The successful use of a probiotic to promote sustainable aquaculture will be dependent on various factors such as defining ecological processes to be affected and naturally dominant species versus desirable alternative species of microorganisms to be added or promoted (Moriarty, 1996b). Further whether we can change a bacterial community depends on knowing enough about the ecological factors that govern species composition, including specific growth rate, nutrient composition and concentration, inhibitory interaction and the type of bacteria already present. It is necessary to both manipulate physico-chemical factors to alter microbial species composition and to alter rate of metabolic activity by adding selected species to carry out particular function at faster rate than those present in the given system.

According to studies of Rosenfeld & Zobell (1947), marine environment is a reservoir of micro-organism able to produce anti-microbial substance (antibiotics) with a inhibitory effect. Even though there are several reports of bacteria with inhibitory effects isolated from sea water. The main purpose of these mostly have been to characterise the

specific antibiotic of bacterium produced. Microbiology of intestinal tracts of marine and fresh water fishes has been investigated by many researchers; few studies have addressed by the production of inhibitory components by these bacteria, but a detailed study of the microbial ecology of indigenous *Bacillus and lactobacillus* from marine environment and the evaluation of their probiotic potential is lacking in literature. So in order to fill this lacuna the present study was conducted to elucidate the frequency of occurrence of *Bacillus and Lactobacillus* species in the aquaculture environment and to investigate the antagonistic properties of the isolates against the pathogenic *Vibrio harveyi*. The objectives of the present study – "**Role of *Bacillus and Lactobacillus* from marine environment for sustainable aquaculture practices."**

To study the eco-physiology of bacillus and lactobacillus from marine environment.

1) Isolation, identification and characterisation of Bacillus and Lactobacillus species from aquaculture ponds.
2) To study the percentage composition of Bacillus and Lactobacillus spp from the culture environment.
3) To study species composition of Bacillus and Lactobacillus from the two culture ponds.

4) To study their biochemical potentials.

5) Statistical inference of the bacterial isolates with environmental parameters.

6) To investigate the effect of L*actobacillus acidophilus and Bacillus subtilis* as separate compounded feed on the growth and survival of *penaeus monodon* juveniles used for the study.

So instead of going for commercial probiotics which imposes a high cost of production to the farmers, they could manipulate the species composition by seeding a large number of desirable strains of bacteria (*Bacillus and Lactobacillus*) by giving chance a helping hand.

2. REVIEW OF LITERATURE

Intensive aquaculture demands the application of microbial biotechnology limited to the rapidly developing science in microbial ecology in order to become a truly susceptible industry. With the decline in capture fisheries, aquaculture is the only way to fulfil the growing global demand for fish. Environmental problem and disease attack have been the major cause of mortality in shrimp larviculture (Wyban & Swency, 1991; Wilkenfeld, 1992) and fish hatcheries (Grisez and ollevier, 1995) and grow out. (Boyd and Clay, 1998)

Application of antibiotics as prophylactics in large quantities is widely followed to control the pathogen and to improve the production of larvae (Baticados et. al., 1990) and shrimps even when pathogens are not evident. Periodic use of commercially available antibiotics has created strains resistant to the commonly used antibiotics. Periodic use of commonly used antibiotics has only been marginally successful in solving the problem, but emerging legislation is increasingly restricting their usage (Bangen et. al., 1994; Barg & Lavilla-Petago, 1996; Reilly and Kaferstein, 1997) which not only raises the cost of production but also the effectiveness of treatment, and a risk for the natural environment (Sou et. al., 1999) in promoting the transfer of antibiotic resistance to human pathology (Moriarity, 1999). Many of the pathogen appear to have mutated

to more virulent forms than were present decade ago and thus even when the shrimps are not stressed, by poor water quality, they succumb to attack. As per Moriarty (1999), the solution lies in the field of microbial ecology and not in the field of pharmacology, i.e., in developing new antibiotics or vaccines. Shrimp farmers have to learn to live with a complex community of microbes and manage them. One such method that is gaining acceptance within the industry is the use of probiotic flora to control potential pathogen. *Lactobacillus* as nutritional probiotics and *Bacillus* as pond probiotics plays a main role for sustainable aquaculture practices.

Probiotics: -

The word probiotics is derived from two Greek words. 'Pro' and 'Bios' Which means for life. The Elich Metchikooff's work at the beginning of this century (1908) is regarded as the first research in this concept of microbial manipulation. Probiotics is described as beneficial microorganism ingested with aim of promoting good health and intestinal microbial balance (Lilly et. al; 1965; Sperti1971; Parker, 1974) while Fuller (1989) defined nutritional probiotics as a live microbial feed supplement which beneficially effects the host animal by improving its intestial microbial balance.

Earlier the beneficial effects of Probiotics were studied only in farm animals or humans but later it was extended to aquaculture industry also. According to Kogasa(1986) probiotic in aquaculture is relatively a new concept with, interest in treatment with friendly bacteria increasing rapidly in aquaculture. Jerry (1998) defined probiotics, particularly in aquaculture as single or mixed culture of selected strain of bacteria. Those are used in culture to modify and manipulate the microbial community in water and sediment, reduce or eliminate selected pathogenic spp of microorganism and generally improve growth survival of the targeted species.

Probiotics is defined as beneficial microorganism that are administered in such a way as to enter the gasto-intestinal tract and be kept alive with the aim of improving health and its microbial balance. In the intestine (Ruiz etal, 1998; Gatesoupe, 1999; Gram et. al, 1999). Irianto and Austin (2002) defined probiotics as microorganisms or their product with health benefit to the host, have found use in aquaculture as means of disease control, supplementing or even in some case replacing the use of anti microbial compounds.

Microbes as Nutritional probiotics

The initial major purpose of using probiotic is to maintain or re-establish a favourable relationship between friendly and pathogenic microorganisms that constitute flora of intestinal or skin mucus of fish.

Antagonism to pathogen

A successful probiotic is expected to have considerable potential properties. Antagonism to pathogen is one such property the inhibitory effect can in many cases be due to low pH and primary metabolites (Brink et. al; 1987) by producing antimicrobial substance like siderophores & organic acids (Hentges 1983; Gram and MeF Chuorsin; 1996) or H_2O_2 (Ringo and Galesoupe; 1998)

Colonisation of the microbes

Adhesion is another important criteria for a probiotic flora because it is considered prerequisite for colonisation, and it is a host specific phenomenon. Adhesion varies between strains of the same species and is also influenced by the growth condition & media used. (Fuller, 1973;Lenzner; 1973; Fuller, 1975, 1980, et.al, Beachy, 1981; Metsouka, 1992; McCartney et. al., 1996; Tannock, 1998; Conway et. al., 1987; Olsson et. al., 1992) Probiotic flora should be able to produce important substance like vitamins. (Indrani Karunasagar, 1999). On colonisation of the microorganisms, digestive tract *lactobacillus* form a natural ecological barrier and prevent these pathogenic microbe from penetration and reproduction.

Competition for Nutrients

Competition for nutrients is another property of probiotic. Iron is required by most organisms and its availability in animal tissue may be virulent factor for pathogen. The competition for carbon sources between the gut flora and *shigella flexineri* (a pathogen) have been studied by Freter, (1962), Smith et. al., (1993) in *P.indicus*.

Bacterial toxins and enzyme

Probionts are said to influence the activity of useful enzymes. eg: β-galactosidase and also alleviate lactose intolerance. Some are able to produce metabolites which can neutralize bacterial toxins. Studies have shown that *Lactobacillus acidophilus* when fed to human suppressed the activity of β-glucuronidose, nitro-reductase and azo-reductase.

Immunity Enhancement

The stimulation of immune system by probiotic lactic acid bacteria is still in dispute in many reports (Tannock, 1997; Gill, 1998; Powel et. al., 1998; McCracken, 1999), but Rengipipat et. al., (2000) reported that the immune response were more pronounced with probiotics treated shrimp when compared with the control.

The practical use of probiotics emphasizes that the microorganisms should have viability for long period under storage on a large scale use

(eg. for industrial purpose) and should be able to survive in the intestine, exhibiting resistance to low pH and other antibacterial influence in the gut. (Fuller 1987, 1991 & 1992). The host should gain beneficially from harbouring the probionts.

In Fish

The most commonly used probiotic in animal nutrition are lactic acid bacteria (*L.bulgaricus, L. acidophilus, L. sporogenes, L. casei, L. salivarus, L. plantarium*). They have been tested as nutrient probiotic in warm blooded animals. Attempts have also been made to use lactic acid bacteria as antagonistic to fish pathogen (Gatesoupe, 1994; Mohammed, 1995; Joborn et. al., 1997; Kjlleberg et. al., 1997 and Rupam Sharma, 1999).

Selected viable counts of lactic acid bacteria often composed of *L.acidophilus and S. faecium* or strains of bacilli used at length and at a high dose modify gut flora of turbot larvae, Wolfer (1986); Kozasa (1988) and or increase zoo technical performance (Vanbellele et. al., 1989 or increase survival, size, uniformity and growth rate of marine fish larvae and attempts have also been made to use lactic acid bacteria, antagonistic to pathogen. (Gatesoupe, 1994; Joborn et. al., 1997; Kjlleberg et. al., 1997). Lactic acid bacteria are rarely present in juvenile fish reared on

artificial feed but may become dominant in the intestinal flora, if they are supplemented in the feed. (Robertson et. al., 1999).

Several studies showed that lactic acid bacteria are a part of natural intestinal flora of healthy fish and they often produce bacteriocin which may inhibit the growth of fish pathogen in the intestine. (Austin et. al., 1988; Gatesoupe 1994; Jankauskiene 1995; Gildberg et. al., 1997; Ringo et. al., 1998; Jankauskiene 2000 a & b).

In Shrimp

The studies of Maeda and Nogami (1989) revealed that by applying bacterial strains possessing vibriostatic activity in aquaculture, a biological equilibrium between competing beneficial and deleterious micro-organisms can be maintained and the Vibrio species which frequently causes large scale damage to larval production can be decreased i.e., a natural resistance of the shrimp to disease can be provided, the survival and growth rate can be enhanced.

Maeda et. al., (1994); Garriques et. al., (1994); Grifith, (1995); Gomezgil, (1995) outlined various developments made in the nutritional use of probiotics in developing shrimp immunity. Studies by Sridhar Chandrashekar (1996) evaluated effect of feeding bacterial biomass strain of bacillus promoted survival rate to 64-70% in larvae of *P.indicus*, while

B.licheniformis compounded & fed to Post larvae of *P.indicus* showed enhanced growth and survival in shrimp.

The studies on *P.indicus* juveniles Ajitha et. al., (1997) that specific growth rate; survival rate and from specific immune response can be enhanced by feeding *P.indicus* juveniles with 4 LAB cultures. (*L.acidophilus, L.bulgaricus 56&57, L.cremoris*), the effect of probiotic bacterium *L.plantarum* on the systems on Penaeid shrimp. *P.indicus* larvae against *Vibrio harveyi* was studied by Uma et. al., (1999).

Probiotic incorporated in live feed

Indirect feeding of the animal is done through live feed like artemia and nauplii; rotifer etc. Bogaurt et. al., (1988) stated that the growth rate of turbot (*s.maximus*) were improved when rotifer fed with live lactic acid bacteria are given as live feed. Gatesoupe (1989&1991 a,b); Bogaurt (1993); Gatesoupe (1993); Douillet (1996), demonstrated that multiple probiotic application in rotifer culture can reduce coefficient of variation in production compared to the control treatments. *Lactococcus Lactis* enhanced the growth of gut flora.

Bio-encapsulation:

Bio-encapsulation method using artemia nauplii was proposed as an innovative way of delivery to the shrimp larvae (Mohney et. al., 1989)

this concept was modified and extended to feed fish larvae with bio-encapsulated probionts in live feed, Gatesoupe (1991). Bio-encapsulated lactic acid bacteria when introduced in turbot larvae improved their growth and survival (Garcia-de-la-banda et. al., 1992), Uma et. al., 1999 also reported that the dietary value artemia nauplii as well as the disease resistance of shrimp larvae can be improved with bio-encapsulated probiont

Bacteria in micro algae

Arvendano et. al., (1999) suggested that feasibility of incorporating bacteria into axenic micro algal culture to be used as vector for the introduction of bacteria antagonistic to unwanted pathogen in culture of aquatic species.

Bacillus spore and live feed

Bacillus species spores as food additive for rotifer (*B plicatilis*) improved the bacterial environment & dietary value of larval turbot (Gatesoupe 1989)

Environmental or pond probiotics

Water quality control in intensive fish culture system become essential due to the accumulation of harmful metabolites that inhibit fish

growth and kills it, hence time has come to a find an alternative to economic use of water with and eye over it quality through ecofriendly technologies such as biofilteration & bioremediation. Bioremediation using probiotics are significant management tools in intensive culture system.

Bioremediation methods involving probiotics

Porubean (1991) reported on two attempt to improve pond water quality and production yield of *P.monodon* with bacterial treatment micro organisms can be the safe alternative to overcome the said problem. Studies on this aspects was conducted in larval rearing of shrimp, Crab and oyster by (Maeda 1991-1999; Douillet et al 1994)

The probiotic especially Bacillus spp. added directly to medium can modify the microbial composition of the water and sediments; increase species diversities, minimise the effect of, if not eliminate the pathogen directly and increase prawn survival. Also they are most efficient in breaking down polymers as they are rich in proteases, amylases, lipases and cellulases since their natural habitat is sediment. Certain strains compete with the bacterial flora naturally present in the organic matter thereby resulting in less accumulation of slime or organic matter, better penetration of oxygen and better environment. (Moriarty, 1996)

Phosphate solubilising bacteria like *Klebsiella pneumonia* & *Bacillus pumilus* have been observed to be good bioremediating agents by De Souza et. al., 1996. Studies by Chandrika (1996) revealed that seven bacillus species. (*B.cereusmycoides, B.megaterium, B.mucosis, B.agglomerates, B.cartilaginous, B.idionrus and B.intricatus*) showed antagonism, proteolytic and catalytic activity and so they could be used for bioremediation / phytoremediation in aquaculture.

Bacillus isolated from intestinal micro flora

Bacteria from the intestinal microflora when isolated and added to larval culture water enhanced the survival rate, growth, disease resistance and health of the larvae. (Wang Xianhong et. al., 1997)

Commercial Probiotic

Recently Moriarty (1998) proposed that bacterial mixtures contributed by water or water additives may have beneficial effect on aquaculture products.

Queroz & Boyd (1998) reported that commercially prepared mixtures of bacillus species mixed into the rearing water increased survival and production of channel cat fish. (Cetalurid Pumctatus)

Laurent Verschauere et. al., (1999) also studied that probiotic based on single strain are less effective than mixed culture in a changing environment. When a beneficial bacterium will dominate the associated microbiota when several bacteria are administered. Ashraf Ali (2000) reported that challenge study had showed that the growth of Aeromonas spp was inhibited in intestinal contents of Add-B treated fish.

According to Bright Singh et. al., (2001) some bacillus spores, Rosebacter sp., Alteromonas and pseudomonas are antagonistic against fish and shell fish pathogen. While studies of Ravichandran et. al., (2001) indicated that probiotics are effective in maintaining soil-water interface by improving the environmental quality.

Isolation of Bacteria from Marine Environment

Bacterial spp. such as *Bacillus* and *Lactobacillus* isolated from marine environment has probiotic effect. Many studies regarding this has been done. Isolation of Bacillus strain from fish (Hamid et. al., 1978; Strom olafsen, Neduhila and Westholf, 1995, Sadhukhan et. al., 1998) from crustacean (Austin & Allen, 1982; Sharmila et. al., 1996; Sugita et. al., 1996a) and bivalve. (Sugita et. al., 1981) in ordinary media by spread plate technique. These natural intestinal flora may actively produce substances that may inhibit or kill the pathogens (Sugita et. al., 1996).

Bacteria isolated from the intestinal microflora of shrimp when isolated and added to larval culture water enhanced the survival rate, growth, disease resistance and health of the larvae. (Wand Xianhong et. al., 1997). Spore of *Bacillus toyoi* isolated from the soil reduced the mortality of Japanese catfish infected by Edwardsiella sp (Kozasa, 1986). The same feed additive was found to increase the growth rate of yellow tail. Maeda and Liao (1992) reported about bacterial strains extracted from soil used as pond probiotics.

Kennedy et. al., (1998) isolated a strain of *B.subtilus* from the common snook (*C.undecimalis*) which when isolated into the rearing water resulted in apparent elimination of Vibrio species.

Microbial ecology of Bacillus and Lactobacillus in aquaculture ponds

It is most accepted that probiotics in ponds probably act through competitive exclusion. According to Moriarty (1996 a) the species composition of a microbial community such as that in a pond will be determined partly by chance and partly by physiological factors that allow a species to grow and divide more rapidly than others, and thus dominate numerically. Chance favour these organism that happen to be in the right place at the right time to respond to a sudden increase in nutrients.

Boyd (1995) noted that bacteria are ubiquitous their spores and vegetative bodies occur in almost all natural environment. The major factor affecting their abundance and activity are temperature, pH, oxygen supply, moisture supply and amount and type of substrate. Bacterial activity is favoured by warmth (25-30°C) and above neutral pH (7.5-8.5) plenty of oxygen low. C:N ratio (L1:15) and a substrate consisting of easily decomposable organic matter. In shrimp ponds the major factor affecting bacterial activity is usually the dissolved oxygen supply.

In 1872, Ferdinand Cohn, a student of Robert Koch, recognised and named the bacterium *B.subtilis*. The first bacillus, *B.anthracis* was isolated in pure culture and shown to possess spore (Koch 1876) and was the first bacterium used for the preparation of an attenuated vaccine (Pasteur 1881).

In 1934, Veddar isolated several endospore forming bacterial strains that were able to grow at pH value upto 10, but not below pH 8.6. Since obligately alkalophilic bacteria were not known at that time Veddar (1934) described his isolate as a new species of *B.alkalophilus*. There have been several reports that the spores of bacillus spp differ from their vegetative cells in cytochrome content (Keilinad Harfree, 1949) and cyanide sensitivity (Spence & Powel, 1957; Hadrisuka et. al., 1956). In

1956, Nakada, Masushiro and Miwalani studied on the development of aerobic spore forming bacteria. The extensive literature dealing with the various aspect of sporogenesis has been received by Cook (1932), Knaysi (1948), William (1952), Steadman (1956) and Halvorson (1957). Protoplasmic differences between mesophilis and thermophilises were studied by Koffer (1957).

When Heno and Wilson (1958); isolated N_2 fixing bacillus strains showing a typical pattern of classification of *B.polymyxa*, many other species have been identified as variants of this species. *Bacillus subtilis* is a ubiquitous soil micro organism that contribute to nutrient cycling when biologically active due to the various enzyme produced by members of the species. Bacillus occur at the levels of 10^6-10^7 /gm of soil (Alexander, 1977) in the environment. However unless the soil has been recently amended with organic matter providing readily utilisable nutrients. The bacillus exist in the endospore stage. It is thought that 60-100% of soil bacilli population exist in the inactive spore state (Alexander, 1977).

Zobell & Upham (1944) isolated several species of the genus bacilli from the marine mud around pacific coast. Studies of Wood (1959) showed that genus bacillus predominated and formed 20% from surface water, 39.5% and 45% from bottom sediments in the lake Macquire . Colwell & Liston (1960) isolated only 6% bacillus from Chesapeake Bay

out of 229 heterotroph isolated. Rodina (1972) has described various species of Bacillus with different isolation of media. While Buck (1976) isolated 7% bacillus from Connecticut river in the pre-thermal and thermal discharge periods. Chandrika (1983) isolated 36 bacillus strains from Cochin backwater which showed high proteolytic, ureolytic and caseinolytic activity. 7 species of bacillus including pink bacillus with central spores was isolated from sediments of seasonal ponds. (Pokkali & perennial ponds). [Chandrika (1999)]

Bacillus cereus was identified by Puchenkova (1994) from bacteriofors and biosediments produced by mussels and system in Sonther Ruch Strait. Area commercial mollusc culture. Nedoluka et. al., (1997) isolated 7% bacillus species from fish samples collected from 3 aquaculture system.

A broad spectrum mercury resistant bacterial strain isolated from contaminated water was identified as *Bacillus pasteurii* by Pahan et. al., 1996. Huang (1999) also reported *B.megatarium* from mercury polluted sediments. The surface water collected in September-October from a fresh water marsh habitat exhibited that majority of the isolates were gram positive rods (51%) out of which 35% belonged to bacillus species alone. (Smith et. al., 1998)

The role of bacillus in naphthalene biodegradation was studied by Zhuang et. al., and found *Bacillus naphthovorans* as the dominant cultivable naphthalene – degrading organism from oil contaminated tropical marine sediments. The ability of bacillus species to survive at very high temperature was revealed by study of Mevel (1998,2000). He isolated thermophilic hetrotrophic nitrifers from deep sea hydrothermal vents that could grow at 65°C. Kahlil et. al., 1998 isolated a thermo-tolerant bacteria from thermal springs along the Jordan rift valley. Here the optimal temperature was .50°C.

Besides soil, bacillus species was isolated from other environment such as the skin and mucus of horse mackerel (*Trachurm saponicus*) [Asakawa et. al., 1988). Ivanova et. al., (1999) isolated 16 representatives of the genus bacillus from seawater samples, sponges & unidentical sponge, ascidians, soft corals etc of Japan, Okholsk sea. He found that 11 out of 16 isolates belong to *B.subtilis* phenotype, one strain of *B.pumctus* and 4 alkaliphilic strains.

Biochemical activity during sporulation of *B.cereus* was studied by Krishnamurthy, Gottakola & Orin Halverson (1959). Effect of temperature on the rate of germination in *B.cereus* was described by George Knaysi (1963). Gray and his coworkers used immuno fluorescence (IF) for studies on the distribution growth and spore formation and germination of

B.subtilis in soil. Hill and Gray (1967); Siala et. al., (1974); Siala and Gray (1975) were able to differentiate between the spore and the vegetative cells of this organism insitu using fluorescent antibodies (FAS) prepared against the respective antigen.

It was shown that *B.subtilis* is present mainly in the vegetative form in an acid horizon of the same soil profile. Cooney et. al., (1997) studied the media dependence of commitment in *B.subtilis*. Isolation, characterisation and mapping of *B.Subtilis* 168 germination mutant are discussed by Trowsdale and Smith, 1975. Effect of temperature on the rate of germination in *Bacillus cereus* was discussed by George kansi; (1963). Aunstrup et. al., (1972) studied the proteus from alkalophilic *bacillus spp.* Chemical composition of spore and spore structure was studied by Murret (1969). Sporulation in *B.subtillus*. Genetic analysis of oligosporogenous mutants was studied by Coote (1972).

Chandramohan (1971) discussed some aspects on the distribution of phosphobacteria in marine environment at Portonova. In their studies preliminary staining and biochemical studies revealed that majority of the isolates belong to one genus Bacillus. Wolf and Barker (1968) gave the biochemical aid to the identification of a species and Knight (1955) worked on the minimal nutritional requirements of some species in the genus bacillus.

Gordon and Clarke (1952) used ammonium salt sugars (A.S.S) for their work on Bacillus species some of which ferment of others oxidise carbohydrate purification properties and regulation of amylase produced by a thermophylic bacillus species was discussed by Srivastava et. al., (1980). Bacitracin and protease production in relation to sporulation during exponential growth of *B.licheniformis* on poorly utilised carbon and nitrogen sources was found by Gibson et. al., (1981). Dham et. al., (1998) reported that casein hydrolysate and NH_3Cl supported the good growth and xylanase production by Bacillus species. Purva sony et. al., (1998) studied the optimisation of cultural condition for the alkaline protease production from an alkalophylic bacillus species and its further characterisation to check its thermo-stability pattern. Pradeep sidhu et. al., (1998) studied the effect of cultural condition on extracellular alkaline lipase production by Bacillus species RS-12 and it characterisation. Adhesh kumar et. al., (2002) reported the optimisation of condition for production of neutral and alkaline protease from species of Bacillus and Pseudomonas.

Isolation of Lactobacillus

Forty seven strains of homo-fermentative rod shaped and 5 hetro-fermentative sphere shaped lactic acid bacteria were isolated from 4 kinds

of fermented fish in Thailand. Four strains were identified as *L.pentosus* and one strain of *L.plantarum* by Tanasupawat et. al., (1998). Muella et. a;., (1999) isolated *L.lactis* from fish fellet and minced fish. Studies by Ringo et. al., (1998) revealed that lactic acid bacteria dominated among the gram positive bacteria detected in all regions of fish fed the PUFA supplemented diet and the frequency of lactic acid bacteria was highest in the digestive tract of fish. Studies of Fuselli et. al., (1998) found that Lactobacillus spp specifically *L.casei* and *micrococcus* spp dominated in the bacterial flora of marinated and stored anchovy.

Occurrence and distribution of lactic acid bacteria in fresh and frozen fishes of national trade, fresh marine fishes as well as brackish and aquaculture farm fishes were studied by Nair et. al., (1997) and reported that LAB count were always 1-2 log cycle less in fishes from internal trade while fresh and brackish water fishes showed high count of LAB. About 90% of LAB isolated were lactobacillus strains. The review of Ringo et. al.,)1998 evaluates that lactic acid bacteria belong to the normal microbiola of gastro intestinal tract in healthy fish and the population level is effected by nutritional and environmental factors. It has also been reported that the lactic acid bacteria isolated from gastro intestinal tract can act as probiotic.

Zakaria et. al., (1998) studied the proteolytic activity of lactic acid bacterium. (*L.paracasei* strain) on treatment with minced scampi waste

supplemented with glucose about 77.5-61.0% of protein and calcium present in the waste was solubilised.

L.plantarium and *L.bulgaricus* act as good fermenters for the preparation of fish silage from fish processing waste (Yoon et. al., 1997; Faid et. al., 1997). Studies of Gancel et. al., (1997) revealed that out of seventy eight strains isolated from fillets of vacuum packed snoked and salted herring showed that all the isolates belong to genus Lactobacillus. CO_2 production and arginine degradation were characteristic of the isolates. Jankauskiene et. al , (1996) studied intestinal tract of 65 carps and found that the lactoflora of intestinal tract of carps could be improved by the immunological and resistance of fish to various infection.

The use of probiotics in shellfish farming shows signs of becoming success. However, a great deal of research is still needed to certify this. One important unanswered question is the destiny of probiotic candidates in skin and gastro-intestinal tract. The forgoing literature provides evidence that certain types of microorganisms have the capacity to protect against fish and shellfish pathogens.

3. MATERIALS AND METHODS

Ecophysiology of Bacillus and Lactobacillus

The study was conducted for a period of 12 months from April 2001 – March 2002. Two perennial polyculture ponds located along 10°00`N 76°135`E and 10°10` 76° 12.5`E in Vypeen islands were selected for the study of monthly occurrence, distribution and seasonal variation Bacillus and Lactobacillus spp isolated from water sediment and shrimp (penaeus indicus) intestine. (Fig1)

Probiotic effect of the most prominent spp isolated from these site was also studied. Estimation of physico- chemical parameters were carried out to study the effect of ecological parameters favouring the growth and distribution of these microbes in aqua culture ponds at Vypeen island.

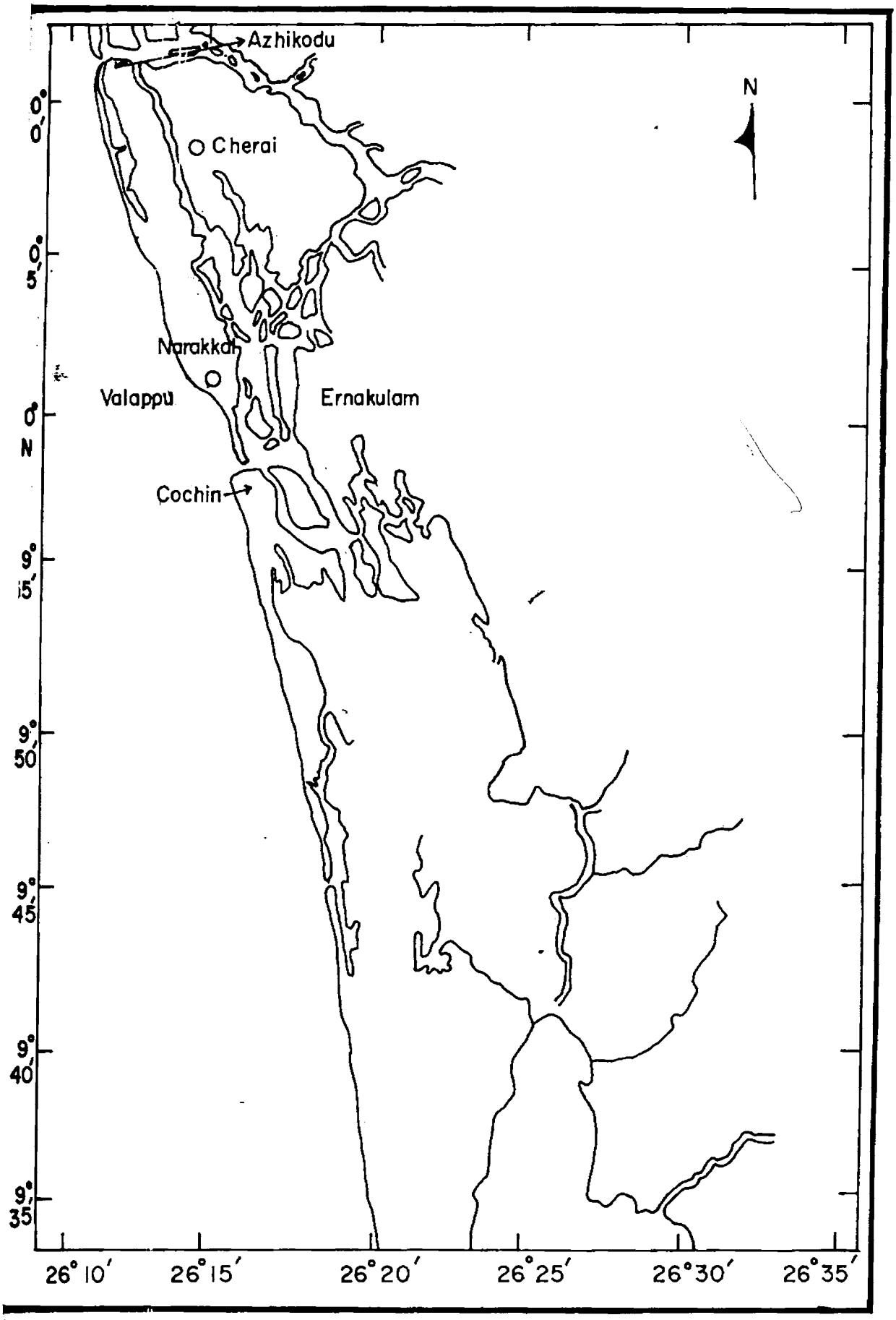


Fig 1-Map showing in the study area

STUDY AREA (Plate 1 and 2)

Station-1	Ajanta co-operative farm Valappu. Vypeen Island.	1ha/1.5m	*Penaeus indicus* *Chanos Chanos* *O. mossambicus* *P. monodon*
Station-2	Private firm at Cherai	8 ha/1m	*Penaeus indicus* *P.monodon* *Scylla serrata* *Etroplus suratensis*

The pond fixed as first station was fertilised once a year and it was fertile with clayey soil and luxuriant growth of Phyto plankton. The second pond was shaded with over growth of trees around. The water appeared darker, productivity was comparatively lesser. The soil was black & muddy due to extensive fermentation was going on by Sulphur reducing bacteria (SRB). Both ponds were of modified extensive type in which the cultured fishes were fed naturally as the water exchange was influenced by strong tidal influx and outflow.

Plate1 Aquaculture pond at (Valappu)1ha/1.5m

Plate 2 Aquaculture pond at (Cherai) 0.8 ha/1m

Sample Collection :

Regular monthly samples were collected from both the stations for the study. The shrimp collected by cast nettings were transported to the laboratory insterile polythene bags in live condition. Water was collected in sterile plastic bottles and sediment in sterile polythene covers for quantitative and quantitative analysis of Bacillus and Lactobacillus species.

The dorsal surface of the body cavity of about 4-5 shrimps was cut open aseptically and the intestine along with hepatopancrease was removed in to Sterile petridish Approximately about I gram of the sample was taken for the study.

Bacteriological Investigation

Both qualitative bacteriological and investigation analysis of total Bacterial count (TPC), Total bacillus count (TBC) and total lacto bacillus count (TLC) were investigated.

Quantitative Analysis

The total heterotrophic bacteria was estimated by serial dilution technique and pour plating method (Rodina, 1972). Approximately 1gm of the sample (water, sediment and shrimp intestine) was taken

separately in a mortar and pestle and ground well with 99ml of aged sea water.

After thorough shaking for 30 minutes at 250 rpm in a bacteriological shaker, serial dilutions were prepared according to the standard procedures. 1ml of the inoculum was pour plated in sterile petridishes (10cm diameter) with Zobells marine agar (Hi-media). These plates were incubated at room temperature for 24 hours and the total bacterial count was estimated (TPC).

Quantification of Bacillus

To enumerate the spore forming Bacillus species present in the shrimp intestine, about 1gm of the samples were taken in a mortar, ground well with 99 ml of aged sea water. The suspension was taken in a 150 ml conical flask, shaked well in a bacteriological shaker at 250 rpm for 20 minutes. The conical flask was then placed in water bath set at 80°C for 10 minutes (Rodina 1972).

Inoculum from the pasteurised suspension was used for pour plate method. The culture medium used was a combination of Nutrient agar mixed with 1% glucose, 7% proteose peptone. The pH of the medium was maintained at 7.0-7.2. The plates were incubated at 25-30°C for 24-48 hours. The colonies were identified by Gram's staining method and phase

contrast microscopy. The colonies suspected to be bacillus strains were isolated and maintained in semi solid nutrient agar in tubes at 4°C for further phenotyphic and biochemical characterisation.

The biochemical characterisation was done as per the scheme followed in **Bergy's manual of determinative bacteriology (1974).** The strains isolated were characterised by conventional microbiological methods, morphology of vegetative cells, sporangium shape, position of spores, Nitrate reduction test, anaerobic growth and gas production in glucose, degradation of starch, urea, casein, gelatin, tyrosine acid from D-arabinose, D-xylose, D-glucose, mannitol, utilisation of citrate, ammonia and Voges proskauer, growth at 45°C and 65°C; growth at pH 5.7 and 7% NaCl.

Quantification of Lactobacillus

MRS (Mann Rogosa Sharpe) medium both as broth or agar was used for the isolation and maintenance of Lactobacillus present in the sample (sediment, water and shrimp intestine). (Harrigan & McCance; 1976). A suspension of 100 ml was made from 1 gm of the sample mixed with 99 ml of aged seawater which after shaking at 250 rpm for 20 minutes, inoculation were made by serial dilution and pour plate method using MRS media. The plates were incubated at 30°C for 24-48 hours in

an anaerobic condition. The strains isolated by plating and grains straining maintained in broth at 4°C in tubes for phenotypic characterisation. The characterisation scheme followed were as per **Bergy's manual of determinative bacteriology (1974).**

The nitrate reduction test, arginine hydrolysis, growth at 15°C and 45°C, Acid from arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melibiose,, melizitose, raffinose, ribose, rhaminose, salicin, sucrose, trehalose, xylose, esculin, sorbitol and gas from glucose were some of the biochemical test done for Lactobacillus identification.

Sediment sample used for Bacteriological investigation

1 gm of the sample was taken in a mortar, ground well using 99 ml of aged sea water and was thoroughly shaken in a bacteriological shaker. This suspension was used for further analysis of TPC, Bacillus and Lactobacillus species.

Water sample

50 ml of water sample collected asceptically from the sampling site was filtered through a (0.02 μ sartorious) membrane filter. The filter paper was later transferred into 99 ml of sterile aged sea water. This was thoroughly shaken in a bacteriological shaker and from this serial dilution

were prepared which was used for determination of TPC, bacillus and lactobacillus species.

Microbial identification of DNA finger printing method (MIS)-Sherlock

Streaking Plates

The quadrant steak pattern is recommended for culturing cells on plates for identification by Sherlock MIS.

Incubation

The standard incubation condition for aerobes (TSBA 40) are the following:

- 28±1°C temperature
- 24±2 hrs time

Preparing Extracts (5 basic steps)

Harvesting

Remove the cultured cells from the plates by gently scraping the surface of the culture media with the sterile 4mm incubating loop. A back and forth motion while slightly rocking the loop is useful in picking of the cells.

Insert the loop with the cells into a clean, dry screw cap cultured tube. Wipe the cells of the loop and on to the inner surface of the culture tube within 10mm of the bottom of the culture tube. Remove and sterilise the loop.

Saponification

Pipette 1.0±0.1 ml of reagent 1, the methanolc base, in to each of the culture tubes in the batch. Tightly seal each tube with the clean Teflon line screw cap. Vortex the tube for 5-10 seconds. Place are a rack of the batched sample tunes into a boiling or circulating water bath at 95-100°C. After 5 mins remove the tubes from the billing water and cool them slightly. Vortex each tube for 5-10 seconds return the tubes to water bath for an additional 25 mins. After a total of 30 minutes of saponification in the water bath, remove the set of rack of tubes in a pan of cold tap water to cool.

Methylation

Add 2 ± 0.1ml of reagent 2. the methylation reagent to each tube. Vortex the solution for 5-10 seconds.

Extraction

Fatty acid methyl esters are removed from the acid aqueous phase and transferred to an organic phase with a liquid extraction procedure.

Uncap each tube, then add 1.25 ± .1ml of reagent 3, the extraction solvent to each tube. Plates batch of tubes in a laboratory rotate and gently mix end over end for 10 minutes using a clean Pasteur pipette for each sample, remove and discard the aqueous phase.

Base wash

A dilute base solution is added to the sample preparation tube to remove free fatty acid s and residual reagents from the organic extracts. Resudual reagents will damage the chromatographic systems, resulting in tailing and loss of the hydroxyl fatty acid methyl esters. Add 3±0.1 ml of reagent 4, the base wash, to each tube, tightly cap and gently rotate the tubes end over end for 5 minutes. Brief centrifugation (3 minutes at 2000rpm) is recommended to clarify the interface between the phases when an emulsion is present

Measurement of hydrological parameters

Temperature:

Temperature of the water was noted in the field during sampling by using a mercury high precision thermometer of ± 0.1°C at 11am on every sampling day.

Hydrogen Ion Concentration:

pH value of water was determined using a digital pH meter. The instrument was calibrated with the help of pH buffers. (4.2 & 9.1)

Salinity:

Salinity was estimated by following the Mohr titration method (Strickland and Parsons; 1968). Here 10 ml of water sample was titrated against 0.14N silver nitrate solution with 10% potassium chromate as an indicator.

$$\text{Salinity} = \frac{\text{Volume of silver nitrate for 10 ml sample} \times \text{salinity of std seawater}}{\text{Volume of silver nitrate for 10 ml std sea water}}$$

Dissolved Oxygen:

Dissolved oxygen in the water sample was determined using Winkler method. (Strickland and Parson; 1968). The water sample with reagent (Winkler A and B and 1 ml Con H_2SO_4) was titrated against 0.01N sodium thio sulphate solution using starch as an indicator.

$$\text{Oxygen (ml/l)} = \frac{V1 \times N \times 8 \times 1000 \times R \times 0.698}{V2}$$

V1 - volume of sodium thiosulphate

N - normality of sodium thiosulphate

V2 - volume of sample

R - concentration factor

0.688 - to convert from ppm to ml of O_2/l

8 - equivalent weight of oxygen

Nutrients

Nutrients such as nitrite was estimated by the Azo-dye method (Bend Schneider and Robinson, 1952) while nitrate was estimated following the method of Mullin and Riley (1955). The absorbance of both nitrite and nitrate was measured at 545 nm.

Phosphate was determined by method described by Murphy and Riley (1962) and followed by Strickland and Parsons (1968). While ammonia was determined following the phenol hypochlorite method. (solarzano, 1969). The absorbance of sample was measured at 885 nm for phosphate and 640 nm for Ammonia. The sample values were expressed in ug-at/l for all the nutrients.

The results obtained through the investigation were statistically located by suitable statistical methods. The analysis of variance was evaluated to find out the level of significance among the stations in different months. Correlation analysis was carried out to ascertain the association of various hydrographic parameters with the counts of bacillus and lactobacillus and total plate count.

Correlation coefficient was worked out by using SYSTAT version 7.0 to study the relationship between the dominant species of *Bacillus and Lactobacillus* and the hydrographic factors. Species diversity index

(Shannon and Weaver, 1963), Index of dominance (Simpson, 1949), Species richness index (Menhimick, 1964) and Evenness index (Pie Lou, 1966) were calculated using the follwing expression.

Index of dominance (C) = $\Sigma (ni/N)^2$

Where ni = important value for each species.

N = total of important values.

Species richness of variety indices (δ)= (S-1)/logN

Where S = number of species

N = number of individuals.

Shannon-Weaver's index of species diversity (Π)

Π = $\Sigma(ni/N) \times \log(ni/N)$

Where ni = important value for each species.

N = total important values

Evenness index (e) = $\Pi/\log S$

Π = Shannon-weaver index

S = number of species.

To facilitate interpretation, the data obtained were analysed season wise as follows: February-May (Pre-monsoon), June-September (Monsoon) and October to January (Post-Monsoon).

Probiotic study of Bacillus and Lactobacillus

The most prominent species of (*Lactobacillus acidophilus and Bacillus subtilis)* Bacillus and Lactobacillus isolated from the two sampling sites was used for the study of their Probiotic effect in shrimp culture.

The experiment was conducted in the hatchery of CMFRI. The most viable strains of *Lactobacillus acidophilus and Bacillus subtilis*, prominently occurred in testing sampling sites were selected as the pro-bionts for the feeding experiment after their antagonistic activity. The cultures were maintained as agar slope under refrigeration using enrichment medias.

Agar	-	2%
Proteose peptone	-	0.7%
NaCl	-	0.5%
Beef extract	-	1%
Glucose	-	1%

GPP media for bacillus and MRS media for Lactobacillus.

Plate 3 ***Bacterial Biomass of Bacillus subtilis & Lactobacillus acidophilus***

Plate 4 Experimental Set up

Preparation of Bacterial Biomass (Plate 3)

The cultures were subcultured for accumulation of bacterial biomass, the bacillus and lactobacillus strains were grown aseptically in 10 ml nutrient broth for 18 hours at room temperature (28±2°C). 5 ml of the log phase culture were then asceptically transferred into 250 ml of the enrichment media and kept in a rotary shaker at 150 rpm for 18-24 hours at room temperature. The cells were harvested by centrifugation at 8000 rpm for 15 mts at 4°C. The culture purity and identity were routinely checked during this investigation. The harvested cells were adjusted to a final concentration of 10^7 cells/ml using sterile physiological saline (0.85% NaCl) after repeated washing with the same and stored at 4°C. This biomass was used directly in feeding experiments for feeding shrimp after incorporation into the feed mix.

Feed preparation

A typical shrimp feed was used for the feeding experiment. (New 1989).

Percentage composition of ingredients

Ingredients	**percentage composition**
Fish meal	10 gm
Prawn meal	20 gm
Groundnut oil	15 gm
Cake	10 gm
Clam meat	10 gm
Soya flour	10 gm
Oil	6 gm
Wheat flour	29 gm
Fish oil	3 gm
Vegetable oil	3 gm

The feed was prepared as moist dough twice a week in the laboratory. The two pro-bionts *Bacillus subtilis* and *Lactobacillus acidophilus* were separately incorporated with the feed in the ratio 1:3 (wet

weight) at 10^7 cell/ml concentration. The control feed was devoid of bacterial biomass. The mixtures were spread out and in 3 trays dried for 1-2 hours at 37°C stored in 3 plastic bags at 4°C until use.

The 3 types of experimental feed used are one with *Bacillus subtilis* (T_1B).

The second one with *lactobacillus acidophilus* (T_2L)

The third one in the control tub (T_3C) devoid of bacterial biomass.

Experimental Setup (Plate 4):

About 200 nos of *penaeus monodon* juveniles of size (5.1 $\pm$ 0.5 cm) length and (0.78 $\pm$ 0.1g) weight were purchased from cochin aqua hatchery and transported to C.M.F.R.I hatchery in well aerated oxygen bags. The animals were acclimatized for a week on the control feed prior to start of the experiment.

The shrimps of 180 nos after a week was suggested into 12 plastic tubs of size 50-70 l capacity arranged in vertical racks. Each tub contain about 12-15 number of shrimps provided with sterilized seawater with good aeration in each tub. The water used for the experiment was filtered using bolting silt of 40μ mesh size and then through sterilized absorbent cotton.

The three treatments such as T_1B, T_2L and T_3C were replicated using 4 tubs each. The shrimps were fed at 10 to 12% body weight per day in two divided doses in the morning and evening. The experimental duration was 90 days. The culture system was cleaned every day by siphoning out the faecal matter and feeding waste. $1/4^{th}$ of the water was changed each day and whole water exchange was done once in 2 days.

The following measurements were made every 30 days for 90 days. Shrimp live weight, length and survival; total bacteria count (Zobells marine agar), Bacillus count (G.P.P media), lactobacillus count (M.R.S agar); Vibrio species count (Tryplic soy agar) from shrimp intestine and faeces of the rearing tank water.

Physico-chemical parameters of the water was also monitored during the entire experiment. Weakly water quality measurements included Ammonia, Nitrite, Nitrate, Phosphate, Temperatures, Dissolved oxygen, pH, and salinity as described by Strickland and Parson (1972)

4. RESULTS

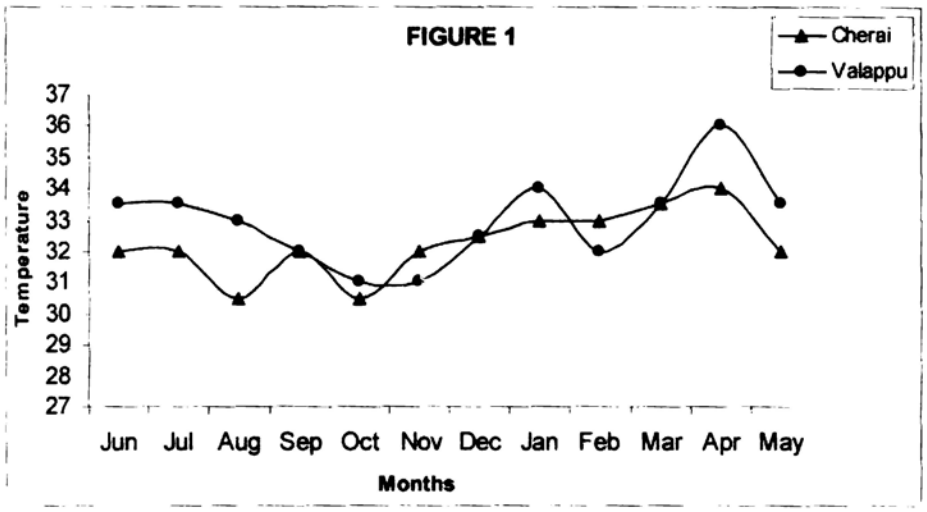
4.1 Quantification of Total Plate Count, Bacillus and Lactobacillus

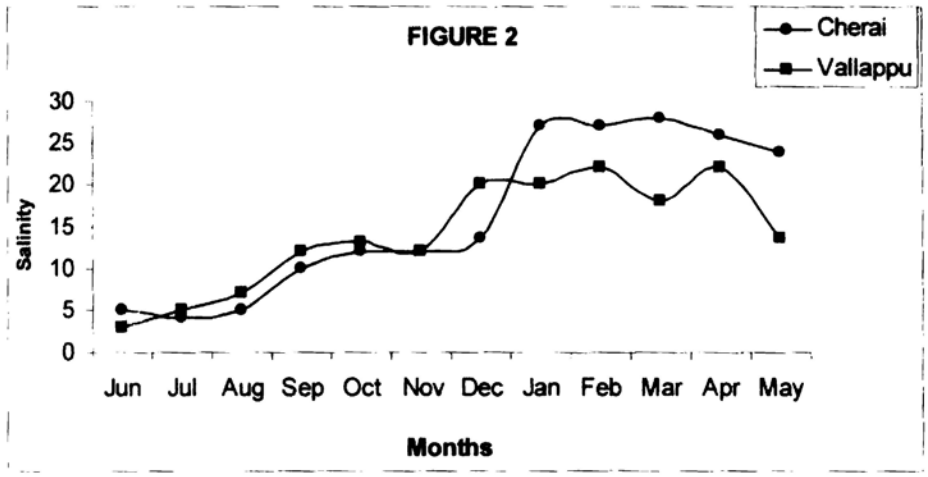
4.2 Bacterial profile of water, sediment and penaeus monodon from culture ponds

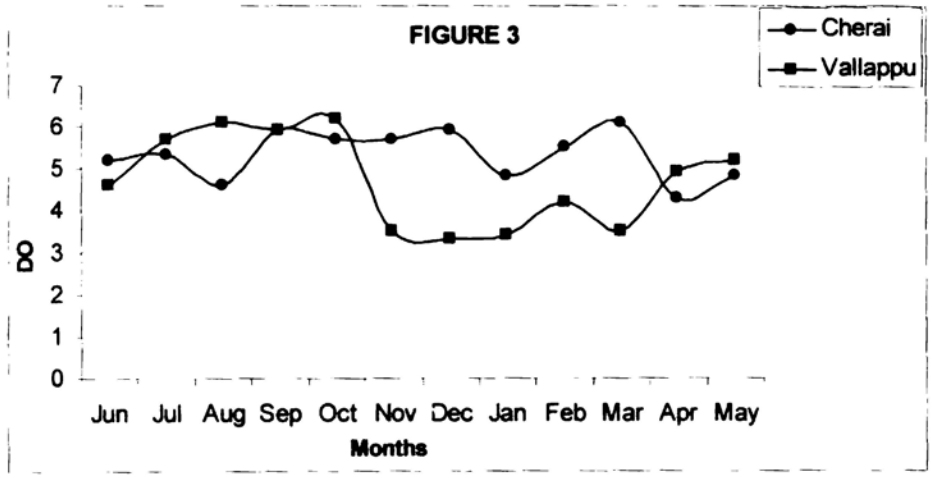
The total plate count (TPC) of water from Cherai station ranged from a maximum of $252x10^3$/ml in the month of September and April 2001 to a maximum of $104x10^3$/ml in the month of August 2001 (fig 10). December and March also should high values ranging from $244x10^3$/ml and $240x10^3$/ml due March. Whereas in Valappu station (Fig 13), the maximum value was recorded in the month of August 2001 $24.8x10^4$/ml and minimum value of $12.8x10^4$gm/m was recorded during November and January.

TPC of sediment from Cherai station recorded a maximum value during the month of October 2001 ($220x10^3$/gm) while a minimum value was recorded during August and December 2001. ($124x10^3$/gm) (Fig 10). While TPC of sediment sample (fig 13) at Valappu recorded a maximum of $18x10^4$/ml during the month of March 2001 while a minimum was recorded in December 2001 ($991x10^4$/ml). TPC during July, Aug, Sept, and October showed only very slight variations when compared to the months of March 2002.

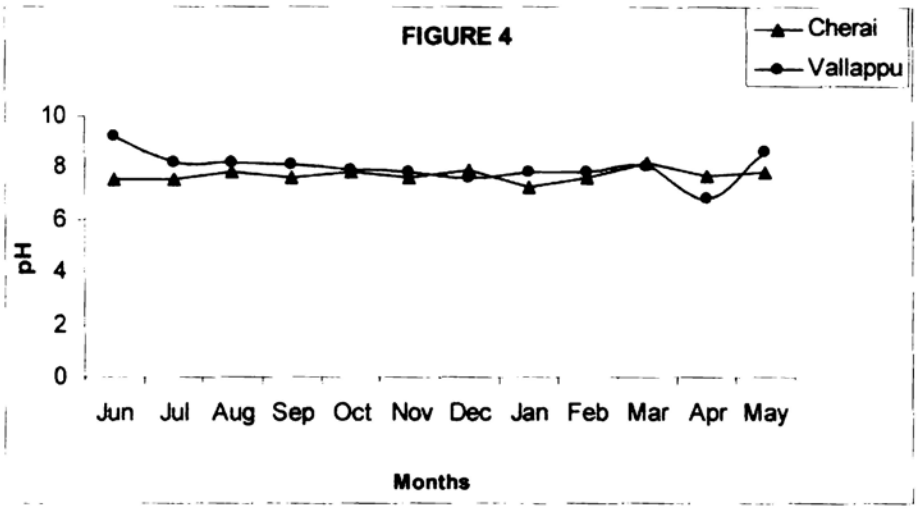
Figs. 1-3: Annual variations of Temperature, salinity and dissolved oxygen concentrations at Cherai and Valappu stations (2001-2002)

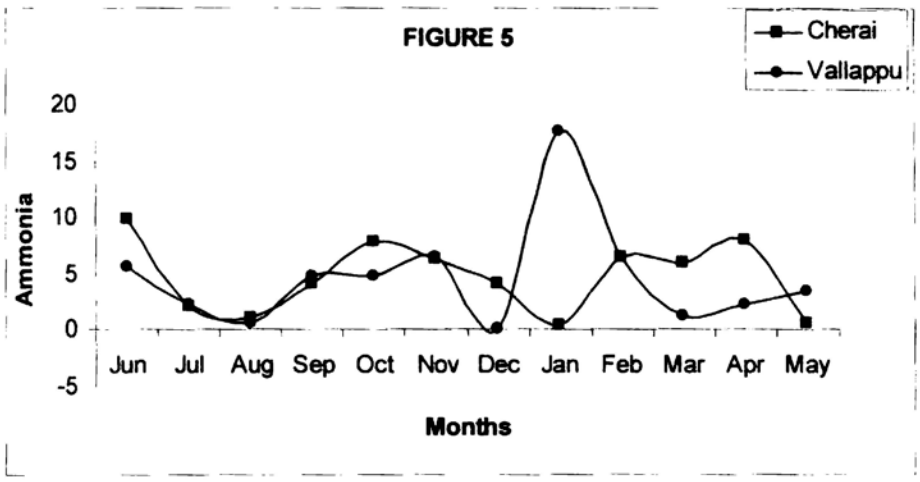


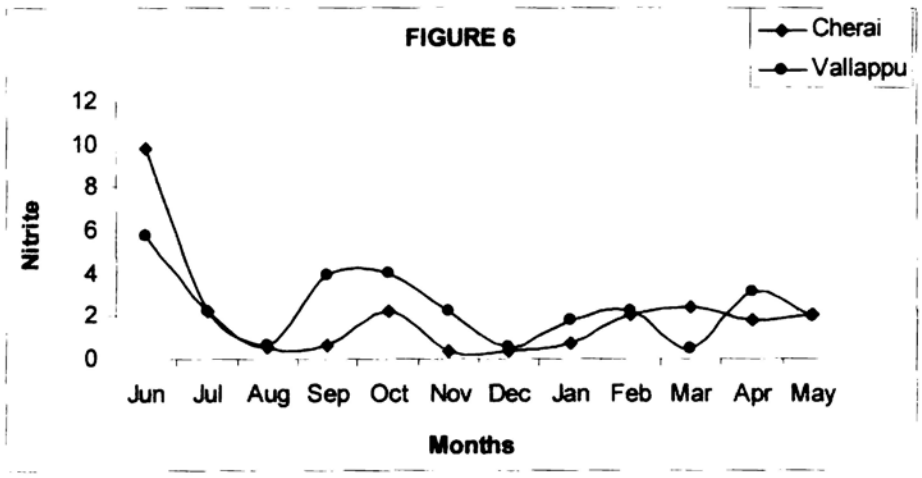




Figs. 4-6: Annual variations of Ammonia, Nitrite and Nitrate concentrations at Cherai and Valappu stations







TPC of shrimp intestine recorded comparatively lesser values than water and sediment in Cherai station during the month of January 2002. (Fig 10) TPC recorded was $160x10^3$/gm while a minimum of $98x10^3$/gm was recorded during March 2002. TPC of the shrimp intestine of Valappu station showed a high increase in the month of March when compared to the other months.

TPC of shrimp intestine (Fig 13) of Valappu showed a very high increase in the month of March when compared to the other months ($26.8x10^4$/ml) while all other months showed comparatively less counts, the lowest count was recorded during January 2002 ($72.8x10^3$ / ml).

4.3 Quantitative analysis of Bacillus from water sediment and shrimp intestine

Total bacillus count (TBC) in water at Cherai station (fig 11) showed a maximum of $22x10^3$ /ml during the month of September while the lowest count was recorded in June, July and December $1\text{-}2x10^3$ /ml. TBC count of water during the month of April was very low in Valappu when compared to Cherai, even though the maximum count was recorded in April (Fig 14) $8.2x10^4$ /ml. No Bacillus isolates were recorded in the month of June and August 2001 and January 2002.

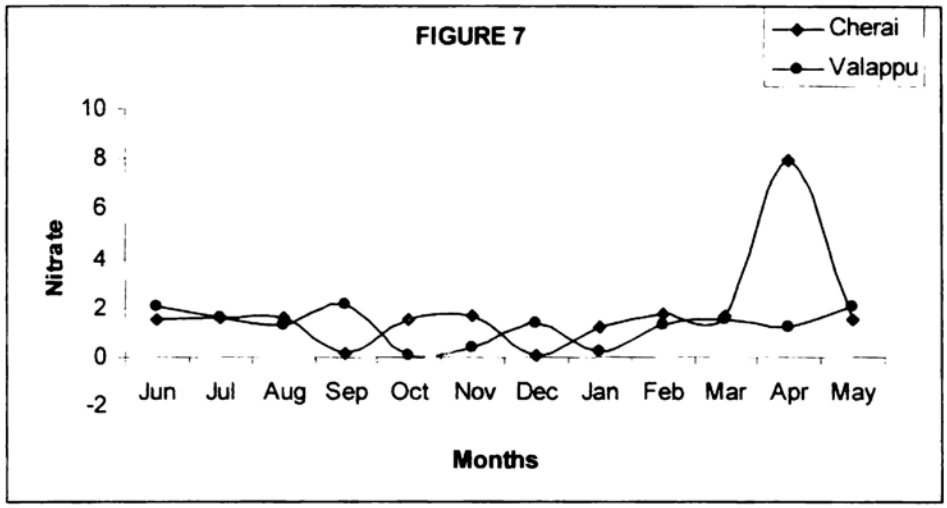
TBC count in sediment sample at Cherai (fig 11) recorded a maximum value ($50x10^3$) in the month of June when compared to the TBC from water and shrimp intestine. TBC was not recorded during August and November. April and May showed a static count ($10x10^3$/ml) which increased during June, but reduced to very low count in August and September while December 2001 also recorded very high count (fig 11). The TBC was maximum in sediment at Valappu during September ($8.8x10^4$) while in August and October no bacillus was isolated.

TBC in shrimp intestine at Cherai (fig 11) and Valappu (fig 14) showed great variations. The TBC was very low $2x10^3$ /ml in April at Cherai while a maximum of $38x10^3$ /ml was recorded in May. No TBC was recorded during December and January while in Valappu (fig 14) TBC in shrimp intestine was lower than Cherai during April while May, June months showed considerable increase $8.0x10^4$ and $7.9x10^4$ /ml where the maximum was in May. August, December and February showed zero count (fig 14).

4.4 Quantitative Analysis of Lactobacillus from water sediment and shrimp intestine

Total lactobacillus count (TLC) in the water sample (fig 12) at Cherai was $12x10^3$ /ml in April which decreased to almost nil in May but by July the count increased to a maximum of $200x10^4$ which decreased

Figs. 7-8: Annual variations of Nitrate and Phosphate variations at Cherai and Valappu stations



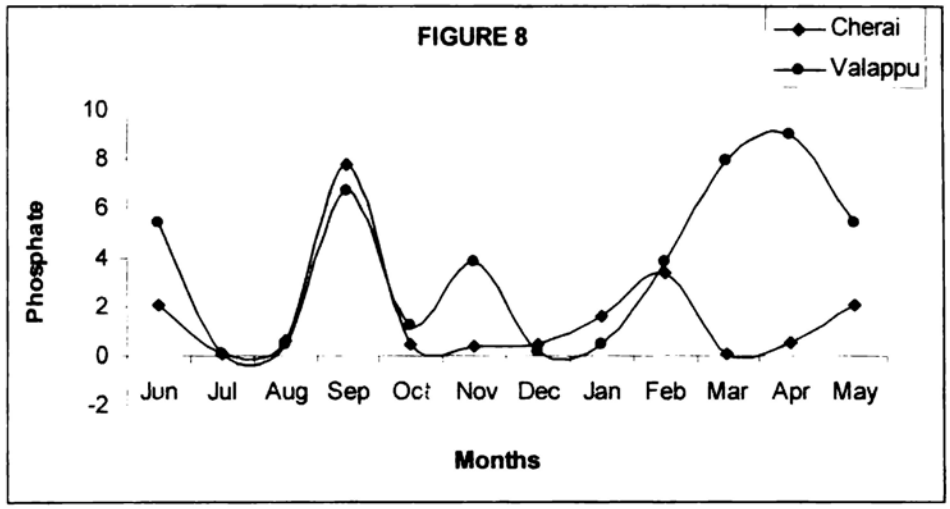
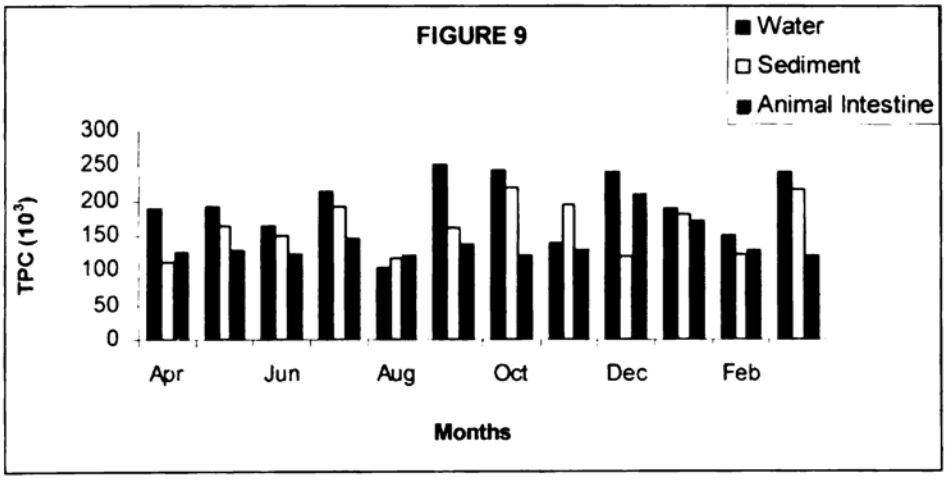


Fig. 9: Annual variations of TPC in water, Sediment and Animal intestine at Cherai station



during October and November. In March TLC was negligible while in Valappu the TLC in water sample (fig 15) showed very high values in April 2001 and July 2001, while February showed the least count ($9x10^3$). No TLC was recorded during January and March.

TLC in the sediment sample at Cherai recorded a maximum of $200x10^3$ in the month of May while June-July showed very low values minimum of $1x10^3$ was recorded during April 2001 while October, November, December recorded no lactobacillus count. In Valappu TLC in the sediment (fig 15) had the maximum count in July and $100x10^3$/ml and a minimum of $2x10^3$/ml during January. Certain months recorded very low lactobacillus counts.

TLC of shrimp intestine from Cherai was very low compared to sediment and water sample, a maximum of $20x10^3$ was recorded in May while rest of the months showed very low values (fig 12). In Valappu also the TLC was very low, a maximum of $15x10^3$/ml was recorded in May while rest of the months showed very low values (fig 15). The results as whole states that the occurrence of the lactobacillus was lower than bacillus species in both the stations.

Diversity indices of Bacillus and Lactobacillus spp in Valappu showed that index of dominance was higher in the post monsoon period.

Species richness was also higher during the post monsoon period while diversity index and evenness index exhibited high values during monsoon and pre-monsoon period (Table 35). Diversity indices in Cherai showed that index of dominance and species richness index was higher during the post monsoon period while diversity index and evenness index showed values in monsoon period (Table 36).

4.5 Hydro biological Parameters

At Cherai the maximum temperature (fig 2) was recorded in April 2001 (34°C) and a minimum of 30.5°C was recorded in August 2001.While Valappu had a maximum of 31°C during November (fig 2).

Salinity reached a maximum of 28ppt at Cherai during March and April (fig 3) while a minimum of 4ppt was recorded during July. Valappu showed maximum salinity during April 2001 (24ppt) while the lowest salinity of 3.5ppt was recorded in June 2001 (fig 3).

Dissolved oxygen was highest in March 2001 (6.4mg/l) and December (6mg.l) and a lowest of 4.2mg/l during April 2001 from Cherai (fig 4) while Valappu showed the highest range of D.O in October 2001 (6.5mg/l) and a minimum of 3.2mg/l in December (fig 4).

pH was recorded maximum during June 2001 (9.2) at Valappu station while a minimum of (7.1) was recorded in April 2001 (fig 5). Cherai

Fig.10: Annual variations of Bacillus in water, Sediment and Animal intestine at Cherai station

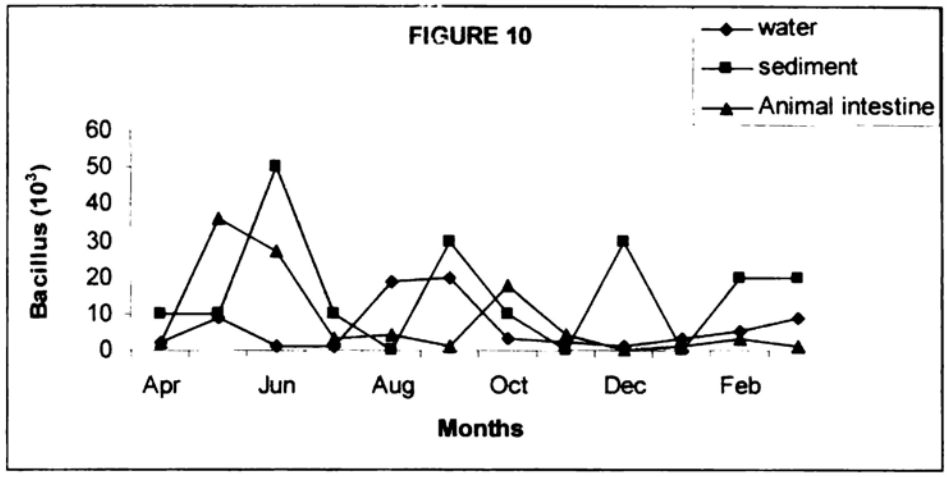


Fig. 11: Annual variations of Lactobacillus in water, Sediment and Animal intestine at Cherai station

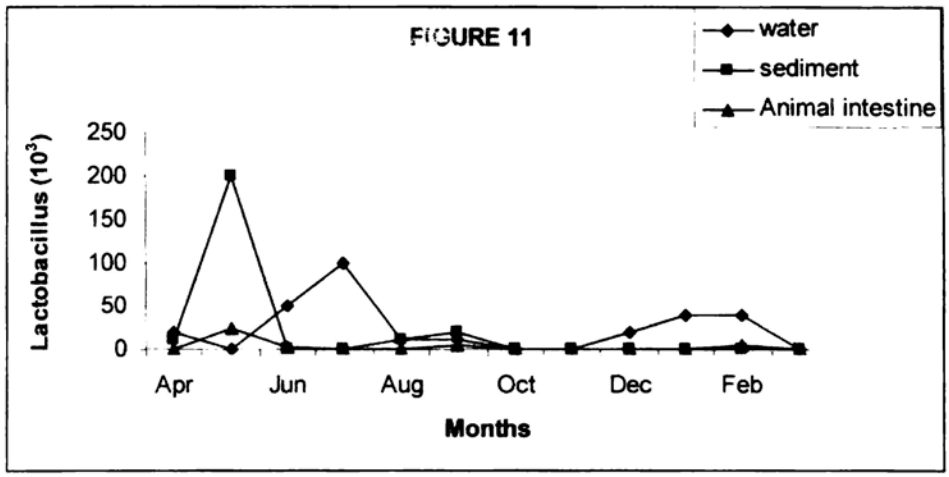
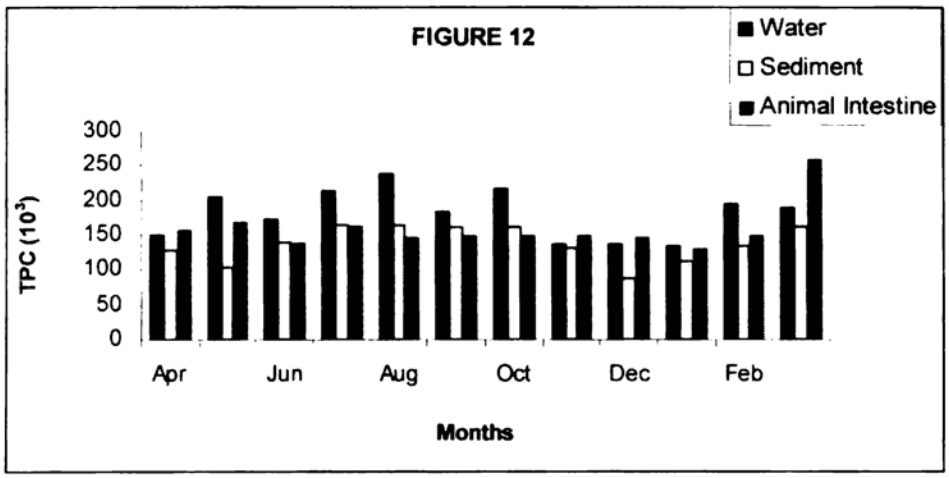


Fig. 12: Annual variations of TPC in water, sediment and Animal intestine at Valappu station



recorded a maximum of 8.4 during March 2002 while a minimum of 7.4 was recorded in January 2002.

Annual variation of nutrient values showed (fig 6) that NH_3 recorded highest value in January 17.6μg/l at Valappu while Cherai recorded lowest value in January (0.45 μgatm/l). Valappu recorded lowest value in December 2001(0.09 μgatm/l) while Cherai recorded highest value is same (2001) 9.8 μgatm/l (fig 6).

Phosphate recorded a maximum 8.9mgat/l in April 2001 at Valappu (fig 9) and a maximum of 0.04 μgatm/l in July 2001. Cherai recorded highest value 7.92mgat/l in September 2001 (fig 9). While the lowest value was recorded in April 2001 (0.04μgatm/l) (fig 9).

Highest value of (7.92μgatm/l) nitrate was recorded from Cherai station (fig 8) in April 2001 while the lowest value was recorded and December (0.097μgal/l).Valappu showed a maximum of 2/16μgatm/l in September 2001(fig 8) while a maximum of (0.096μgatm/l) in November 2001.

Nitrite showed a highest values in March 2001 (2.34gmat/l) from Cherai station (fig 7) while a maximum of 0.37mgat/l in November December of 3.96Mgat/l. Valappu recorded the highest value in October 2001 while a maximum of 2001(fig 7) 0.48μgatm/l was recorded in March 2002.

Fig. 13: Annual variations of Bacillus in water, sediment and Animal intestine at Valappu station

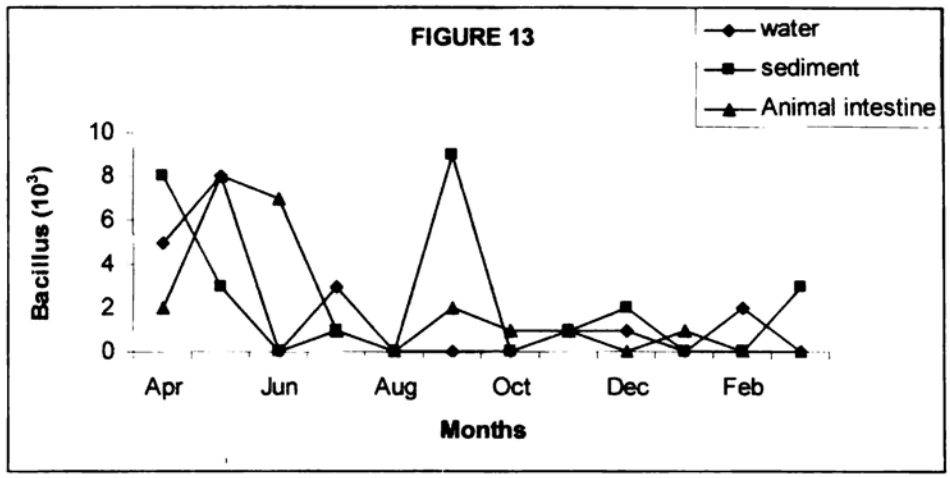
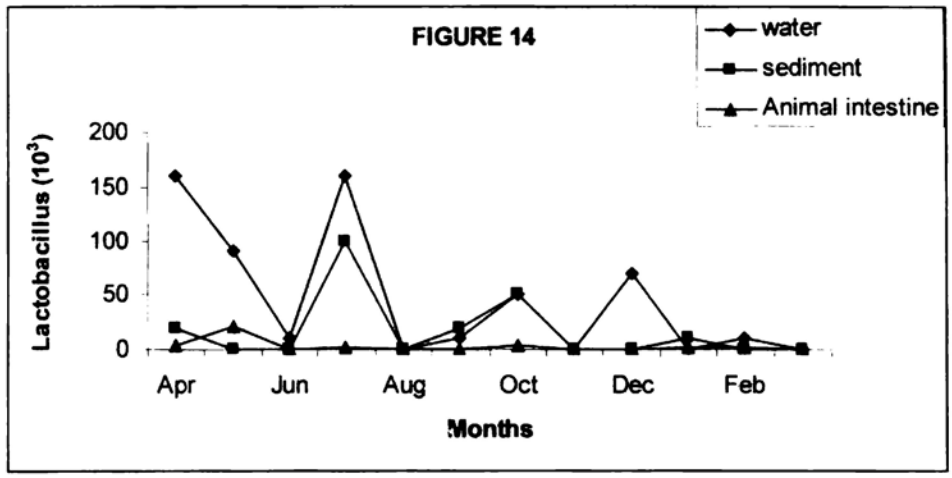


Fig. 14: Annual variations of Lactobacillus in Water, Sediment and Animmal intestine at Valappu station



4.6 To study the relation between TPC, TBC and TLC with the eight environmental parameters at Cherai and Valappu

Pearson correlation matrix were formed to find the amount of interdependency of the Variables (Temperature, Salinity, Dissolved Oxygen, pH, ammonia, nitrate, nitrite, phosphate) cited above table1. The correlation matrix between TPC of water and the environmental parameters studied from Cherai (table 1) and (table 4) showed that total bacterial count of water was significantly positively correlated with dissolved Oxygen and Phosphate ($P< 0.05$), while matrix of correlation of the total Bacillus count (TBC of water environmental parameter of Cherai stations showed that (Table2, Table4), TBC was significantly negatively correlated with temperature ($P<0.05$) and while there were no positively significant correlation with the any of the environmental parameters.

Matrix correlation of the total lactobacillus count of water and the environmental parameters at Cherai station revealed that no significantly positive correlation existed between TLC and the environmental parameters (table3 and table4) but TLC is significantly negatively correlated with pH at 5% level.

Correlation matrix of total plate count of sediment and the environmental parameters of Cherai (table5 & table7) shows that TPC is significantly negatively correlated to nitrate at 5%. Matrix correlation of

Tabel:1 Pearson correlation matrix of TPC at Cherai station (WATER)

	TPC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
TPC	1.000								
TEMP	-0.028	1.000							
SALINITY	0.117	0.327	1.000						
DO	0.544	-0.076	-0.130	1.000					
PH	0.294	-0.182	0.168	0.362	1.000				
NH3	0.114	0.491	-0.100	0.243	0.163	1.000			
NO2	0.211	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.189	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	0.561	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table:2 Pearson correlation matrix of Bacillus at Cherai station

	Bacillus	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
Bacillus	1.000								
TEMP	-0.539	1.000							
SALINITY	-0.134	0.327	1.000						
DO	0.002	-0.076	-0.130	1.000					
PH	0.240	-0.182	0.168	0.362	1.000				
NH3	-0.387	0.491	-0.100	0.243	0.163	1.000			
NO2	-0.311	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.253	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	0.422	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

total Bacillus count in sediment and the environmental parameters of Cherai station showed a significantly positive correlation between TBC and Ammonia at 5% level (table8 & table5). While a negative correlation matrix of total lactobacillus count (TLC) in the sediment sample and the environmental parameters of the Cherai station revealed (table9 & table 5) that no significant correlation existed between TLC and environmental parameters.

4.7 Correlation matrix in Cherai

Pearsons correlation matrix of TPC in the shrimp intestine and environmental parameters at Cherai (table6 & table13) showed that no significant positive correlation ship existed between TPC of s1 as the environmental parameters 1% level.

Correlation matrix of the total bacillus count (TBC) in the shrimp intestine and the environmental parameters at Cherai (Table 14 & Table 6) showed no significant correlation between TBC and any of the environmental parameters.

Correlation matrix of the total lactobacillus count in the shrimp intestine and the environmental parameters at Cherai (table15& table6) showed that no significant correlation existed between TLC and the environmental parameters.

Number of observations: 12

Table:3 Pearson correlation matrix of Lactobacillus at cherai station

	LB	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LB	1.000								
TEMP	0.248	1.000							
SALINITY	-0.296	0.327	1.000						
DO	-0.157	-0.076	-0.130	1.000					
PH	-0.566	-0.182	0.168	0.362	1.000				
NH3	-0.123	0.491	-0.100	0.243	0.163	1.000			
NO2	0.266	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.011	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	-0.347	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

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Tabel:7 Number of observations: 12

Pearson correlation matrix of TPC at Cherai station (sediment)

	TPC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
TPC	1.000								
TEMP	0.152	1.000							
SALINITY	0.015	0.327	1.000						
DO	0.206	-0.076	-0.130	1.000					
PH	-0.134	-0.182	0.168	0.362	1.000				
NH3	-0.339	0.491	-0.100	0.243	0.163	1.000			
NO2	-0.503	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.348	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	-0.272	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table: 8 Pearson correlation matrix of Bacillus at Cherai station (sediment)

	Bacillus	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
Bacillus	1.000								
TEMP	0.479	1.000							
SALINITY	-0.220	0.327	1.000						
DO	0.385	-0.076	-0.130	1.000					
PH	0.089	-0.182	0.168	0.362	1.000				
NH3	0.508	0.491	-0.100	0.243	0.163	1.000			
NO2	0.238	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.028	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	0.307	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table: 9 Pearson correlation matrix of Lcatobacillus at cherai station (sediment)

	LB	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LB	1.000								
TEMP	-0.144	1.000							
SALINITY	0.247	0.327	1.000						
DO	-0.260	-0.076	-0.130	1.000					
PH	0.167	-0.182	0.168	0.362	1.000				
NH3	-0.355	0.491	-0.100	0.243	0.163	1.000			
NO2	0.264	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.027	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	-0.070	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table: 13 Pearson correlation matrix of TPC at Cherai station (Intestine)

	TPC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
TPC	1.000								
TEMP	-0.436	1.000							
SALINITY	-0.002	0.327	1.000						
DO	0.471	-0.076	-0.130	1.000					
PH	0.080	-0.182	0.168	0.362	1.000				
NH3	0.058	0.491	-0.100	0.243	0.163	1.000			
NO2	0.294	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.314	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	0.394	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table: 14 Pearson correlation matrix of Bacillus at cherai (Intestine)

	Bacillus	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
Bacillus	1.000								
TEMP	-0.047	1.000							
SALINITY	-0.095	0.327	1.000						
DO	-0.216	-0.076	-0.130	1.000					
PH	0.012	-0.182	0.168	0.362	1.000				
NH3	0.099	0.491	-0.100	0.243	0.163	1.000			
NO2	0.467	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.076	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	0.048	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table: 15 Pearson correlation matrix of LB at Cherai (Intestine)

	LB	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LB	1.000								
TEMP	-0.112	1.000							
SALINITY	0.247	0.327	1.000						
DO	-0.262	-0.076	-0.130	1.000					
PH	0.001	-0.182	0.168	0.362	1.000				
NH3	-0.456	0.491	-0.100	0.243	0.163	1.000			
NO2	0.136	0.179	0.226	-0.040	0.193	0.334	1.000		
NO3	-0.137	0.456	0.350	-0.629	0.017	0.364	0.280	1.000	
PO4	-0.063	-0.326	-0.153	0.535	0.177	0.306	0.111	-0.307	1.000

Number of observations: 12

Table: 16 Pearson correlation matrix of TPC (Vallappu in water)

	TPC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
TPC	1.000								
TEMP	-0.374	1.000							
SALINITY	-0.523	0.266	1.000						
DO	0.672	-0.206	-0.530	1.000					
PH	0.403	-0.375	-0.751	0.220	1.000				
NH3	0.129	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.007	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.616	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	-0.003	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 17 Pearson correlation matrix of Bacillus (Valappu in water)

	BAC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
BAc	1.000								
TEMP	0.554	1.000							
SALINITY	0.106	0.266	1.000						
DO	0.128	-0.206	-0.530	1.000					
PH	-0.074	-0.375	-0.751	0.220	1.000				
NH3	0.075	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.084	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.025	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.436	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 18 Pearson correlation matrix of Lactobacillus (Valappu in water)

	LBC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LBc	1.000								
TEMP	0.460	1.000							
SALINITY	0.031	0.266	1.000						
DO	0.287	-0.206	-0.530	1.000					
PH	- 0.333	-0.375	-0.751	0.220	1.000				
NH3	-0.077	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.186	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.138	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.307	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 22 Pearson correlation matrix of TPC (Valappu-animal intestine)

	TPC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
TPC	1.000								
TEMP	-0.337	1.000							
SALINITY	-0.511	0.266	1.000						
DO	0.611	-0.206	-0.530	1.000					
PH	0.201	-0.375	-0.751	0.220	1.000				
NH3	-0.041	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.274	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.511	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	-0.158	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table:23 Pearson correlation matrix of Bacillus (Valappu-intestine)

	BAC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
BAC	1.000								
TEMP	-0.170	1.000							
SALINITY	-0.400	0.266	1.000						
DO	0.202	-0.206	-0.530	1.000					
PH	0.411	-0.375	-0.751	0.220	1.000				
NH3	0.031	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.496	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.551	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.302	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 24 Pearson correlation matrix of Lactobacillus (Valappu-intestine)

	LBC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LBC	1.000								
TEMP	0.210	1.000							
SALINITY	0.056	0.266	1.000						
DO	0.213	-0.206	-0.530	1.000					
PH	0.219	-0.375	-0.751	0.220	1.000				
NH3	-0.101	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.079	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.018	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.552	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 19 Pearson correlation matrix of TPC (valappu-sediment)

	TPC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
TPC	1.000								
TEMP	-0.150	1.000							
SALINITY	0.109	0.266	1.000						
DO	-0.172	-0.206	-0.530	1.000					
PH	0.004	-0.375	-0.751	0.220	1.000				
NH3	-0.191	0.191	-0.024	-0.163	0.075	1.000			
NO2	- 0.320	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.136	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.116	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 21 Pearson correlation matrix of Bacillus (Valappu-sediment)

	BAC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
BAC	1.000								
TEMP	0.087	1.000							
SALINITY	0.347	0.266	1.000						
DO	0.084	-0.206	-0.530	1.000					
PH	-0.430	-0.375	-0.751	0.220	1.000				
NH3	-0.095	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.341	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.306	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.514	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 22 Pearson correlation matrix of Lactobacillus (Valappu-sediment)

	LBC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LBC	1.000								
TEMP	-0.019	1.000							
SALINITY	-0.348	0.266	1.000						
DO	0.507	-0.206	-0.530	1.000					
PH	-0.047	-0.375	-0.751	0.220	1.000				
NH3	0.326	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.383	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.400	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	-0.118	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 21 Pearson correlation matrix of Bacillus (Valappu-sediment)

	BAC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
BAC	1.000								
TEMP	0.087	1.000							
SALINITY	0.347	0.266	1.000						
DO	0.084	-0.206	-0.530	1.000					
PH	-0.430	-0.375	-0.751	0.220	1.000				
NH3	-0.095	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.341	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.306	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	0.514	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Number of observations: 12

Table: 22 Pearson correlation matrix of Lactobacillus (Valappu-sediment)

	LBC	TEMP	SALINITY	DO	PH	NH3	NO2	NO3	PO4
LBC	1.000								
TEMP	-0.019	1.000							
SALINITY	-0.348	0.266	1.000						
DO	0.507	-0.206	-0.530	1.000					
PH	-0.047	-0.375	-0.751	0.220	1.000				
NH3	0.326	0.191	-0.024	-0.163	0.075	1.000			
NO2	0.383	0.192	-0.147	0.509	-0.013	0.225	1.000		
NO3	0.400	-0.179	-0.434	0.643	0.316	0.090	0.552	1.000	
PO4	-0.118	0.121	0.076	0.299	0.028	-0.470	0.456	0.463	1.000

Table 4: Correlation coefficients of TPC, *Bacillus* and *Lactobacillus* count with selected physico-chemical parameters of water at Cherai Station.

Parameters	TPC	*Bacillus*	*Lactobacillus*
Temperature (°C)	0.172	-0.326	0.123
Salinity (ppt)	0.117	-0.134	-0.285
Dissolved Oxygen (mg/l)	0.544*	0.002	-0.157
pH	0.294	0.240	-0.566*
Ammonia (µg at./l)	0.114	0.508*	-0.123
Nitrite (µg at./l)	0.212	-0.309	0.266
Nitrate (µg at./l)	-0.189	-0.253	-0.011
Phosphate(µgat./l)	0.561*	0.422	-0.347

(All statistically significant values are marked with *; If *, $p<0.05$; if **, $p<0.01$)

Table 5: Correlation coefficients of TPC, *Bacillus* and *Lactobacillus* count with selected physico-chemical parameters of sediment at Cherai Station.

Parameters	TPC	*Bacillus*	*Lactobacillus*
Temperature (°C)	-0.186	0.129	-0.087
Salinity (ppt)	0.015	-0.159	0.229
Dissolved Oxygen (mg/l)	0.206	0.385	-0.279
pH	-0.134	0.089	0.141
Ammonia (µg at./l)	-0.312	0.427	-0.426
Nitrite (µg at./l)	-0.503*	0.239	0.294
Nitrate (µg at./l)	-0.348	-0.228	-0.049
Phosphate(µgat./l)	-0.272	0.307	-0.098

(All statistically significant values are marked with *; If *: $p<0.05$; if **: $p<0.01$)

Table 6: The correlation coefficients of intestinal TPC, *Bacillus* & *Lactobacillus* count with selected environmental parameters at Cherai Station.

Parameters	TPC	*Bacillus*	*Lactobacillus*
Temperature (°C)	-0.171	-0.349	-0.059
Salinity (ppt)	-0.002	-0.095	0.249
Dissolved Oxygen (mg/l)	0.471	-0.216	-0.415
pH	0.080	0.012	0.083
Ammonia (µg at./l)	0.058	0.099	-0.372
Nitrite (µg at./l)	0.294	0.467	0.224
Nitrate (µg at./l)	-0.314	-0.076	-0.121
Phosphate(µgat./l)	0.394	0.048	-0.041

(All statistically significant values are marked with *; If *, $p<0.05$; if **, $p<0.01$)

Table 10: Correlation coefficients of TPC, *Bacillus* and *Lactobacillus* count with selected physico-chemical parameters of water at Valappu Station.

Parameters	TPC	*Bacillus*	*Lactobacillus*
Temperature (°C)	-0.225	0.402	0.393
Salinity (ppt)	-0.476	0.163	0.031
Dissolved Oxygen (mg/l)	0.836**	0.028	0.134
pH	0.401	-0.100	-0.333
Ammonia (µg at./l)	-0.246	-0.129	-0.077
Nitrite (µg at./l)	0.052	0.059	0.186
Nitrate (µg at./l)	0.361	-0.135	0.138
Phosphate(µgat./l)	0.019	0.451	0.307

(All statistically significant values are marked with *; If *, $p<0.05$; if **, $p<0.01$)

Table 11: Correlation coefficients of TPC, *Bacillus* and *Lactobacillus* count with selected physico-chemical parameters of sediment at Valappu Station.

Parameters	TPC	*Bacillus*	*Lactobacillus*
Temperature (°C)	0.144	0.456	-0.163
Salinity (ppt)	0.165	0.347	-0.371
Dissolved Oxygen (mg/l)	-0.197	-0.061	0.488
pH	-0.030	-0.430	-0.047
Ammonia (µg at./l)	-0.377	-0.096	0.326
Nitrite (µg at./l)	-0.378	0.341	0.383
Nitrate (µg at./l)	-0.011	0.306	0.400
Phosphate(µgat./l)	0.127	0.514*	-0.118

(All statistically significant values are marked with *; If *, $p<0.05$; if **, $p<0.01$)

Table 12: Correlation coefficients of intestinal TPC, *Bacillus* and *Lactobacillus* count with selected environmental parameters at Valappu Station.

Parameters	TPC	*Bacillus*	*Lactobacillus*
Temperature (°C)	-0.246	0.049	0.153
Salinity (ppt)	-0.511*	-0.400	0.056
Dissolved Oxygen (mg/l)	0.029	0.198	0.205
pH	0.201	0.411	0.219
Ammonia (µg at./l)	-0.041	0.031	-0.101
Nitrite (µg at./l)	0.274	0.496	0.079
Nitrate (µg at./l)	0.511*	0.551*	0.018
Phosphate(µgat./l)	-0.158	0.302	0.552*

(All statistically significant values are marked with *; If *, $p<0.05$; if **, $p<0.01$)

4.8 Correlation matrix at Valappu

Correlation matrix of total bacterial count of TPC in water and the environmental parameters at Valappu (table16 & table10) showed that there existed a significantly positively correlation with dissolved oxygen and Nitrate 1% level and significantly negative correlation ($P<0.05$) existed between salinity and TPC.

Correlation matrix of the total bacillus count (TBC) in water and environmental parameters at Valappu showed that a significantly positive correlation existed between temperature and TBC count at 5% level only (table17 &10).

Matrix correlation total lactobacillus count in water and the environmental parameters at Valappu showed that no significantly relationship existed between Lactobacillus count and any of the environmental parameters (table18 & 10).

Matrix correlation of total bacterial count sediment with environmental parameters at Valappu (table19 & 11) showed that no significant relationship existed between any of the environmental parameters and total plate count.

Matrix correlation of total bacillus count in sediment and environmental parameters at Valappu showed significantly positive correlation existed between TBC and Phosphate at 5% level (table 20 & 11). Matrix correlation of total lactobacillus count in sediment and environmental parameters at Valappu showed 5% level of significance with dissolved oxygen (table 21 & 11).

Matrix correlation of TPC of shrimp intestine with environmental parameters at Valappu showed that TPC was highly significantly positive correlation with oxygen at $P<0.01$ as nitrate at $P<0.05$ while salinity was significantly negatively correlated at 5% level (table22 & 12). Matrix correlation of Bacillus count in the shrimp intestine showed no significant correlation with the environmental parameters (table 23 & 12). Matrix correlation of total lactobacillus count in shrimp intestine with environmental parameters at Valappu showed a significantly positively correlation with phosphate only at 5% level. (Table 24 & 12)

4.9 Biochemical reactions of bacillus species isolated from Cherai

All the strains isolated from Cherai were actively motile, gram positive spore forming rods, with oval shaped spores except for *B.sphaericus* with round spores (table 25). 100% of the strains of *B.subtilis, B.pumilis, B.megaterum, B.firmus* and *B.licheniformis* had

Sl. No.	Biochemical Tests	*B.subtilis (n=18)*	%	*B.pumilus (n=11)*	%	*B.polymyxa n=6*	%	*B.megaterulm n=10*	%	*B.circulans n=7*	%
1	Gram reaction	0	100	+	100	+	100	+	100	+	100
2	Motility	+	100	+	100	+	100	+	100	+	100
3	Spore shape	oval	100	oval	100	oval	100	oval	100	oval	100
4	Spore position	central	100	central	100	terminal	100	central	100	uvt	100
5	Growth at 45° c	+	100	+	100	d	83.3	d	100	d	100
6	Growth at 65° c	--	83.3	--	81.8	--	66.6	--	100	--	100
7	Growth at ph 5.7	+	83.3	+	63.6	+	83.3	--	71.4	d	71.4
8	Growth at 7% Nacl	+	77.7	+	81.81	--	66.6	+	71.4	d(--)	71.4
9	Catalase	+	100	+	100	+	100	+	100	+	100
10	Oxidase	-	92.3	--	100	--	100	--	100	--	100
11	Indole	--	100	--	100	--	100	--	100	--	100
12	Arginine dihydrolase	--	77.7	.		.		.		.	
13	L.V. reaction	--	100	--	100	.		--	100	d	57.14
14	Nitrate	+	72.2	--	63.6	+	50	d	40	d	71.4
15	Acid from glucose	+	100	+	90.9	+	100	+	90	+	71.4
16	Acid from arabinose	+	77.7	+	54.5	+	83.3	d	50	+	71.4
17	Acid from mannitol	+	61.11	+	36.36	+	33.3	d	50	+	71.4
18	Acid from xylose	+	77.7	+	81.81	+	100	d	90	+	71.42
19	Gas from glucose	--		--		+	83.33	--		--	
20	Anaerobic Growth in glucose	--	83.33	--	72.7	+	83.33	--	80	d	57.14
21	Citrate	+	55.5	+	54.5	--	66.6	+	80	--	100
22	Starch	+	100	--	90.9	+	100	+	100	+	28.5
23	Gelatin	+	100	+	90.9	+	83.33	+	80	+	85.71
24	Caesin	+	88.8	+	72.7	+	50	+	70	d	0
25	Urease	d	33.33	--	63.63	+	0	d	20	--	42.8
26	Voges Proskauer	+	61.11	+	54.54	+	50	--	70	--	42.8
27	Tyrosine decomposition	--	38.8	--	63.6	--	50	d	10	--	57.14

Biochemical Tests	*B.Firmisn=6*	%	*B.brevis n=4*	%	*B.coagulans n=3*	%	*B.sphaericus n=3*	%	*B.lichenIformisn =3*	%	*B.stearo thermophilus n=2*	%
Gram reaction	+	100	+	100	+	100	+	100	+	100	+	100
Motility	+	100	+	100	+	100	+	100	+	100	+	100
Spore shape	oval	100	oval	100	oval	100	round	100	oval	100	oval	100
Spore position	central	100	uvt	100	uvt	100	terminal	100	central	100	terminal	100
Growth at 45° c	d	83.3	+	100	+	100	d	100	+	100	+	100
Growth at 65° c	--	100	d	100	d	66.6	--	100	--	100	+	100
Growth at ph 5.7	--	83.3	+	100	+	66.6	d	100	+	66.6	--	100
Growth at 7% Nacl	+	66.6	--	50	--	100	d	100	+	66.6	--	100
Catalase	+	100	+	100	+	100	+	100	+	100	d	50
Oxidase	--	100	--	100	--	100	--	100	+	100	--	100
Indole	--	100	--	100	--	100	--	100	--	100	--	100
Arginine dihydrolase					--	33.3			+			
L.V. reaction	--	100	--	100	--	100	--	100	--	100	d	0
Nitrate	+	33.3	d	75	d	66.6	--	100	+	100	d	100
Acid from glucose	w	66.6	d	75	(d) +	66.6	--	100	+	100	+	100
Acid from arabinose	d	66.6	--	25	d	33.3	--	66.6	+	66.6	d	0
Acid from mannitol	+	50	d	50	d	33.3	--	33.3	+	33.3	d	100
Acid from xylose	d	66.6	--	100	d	66.6	--	100	+	100	d	50
Gas from glucose	--		--		--		--		--		--	
Anaerobic Growth in glucos	--	83.3	--	0	+	100	--	33.3	+	33.3	+	100
Citrate	--	33.3	d	25	d	0	d	0	+	66.6	d	50
Starch	+	100	--	25	+	100	--	66.6	+	100	+	100
Gelatin	+	100	+	100	--	66.6	+	66.6	+	100	--	100
Caesin	+	100	--	75	d	0	d	0	+	66.6	d	100
Urease	--	50	--	50		33.3	d	33.3	d	66.6	--	50
Voges Proskauer	--	33.3	--	25	--	33.3	--	66.6	+	66.6	d	50
Tyrosine decomposition	d	0	+	0	--	100	--	33.3		0	--	50

central spores while *B.sphaericus, B.polymyxa*, *B.stearothermophilus* had their spores terminal in position and the rest of the strains had their spores either central, terminal or sub-terminal in position.

All the strains vigorously produced the enzyme catalase except for 50% of *B.stearothermophilus*. Cytochrome oxidase was produced by 92.3% of *Bacillus subtilis* only. Indol was produced by none of the strains.Nitrate was reduced to nitrite by 100% of *B.licheniformis*, 72% of *B.subtilis*, 50% of *B.polymyxa* and 33% of *B.firmus*. While a weak reaction was exhibited by *B.megaterum* (40%), *B.circulans* (71.4%), *B.brevis* (75%), *B.coagulans* (66.6%), *B.stearothermophilus* (100%) while other strains did not reduce other strains.

4.9.1 Temperature tolerance

B.subtilis (100%), *B.pumilus* (100%), *B.brevis* (100%), *B.coagulans* (100%), *B.licheniformis* (100%), *B.stearothermophylus* (100%) had a very high tolerance at 45°C. while 100% of *B.megaterum*, *B.circulans*, *B.sphaericus*, 83.3% of *B.polymyxa* and *B.firmus* showed very slow growth. Active growth at 65°C was exhibited by all the strains of stearothermophilus. While only a few strains of *B.subtilis* (16.7%), *B.pumilus* (18.2%), *B.polymyxa* (33.4%) showed a positively tolerance at 65°C.

4.9.2 Salinity tolerance

A very high affinity for NaCl was exhibited by *B.subtilis* (77.7%), *B.pumilus* (81.81%), *B.megaterum* (71.4%), *B.firmus* (66.6%) and *B.licheniformis* (66.6%) while a low tolerance was exhibited by 50% of *B.brevis*, 33.4% of *B.polymyxa*. Arginine was hydrolysed by *B.licheniformis* (100%), *B.subtilis* (22.3%) and 66.7% of *B.coagulans* only, 42.86% of *B.circulans* only showed a positive reaction for licithovitelline.

4.9.3 Fermentation of carbohydrates

Except for 100% of *B.sphaericus*, 33.4% of *B.coagulans*, 22.6% of *B.circulans*, 9.1% of B.pumilus and 10% of *B.megaterum* all the strains were found to ferment glucose. 77.7% of *B.subtilis*, 54.5% of *B.pumilus*, 71.4% of *B.circulans*, 83.3% of *B.polymyxa*, 66.6% of *B.licheniformis*, 75% of *B.brevis*, 33.4% of *B.sphaericus* were able to ferment arabinose while the rest of the strain could not ferment the carbohydrate. Mannitol was fermented by almost all the strains except for 33.3% *B.sphaericus*, 50% of *B.brevis*, 66.7% of *B.licheniformis* and *B.polymyxa* 50% of *B.firmus*, *B.megaterum*, 63.4% of *B.pumilus*, 38.99% *B.subtilis* and 28.6% *B.circulancs*. Xylose was utilised by 100% of *B.polymyxa*, *B.licheniformis*, 81.81% of *B.pumilus*, 77.7% of *B.subtilis*, 71.42% of *B.circulans*, while 90% of *B.megaterum*, 66.6% *B.firmus*, 66.6% of *B.coagulans* and 50% of

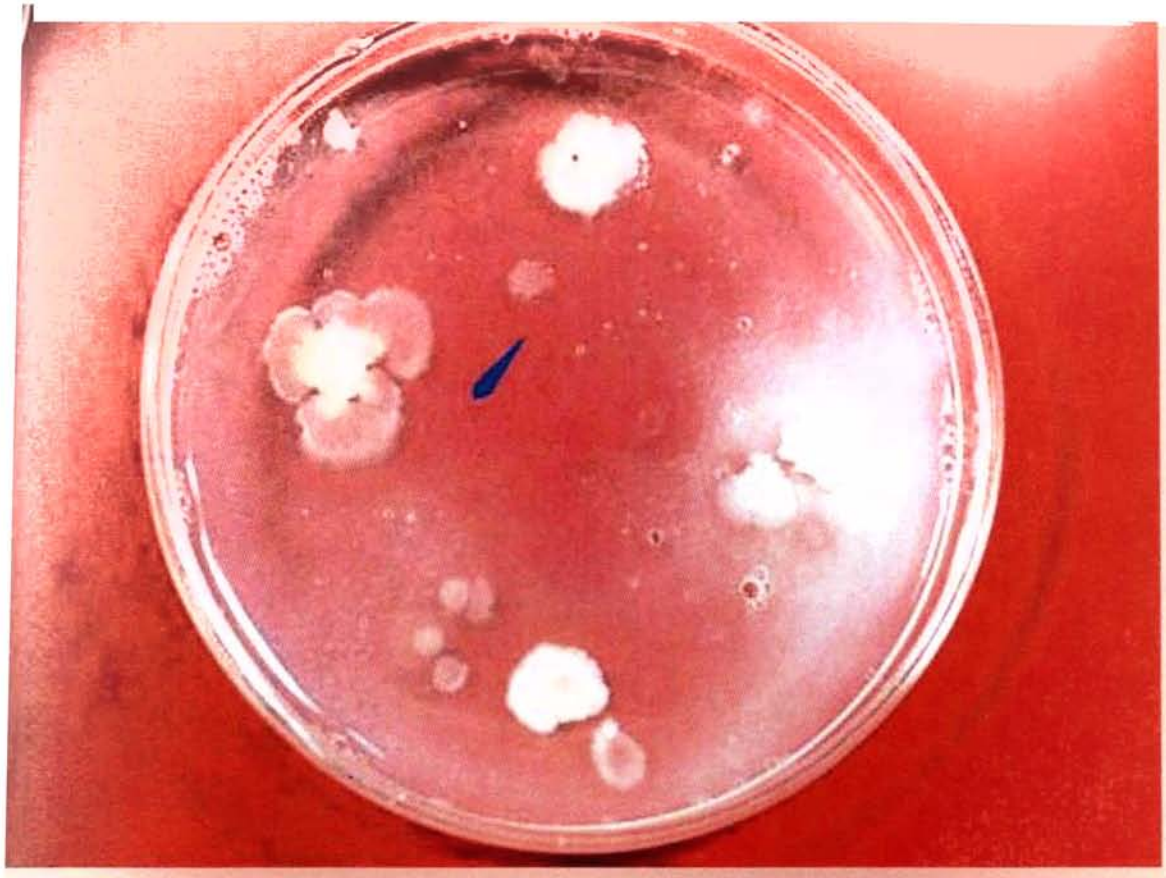

Plate 19 Tyrosine Hydrolysis of Bacillus spp

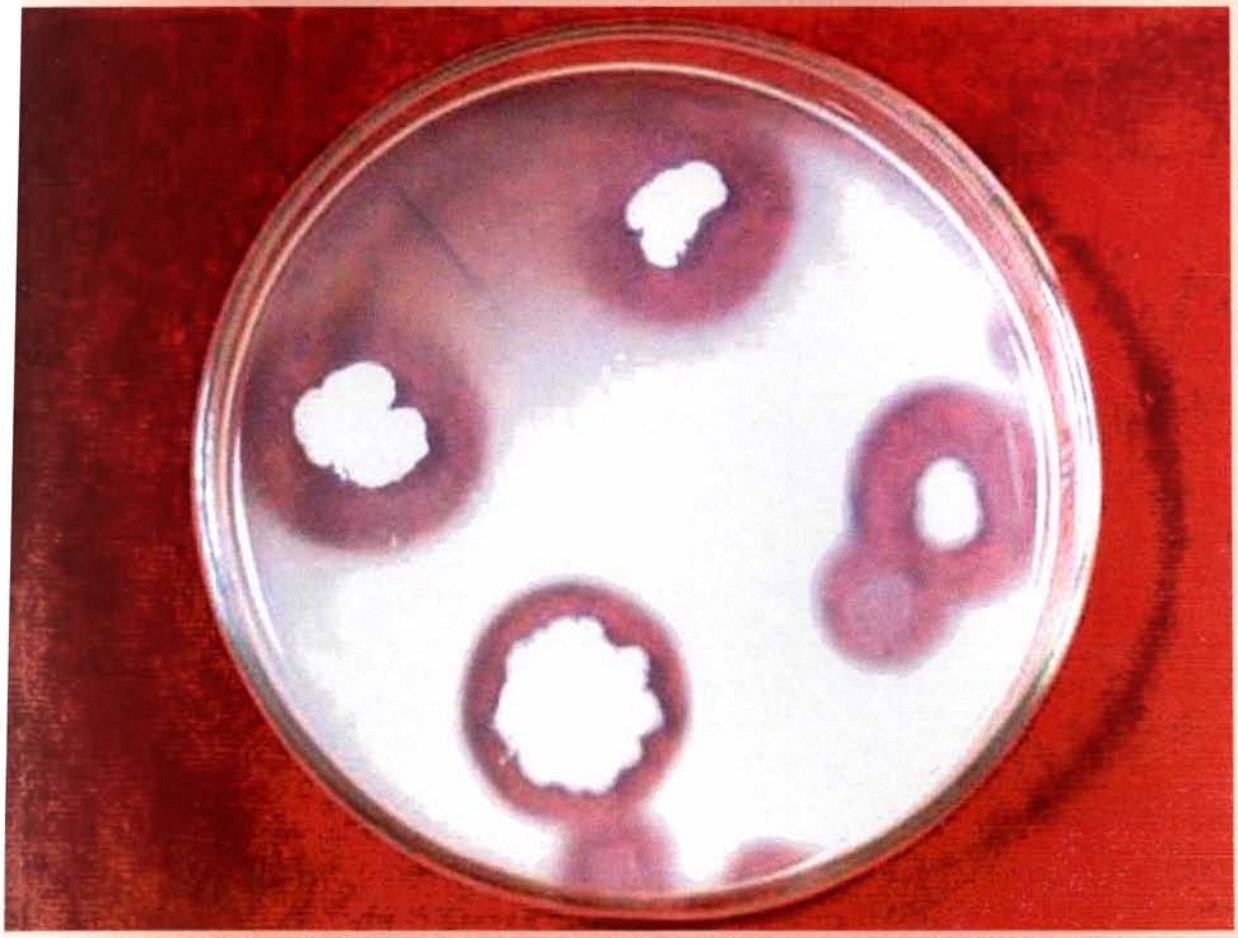

Plate 20 Gelatin Hydrolysis Exhibited by Bacillus spp

B.stearothermophilus differ in the fermentation of the carbohydrate. None other then 83.3% of *B. polymyxa* was able to produce gas from glucose.

4.9.4 Gelatinolytic Activity

B.firmus, B.brevis, B.licheniformis 100% of *B.subtilis*, 90.9% of *B.pumilus*, 80% of *B.megaterum*, 85.71% of *B. circulans*, 83.3% of *B.polymyxa*, 66.6% of *B.coagulans* and 33.4% of *B.sphaericus* had a very good gelatin hydrolysing capacity. While the rest of the strains could not hydrolyse gelatin.

4.9.5 Caseinolytic activity

Casein was utilised by 88.8% of *B.subtilis*, 72.7% of *B.pumilus*, 70% of *B.magetrum*, 50% of *B.polymyxa*, 100% of *B. firmus* and 66.6% of *B. licheniformis*, while the rest of the strains either differed in their reaction or did not show any reaction.

4.9.6 Amylolytic Activity

Starch was hydrolysed by almost all the strains except for 90.9% of *B.pumilus*, 71.5% of *B. circulans*, 75% of *B.brevis* and 33.4% of *B.sphaericus*.

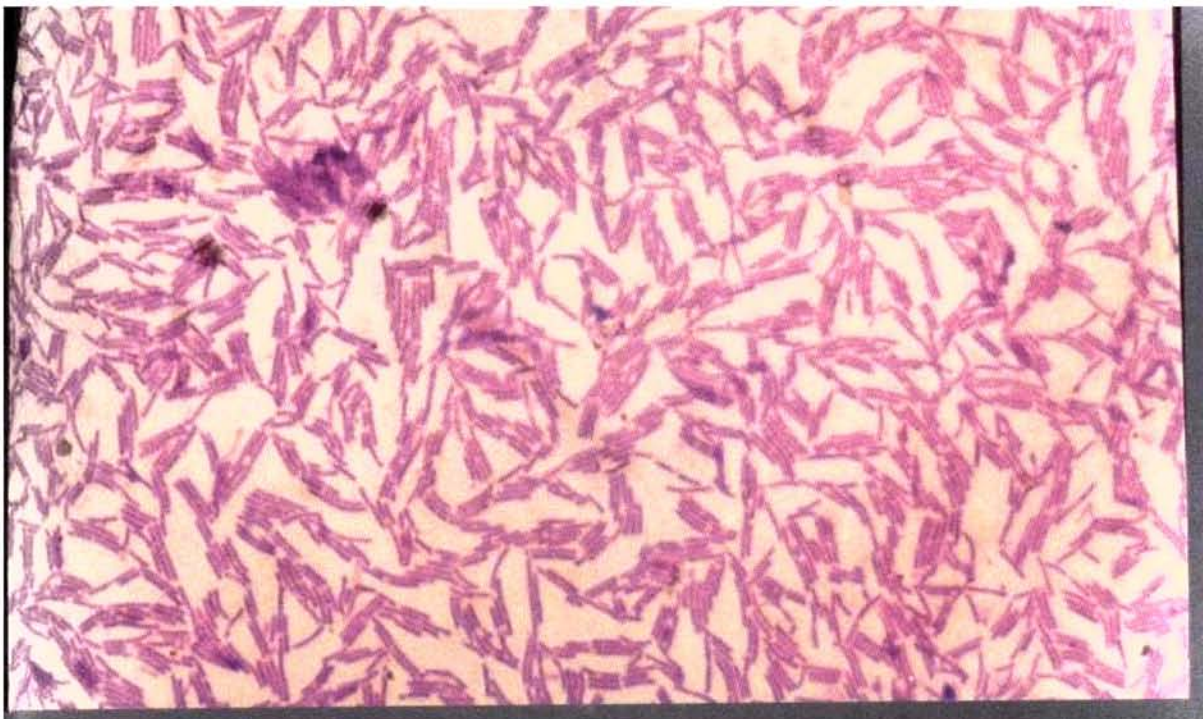

Plate 13 Microscopic Examination of Bacillus Subtilis

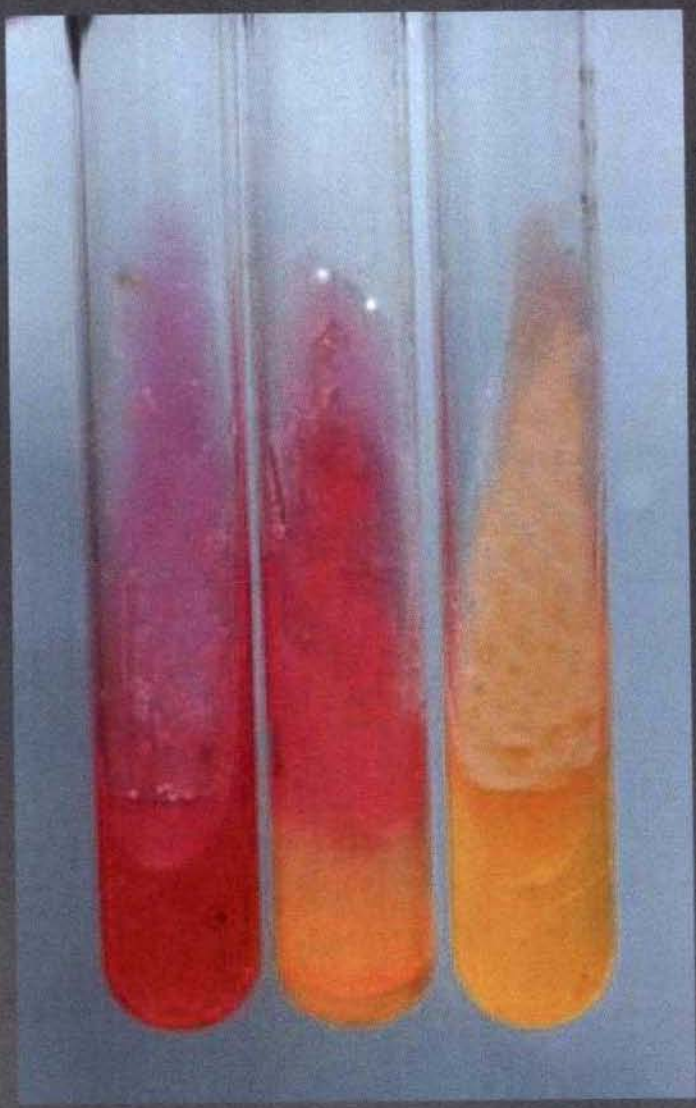

Plate 14 Ureolytic Activity of Bacillus spp

4.9.7 Citrate as sole carbon source (Plate 15)

55% of *B. subtilis*, 54.5% of *B. pumilus*, 80 % of *B.megaterum*, 33.4% of *B.polymyxa*, 66.7% of *B. firmus*, and 66.6% of *B. licheniformis* utilised citrate as the sole source of carbon.

4.9.8 Ureolytic Activity (Plate 14)

50% of *B.brevis*, 33.3% of *B.coagulans*, 66.7% of *B.sphaericus*, 33.4% of *B.licheniformis*, 50% of *B.stearothermophilus*, 66.7% *B.subtilis*, 63.6% of *B.pumilus*, 80% of *B megaterum*, 42.8% of *B.circulans*, 100% of *B. polymyxa* and 50% of *B.firmus* failed to hydrolyse urea while 33.3% of *B. subtilis*, 20% of *B.megaterum*, 33.3% of *B.sphaericus* and 66.6% of *B. licheniformis* differ in their hydrolysis of Urea.

4.9.9 Voges Proskauer Reaction

61.1% Of *B.subtilis*, 54.5% of *B.Pumilus*, 30% of *B. megaterum*, 57.2% of *B.circulans*, 50% of *B. polymyxa*, *B.stearothermophilus*, 66.7% of *B. pumilus*, *B.coagulans*, 75% of *B. brevis*, 33.4% of *B. sphaericus* and 66.6% of *B.licheniformis* were found to produce acetyl methyl carbinol while the rest of the strains failed to do so. Tyrosine was decomposed by 61.2% of *B. subtilis*, 36.4% of *B. pumilus*, 42.86% of *B. ciruclans*, 50% of *B. polymyxa*, *B. stearothermophilus* and 66.7% of *B.sphaericus* while all the other strains failed to decomposing tyrosine.

4.10 Biochemical Reactions of Bacillus Species isolated from Valappu.

All the strains isolated from Valappu (table26) were actively motile gram positive rods with oval spores, except for *B.sphaericus* with round spores. All the strains of *B. subtilis*, *B. megaterum*, *B. firmus*, *B. pumilus*, *B. lecheniformis*, and *B.circulans* had central spores while *B. sphaericus*, *B. stearothermophilus* and *B.macerans* had terminal spore and the position of spores in the case for *B. alvei*, *B.circulans*, *B.brevis*, *B. coagulans*, and *B.polymyxa* were either central, terminal or subterminal in position.

4.10.1 Tolerance of temperature, pH and salinity on growth

100% of *B.stearothermophilus* and *B. brevis*, 83% of *B. coagulans*, 80% of *B.licheniformis*, 69.2% of *B. subtilis*, 23.9% of *B. sphaericus*, 68.4% of *B.megaterum*, 68.7% of *B. firmus*, 75% of *B. pumilus* and *B. alvei*, 37.5% of *B. circulans*, 25% of *B.polymyxa*, 33.4% of B.macerans could tolerate 45°C while rest of the strains had no growth at 45°C. Except for 100% of *B.stearothermophilus*, 40% of *B.licheniformis*, 14.3% of *B. brevis* all the other strains failed to grow at 65°C.

Table 28 BIO-CHEMICAL PROFILE OF BACILLUSSPECIES ISOLATED FROM STATION 1(VALAPPU) and their percentage composition

Sl. No.	Biochemical Tests	B. subtilis(n=26) %		B.sphaericus(n=21) %		B.megaterium (n=16) %		B.firmus(n=16)%		B.cereus(n=16) %		B.alvei(n=8)%		B.circulans(n=8) %	
1	Gram reaction	+	100	+	100	+	100	+	100	+	100	+	100	+	100
2	Motility	+	100	+	100	+	100	+	100	+	100	+	100	+	100
3	Spore shape	oval	100	round	100	oval	100	oval	100	oval	100	oval	100	oval	100
4	Spore position	central	100	terminal	100	central	100	central	100	central	100	central,terminal or subterminal	100	uvt	100
5	Growth at 45° c	+	69.2	d(-)	76.1	d(+)	68.4	d(+)	68.7	+	75	d(+)	75	d(--)	62.5
6	Growth at 65° c	--	100	--	100	--	100	--	100	--	100	--	100	--	100
7	Growth at ph 5.7	+	65.38	d(-)	66.6	+	78.9	--	68.7	+	87.5	--	62.5	d(--)	50
8	Growth at 7% Nacl	+	65.3	d	47.6	+	52.6	+	56.2	+	87.5	--	75	d	75
9	Catalase	+	100	+	100	+	100	+	100	+	100	+	100	+	100
10	Oxidase	+	88.4	--	100	--	100	--	100	--	100	--	100	--	100
11	Indole	--	100	--	100	--	100	--	100	--	100	+	100	--	100
12	Arginine dihydrolase	--	76.9												
13	L.V. reaction	--	100	--	100			--	100	--	100	--	100		
14	Nitrate	+	84.6	--	71	d(+)	52	+	68	-	56.25	--	75	d	87
15	Acid from glucose	+	100	--	90.4	+	89.4	w	68.7	+	81.2	+	75	+	100
16	Acid from arabinose	+	50	--	71.4	d	42.1	d	31.25	+	62.5	--	25	+	37.5
17	Acid from mannitol	+	46.15	--	76.19	d	31.57	+	37.5	+	12.5	--	62.5	+	37.5
18	Acid from xylose	+	42.3	--	76.19	d	47.36	d	62.5	+	43.75	--	37.5	+	75
19	Gas from glucose	--	53.8	--	52.6	--	73.6	--	93.75	--	68.75	--	62.5	--	75
20	Anaerobic Growth in glucose	--	69.2	--	71	--	47.3	--	43.7	--	56.2	+	62.5	d	50
21	Citrate	+	76.9	d	80.9	+	94.7	--	68.75	+	81.25	--	37.5	--	75
22	Starch	+	100	--	57.14	+	73.68	+	87.5	--	62.5	+	50	+	37.5
23	Gelatin	+	100	+	57.14	+	89.47	+	62.5	+	31.25	+	62.5	+	75
24	Casein	+	92.3	d	90.4	+	89.47	+	100	+	81.25	+	100	d	62.5
25	Urease	d	34.6	d	47.6	d	52.63	--	75	--	31.25	--	50	--	37.5
26	Voges Proskauer	+	73.07	--	66.66	--	89.4	--	68.75	+	43.75	+	50	--	75
27	Tyrosine decomposition	--	80.7	--	71.4	d	0	d	62.5			d	0	--	50

Table 26 BIO-CHEMICAL PROFILE OF BACILLUSSPECIES ISOLATED FROM STATION 1(VALAPU) and their percentage composition

Biochemical Tests	*B.brevis(n=7)*	%	*B.coagulene(n=6)*	%	*steorothermophilius n=5*		*B.lichenefor (n=5)*	%	*B.polymyxa(n=4)*	%	*B.mecerans(n=3)*	%	*B.cereus var mycoides(n=1)*	%
Gram reaction	j+	100	+	100	+	100	+	100	+	100	+	100	+	100
Motility	+	100	+	100	+	100	+	100	+	100	+	100	--	100
Spore shape	oval	100	oval	100	oval	100	oval	100	oval	100	oval	100	oval	100
Spore position	central,terminal or subterminal	100	uvl	100	terminal	100	central	100	uvl	100	terminal	100	central	100
Growth at 45° c	d(+)	100	+	83	+	100	+	80	--	75	d(--)	66.6	d--	0
Growth at 65° c	--	85.7	d	33.3	+	100	--	60	--	100	--		--	100
Growth at ph 5.7	d(+)	85.5	+	83.3	--	80	+	60	+	75	--		+	100
Growth at 7% Nacl	--	57.1	--	66.6	--	80	+	60	--	100	d(--)	66	d--	100
Catalase	+	100	+	100	d	25	+	80	+	100	+	66.6	+	100
Oxidase	--	100	--	100	--	100	+	100	--	100	--	100	--	100
Indole	--	100	--	100	--	100	--	100	--	100	--	100	--	100
Arginine dihydrolase			--	66.6			+	80						
L.V. reaction	--		--	100	d	60	--	100	--	100	--	:00	+	100
Nitrate	d	71.4	d(--)	83.3	d(--)	80	+	100	+	100	+	66.6	+	100
Acid from glucose	d(+)	71.4	d(+)	100	d(+)	80	+	80	+	100	+	66.6	+	100
Acid from arabinose	--	71.4	d	83.3	--	40	+	40	+	75	+	100	--	100
Acid from mannitol	d	28.5	d	16.6	--	80	+	40	+	50	+	33.3	--	0
Acid from xylose	--	57.14	d	50	--	80	+	80	+	25	+	66.6	--	0
Gas from glucose	--	85.7	--	66.6	--	100	--	60	+	75	+	66.6	--	100
Anaerobic Growth in glucose	--	42.8	+	66.6	+	80	+	40	+	75	+	33.3	+	100
Citrate	d	57.14	d	83.3	--	100	+	60	--	50	--	33.3	+	100
Starch	--	42.8	+	83.3	+	60	+	60	+	75	+	66.6	+	100
Gelatin	+	71.4	--	71.4	d	80	+	80	+	75	+	66.6	+	0
Casein	+	28.5	d	33.3	d	40	+	60	+	50	--	66.6	+	0
Urease	--	28.5	--	50	--	60	d	20	+	25	--	66.6	d	100
Voges Proskauer	--	28.5	d	16.6	--	40	+	40	+	50	--	33.3	+	100
Tyrosine decomposition	+	57.14	--	50	--	40	--	60	--	25	--	33.3	+	0

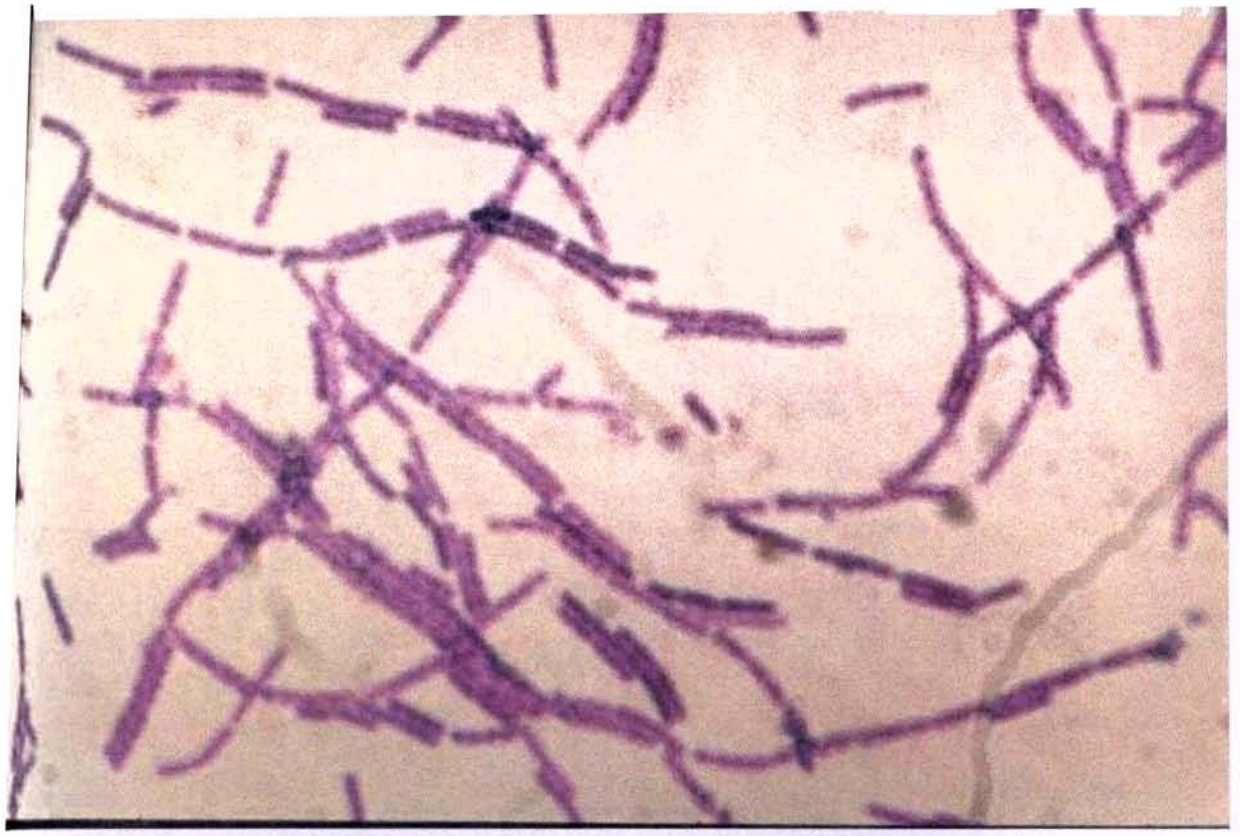

Plate11 Rods of Bacillus spp showing alignment

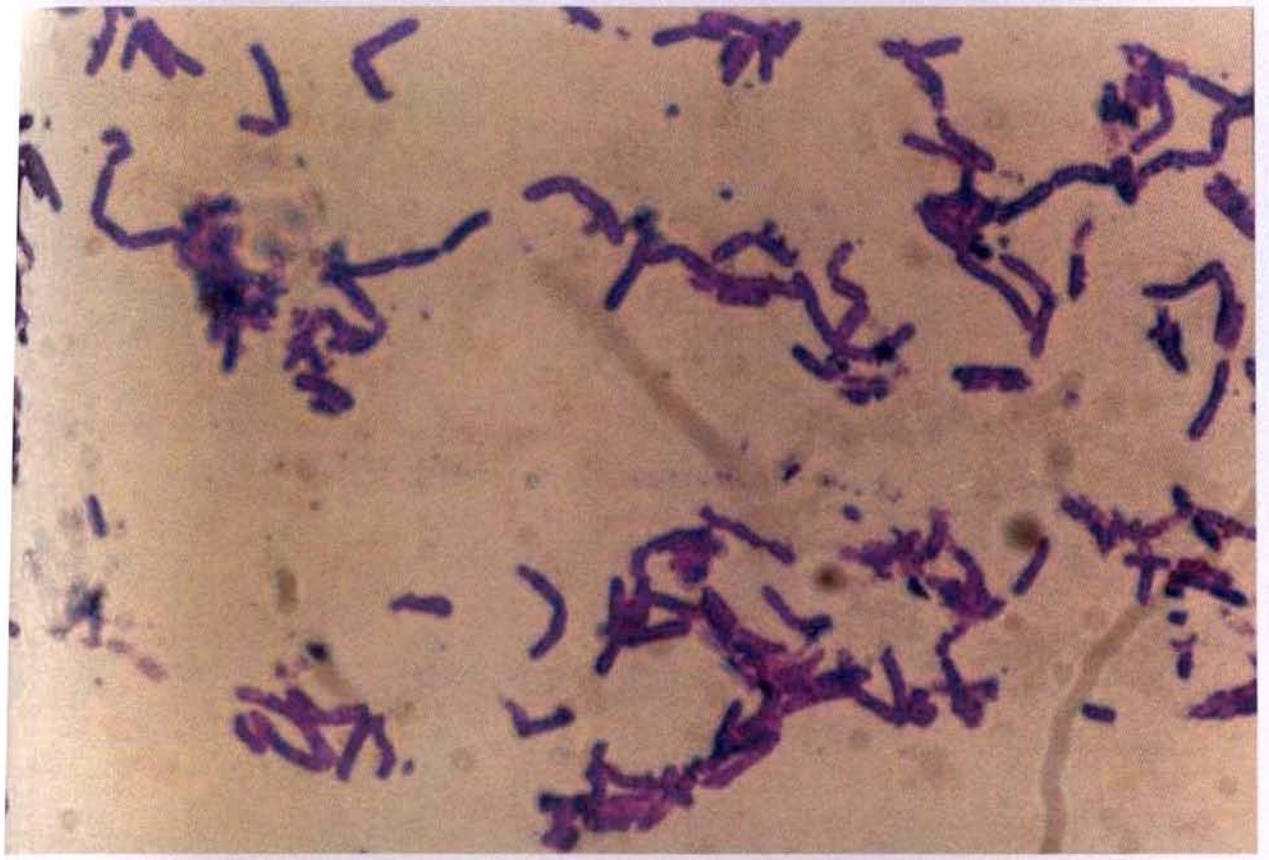

Plate12 Bacillus Species showing the Dividing Phase

4.10.2 Effect of pH on growth

100% of *B. cereus*, 65.38% of *B.subtilis*, 78.9% of *B. megaterum*, 87.5% of *B.pumilus*, 85.5% of *B. brevis*, 83.3% of *B.coagulans*, 60% of *B. licheniformis*, 75% of *B. polymyxa*, 33.4% of *B. sphaericus*, 31.3% of *B. firmus*, 37.5% of *B. alvei*, 50% of *B. circulans*, 20% of *B.stearothermophilus*, and *B.macerans* had good growth at 5.7 pH while rest of the strains had no tolerance at 5.7pH.

4.10.3 Sodium- Chloride Tolerance

65.3% of *B.subtilis*, 52.6% of *B.megaterum*,20% of *B.stearothermophilus*, 60% of *B.licheniformis*, 34% of *B. mecarans* showed great tolerance to 7% of NaCl while rest of the strains showed a negative tolerance.

4.10.4 Production of Catalase, Oxidase and Indole

All the strains were able to produce the enzyme catalase except 20% of *B.Licheniformis*, 33.4% of *B. macerans* and 75 % of *B. stearothermophilus*. Except 100% of *B. licheniformis*, all the other strains were not able to produce Cytochrome Oxidise. Indole production was found in 100% of *B. alvei* while other strains fail to produce indole.

4.10.5 Arginine Hydrolysis (Plate 16)

The amino acid arginine was hydrolysed by 80% of B. licheniformis, 23.1% of B. subtilis and 33.4% of B. coagulans only. Except 100% of B. series and 40% of B. stearothermophilus rest of the other strains failed to show any reaction with arginine fermentation of glucose was very high in all the strains of *B. subtilis*, *B. circulans*, *B. coagulans*, *B. polymyxa* and *B. cereus* (mycoides) followed by B. megaterum (89.4%), *B.pumilus* (81.2%) . 80% of *B. licheniformis*, 66.6% of B. *macerans* and 75% of *B. alvei*. The rest of the strains either weakly fermented or did not ferment glucose at all.

Fermentation of Arabinose was very high for *B. macerans* (100%), *B.alvei* and *B. polymyxa* (75%), *B.subtilis* (50%) and *B. pumilus*. (62.5%) *B.stearothermophilus* (60%) while a medium reaction was exhibited by *B. brevis* (28.6%), *B. circulans* (37.5%). B. megaterum (42.1%), *B. firmus* (31.2%) and *B. coagulans* (83.3%).

Fermentation of mannitol was exhibited only by 46.15% of *B.subtilis*, 37.5% of *B. firmus* and *B. circulans* 40% of *lechenifirmus*, 50% of polymyxa and 33.3% of macerans. While B. sphaericus (76.19); B. elvei (62.5%), *B.stearothermophilus* (80%) failed to ferment mannitol. Weak reaction was exhibited by *B. brevis* (28.5%) B. *coagulans* (16.6%), B. megaterum (31.57%).

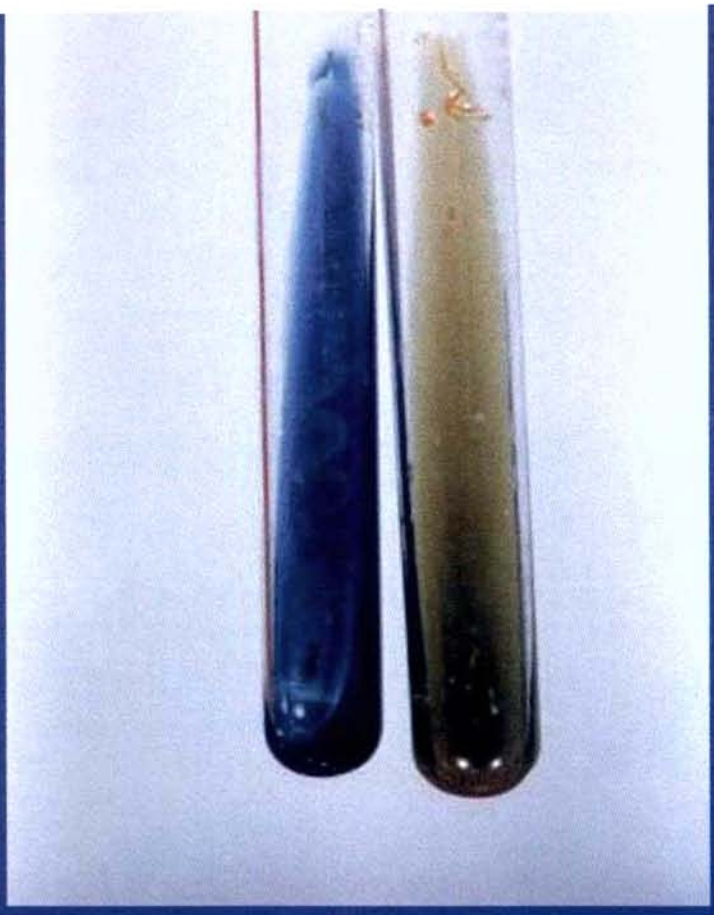

Plate 15 Citrate Utilization by Bacillus spp

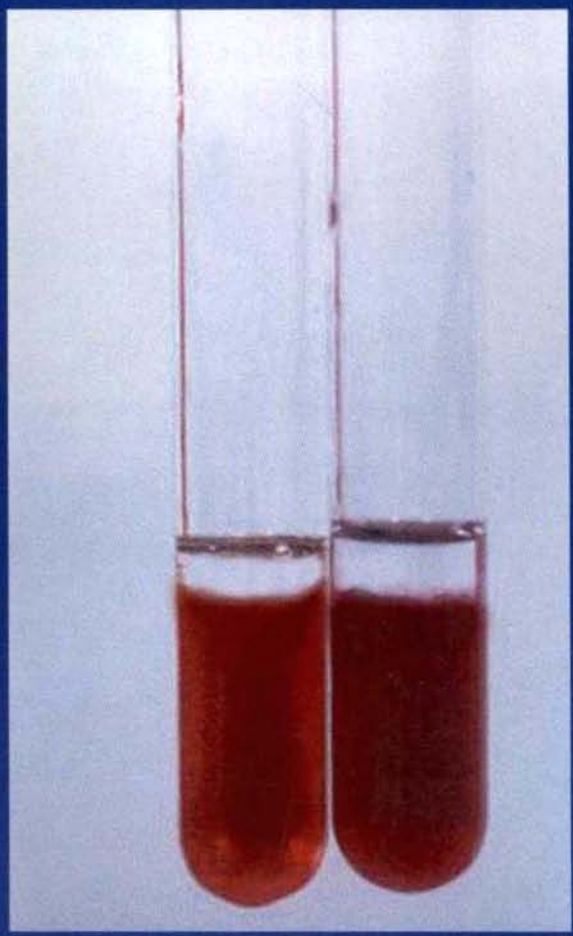

Plate 16 Arginine Hydrolysis of Bacillus spp

Xylose was highly fermented by *B. circulans*(75%), *B. lichenifirmus* (80%) and *B. macerans* (66.6%) followed by *B.pumilus* (43.75%), *B. subtilis* (42.3%) and *B.polymyxa* while a weak fermentation was shown by *B. megterum* (47.36%) *B.firmus* (62.5%), *B. coagulans* (50%) and the rest of the strains failed to do so. Production of gas from glucose was high in *B. polymyxa* (75%), *B. macerans* (66.6%) while most it was either low or negligible in other strains.

4.10.6 Gelatinolic activity: -

Gelatin hydrolysing was very high in *B.subtilis* (100%), *B. megaterum* (89.47%). *B.stearothermophilus* and *B. licheniformis* (80%), *B. polymyxa* and *B.circulans* (75%), *B. brevis* (71.4%), *B. macerans* (66.6%) and *B. firmus*(62.5%). While only a medium activity was exhibited by most of the other strains and *B.coagulans* (71.4%) did not hydrolyse gelatin. (Plate 20)

4.10.7 Citrate utilization:

Citrate as a sole source of carbon was utilised by more then 50% of the strains of *B. subtilis, B. sphaericus, and B. megaterum, B. pumilus, B. coagulans, B.licheniformis* and 1005 of B mycoides. While 68.75% of *B. firmus*, 75% of *B.circulans*, 100% of *B.Stearothermophilus*, 57.14% of *B.*

brevis; 50% of B.*polymyxa*, *B. alvei* (37.5%) and *B. macerans* (33.3%) did not utilize citrate.(Plate 15)

4.10.8 Amylolytic activity: -

Amylolytic activity was very high in 100% of *B. subtilis and B. cereus*, 73.68% of *B.megaterum*, and 87.5% of *B. firmus*, 83.3% of *B. coagulans*, 66.6% *B. macerans* and 755 of B. polymyxa while in strains amylolitic activity was less than 50% and certain other failed to hydrolyse starch.

4.10.9 Caseinolytic Activity

Casein was hydrolysed by 100% of *B.firmus* and *B. alvei* more than 80% *B.subtilis, B. sphaericus, B. megaterum, B. pumilus*, more than 50% of *B. circulans, B. licheniformis, B. macerans* and a few strains of *B. brevis, B. coagulans, B.stearothermophilus.* While the rest of the strain did not exhibit caesinolytic activity.

4.10.10 Ureolytic Activity

Very high Ureolytic activity was recorded by species like *B. brevis*(71.5%), *B.circulans* 62.5%, *B. megaterum* 52.63% and *B. pumilus* 68,75%. *B. sphaericus* 47.6%, *B. subtilis* 34.6%, *B. macerans* 33.4%, *B.*

firmus 25% and *B. licheniformis* showed only a medium ureolytic activity. (Plate 14)

4.10.11 Voges Proskauer Reaction: -

Acetyl methyl carbinol production was 100% for *B. cereus*, 71.5% for *B.brevis*, 73.04% for *B. subtilis* and 50% for *B. alvei* and 66.7% of *B. macerans* while in the case, *B.sphaericus*, *B. firmus*, *B. pumilus*, *B. circulans*, *B. coagulans*, *B.licheniformis* acetyl methyl carbinol production was less than 50%.

4.10.12 Tyrosine Composition

Tyrosine degradation was very high in species like B. polymyxa75%, B macerans 66.7%, and *B. stearothermophilus* 60%, *B. brevis* 57.14%, and *B. firmus* (62.5%) while a low degradation was exhibited by species like *B. subtilis* 19.3%, *B.sphaericus* 28.6%, *B. licheniformis* 40% and *B. pumilus* 43.75%. (Plate 19)

4.11 Percentage composition of Bacillus species isolated from from the two sampling sites. (Cherai and Valappu)

The total percentage composition of bacillus species isolated from the two sampling sites given in table (27, 28, 29). The intensity of the

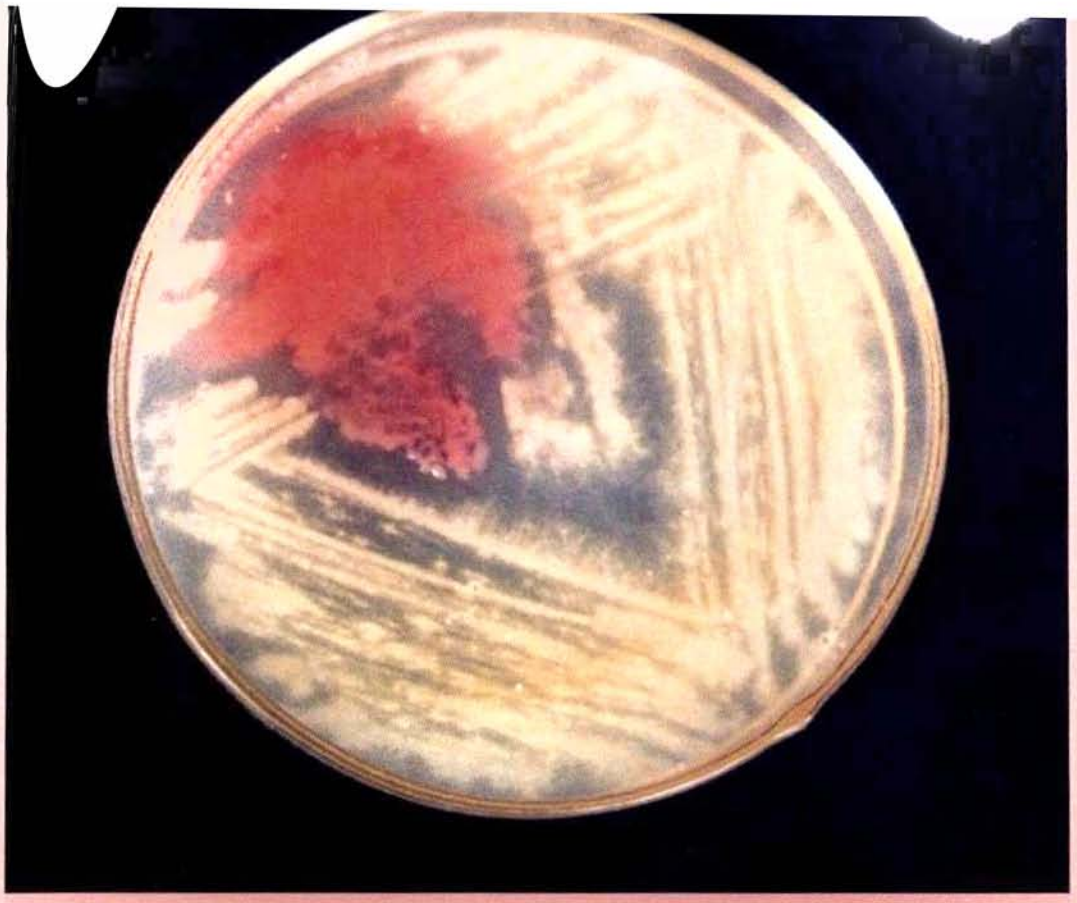

Plate 9 Acetoin Production by Bacillus licheniformis

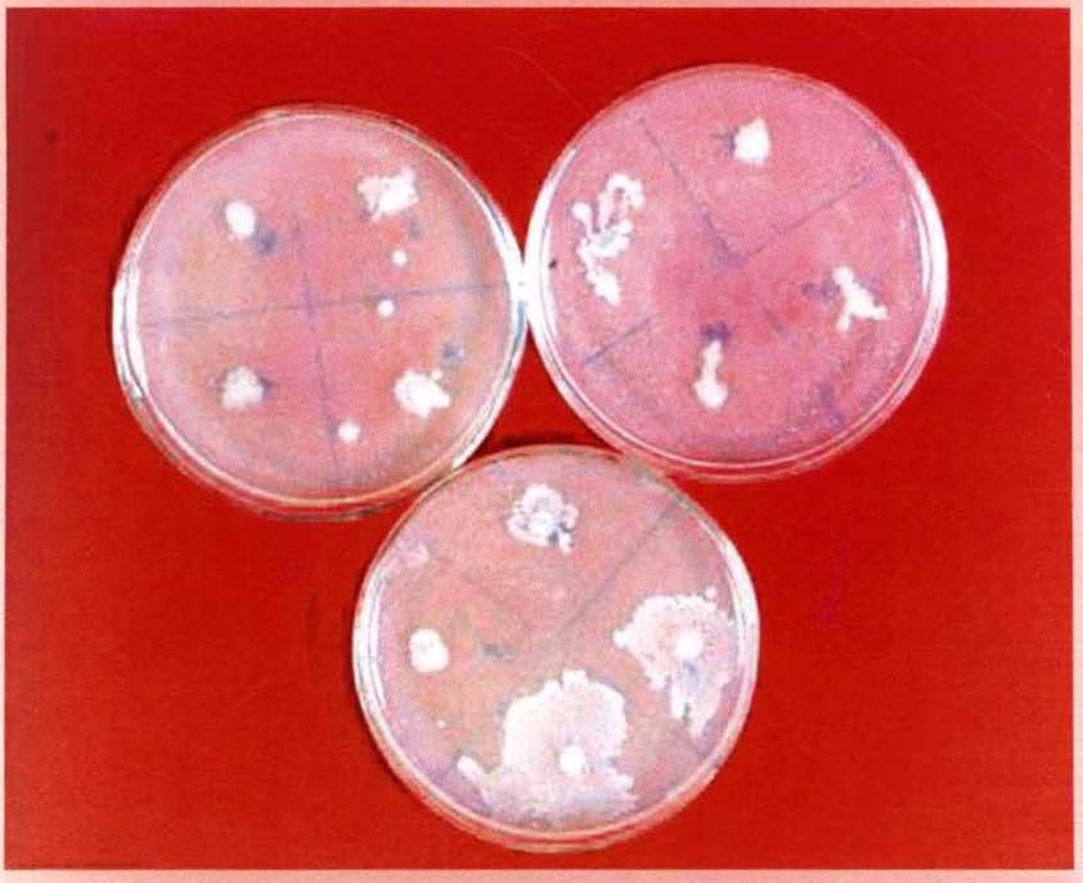

Plate 10 Colonies of Different Bacillus Species

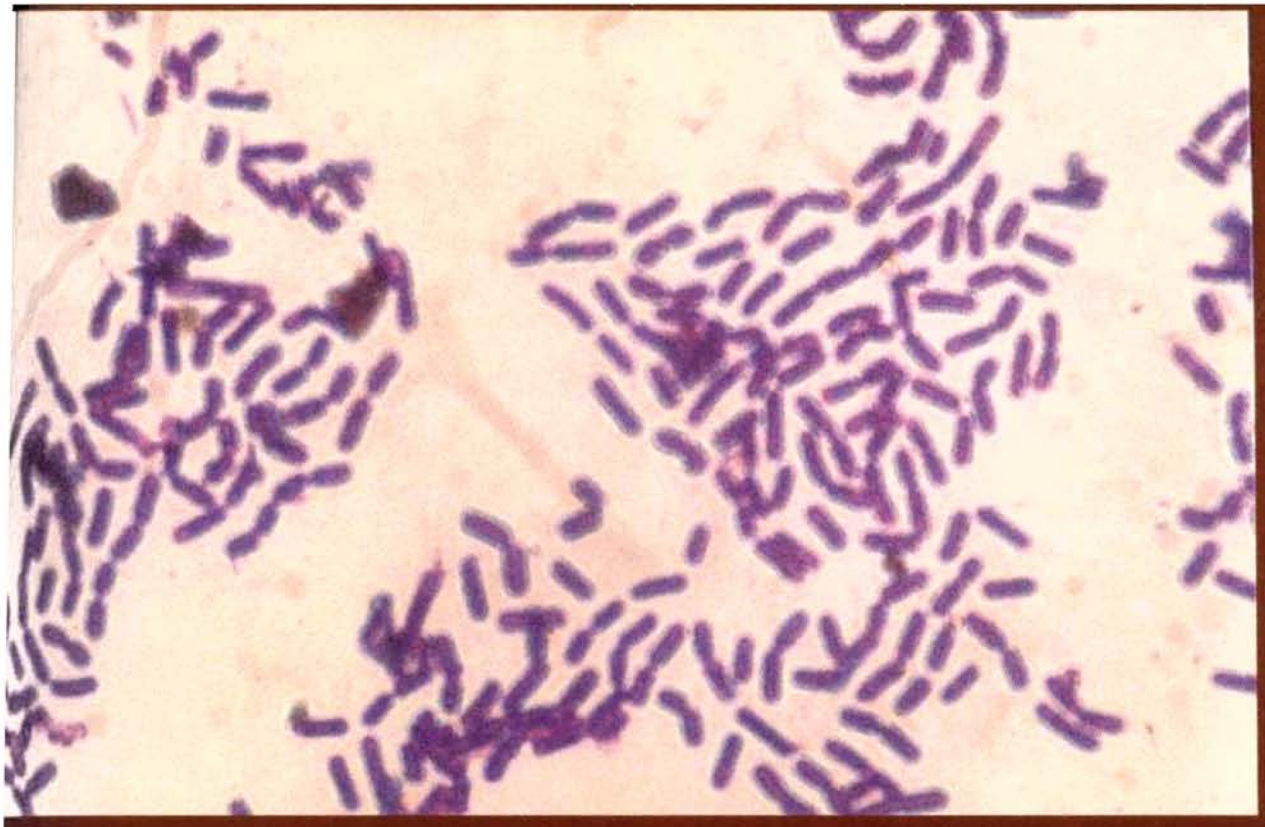

Plate 21 Microscopic Examination of Bacillus Subtilis

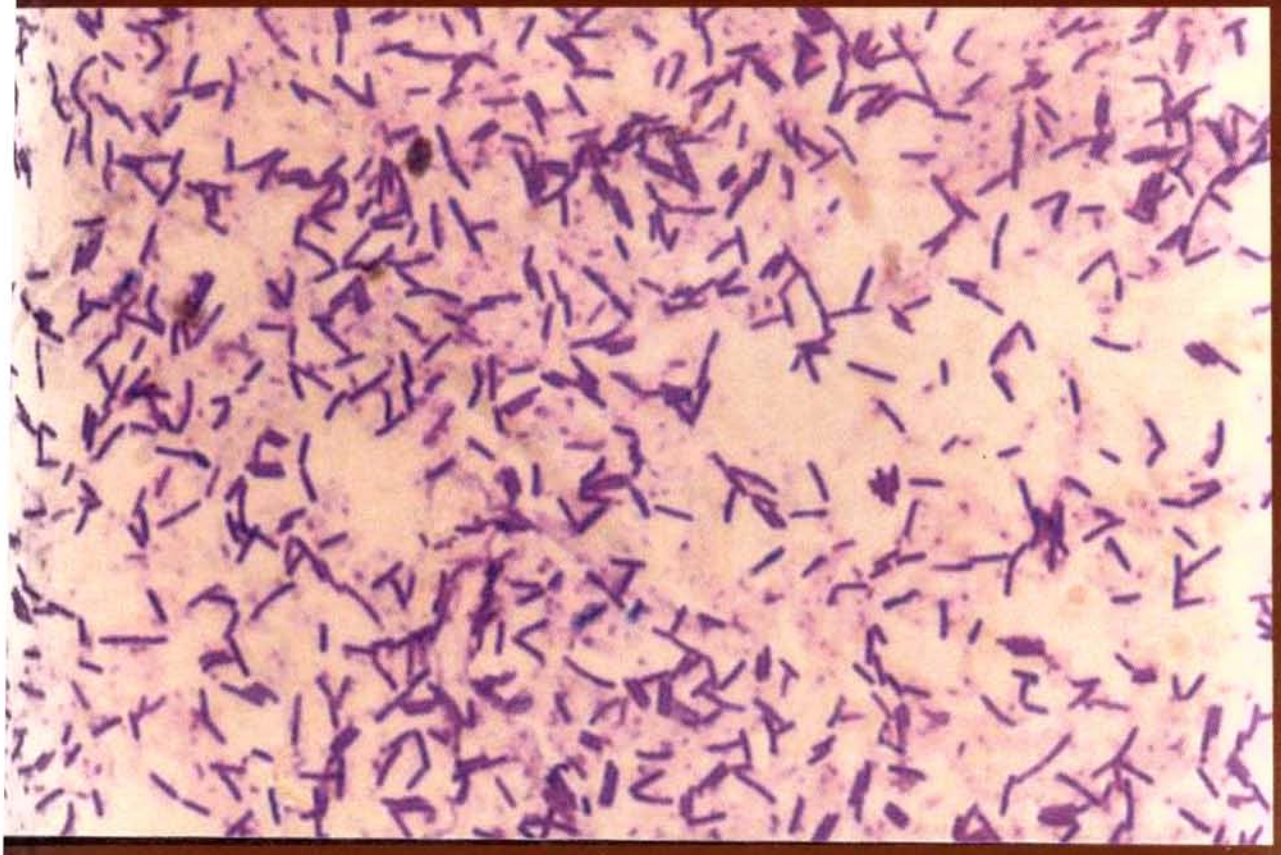

Plate 22 Microscopic Examination of Bacillus pumilus

bacillus species as well as the diversity of the species isolated from both the sites differed to a certain extent.

Bacillus subtilis was the most frequently isolated species from both the stations. 24.65% from Cherai and 17.93% from Valappu). *Bacillus subtilis, B.sphaericus, B. megaterum, B. firmus and B.pumilus* were the five predominant species isolated through the period of the study from Valappu while *B.subtilis, B.pumilus, B. megaterum* were three dominant species isolated from Cherai station total of 14 species of Bacillus were isolated from Valappu while only 11 species were isolated from Cherai.

B. sphaericus 14.48% occurred next to *B. subtilis* in Valappu while only 4.10% of *B. sphaericus* were isolated from Cherai station 11.03% of *B.firmus* was isolated from Valappu. While only 8.21% of *B. firmus* were isolated from Cherai. *B.alvei, B. macerans*, and *B. cereus* (mycoides) was isolated only from Valappu. While these three species was not awaited from Cherai station *B. alvei* 5.51%, *B.macerans* 2.06% and *B. cereus* (mycoides) 0.68%, were found. 8.21% of *B.polymyxa*, was reported from Cherai while Valappu contained only 2.75% of *B.polymyxa*. Percentage occurrence of *B. circulans* 9.58% was higher in Cherai while Valappu had only 5.51% of *B. circulans* (Table 28 and 29) (Plate 10)

Table: 29 Percentage Composition of *Bacillus Species* isolated from Valappu

Species	Total no.of Isolates n=145	Percentage
B.sublities	n=26	17.93
B.sphaericus	n=21	14.48
B.megaterium	n=19	13.1
B.firmis	n=16	11.03
B.pumilus	n=16	11.03
B.alvei	n=8	5.51
B.circulans	n=8	5.51
B.brevis	n=7	4.82
B.coagulans	n=6	4.13
B.stearothermophilus	n=5	3.44
B.licheniformis	n=5	3.44
B.polymyxa	n=4	2.75
B.macerans	n=3	2.06
B.cereus mycoides	n-1	0.68

Table: 28 Percentage Composition of *Bacillus Species* isolated from cherai

Species	Total No. of Isolates n=73	Percentage
B.sublities	n=18	24.65
B.pumilus	n=11	15.06
B.megaterium	n=10	13.69
B.circulans	n=7	9.58
B.polymyxa	n=6	8.21
B.firmis	n=6	8.21
B.brevis	n=4	5.47
B.coagulans	n=3	4.1
B.sphaericus	n=3	4.1
B.licheniformis	n=3	4.1
B.stearothermophilusn=2	n=2	2.73

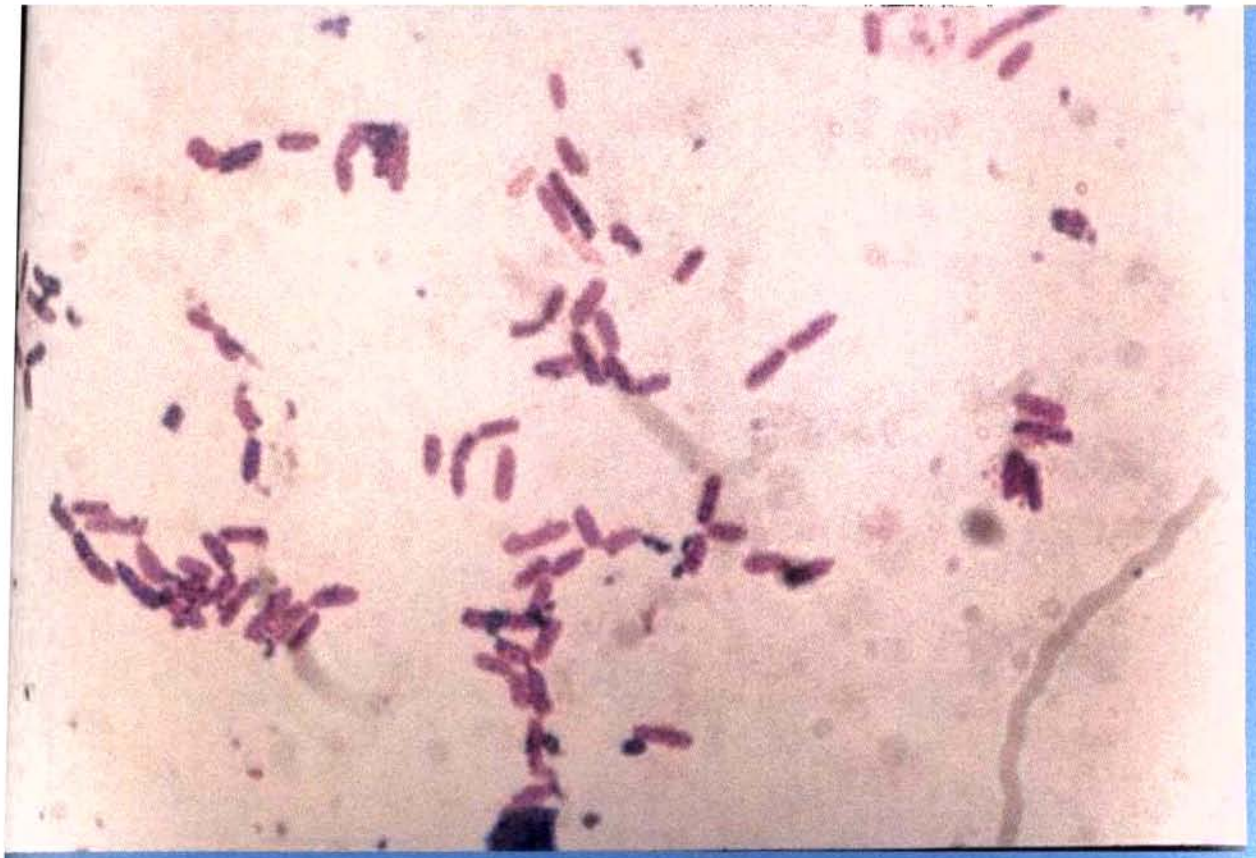

Plate7 Microscopic Examination of Bacillus licheniformis

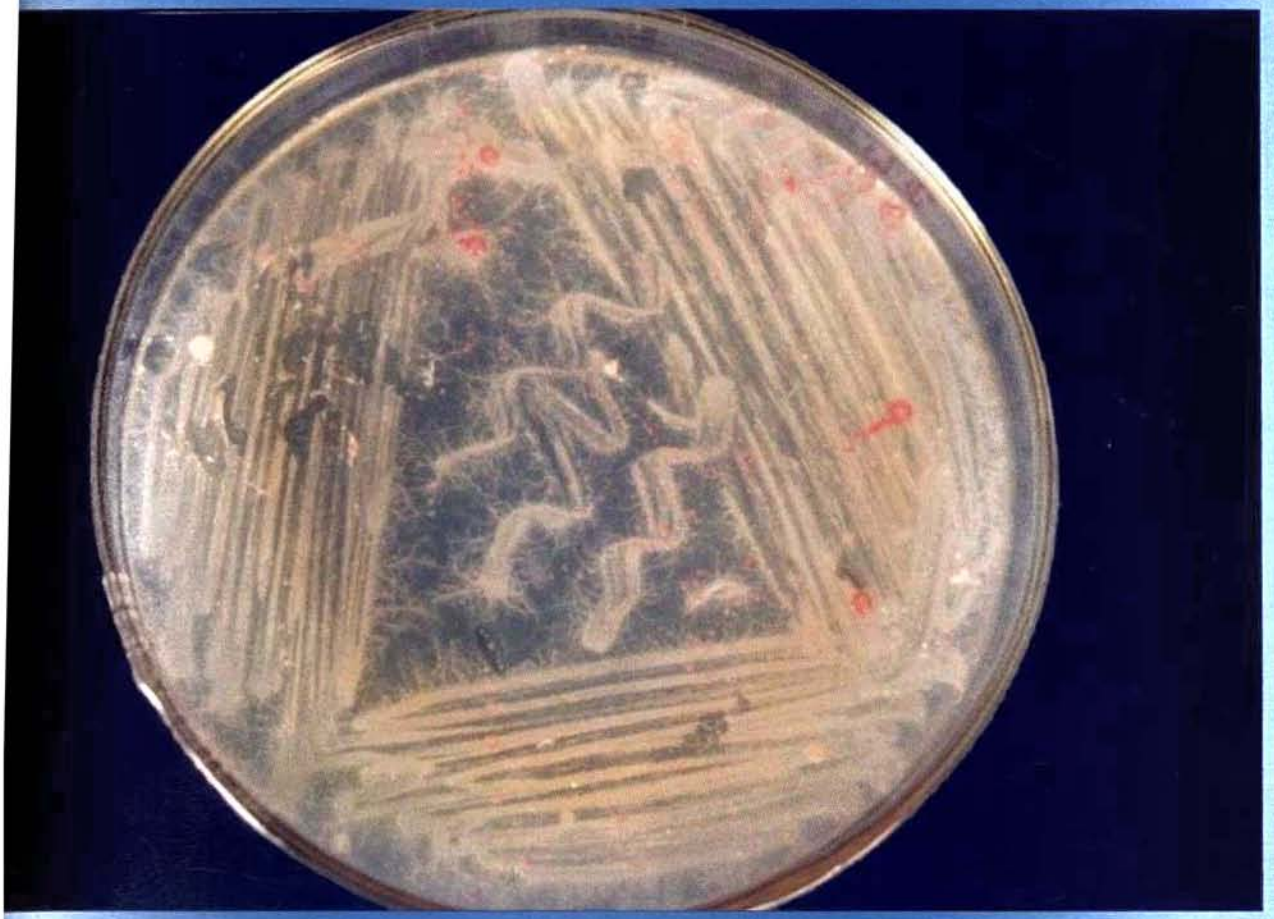

Plate 8 Colony of Bacillus licheniformis

Percentage Composition of Bacillus Species isolated from Valappu and cherai

Total number of isolates--n=218

Species	Isolates	Percentage
B.subtilis	n=44	20.18
B.sphaericus	n=24	11
B.megaterium	n=29	13.3
B.firmis	n=22	10.09
B.pumilus	n=27	12.38
B.alvei	n=8	3.66
B.circulans	n=15	6.88
B.brevis	n=11	5.04
B.coagulans	n=9	4.12
B.stearothermophilus	n=7	3.21
B.licheniformis	n=8	3.66
B.polymyxa	n=10	4.58
B.macerans	n=3	1.37
B.cereus mycoides	n-1	0.45

4.11.1 Microbial identification by DNA fingerprinting method

The harvested and extracted cells were loaded to Sherlock MIS which compared the fatty acid of the loaded strain to the build in library of the strains in the software. The identified species are Bacillus subtilis with a peak match of 0.543, Bacillus licheniformis 0.459, Bacillus pumilus 0.605.

4.12 Biochemical reactions of B. lactobacillus species isolated from Cherai station

All the lactobacillus species (table30) isolated from Cherai were mostly non-mobile, catalase negative, non-sporogenous, rods, which were positive in gram staining. A few strains i.e.; 14.3% of *L. casei* and 25% of *B.coryniformis* produced the enzyme catalase which may sometimes be pseudo catalase. Nitrate reduction capacity was seen only in 25% of *L. buchenerii* while all the other strains were not able to reduce Nitrate to Nitrite.

4.12.1 Temperature Tolerance

Except 100% of *L. fermentum*, 20% of *L. acidophilus*, 25% *L. brevis*, 20% of *L.buchenerii*, 25% of *L.brevis*, 20% of *L.buchenerii*, 25% of *L.coryniformis*, 42.8% of *L.casei* and 22.3% of *L.curvatus* all the strains were able to grow actively at 15°C while only 92.3% of *L.fermentum*, 90% of *L.acidophilus* and 11.2% of *L.curvatus* could tolerate 45°C.

2264.30A [1277] A.I.25

me: DATA1 File: E042264.30A Seq Counter: 9 ID Number: 1277
: Samp Bottle: 8 Method: TSBA40
ted: 2/26/2004 1:36:08 PM
ted By: mohan
ple ID: A.I.25

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
.628	4.421E+8	0.025	----	7.021	SOLVENT PEAK	----	< min rt	
733	11769	0.021	----	7.233		----	< min rt	
7.17	647	0.025	----	8.004		----	< min rt	
7.897	1008	0.032	----	8.185		----	< min rt	
11.704	271	0.026	1.038	12.001	12:0	0.13	ECL deviates 0.001	Reference -0.004
11.892	1109	0.028	1.019	12.614	13:0 ISO	0.52	ECL deviates 0.000	Reference -0.005
11.902	287	0.030	1.016	12.701	13:0 ANTEISO	0.13	ECL deviates -0.001	Reference -0.005
12.682	2319	0.033	0.994	13.618	14:0 ISO	1.06	ECL deviates -0.001	Reference -0.005
12.901	1032	0.036	0.986	14.000	14:0	0.47	ECL deviates 0.000	Reference -0.004
13.155	62488	0.036	0.976	14.624	15:0 ISO	28.12	ECL deviates 0.001	Reference -0.004
13.293	88675	0.036	0.974	14.714	15:0 ANTEISO	39.85	ECL deviates 0.001	Reference -0.004
13.370	940	0.039	0.966	15.386	16:1 w7c alcohol	0.42	ECL deviates -0.001	
13.770	2093	0.038	0.963	15.627	16:0 ISO	0.93	ECL deviates 0.000	Reference -0.005
13.832	3100	0.038	0.963	15.665	unknown 15.669	1.38	ECL deviates -0.004	
13.985	1044	0.040	0.962	15.757	16.1 w11c	0.46	ECL deviates 0.000	
14.387	2786	0.038	0.959	16.000	16:0	1.23	ECL deviates 0.000	Reference -0.005
14.899	1231	0.040	----	16.297		----		
15.057	3882	0.045	0.956	16.389	ISO 17:1 w10c	1.71	ECL deviates 0.001	
15.211	1664	0.038	0.955	16.478	Sum In Feature 4	0.73	ECL deviates 0.002	17:1 ISO I/ANTEI B
15.473	16491	0.042	0.954	16.630	17:0 ISO	7.25	ECL deviates 0.000	Reference -0.006
15.634	26772	0.040	0.953	16.724	17:0 ANTEISO	11.77	ECL deviates 0.001	Reference -0.005
15.864	1606	0.043	0.943	17.998	18:0	0.70	ECL deviates -0.002	Reference -0.009
16.246	1108	0.042	----	18.216		----		
16.416	827	0.045	----	18.313		----		
16.976	2019	0.044	0.938	18.634	19:0 ISO	0.87	ECL deviates 0.000	Reference -0.007
17.144	2867	0.044	0.938	18.730	19:0 ANTEISO	1.24	ECL deviates -0.001	
17.658	1614	0.047	----	19.603		----		
17.846	2354	0.044	0.924	20.001	20:0	1.00	ECL deviates 0.001	Reference -0.008
----	1664	---	----	----	Summed Feature 4	0.73	17:1 ISO I/ANTEI B	17:1 ANTEISO B/1

ECL Deviation: 0.001 Reference ECL Shift: 0.006 Number Reference Peaks: 14
Total Response: 228579 Total Named: 223799
Percent Named: 97.91% Total Amount: 216812

Matches:

Library	Sim Index	Entry Name
TSBA40 4.10	0.543	Bacillus-subtilis*
	0.378	Bacillus-licheniformis* (Bacillus subtilis group)
	0.278	Bacillus-marinus*

204.48A [1269] A.I.31

me: DATA1 File: E042204.48A Seq Counter: 17 ID Number: 126
:Samp Bottle: 15 Method: TSBA40
ted: 2/20/2004 5:19:51 PM
ed By: mohan
le ID: A.I.31

k:

IT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
50	4.359E+8	0.025	----	7.023	SOLVENT PEAK	----	< min rt	
35	11369	0.021	----	7.233		----	< min rt	
19	529	0.026	----	8.006		----	< min rt	
98	789	0.028	----	8.186		----	< min rt	
34	145	0.021	----	8.806		----	< min rt	
15	131	0.020	1.140	10.000	10:0	0.06	ECL deviates 0.000	Reference 0.000
18	417	0.032	1.040	12.000	12:0	0.19	ECL deviates 0.000	Reference -0.002
47	1217	0.029	1.019	12.613	13:0 ISO	0.53	ECL deviates -0.001	Reference -0.003
17	2163	0.033	0.992	13.618	14:0 ISO	0.93	ECL deviates -0.001	Reference -0.003
26	1347	0.036	0.984	14.000	14:0	0.57	ECL deviates 0.000	Reference -0.002
42	104344	0.036	0.974	14.625	15:0 ISO	43.83	ECL deviates 0.002	Reference -0.001
99	68996	0.037	0.972	14.714	15:0 ANTEISO	28.94	ECL deviates 0.001	Reference -0.002
75	2823	0.039	0.961	15.626	16:0 ISO	1.17	ECL deviates -0.001	Reference -0.004
39	3268	0.038	0.960	15.665	unknown 15.669	1.35	ECL deviates -0.004	
94	4637	0.038	0.957	15.999	16:0	1.91	ECL deviates -0.001	Reference -0.004
68	1094	0.041	----	16.298		----		
31	24497	0.041	0.951	16.630	17:0 ISO	10.06	ECL deviates 0.000	Reference -0.003
42	18490	0.041	0.951	16.724	17:0 ANTEISO	7.58	ECL deviates 0.001	Reference -0.003
24	1757	0.042	0.941	17.999	18:0	0.71	ECL deviates -0.001	Reference -0.005
56	861	0.041	----	18.217		----		
86	1910	0.044	0.935	18.635	19:0 ISO	0.77	ECL deviates 0.001	Reference -0.004
54	1505	0.042	0.935	18.731	19:0 ANTEISO	0.61	ECL deviates 0.000	
49	1233	0.045	----	19.604		----		
54	1951	0.045	0.920	20.000	20:0	0.77	ECL deviates 0.000	Reference -0.005

Deviation: 0.001 Reference ECL Shift: 0.003 Number Reference Peaks: 14
Response: 242639 Total Named: 239452
nt Named: 98.69% Total Amount: 231772

hes:

ary	Sim Index	Entry Name
A40 4.10	0.459	Bacillus-licheniformis* (Bacillus subtilis group)
	0.402	Bacillus-pumilus-GC subgroup B* (other than type strain)
	0.308	Staphylococcus-chromogenes
	0.266	Staphylococcus-sciuri

```
Injection Date   : 2/20/2004 3:19:39 PM
Sample Name      :  1265                          Location : Vial 11
Acq. Operator    : Mohan                             Inj :  1
                                                 Inj Volume : 2 µl
Method           : C:\HPCHEM\1\METHODS\$MIDI$A.M
Last changed     : 2/20/2004 3:16:26 PM by Mohan
MIDI Aerobe method saved on ChemStation Version 4.02
Switched to new integration algorithm 11-Nov-98
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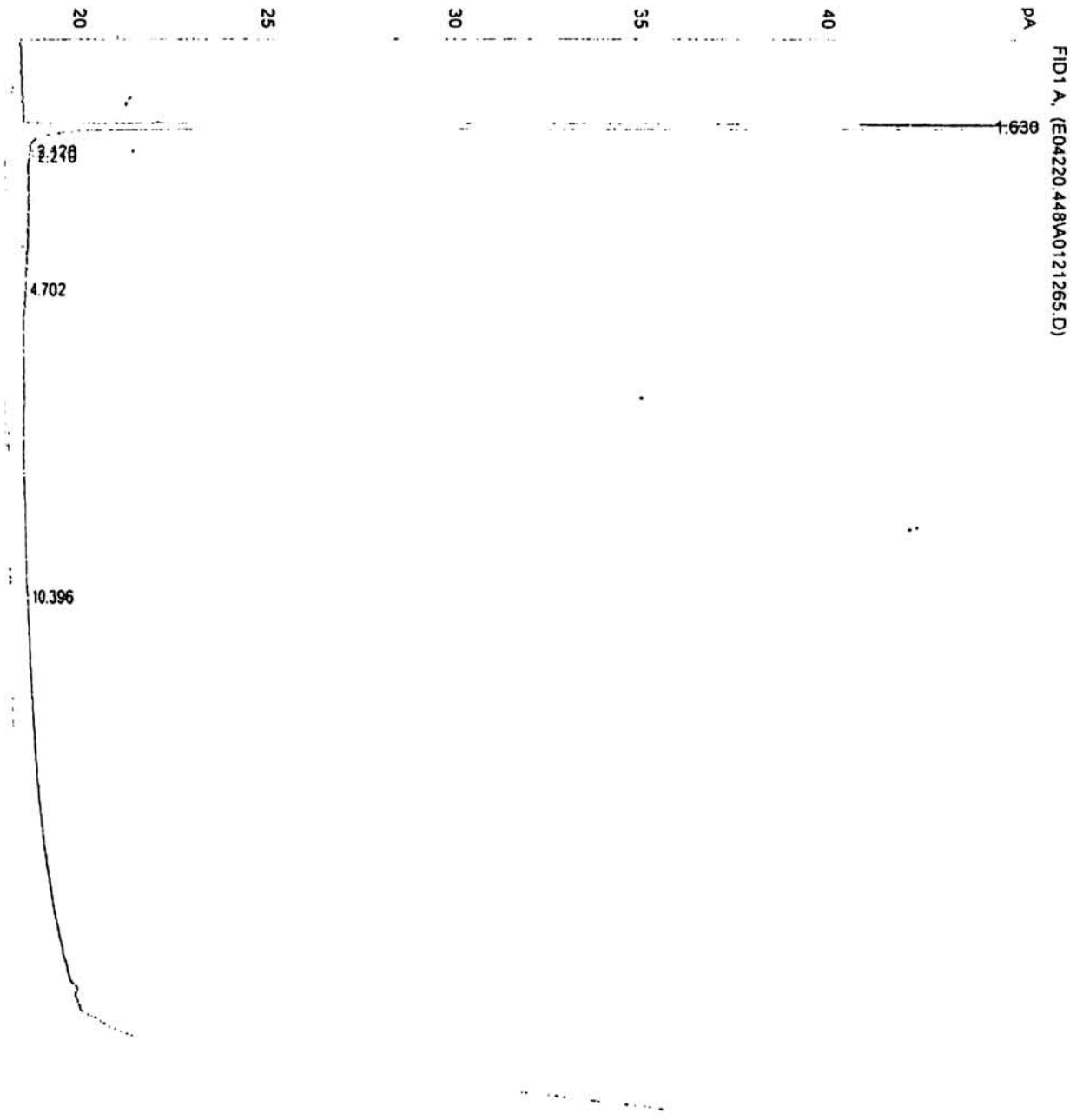


220L4SA [1266] A.I.17

ame: DATA1 File: E04220L4SA Seq Counter: 13 ID Number: 1266
e: Samp Bottle: 12 Method: TSBA40
ated: 2/20/2004 3:41:01 PM
ated By: mohan
ple ID: A.I.17

file:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
929	4.381E+8	0.024	----	7.025	SOLVENT PEAK	----	< min rt	
234	13663	0.020	----	7.236		----	< min rt	
1019	510	0.022	----	8.007		----	< min rt	
1207	878	0.025	----	8.184		----	< min rt	
1522	64	0.013	----	8.816		----	< min rt	
1800	271	0.037	1.087	11.001	11:0	0.18	ECL deviates 0.001	Reference -0.004
1807	261	0.025	1.042	11.999	12:0	0.17	ECL deviates -0.001	Reference -0.006
1906	568	0.028	1.020	12.612	13:0 ISO	0.35	ECL deviates -0.002	Reference -0.007
1989	1339	0.033	0.992	13.619	14:0 ISO	0.81	ECL deviates 0.000	Reference -0.005
2007	1397	0.034	0.984	14.000	14:0	0.84	ECL deviates 0.000	Reference -0.006
2163	98785	0.035	0.973	14.625	15:0 ISO	58.76	ECL deviates 0.002	Reference -0.004
2300	35216	0.038	0.971	14.715	15:0 ANTEISO	20.92	ECL deviates 0.002	Reference -0.004
2377	3956	0.039	0.960	15.627	16:0 ISO	2.32	ECL deviates 0.000	Reference -0.006
2494	325	0.027	0.958	15.757	16:1 w11c	0.19	ECL deviates 0.000	
2595	5791	0.039	0.956	15.999	16:0	3.38	ECL deviates -0.001	Reference -0.007
2669	675	0.041	0.952	16.390	ISO 17:1 w10c	0.39	ECL deviates 0.002	
2753	13478	0.041	0.950	16.630	17:0 ISO	7.83	ECL deviates 0.000	Reference -0.006
2844	6264	0.040	0.949	16.723	17:0 ANTEISO	3.64	ECL deviates 0.000	Reference -0.006
3367	386	0.029	0.941	17.823	18:1 w7c	0.22	ECL deviates 0.000	

L Deviation: 0.001 Reference ECL Shift: 0.006 Number Reference Peaks: 11
al Response: 168713 Total Named: 168713
cent Named: 100.00% Total Amount: 163569

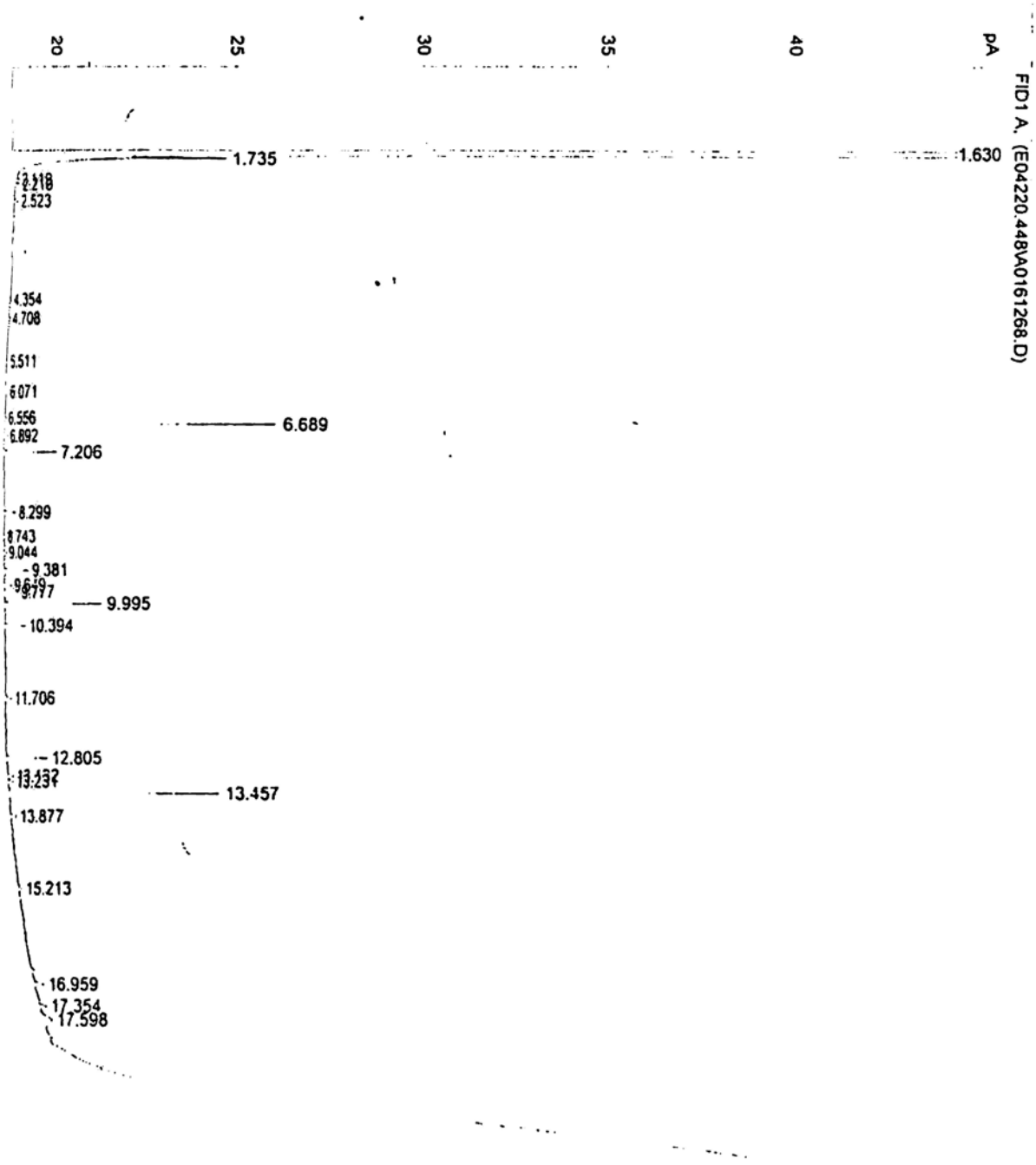
ches:

Library	Sim Index	Entry Name
SBA40 4.10	0.605	Bacillus-pumilus-GC subgroup B* (other than type strain)
	0.350	Staphylococcus-sciuri

Sequence ID: [illegible]

---

ction Date : 2/20/2004 4:58:29 PM
le Name : 1268 Location : Vial 14
Operator : Mohan Inj : 1
Inj Volume : 2 µl
od : C:\HPCHEM\1\METHODS\$MIDI$A.M
changed : 2/20/2004 4:55:09 PM by Mohan
Aerobe method saved on ChemStation Version 4.02
ched to new integration algorithm 11-Nov-98



Sl No	Biochemical Tests																	
1	Gram reaction	+	100	+	100	+	100	+	100	+	100	+	100	+	100	+	100	+	100
2	Motility	--	100	--	100	--	84.6	--	77.7	--	100	--	85.7	--	100	--	100	--	100
3	Catalase	--	100	--	100	--	100	--	100	--	100	--	85.7	--	100	--	75	--	100
4	Growth at 15° c	--	80	+	100	--	100	+	77.7	+	75	+	57.14	+	80	+	75	+	100
5	Growth at 45° c	+	90	--	100	+	92.3	--	88.8	--	100	--	100	--	100	--	100	--	100
6	Nitrate reduction	--	100	--	100	--	100	--	100	--	100	--	100	--	75	--	100	--	100
7	Arginine hydrolysis	--	100	--	94.4	+	84.6	--	100	+	87.5	--	71.4	--	100	--	100	+	50
8	Acid from Arabinose	--	70	--	100	d	69.2	--	44.4	+	75	--	100	+	80	--	75	+	100
9	Cellobiose	+	90	+	83.3	--	100	--	44.4	--	100	--	100	+	80	--	75	+	100
10	Fructose	+	60	+	88.8	+	76.9	+	100	+	100	+	100	+	80	+	100	0	
11	Galactose	+	60	+	77.7	+	92.3	+	88.8	w	62.5	+	85.71	w	40	+	50	+	100
12	Glucose	+	100	+	100	+	100	+	100	+	100	+	100	+	100	+	100	+	100
13	Gluconate	--	75	+	83.3	+	76.9	+	55.5	-	87.5	+	71.4	+	40	+	50	+	50
14	Lactose	+	70	+	94.4	+	100	d	44.4	w	50	w	57.14	w	40	--	100	--	100
15	Maltose	+	80	+	100	+	84.6	+	100	+	87.5	(d)	57.14	+	80	+	75	+	0
16	Mannitol	--	100	+	100	--	100	--	77.7	w	62.5	+	85.71	w	60	+	25	--	100
17	Mannose	+	85	+	88.8	w	61.53	+	100	--	100	+	71.4	--	100	+	75	0	50
18	Melibiose	d	60	+	94.4	+	76.9	--	88.8	+	87.5	--	100	+	80	d	25	--	100
19	Raffinose	d	50	+	83.3	+	38.4	--	100	w	50	--	100	--	80	d	50	--	100
20	Ribose	--	100	+	100	+	61.5	+	88.8	+	75			+	100	--	100	+	50
21	Rhamnose	--	65	--	77.7	--	84.6	--	55.5	--	100			--	20	+	100	0	100
22	Salicin	+	90	+	50	--	46.1	+	66.6	--	62.5	+	85.71	--	60	d	50	d	50
23	Sorbitol	--	75	+	50	--	100	--	88.8	--	62.5	+	71.4	--	100	d	25	--	50
24	Sucorose	+	100	+	94.4	+	100	--	100	d	62.5	d	57.14	d	20	+	75	+	50
25	Trehalose	+	55	+	100	d	76.9	--	88.8	--	37.5	+	71.4	--	80	--	100	0	100
26	Xylose	--	70	d	88.8	d	69.2	+	33.3	d	75	--	100	d	40	--	100	+	50
27	Esculin	+	80	d	55.5	--	100	--	33 3	d	50	+	71.4	d	40	d	50	+	50
28	Gas from Glucose	--	100	--	100	+	76.9	+	100	+	100	--	85.71	+	100	--	75		0

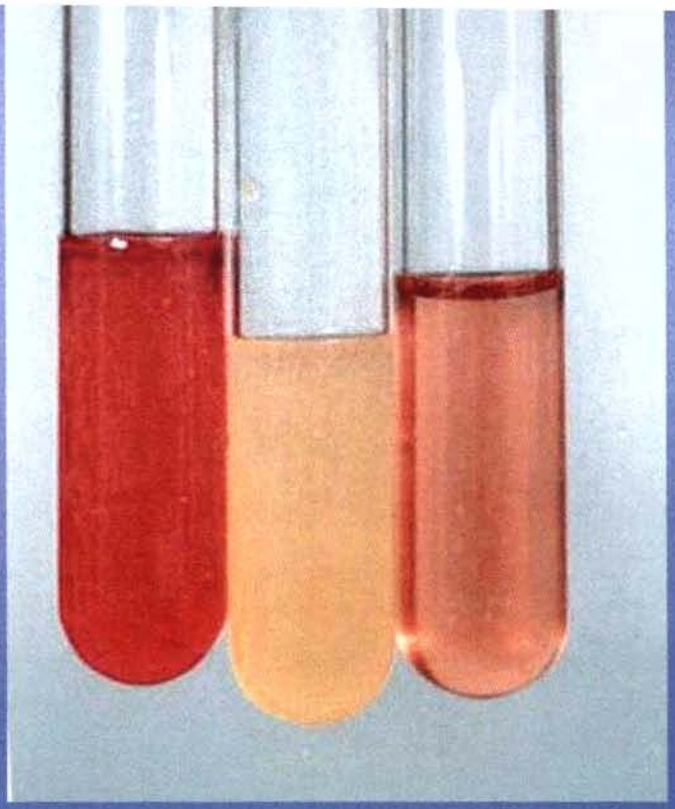


Plate 17 Glucose fermentation by Lactobacillus spp

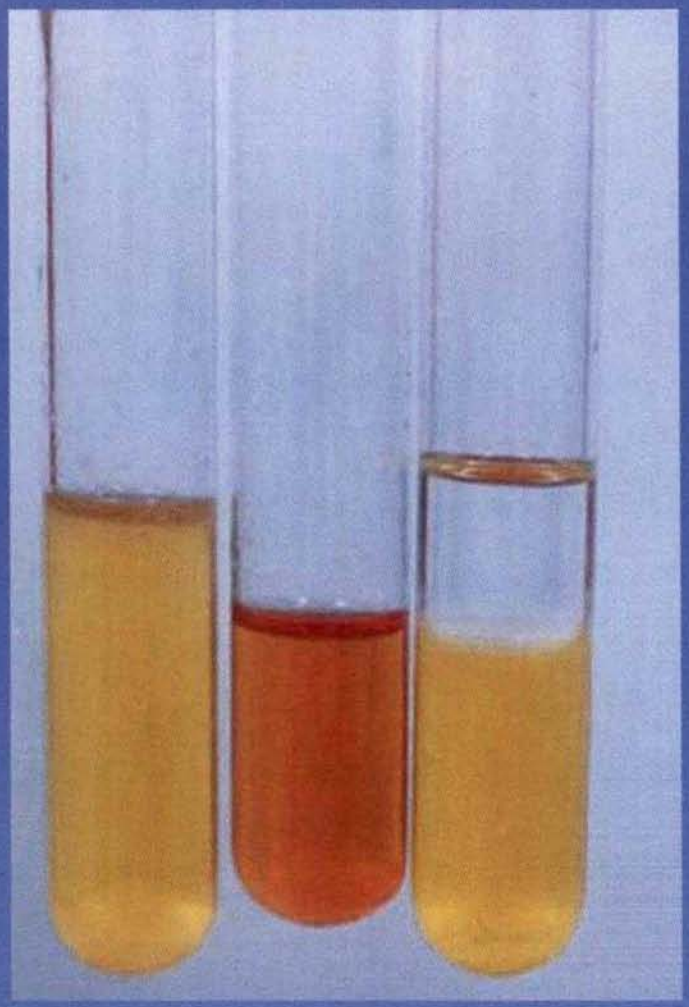


Plate 18 Anaerobic fermentation of Glucose by Bacillus spp

4.12.2 Fermentation of Carbohydrates

30% of *L.acidophilus*, 30.8% of *L.fermentum*, 44% of *L.curvatus*, 25% of *L.brevis*, 20% of *L.buchnerii*, 25% of *L.coryniformis*, 100% of *L.plantarum* and *L.casei* were unable to ferment arabinose while all other strains produced acid from arabinose, while only 90% of *L.acidophilus*, 83.3% of *L.plantarum*, 20% of *L.buchnerii*, 25% of *L.coryniformis*, 55.6% of *L.curvatus* and 100% of L.coprophilus were able to ferment cellobiose. (Plate 17)

All the strains were able to ferment glucose. Fructose was fermented by 60% of *L.acidophilus*, 88.8% of *L.plantarum*, 76.9% of *L.fermentum*, 100% of *L.curvatus*, *L.brevis*, *L.casei*, *L.coryniformis* and 80% of *L.buchenerii*. Galactose fermentation was reported from 60% of *L.acidophilus*, 77.7% of *L.plantarum*, 92.3% of *L.fermentum*, 88.8% of *L.curvatus*, 85.71% of *L.casei*, 50% of *L.coryniformis* and 100% of *L.coprophilus*, 62.5% of *L.bervis* and 40% of *L.buchnerii* showed weak reaction while all other strains did not ferment galactose. (Plate 6)

Except 75% of *L.acidophilus*, 16.7% of *L.plantarum*, 23.1% of *L.fermentum*, 44.5% of *L.curvatus*, 12.5% of *L.brevis*, 28.6% of *L.casei*, 60% of *L.buchneri*, 50% of *L.coryniformis* and 50% of *L.coprophilus*, all strains ferment gluconate. Lactose fermentation was reported in 70% of L.acidophilus, 94.4% of *L.plantarum*, 100% of *L.fermentum* and 44% of

L.curvatus only 50% of *L.brevis*, 57.14% of *L.casei* and 40% of *L.buchenerii* showed only weak reaction while other strains failed to ferment the sugar. (Plate 5)

Maltose was fermented by 80% of *L.acidophilus*, 100% of *L.plantarum*, 84.6% of *L.fermentum*, 100% *L.curvatus*, 87.5% of *L.brevis*, 57.14% *L.casei*, 80% of *L.buchneri*, 75% of *L.coryniformis*, while no other strains fermented Maltose. Fermentation of mannitol was reported from 100% *L.plantarum*, 85.71% of *L.casei*, 20% of *L.coryniformis*, 22.3% of *L.curvatus* and 62.5% of *L.brevis* and 60% of *L.buchnerii* showed weak reactions. Except 100% of *L.brevis, L.buchneri,* 25% of *L.coryniformis*, 50% of *L.coprophilus*, 28.6% of *L.casei*, 38.47% of *L.fermentum*. All other species were able to ferment Mannose while melibiose was fermented by 94.4% of *L.plantarum*, 76.9% of *L.fermentum*, 87.5% of *L.brevis*, 80% of *L.buchnerii*, 75% of *L.coryniformis*, 60% of *L.acidophilus* and 11.2% of *L.curvatus*.

Raffinose fermentation was repoted in 50% of *L.acidophilus*, 83.3% of *L.plantarum*, 38.4% of *L.fermentum*, 20% of *L.buchnerii* and 50% of *L.coryniformis*, while Ribose was fermented by 100% of *L.plantarum*, 61.5 *L.fermentum*, 88.8% of *L.curvatus*, 75% of *L.brevis*, 100% of *L.buchnerii* and 50% *L.coprophilus*.

Rhamnose fermentation was 100% in the case of *L.coryniformis* followed by 35% of *L.acidophilus*, 22.3% of *L.plantarum*, 15.4% *L.fermentum*, 44.5% of L.curvatus and 80% of *L.buchnerii*. None other than 90% of *L.acidophilus*, 50% of *L.plantarum*, 60.6% of *L.curvatus*, 85.71% of *L.casei*, 53.9% of *L.fermentum*, 37.5% of *L.brevis*, 40% of *L.buchneri* and 50% of *L.coryniformis* and *L.coprophilus* were able to ferment Salicin. While most of the strains, 75% of L.acidophilus, 50% of *L.plantarum*, 100% of *L.fermentum* and *L.buchneri*, 88.8% of *L.curvatus*, 62.5% of *L.brevis*, 75% of *L.coryniformis*, 50% of *L.coprophilus* and 28.6% of *L.casei* showed a negative reaction for sorbitol.

Sucrose was fermented by 100% of *L.acidophilus*, *L.fermentum*, 94.4% of *L.plantarum*, 62.5% of *L.brevis*, 57.14% of *L.casei*, 75% of *L.corynoformis*, 70% of L.buchneri and 50% of L.coprophilus. Trehalose fermentation occurred only in 50% of *L.acidophilus*, 100% of *L.plantarum*, 76.9% of *L.fermentum*, 71.4% of *L.casei*, 11.2% of *L.curvatus*, 62.5% of *L.brevis* and 20% of *L.buchnerii*.

Xylose fermentation was 30% for *L.acidophilus*, 88.8% (weak) *L.plantarum*, 69.2% *L.fermentum*, 33.3% of *L.curvatus*, 75% of *L.brevis*, 40% of *L.buchnerii* and 50% of *L.coprophilus*. While esculin hydrolysis was reported from 60.7% of *L.curvatus*, 50% of *L.brevis*, 71.4% of *L.casei*, 40% of *L.buchnerii* and 50% of *L.coryniformis*.

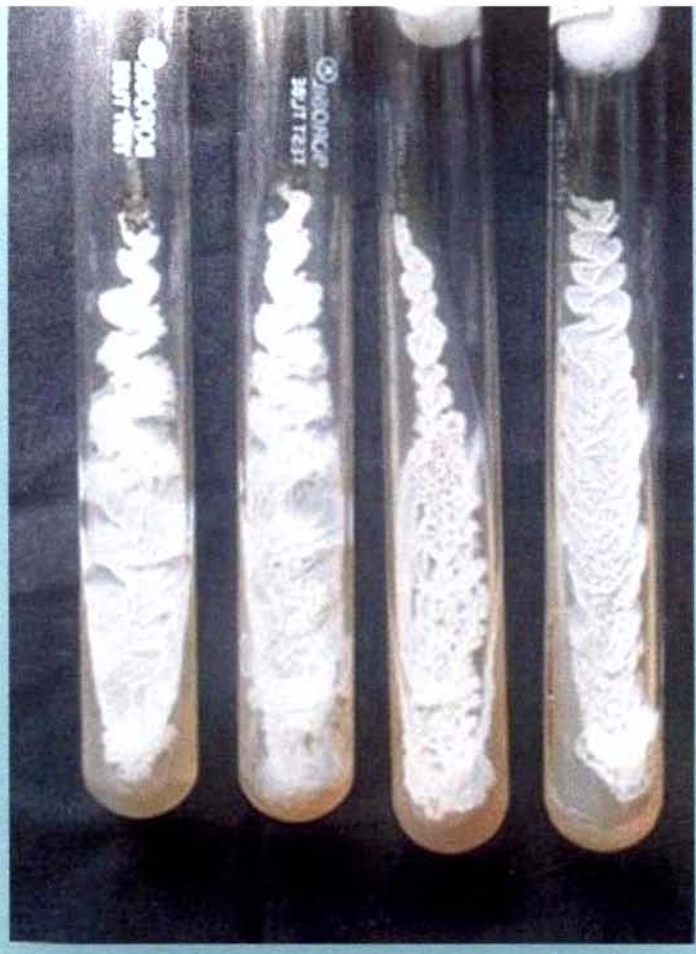


plate 5 Different Species of Lactobacillus

plate 6 Spreading Colonies of lactobacillus

Gas production from glucose was reported only from 76.9% of *L.fermentum*, 100% of *L.curvatus*, 100% of *L.brevis* and 100% of *L.buchnerii*.

4.13 Biochemical reaction of Lactobacillus strains isolated from Valappu.

All the strains of lactobacillus isolated from Valappu (Table 31) were non-motile except 20% of *L.buchnerii* and 6.7% of *L.brevis*, non-sporogenous positive rods which were unable to produce the enzyme catalase except 9.1% of *L.brevis*.

Active growth at 15°C were reported by 100% of *L.curvatus*, *L.butchneri* and *L.casei* followed by 93.75% of *L.plantarum*, 81.8% of *L.brevis*, 75% of *L.coryniformis*, 11.8% of *L.acidophilus*, and 13.4% of *L.fermentum*. While growth at 45°C was reported only by 80% of *L.fermentum*, 43.25% of *L.plantarum* and 9.1% of *L.brevis*. Nitrate was reduced to nitrite by 41.2% of *L.acidophilus*, 56.25% of *L.plantarum*, 53.4% of L.fermentum, 12.5% of *L.curvatus* and 20% of *L.buchnerii*. While Arginine was hydrolysed by 40% of *L.buchnerii* and 18.2/*L.brevis*, 12.5% of *L.plantarum*, 81.8% of *L.brevis*, 40% of *L.buchnerii* and 50% of *L.coryniformis* only. (Plate 5 and 6)

Sl. No	Biochemical Tests	L.acidophilus n=17%		L.plantarum n=16%		L. fermentum n=15%		L. brevis n=11%		L curvatus n=8%		L.buchnire n=8%		L casei n=4%		L.coprohilus n=4%	
1	Gram reaction	+	100	+	100	+	100	+	100	+	100	+	100	+	100	+	100
2	Motility	--	100	--	100	--	93.3	--	100	--	100	--	80	--	75	--	100
3	Catalase	--	100	--	100	--	100	--	90.9	--	100	--	100	--	100	--	100
4	Growth at 15° c	--	88.2	+	93.75	--	86.6	+	81.8	+	100	+	100	+	100	+	75
5	Growth at 45° c	+	100	--	56.75	+	80	--	90.9	--	100	--	100	--	100	--	100
6	Nitrate reduction	--	58.8	--	43.75	--	46.6	--	100	--	87.5	--	80	--	100	--	100
7	Arginine hydrolysis	--	100	--	87.5	+	100	+	81.8	--	100	--	60	--	100	--	50
8	Acid from Arabinose	--	88.2	--	100	d	33.3	+	100	--	87.5	+	100	--	75	--	100
9	Cellobiose	+	100	+	100	--	86.6	--	100	+	62.5	--	60	+	100	--	50
10	Fructose	+	100	+	100	+	93.3	+	100	+	100	+	100	+	100	+	100
11	Galactose	+	94.1	+	93.75	+	100	+	100	+	87.5	+	100	w	50	+	75
12	Glucose	+	100	+	100	+	100	+	100	+	100	+	100	+	100	+	100
13	Gluconate	--	52.9	+	75	+	100	+	63.6	+	100	+	80	+	75	+	100
14	Lactose	+	100	+	93.7	+	80	w	72.7	d	62.5	w	100	w	100	--	50
15	Maltose	+	100	+	87.5	+	73.3	+	63.6	+	62.5	+	100	d	75	+	100
16	Mannitol	--	29.4	+	62.5	--	66.6	w	54.5	--	62.5	w	80	+	100	+	0
17	Mannose	+	64.7	+	37.5	w	53.3	--	100	+	75	--	40	+	100	+	100
18	Melizilose	d	88.2	+	93.75	+	100	+	81.8	--	50	+	80	--	50	d	75
19	Melibiose	d	76.4	+	68.75	+	73.33	+	72.7	--	87.5	+	60	--	100	d	50
20	Raffinose	d	70.5	+	100	+	80	w	54.5	--	100	--	100	--	100	d	75
21	Ribose	--	41.1	+	43.7	+	66.6	+	54.5	+	87.5	+	60	.		--	100
22	Rhamnose	--	100	--	100	--	93.3	--	81.8	--	100	--	100	.		+	75
23	Salicin	+	58.8	+	56.25	--	100	--	100	+	75	--	60	+	50	d	75
24	Sucorose	+	64.7	+	56.25	+	63.9	d	63.6	--	100	d	40	d	25	+	100
25	Trehalose	+	100	+	62.5	d	66.6	--	45.4	--	62.5	--	20	+	75	--	0
26	Xylose	--	82.3	d	50	d	60	d	54.5	+	62.5	d	0	--	100	--	50
27	Esculin	+	35.2	+	87.5	--	46.6	d	36.3	--	75	d	60	+	50	d	50
28	Sorbitol	-	88.2	+	68.75	--	100	--	81.8	--	62.5	--	100	+	100	d	.
29	Gas from Glucose	--	100	--	100	+	53.33	+	72.7	+	62.5	+	40	--	100	--	100

4.13.1 Fermentation of Sugar

11.8% of *L.acidophilus*, 33.3% of *L.fermentum*, 12.5% of *L.curvatus* and 5% of *L.casei* produced acid from arabinose, while other strains not cellubiose was fermented by 100% of *L.acidophilus*, *L.plantarium*, *L.casei*, 62.5% of *L.curvatus*, 40% of *L.buchnerii*, 18.4% of *L.fermentum* and 50% of *L.coryniformis*. Fructose was fermented by most of the strains i.e., 100% of *L.acidophilus*, *L.plantarum*, *L.curvatus*, *L.brevis*, *L.buchenerii*, *L.casei*, and *L.coryniformis* followed by 93.3% of *L.fermentum*, while glucose was fermented by all the strains. Galactose fermentation was reported in 94.1% of L.acidophilus, 93.75% of *L.plantarum*, 100% of *L.fermentum*, *L.brevis*, *L.buchnerii*, 87.5% of *L.curvatus*, 75% of *L.coryniformis*, while 50% of *L.casei* showed a weak reaction. (Plate 17)

Gluconate was fermented by 100% of *L.fermentum*, *L.curvatus*, *L.coryniformis*, 75% of *L.plantarum* and *L.casei*, 63.6% of *L.brevis*, 80% of *L.buchnerii*, while all the other strains did not ferment gluconate. Lactose fermenting strains of lactobacillus species include 100% of *L.acidophilus*, 93.7% of *L.plantarum*, 80% of *L.fermentum*, 62.5% of *L.curvatus*, and 50% of *L.coryniformis*, 72.7% of *L.brevis*, 100% of *L.buchnerii* and *L.casei* showed weak reactions. Except 12.5% of *L.plantarum*, 26.7% of *L.fermentum*, 36.4% of *L.brevis*, 37.5 of *L.curvatus*, 25% of *L.*casei. all other strains fermented Maltose while mannitol was fermented by 62.5% of *L.plantarum*, 70.6% of *L.acidophilus*, 33.4% of *L.fermentum*, 100% of

L.casei, 37.5% of *L.curvatus*, 54.5% of *L.brevis* and 60% of *L.buchnerii* showed weak reaction. 64.7% of *L.acidophilus*, 37.5% of *L.plantarum*, 75% of *L.curvatus*, 60% of *L.buchnerii*, 100% of L.casei and *L.coryniformis* fermented mannose, while all the other strains failed to ferment mannose.

A negative reaction was reported by 11.8% of *L.acidophilus*, 40.5% of *L.plantarum*, 18.2% of *L.brevis*, 50% of *L.casei* and 25% of *L.coryniformis* in the fermentation of melizitose. Melibiose was fermented by 76.4% of *L.acidophilus*, 68.75% of *L.plantarum*, 73.3% of *L.fermentum*, 72.7% of *L.brevis*, 12.5% of *L.curvatus*, 60% of *L.buchnerii* and 50% of *L.coryniformis*, while raffinose was fermented by 100% of *L.plantarum* followed by 70.5% of *L.acidophilus*, 80% of *L.fermentum*, and 75% of *L.coryniformis*, 54.5% of *L.brevis* showed a weak reaction. Ribose was fermented by 66.6% of L.fermentum, 54.5% *L.brevis*, 87.5% of *L.curvatus*, 60% of *L.buchneri,* while Rhamhose fermentation was reported in a very few strains such as 6.7% of *L.fermentum*, 18.8% of *L.brevis* and 75% of *L.coryniformis*.

Salicin was fermented by 58.8% of *L.acidophilus*, 56.25% of *L.plantarum*, 75% of *L.curvatus*, 50% of *L.casei* and 50% of *L.coryniformis*. Sucrose fermentation was reported in 64.7% of *L.acidophilus*, 56.25% of *L.plantarum*, 63.9% of *L.fermentum*, 63.6% of *L.brevis*, 40% of *L.fermentum*, 63.6% of *L.brevis*, 40% of *L.buchnerii*, 25% of *L.casei* and

100% of *L.coryniformis*. While trehalose was fermented by 100% of *L.acidophilus*, 62.5% of *L.plantarum*, 66.6% of *L.fermentum*, 54.6% of *L.brevis*, 37.5% of *L.curvatus*, 80% of *L.buchnerii* and 75% of *L.casei*.

Xylose was fermented by all the strains other than 82.3% of *L.acidophilus*, 100% of *L.casei*, 50% of *L.plantarum*, 40% of *L.fermentum* and 45.5% of *L.brevis*. Esculin hydrolysis was reported in 35.2% of *L.acidophilus*, *L.plantarum* (87.5%), 53.4% of *L.fermentum*, 36.3% of *L.brevis*, 25% of *L.curvatus*, 60% of *L.buchnerii*, 50% of *L.casei*, and *L.coryniformis*. while the other strains failed to hydrolyse Esculin. Sorbitol was fermented by 100% of *L.casei*, 68.75% of *L.plantarum*, 11.8% of *L.acidophilus*, 31.25% of *L.plantarum*, 18.2% of *L.brevis* and 37.5% of *L.curvatus*. Gas from glucose was reported from 53.3% of *L.fermentum*, 62.5% of *L.curvatus*, 72.7% of *L.brevis*, 53.3% and 40% of *L.buchnerii*.

4.14 Percentage composition of Lactobacillus isolated from the two sampling site Cherai and Valappu

Total of about 166 strains of lactobacillus consisting of 8 species were isolated from the two sites (table 32,33,34) *L. acidophilus* and *L. plantarum* were the two dominants species through out the period of collection from the two sites. (*L. acidophilus*(23.25%) from Cherai and 21.25% from Valappu. *L. plantarum* 20.95% from Cherai and 20% from Valappu.

Table: 32 Percentage composition of lactobacillus species isolated from the sampling sites

species	total isolates n = 166	percentage %
L. acidophilus	37	22.28
L. plantarium	34	20.48
L. fermentum	28	16.86
L. brevis	19	11.44
L. curvatus	17	10.24
L. casei	11	6.62
L. butchnerii	10	6.02
L. coryniformis	6	3.61
L. coprophilus	2	1.2

Table: 33 Percentage composition of lactobacillus species isolated from the cherai

species	total isolates n = 86	percentage %
L. acidophilus	20	23.25
L. plantarium	18	20.93
L. fermentum	13	15.11
L. brevis	8	9.3
L. curvatus	9	10.4
L. casei	7	8.13
L. butchnerii	5	5.81
L. coryniformis	4	4.65
L. coprophilus	2	2.32

Table: 34 Percentage composition of lactobacillus species isolated from the valappu

species	total isolates n = 80	percentage %
L. acidophilus	17	21.25
L. plantarium	16	20
L. fermentum	15	18.75
L. brevis	11	13.75
L. curvatus	8	10
L. casei	4	5
L. butchnerii	5	6.25
L. coryniformis	4	5

L. acidophilus, L plantarum, L. fermentum, L. curvatus, L. brevis, L. casei, were the five predominant species isolated from Cherai while *L. acidophilus, L.plantarum, L. fermentum, L. brevis* and *L. curvatus* dominated in Valappu throughout the period of study. The percentage composition of *L. casei* was 8.13% in Cherai while Valappu had only 5%. The species *L. coprophilus* was present in Cherai while Valappu lacked the species. 15.11% of *L. fermentum* was isolated from Cherai while Valappu had 18.75% *L. brevis* also showed a high percentage (13.75%) from Valappu. Even though the intensity of the Lactobacillus species differed between the two stations, the diversity of the species had no much difference.

4.15 Experimental Study

4.15.1 Water quality parameters

There was no obvious effects of *bacillus* or *lactobacillus* on water quality in probiotic treatment during the experiment. The water quality parameters were maintained as follows throughout the experiment.

Dissolved oxygen	-	4.2 – 6.5mg/l
pH	-	7.4 – 8.0
Temperature	-	25-28 °C
Salinity	-	26-28ppt

Table 35: Diversity indices of Bacillus and Lactobacillus species in Valappu station in different seasons during the period of study (April 2002 – March 2003)

Diversity indices	Monsoon	Post Monsoon	Pre Mosson
Index of dominance	0.201	0.206	0.191
Index of Species richness	3.043	3.673	2.434
Diversity index	0.819	0.711	0825
Evenness index	0.969	0.841	1.060

Table 36: Diversity indices of Bacillus and Lactobacillus species in Cherai station in different seasons during the period of study

Diversity indices	Monsoon	Post Monsoon	Pre Monsoon
Index of dominance	0.183	0.240	0.203
Index of Species richness	1.649	2.606	1.716
Diversity index	0.750	0.699	0.732
Evenness index	0.976	0.898	0.941

Fig 15 **Variation in the no.of isolates of *Bacillus Species* between Cherai & Valappu**

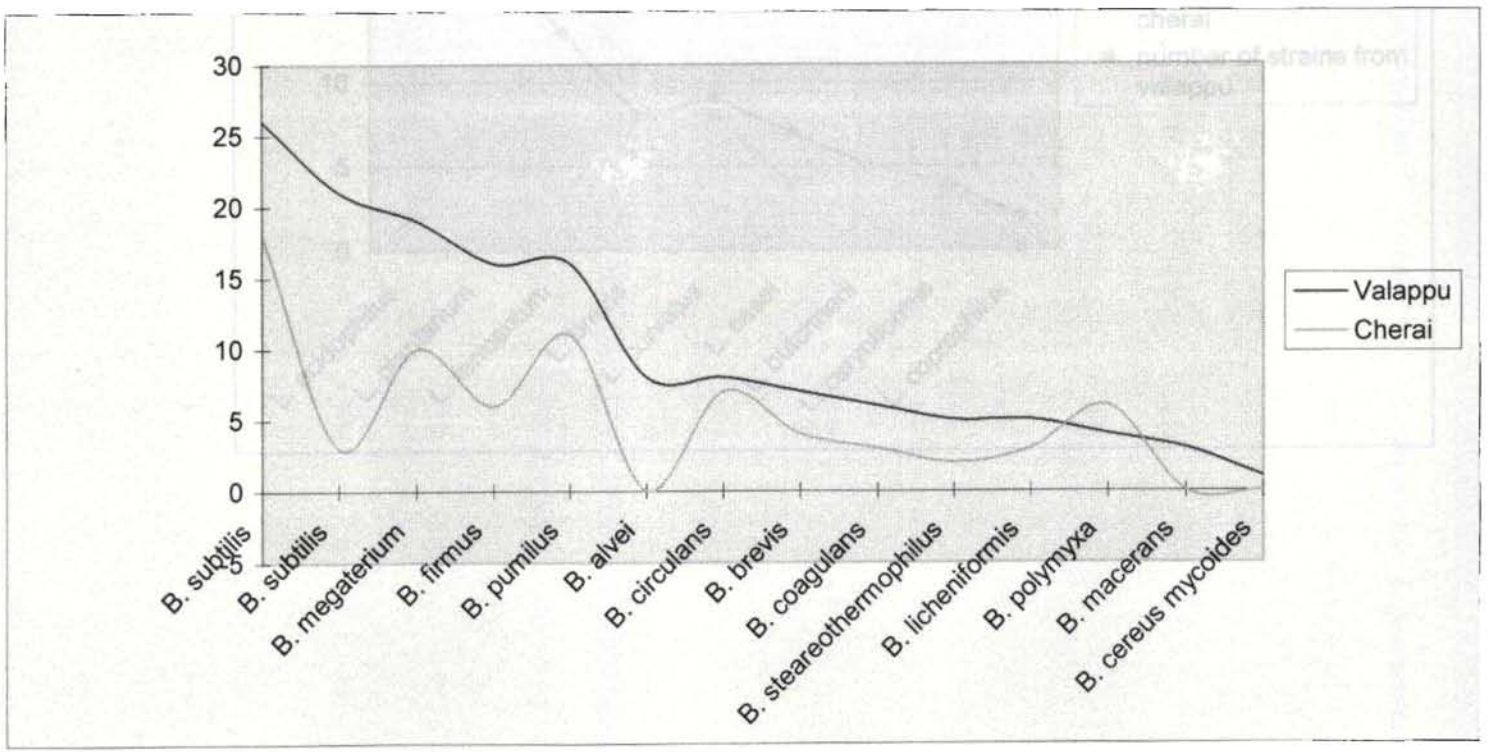


Fig 16 **Variation in the no.of isolates of *Lactobacillus Species* between Cherai & Valappu**

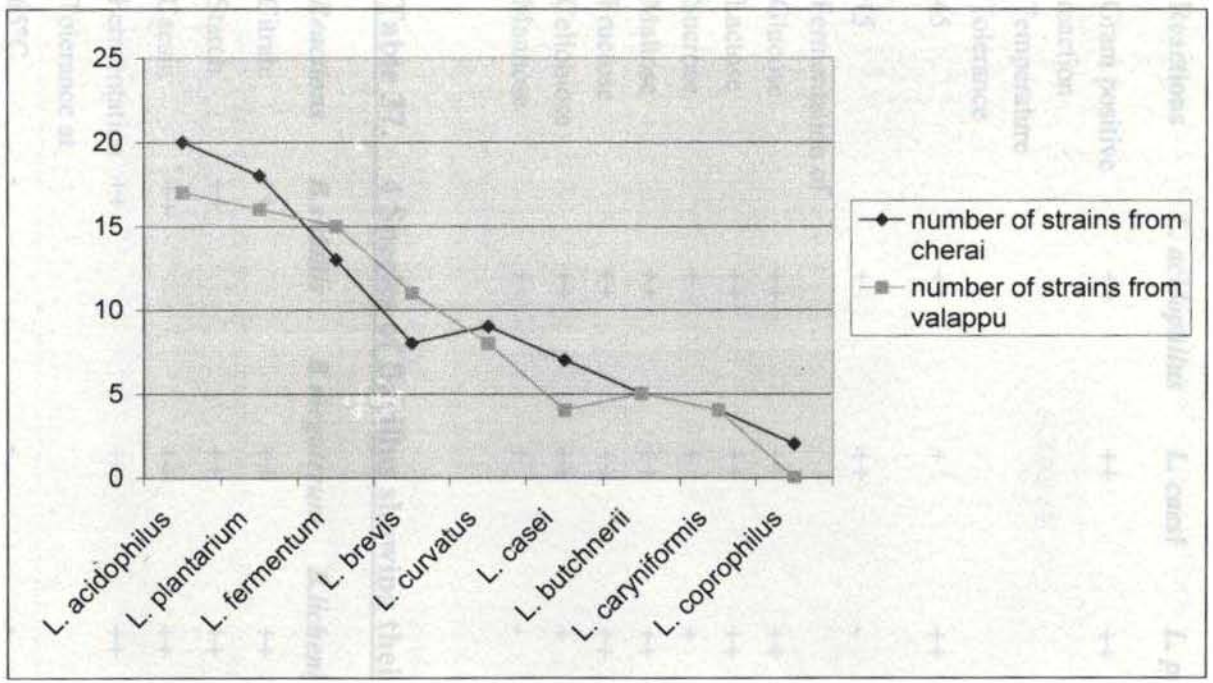


Table 38. 4 species of *Lactobacillus* showing potential character

Reactions	*L. acidophilus*	*L. caesi*	*L. plantarum*	*L. fermentum*
Gram positive reaction	++	++	++	++
Temperature Tolerance				
45	++	+	++	-
15	+	++	-	++
Fermentation of				
Glucose	++	++	++	++
Lactose	++	++	++	+
Sucrose	+	+	+	++
Maltose	++	++	++	++
Fructose	++	++	++	++
Cellobiose	++	++	+	++
Mannose	++	+	-	++

Table 37. 4 Species of Bacillus showing their potential character

Reactions	*B.subtilis*	*B.megaterum*	*B.licheniformis*	*B.polymyxa*
Citrate	++	++	++	-
Starch	++	++	++	++
Caesin	++	++	++	+
Fermentation	++	++	++	++
Tolerance at				
65°C	-	-	-	-
45°C	++	++	++	++
Nacl – 7%	++	++	++	+
Catalase	++	++	++	++

ig. 17. Comparison of growth rate (% increase in weight in gm) of *Penaeus monodon* larvae fed with different feed combinations

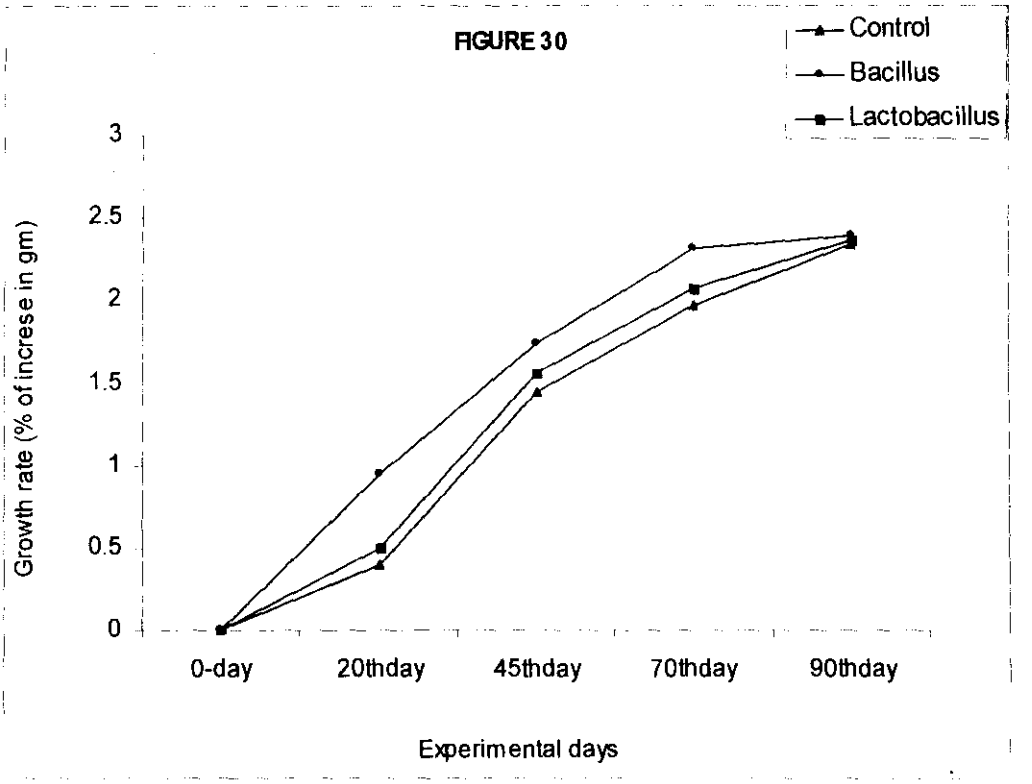


Table 39: ANOVA comparing the growth rate of *P. monodon* larvae fed with different feed combinations

Source	Sum-of-Squares	df	Mean-Square	F-ratio	Level of significance
A	29.577	4	7.394	785.145	p<0.01
B	0.169	2	0.084	8.954	p<0.01
A*B	0.078	8	0.010	1.040	NS
Error	0.283	30	0.009		

A: Between rows; B: Between columns; A*B: Residual error

Fig. 18. Comparison of survival of *P. monodon* larvae fed with different feed combinations

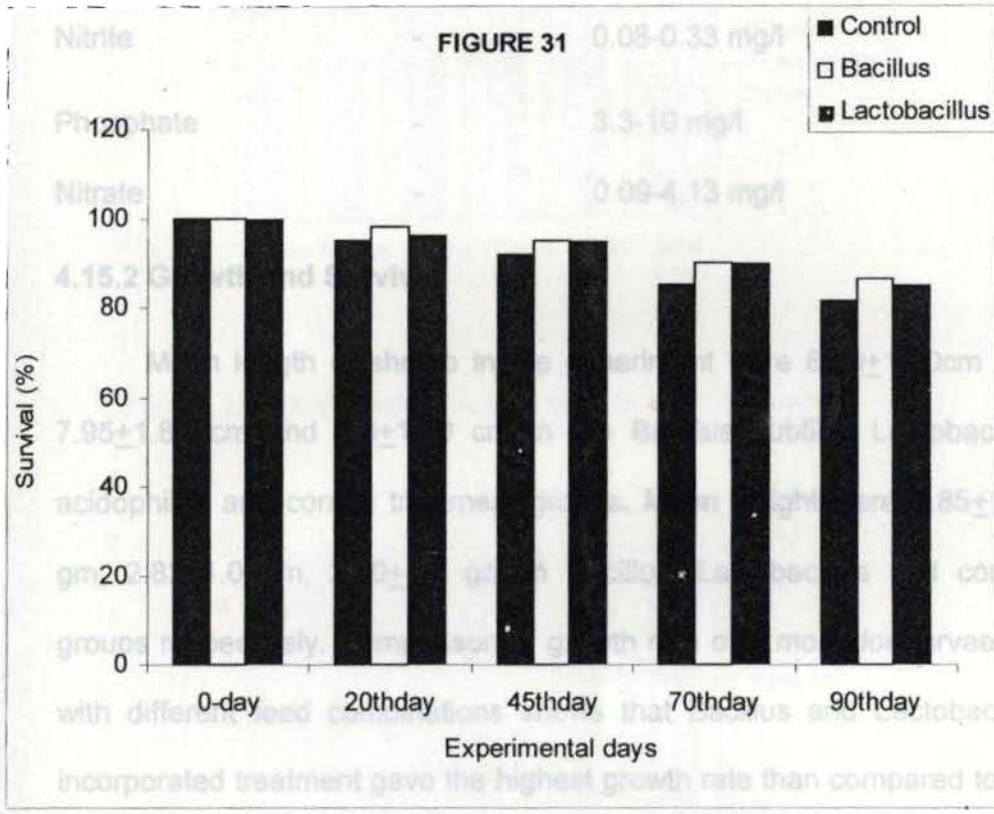


Table 40: ANOVA comparing the survival of P. monodon larvae fed with different feed combinations

Source	Sum-of-Squares	df	Mean-Square	F-ratio	Level of significance
A	1402.130	4	350.533	680.910	p<0.01
B	102.303	2	51.152	99.362	p<0.01
A*B	34.754	8	4.344	8.439	p<0.01
Error	15.444	30	0.515		

A: Between rows; B: Between columns; A*B: Residual error

Ammonia	-	0.0 – 0.42 mg/l
Nitrite	-	0.08-0.33 mg/l
Phosphate	-	3.3-10 mg/l
Nitrate	-	0.09-4.13 mg/l

4.15.2 Growth and Survival

Mean length of shrimp in the experiment were 8.10±1.90cm and 7.95±1.85 cm and 7.8±1.20 cm in the Bacillus subtilis, Lactobacillus acidophilus and control treatment groups. Mean weight were 2.85±1.05 gm, 2.82±1.0 gm, 2.80±1.0 gm in Bacillus, Lactobacillus and control groups respectively. Comparison of growth rate of *P.monodon* larvae fed with different feed combinations shows that Bacillus and Lactobacillus incorporated treatment gave the highest growth rate than compared to the control. The growth performance between Bacillus and Lactobacillus diets showed that Bacillus fed groups exhibited a better growth rate than Lactobacillus fed diets. (Fig 27)

Survival rate also of the Bacillus and Lactobacillus fed animals were higher than the control. Survival rate for the Bacillus when compared to Lactobacillus showed better performance and higher growth rate. (Fig 28). Analysis of variance (Table 40, 41) (ANOVA) showed significant influence of Bacillus subtilis and Lactobacillus acidophilus on the growth rate and survival of the shrimp *P.monodon* juveniles during the period of experiment.

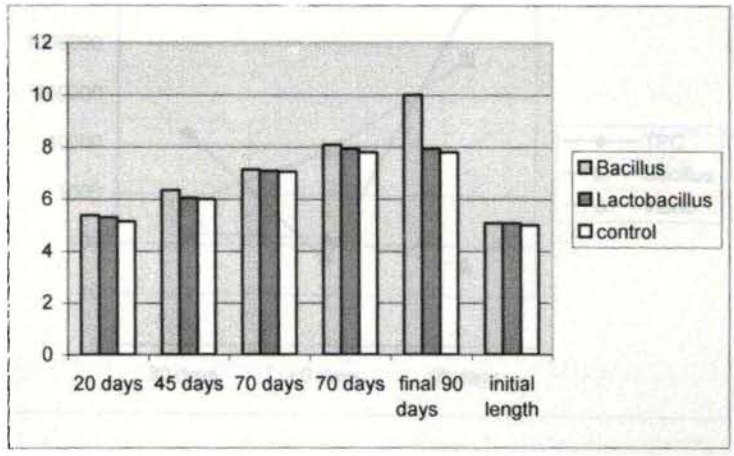


Fig 25. Lengthwise relation of the three treatment Groups

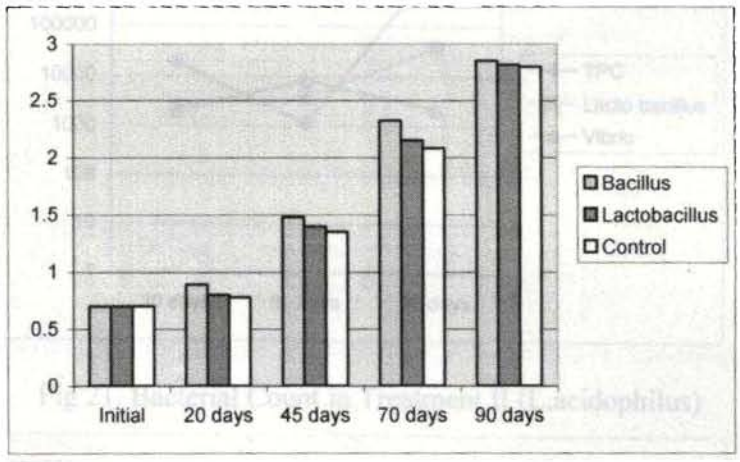


Fig 26. Weight wise relation of the three treatment Groups

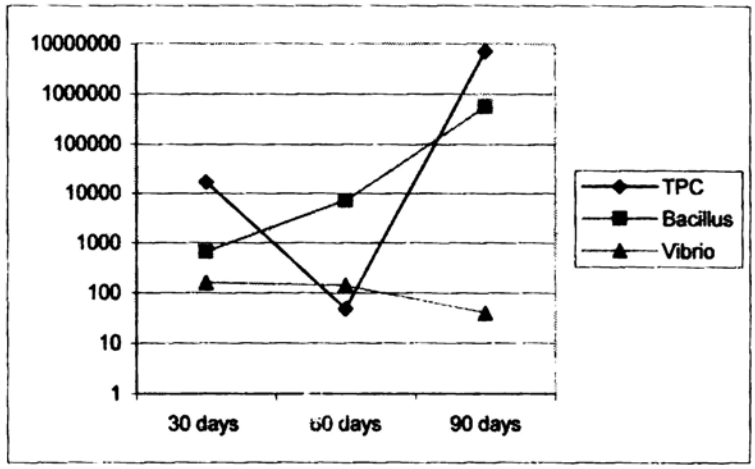


Fig 19. Bacterial Count in Treatment I (B.subtilis)

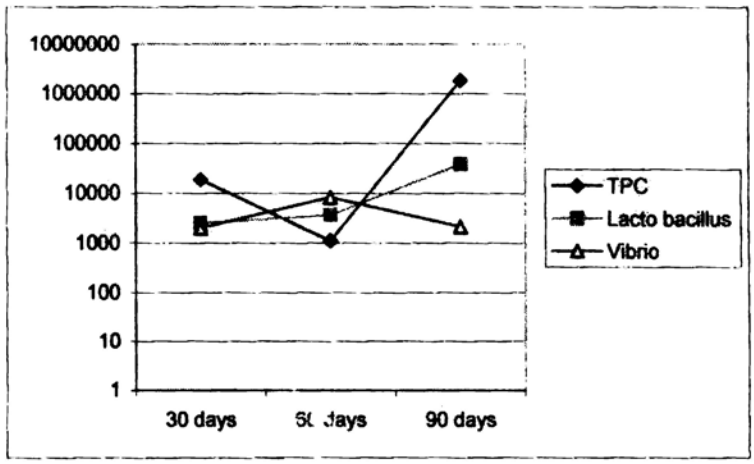


Fig 21. Bacterial Count in Treatment II (L.acidophilus)

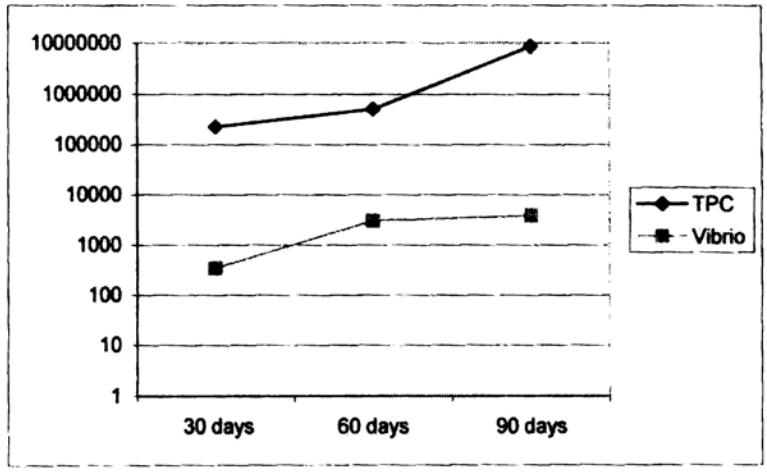


Fig 23. Bacterial Count in Control Tank

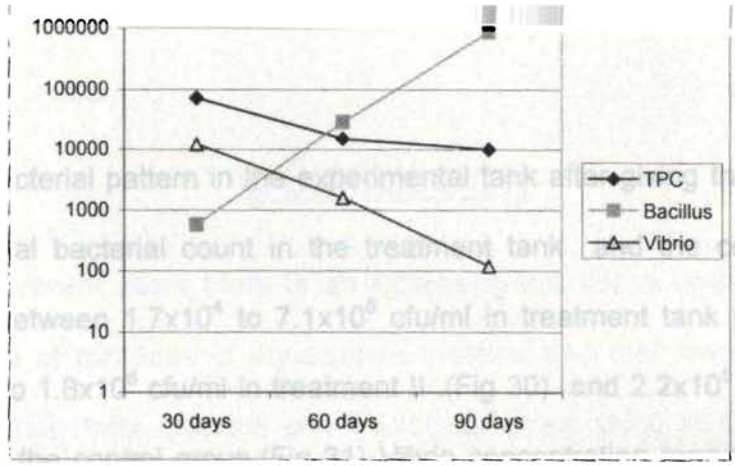


Fig 20. Bacterial Count in the shrimp intestine of treatment I (B.subtilis)

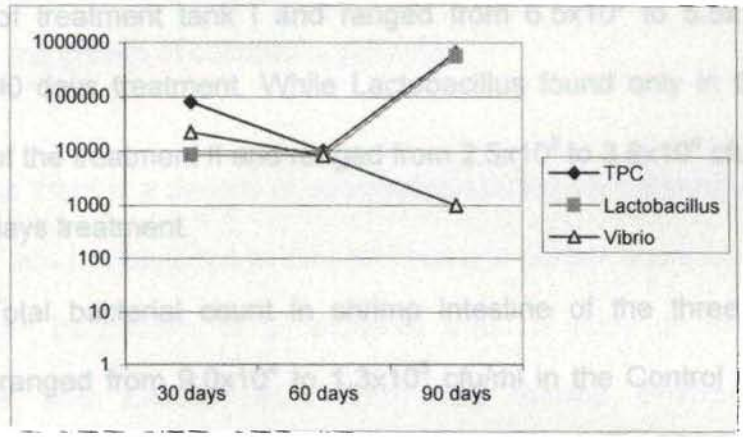


Fig 22. Bacterial Count in the shrimp intestine of treatment II (L.acidophilus)

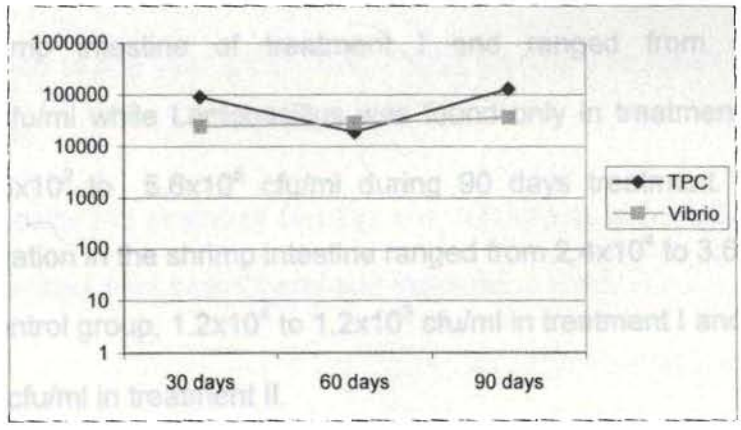


Fig 24. Bacterial Count in the shrimp intestine of Control tank

Bacterial pattern in the experimental tank after giving the probiotic feed. Total bacterial count in the treatment tank and the control tank ranged between $1.7x10^4$ to $7.1x10^6$ cfu/ml in treatment tank I .(Fig 29), $1.9x10^4$ to $1.8x10^6$ cfu/ml in treatment II .(Fig 30) and $2.2x10^5$ to $8.6x10^5$ cfu/ml in the control group.(Fig 31) Vibrio concentration ranged between $1.6x10^2$ to $0.4x10^2$ cfu/ml. Bacillus count was found only on the rearing waters of treatment tank I and ranged from $6.5x10^2$ to $5.5x10^5$ cfu/ml during 90 days treatment. While Lactobacillus found only in the rearing waters of the treatment II and ranged from $2.5x10^2$ to $3.8x10^4$ cfu/ml during the 90 days treatment.

Total bacterial count in shrimp intestine of the three treatment groups ranged from $9.0x10^4$ to $1.3x10^5$ cfu/ml in the Control group. (Fig 34), $7.1x10^4$ to $1.0x10^4$ cfu/mlin the treatment tank I (Fig 32) and $8.60x10^4$ to $2.5x10^4$ cfu/ml in treatment II (Fig 33). Bacillus subtilis was found only in the shrimp intestine of treatment I and ranged from $5.7x10^2$ to $8.6x10^5$cfu/ml while Lactobacillus was found only in treatment II ranging from $8.5x10^2$ to $5.6x10^5$ cfu/ml during 90 days treatment. The Vibrio concentration in the shrimp intestine ranged from $2.4x10^4$ to $3.6x10^4$ cfu/ml in the control group, $1.2x10^4$ to $1.2x10^3$ cfu/ml in treatment I and $2.2x10^4$ to $1.0x10^4$ cfu/ml in treatment II.

5. DISCUSSION

In recent years there is an increasing interest to understand the relevance of microbes in aquaculture systems and their importance for prawn productivity. *Bacillus* and *Lactobacillus* are used in Culture and Production System to manipulate the microbial communities in order to reduce or eliminate, selected pathogenic microorganism like Vibrio and to improve the growth of cultured organisms.

As there is a paucity of information studies on indigenous flora like *Bacillus* and *Lactobacillus* in the perennial and pokkali aquaculture System of Cochin backwaters is un explored area, a study on the "**Role of *Bacillus* and *Lactobacillus* from the marine environment for sustainable aquaculture Practices**" was initiated and regular collection of water, sediment and shrimp intestine was made from two fixed station for a period of one year (2001 April – 2002 March). The result of the study show that:

1. Totally 248 strains of *Bacillus* and 166 strains of *Lactobacillus* were isolated from both Cherai and Valappu.

2. *Bacillus Subtilis* and *Lactobacillus acidophilus* were the most prominent species of Bacillus isolated. The biochemical potential

(proteolytic, amylolytic, caesenolytic, Ureolytic, saccharolytic etc) was found very high.

3. 14 Species of Bacillus (*B.sublitis, B.sphaericus, B.pumilus, B.megataruim, B.firmus, B.circulane, B.alvei, B.polymixa, B.brevis,B.coagulans, B.stearothermophilus, B.licheniformis, B.macurance and B.cereus*(mycoides) and 8 species of *lactobactobacillus(L.acidophilus, L.plantarum, L.fermentum, L.brevis, L.curvatus, L.casei, L.butchnerii, L.cosynifosmil and L.coprophihis*) were isolated from Cherai and Valappu.

4. Statistical inference on the influence of environmental parameters on *Bacillus* and *Lactobacillus*, showed that significant correlation existed between environmental parameters such as salinity, dissolved oxygen, temperature, Phosphate, Nitrite, Nitrate and Ammonia.

5. Selected strains of 4 Bacillus species identified by biochemical potential was subjected to Microbial Identification System (MIS) and the identification was found correct based on the fatty acid profile.

Through out the period of study at Cherai and Valappu showed almost identical physico-chemical parameters such as salinity, temperature, Dissolved oxygen and pH, even though slights variation existed between the two stations which can be attributed to the tidal influence and under water currents to change in the Cochin back water system as the culture ponds are extensions of these estuary.

The temperature of the study area showed considerable seasonal fluctuation and recorded maximum of 34°C in Cherai while Valappu recorded ,36°C and a minimum of 30.5-31°C was recorded from both the stations. The changes in temperature is mainly due to monsoonal influences in these aqua culture pond: Lakshmanan et. al., (1982) reported sharp variation in temperature during onset and withdrawal of South West monsoon, Rheinheimer (1985) also reported that fluctuation in the seasonal temperature causes a change in the distribution of actinomycetes during monsoon which may be due to environmental parameters other than temperature as temperature was recorded low during monsoon. (Fig 2)

Lakshmanan et. al., (1982) also reported that salinity gradient in the northern side of the estuary is lower than the southern side. The present study revealed a gradual increase in the salinity from December to April in Cherai. Maximum of 28ppt was recorded in March a minimum during June

July and August (3-5 ppt). The degree of salinity determines to a particularly large extent the living microbial communities in water. Majority of marine bacteria are halophilic. It is the NA^+ which is the vital necessity for most marine microbes and for some in additional Cl^+. (Macleod,1965, 1968). Salinity which deviates to some degree from the optimum prolongs the generation time in all bacteria (Rheinheimer, 1985). (Fig 4). Minimum salinity at Cherai was recorded during July (4 ppt) while at Valappu during June (3 ppt). This is due to the monsoon and post monsoon rains, which in turn brought down the salinity to low range and there by the bacterial counts.

According to Mayer-Reil (1972) adaptation is largely restricted to within certain salinity ranges characteristic for the particular ecological group ie that is the bacteria with the greatest salt tolerance are as a rule, also the most adaptable. Sridharan and Mohammed Salih (1985) reported wide variation in salinity which may be due to combined action of water movement, tidel variation and mixing. A maximum of 28 ppt was recorded in Cherai in March, April while Valappu showed only 24ppt.

Maximum dissolved oxygen at Cherai was encountered during March. (6.5 mg/l) while Valappu showed maximum during October ((6.5 mg/l) (fig 4) the range of dissolved oxygen observed by Venkitesan et. al., (2001) was from 7-8.4 mg/l whereas in the presence study dissolved

oxygen ranged between (3.5-6.5mg/l). The aquaculture pond at Cherai had a maximum pH of 8.4 during march while Valappu recorded a maximum pH of 9.2 during June. Sing (1986) has observed similar results during his study where he reported that 35.7% of isolates from cultured *P indicus* gave good growth at PH 9. Nellan (1967)has reported that the schelei fjord (Baltic) at a time of vigorous plankton bloom had a pH of 9.5. The large fluctuation of pH affects the composition of bacterial flora. At Valappu and Cherai minimum pH recorded were 7.1 and 7.4 the comparatively higher pH due to higher photo synthetic activity the aquatic vegetation which removed the CO_2 from water column low pH may be due to decomposition of organic matter. (fig 5)

Nitrite Nitrogen values at Cherai ranged between 0.37 µg atm/l – 2.34 µgatm/l and Valappu recorded Nitrite values range in between 0.48mgatm/l –3.96 µgatm/l. the reports of Venkitesan et. Al., (2 001) and Lekhsmanan et. al., (1987) was found consistent to the presence study. Satpathy and Nair (1996) reported a range of 0.19-3.27 reaching almost parallel to the present study. (fig 7)

Nitrate ranged between 0.97. µgatm/l to 7.92 µgatm/l from Cherai station while Valappu recorded a minimum of 0.096 µgatm/l in November and maximum of 2.16 µgatm/l in September. In the present study the

highest nutrient value were noticed during the pre monsoon season while low values were recorded during post monsoon season coinciding with the influx of rainwater. At Cherai a maximum of 9.8 μgatm/l of NH_3 was recorded in June while Valappu recorded a maximum of 17.6mgatm/l in January the lowest values of NH_3 from Cherai and Valappu were recorded in January (0.45mgatm/l) and December (0.09μgatm/l) (fig 8)

Ammonia and Nitrate play and important role in the supply of energy for nitrifying bacteria and the oxygen bound in Nitrate can be used by the numerous flora capable of denitrification under anaerobic condition for the oxidation of organic material. Optimum concentration of nitrate and ammonia were found to enhance bacterial activity were as sub acute or chronic exposure to 1 ppm unionised ammonia or 10 ppm nitrite may result in gill damage, black gills and low level mortalities (Chen et. al., 1990) (fig 6)

At Cherai maximum phosphate values were recorded in September 2001 (7.92μgatm/l) and a minimum in April 2001 (0.04μgatm.l) while Valappu recorded a maximum of 8.9μgatm/l in April and a minimum of 0..4μgatm/l in July the high phosphate contain observed in April at Valappu may be due to the pre monsoon effects as recorded by Lakshmanan et. al., (1987). The activity of heterotrophic microorganisms

causes enrichment of nitrate and phosphate consequently regions where water from the deeper parts which are rich in nutrients well up to the surface show high productivity and a more abundant growth (Rheinheimer, 1985). (fig 9).

In the present study 3 different selective medias were used. Apart from Zobell's marine agar as mentioned in the results for enumeration of TPC, *Bacillus*, *Lactobacillus* flora. It is always better to employ more than one medium to assess the antagonistic strain of *bacillus and lactobacillus* spp than by employing a single media. (Grey and Meyer; 1968).

The annual variation of TPC in water showed a maximum total heterotrophs in the month of September at Cherai ($252x10^3$/ml) and August ($24.8x10^4$/ml) at valappu. (Fig 12 and Fig9). Sediment sample at Valappu recorded a maximum during the month of March 2001 ($18.0x10^4$/gm) while Cherai recorded a maximum in October 2001 (220 x 10^3/gm). The correlation matrix between TPC of water and environmental parameter at Cherai showed that TPC was significantly positively correlated with Dissolved Oxygen and phosphate at 5% level and TPC was significantly correlated with temperature at 1% o level. In Valappu TPC was correlated with dissolved oxygen and nitrate at 1% level, while a negative correlation at 5% existed with salinity. The primary environmental factors influencing the sediment bacteria include moisture, temperature,

acidity, organic matter and inorganic ingredient supply. The influence of season occurs only from the combination of primary determinants. The number and types of *Bacillus and Lactobacillus* is determined by sediment type also. Valappu sediment was of clayey nature rich in organic matter which influenced the highest count of *Bacillus and Lactobacillus*. Alexander (1978) reported that bacterial density is influenced to a large extent by the organic matter content in the habitat. (Fig10 and 13) (Table1)

Valappu recorded the maximum no: of Bacillus and Lactobacillus during the study period which may be due to the tidal effect. Whenever maximum no: of Bacillus and Lactobacillus occurred, variety decreased. (Table 8 and 9). The minimum TPC was observed in monsoon and maximum TPC in post monsoon during the present study was found to be in accordance with the observation of Chandrika (1983). The heterotrophic bacteria of sandy beaches of Goa showed a maximum count of $134.46x10^3$/gm and minimum of $0.47x10^3$/gm in July and October. (Nair and Lokabharathi, 1980). This may be due to the sandy nature of the study area. Venketeswaran et. al., (1989) reported <2-80 cfu/gm in the sediment of the Seto island. The mean total viable counts ranged from $1.85x10^5$ to $6.18x10^6$/g in shrimp; $1.80x10^3$ – $4.50x10^3$/g in rearing water and $1.82x10^6$-$4.72x10^6$/g in sediment. Sharmila et. al., 1996 studied the bacterial flora of penaeid shrimp, *P.indicus*, pond water and sediment in a

semi-intensive surface water collected in September and October(1999) from fresh water marsh habitat showed bacterial density ranging from $9.7x10^5$ to $1.3x10^7$ cells/ml. The majority of 888 isolates were gram positive, of which 35% were bacillus spp. (Smith, T. W; Walker, E. D; Kaufman, M. G.. 1998). Matrix correlation of TPC sediment showed negative correlation at 5% level with nitrate in Cherai, while in Valappu TPC showed no significant correlation with any of the environmental parameters in sediment. (fig 14and15)

TPC of shrimp intestine recorded a maximum during January 2002 ($160x10^3$/gm) while a minimum of $98x10^3$ was recorded during March. While Valappu station showed a maximum in March $26.8x10^4$/gm while January recorded minimum $72.8x10^3$/gm. The temporal and seasonal variations in the different environmental parameters are reflected in the quantitative and qualitative distribution of TPC. (Fig 13)

Correlation matrix of TPC at Cherai showed no significant correlation with any of the environmental parameters. At Valappu a significantly positive correlation existed between oxygen at 1% level and nitrate at 5% while salinity showed a negative correlation at 5% level. (Table 7and13).

This may be probably due to wide fluctuation in the water quality and the nature of pond substrate and organic enrichment in the sediment are the important factors restricting the abundance of TPC.

Singh (1986) observed that an increasing order of TPC from the alimentary canal of *P.indicus*. He has also observed that gram negative bacterial count was higher in intestine than body surface. Similar results were observed by Palaniappa (1982). Chandrasekharan (1985) has found that the bacterial flora varied from 10^6-10^7/cm^2 in the body surface, 10^6-10^8 gm in gills and 10^6-10^8 in the intestinal content of *P.indicus*.

The low number of flora is present contrary to this low no: of flora in the present study indicates stress condition and wherever optimal conditions are obtained because of the dilution, low count has been observed.

Total Bacillus Count (TBC) from Cherai station recorded a maximum ranging between 22.2x10^3-50x10^3/gm while a minimum of 1-2x10^3/gm was recorded. Valappu recorded a maximum of 79x10^3=98x10^3/gm, while a minimum value recorded as 1-2x10^3/gm.

The bacillus counts were higher in Valappu when compared to Cherai and that the highest counts were recorded during monsoon season

as it is found that matrix correlation of Cherai showed that significant correlation existed between total bacillus count and Ammonia at 5% level while at Valappu station 5% correlation existed with temperature, phosphate and nitrate.

This may be the reason for increased count in Valappu when compared to Cherai. Studies of Ostenvick et. al., (2004) revealed that bacillus species from 8 different rivers were 15-1400 cfu/100ml while Binimol (2004) isolated 6.3-24x10^2/gm of bacillus from mangrove sediments. Variations in different environmental parameters and composition of sediments and substrate availability are reflected in the quantitative and qualitative distribution of bacillus. The flora composition exhibited a different picture in the Cherai and Valappu culture ponds.

Total lactobacillus count was recorded maximum in May (210x10^3/gm) at Cherai while at Valappu showed maximum in July (170x^3/gm), rest of the months showed low count in both the station. Matrix correlation of Total bacillus Count at Cherai showed negative correlation at 5% level with pH of water while no correlation existed with other parameters. While in Valappu Total Lactobacillus exhibited 5% significance with phosphate and dissolve oxygen each. (Table 10 and 4)

Studies of Nair et. al., (1997) reported that LAB counts were always 1-2 log cycles in the case fishes from internal trade. Lactobacillus were present in fresh and brackish water fishes. But LAB was not present in ocean fresh fish. Reports of Ringo et. al., (1998) and Halami et. al., (1991) also revealed that lactic acid bacteria dominated in the intestine of fish and fowl.

8 species of lactobacillus were isolated from both Cherai and Valappu. *Lactobacillus acidophilus* dominated in both the station total of (22.28%), the other species *L.plantarum* (20.48%) *L.fermentum* (16.86%), *L.brevis* (11.44%), *L.curvatus* (10.24%), *L.casei* (6.62%), *L.butchenerii* (6.02%), *L.coryniformis* (3.61%) and *L.coprophilus* (1.20%). A total of 166 strains were isolated and identified using Bergy's manual of determinative bacteriology (1974) and Cowan and Steel, (1977) from the two stations. (Table 30)

Forty seven strains of homofermentative rod shaped and 5 heterofermentative sphere shaped lactic acid bacteria were isolated by Tanasupawat et. al., (1998) from 4 kinds of fermented fish. Four strains were identified as lactobacillus pentoses and one strain as *L.plantarum*. About 90% of lactobacillus strains were isolated and characterised from fresh and frozen fish by Nair et. al., (1999).

All these studies in parallel to the present study shows that lactic acid bacteria are candidate species of the gut. They colonise in the gut and produce bacteriocin which may act antagonistic to gram negative fish pathogen. So the need to use antibiotics in future aquaculture can be reduced by periodically administering the flora. Seventy-eight strains of lactobacillus were isolated from fillets of vacuum-packed smoked and salted herring by Gancel et. al., (1997).

Binimol (2004) isolated 4 species of Bacillus – *B.subtilis, B.licheniformis, B.pumilus and B.oleronicus* from Mangalavanam mangrove sediments. 86 Bacillus strains were totally isolated out of which bacillus species such as *B.cereus* and *Bacillus subtilis* representing from all the samples. Nirmala Thampuran recorded 30% *B.subtilis*, 40% *B.coagulans*, 10% *B.alvei*, 5% *B.brevis* and 5% *B.pumilus* from dried, barred and cured fish from canned fish. *B.subtilis, B.pumilus* occurred 20% each, *B.alvei* occurred 40% where as pickled fish harboured 44% *B.subtilis*, 28% *B.coagulans*, 8% B.pumilus and 4% *B.alvei, B.brevis* and *B.megaterum* each.

In the present study about 14 species of bacillus strains i.e., *B.subtilis (44), B.sphaericus (24), B.pumilus (27), B.megaterium (39), B.firmus (22), B.circulans (15), B.alvei (n=8), B.polymyxa (n=10), B.brevis (11), B.coagulans (9), B.steareothermophilus (7), B;licheniformis (8),*

B.maceran (3) and B.cereus (mycoides) (n=1) were isolated from the two stations Cherai and Valappu. (Table 25 and 26)

Ivanova et. al., (1999) isolated 20 aerobic sporeforming Bacillus of which only species of Bacllius, *B.subtilis, B.cerecus, B.licheniformis, B.firmus, B.pumilus, B.mycoides and B.lentus* from the marine environment and in relation to the present study. *B.subtilis* and *B.pumilus* were most abundant species associated with marine environment. This may be so because the strains were able to utilise a wide range of organic compounds were halotolerant and alkalitolerant and reflect their great metabolic flexibility.

Quantitative variations in the different environment parameters and composition of sediments, substrate availability are reflected in the quantitative and qualitative distribution of Bacillus in the present observation. The flora composition exhibited a different picture in Cherai and Valappu aquaculture ponds. *B.subtilis* was dominant species in both the aquaculture pond which suggest that *B.subtilis* has got enzyme potential to degrade and refractory organic compounds received by high organic load through terrestrial inputs and anthropogenic influence in this environment. (Table 37)

The predominance of stress tolerant species getting mutated with adaptive enzymes existed with low microbial diversity wherever optimal

conditions are not met with. The enzyme potential shows an impact on environmental parameters which control the distribution of Bacillacea at the same time indicate the quantum of endurance warranted by the flora to tide over by the range of environmental stress. High microbial diversity of Bacillus species is to stabilise the ecosystem as the ecosystem and the flora is highly fragile.

Several taxonomic studies of bacillus and related species have been conducted by various workers. Ivanova et. al., (1999) isolated 16 representatives of the genus bacillus from seawater samples. Ivanova and Mikhail (1999) isolated 20 aerobic endospore forming bacillus. Binimol (2004) isolated 4 species from mangrove ecosystems. Further problems that arise in making comparisons are that different set of characters may have been used and the same test may not give the same results in different laboratories explaining the variations that occur in the certain tests for the classification of bacterial isolates (Bryant et. al., 1986). The present study helped to establish whether data collected from different times by employing standard techniques were reliable and could be combined to provide a useful taxonomic result.

The procedure for the identification of bacillus was exemplified by Bergy's Manual of determinative bacteriology (1986) and Cowan and Steel's (1977) Manual for identification of medical bacteria. The

comprehensive and practical description of conventional test media and methods set out in Bergy's Manual as appropriate and widely used for the characterisation of most of the bacillus species. All the strains of bacillus from Cherai and Valappu were gram positive rods which were highly motile.

The spore shapes were oval for most of the species except for *B.sphaericus* with round spores and the spores position were either central, terminal or subterminal. In most cases the spores were central in position. In relation to the present study Alexander, 1977 reported that 60-100% of soil bacilli population exist in the inactive spore state. Claus and Berkeley (1986) reported that genus bacillus count of a large no: of diverse rod shaped gram positive bacteria that are motile by peritrichous flagella and are aerobic. The numbers are capable of producing endospores that are highly resistant to unfavourable environmental condition.

79.54% of bacillus from Valappu and 96.9% of bacillus strains from Cherai showed a high tolerance at 45°C while temperature tolerance at 65°C was exhibited only by strain B.stearothermophilus from both stations. (Table 25 and 26) Alkali tolerance of 5% was exhibited by 77.2% of strain from Cherai and 71.95% of strains in Valappu station. Halotolerance at 7% NaCl was exhibited by 69% of strains from Cherai and 73.3% of strains from Valappu. The study shows that bacillus species exhibited a high

salinity, temperature and alkali tolerance which make them suitable as pond probiotics.

All the strains from both Cherai and Valappu produced the enzyme catalase by cytochrome oxidase was produced by 88.4% of *B.subtilis* only. Indole production and Arginine dihydrolysis was weak in all the strains of bacillus. Nitrate was reduced to nitrite by 81.06% of bacillus from Valappu and 67.6% of strains from Cherai station. This shows that Bacillus species exhibited a high enzyme potential capacity. Most species of Bacillus can grow anaerobically at the expense of sugars. They carry out a distinctive fermentation, in which 2,3-butanedoil, glycerol and CO_2 are the major end products accompanied by small amounts of lactate and ethanol. The fermentation can be represented as:

3 glucose ⟶ 2,2,3-butanediol + 2 glycerol + 4 CO_2.

Stainer (1976) reported glucose is initially dissimilated through Embden and Meyer hof. pathway to the level of triose phosphate at which point a metabolic divergent occurs. Pyruvate is formed from part of the triose phosphate from which butanediol and CO_2 are produced. *Bacillus subtilis* cannot grow anaerobically at the expense of glucose as it cannot reduce triose phosphate to glycerol where as *B.licheniformis* can grow anaerobically at the expense of organic substrate when furnished with

nitrate it is the only bacillus with a vigorous denitrifying capacity under anaerobic condition.

In the present study all the strains of *B.subtilis* from Valappu and Cherai fermented glucose while only 85.5% of strain from Valappu and 86.05% of strains from Cherai exhibited a high glucose fermentation capacity. Chandrika (1999) stated that all strains isolated from Cochin backwaters exhibited fermentation of glucose while *Bacillus polymyxa* and *B.macerans* form spores with distinct star shaped profile to the spore. Both are fermentative organisms dissimilating starch and pectin as well as monosaccharides and good growth will occur only in the presence of utilisable carbohydrate like glucose. Another distinctive property of *B.polymyxa and B.macerans* is the ability to fix nitrogen when grown under anaerobic conditions. They are the only Bacillus species known to possess this property.

Arabinose fermentation was exhibited high, by *B.subtilis* (77.7%), *B.circulans (71.4%), B.polymyxa (83.3%), B.pumilus* (54.5%). While only few strains showed arabinose fermentation for other species in Cherai. While xylose and mannitol fermentation was exhibited by 77.7% and 61.11% of B.subtilis, 81.81% and 36.3% of *B.pumilus*, 71.42% of *B.circulans*, 100% and 33.3% of *B.polymyxa*, 100 and 33.3% of *B.licheniformis*, from Cherai station while in Valappu station 100% *B.macerans, 75% B.polymyxa, 40% B.licheniformis, 62.5% of B.pumilus,*

50% *B.subtilis* fermented arabinose, while xylose and mannitol fermentation was low in most of them except 75% of *B.circulans* which exhibited a high xylose fermentation.

According to Chandrika (1999) 95% of bacillus exhibited Gelatin liquefaction and 100% hydrolysis of casein was exhibited by the isolated strains. 83.3% of isolates hydrolysed starch in 24 hours incubation time. While in the present study 89.2% of strains from Cherai and 72.14% of strains from Valappu exhibited a starch hydrolysis capacity showing their amylolytic potential. The starch hydrolysing enzymes are usually inducible but the ability of microorganism to form amylolytic enzymes depends on the type of starch.

In the present study gelatin liquefaction was exhibited by all strains of B.subtilis from both Cherai and Valappu and more than 80% of *B.megaterum, B.steareothermophilus, B.licheniformis* from Valappu station exhibited gelatin hydrolysis while more than 80% of strain of *B.pumilus, B.megaterum, B.circulans, B.polymyxa, B.brevis, B.licheniformis and B.sphaericus* from Cherai exhibited gelatin hydrolysis showing their versatility for proteolytic activity.

In relation to the present study, Hitchfeld and Wood (1996) found that by proteolytic activity of bacillus higher enzyme yield was obtained.

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When protein was suspended in medium than when dissolved in the medium. Chandrika (1983) studied that 36 bacillus from sediments of Cochin backwaters showed high proteolytic, ureolytic and caseinolytic activity. Wood (1959) isolated proteolytic bacillus from surface water and bottom sedimentary 1 metre depth, the genus Bacillus dominated predominated and formed 22% of surface water 39.5% from 1m and 45% from bottom sediments. New species produce specific enzymes are being studied with reclassification of certain species and variants for use as probiotics and DMS-range of bacteria to enhance intensive shrimp fish culture. In the present study casenolytic activity was exhibited by 80.4% of the strain from Cherai while only 78.1% of strains from Valappu exhibited caseinolytic activity. Murchelano and Brown (1970) reported that in all the seasons proteolytic, amylolytic activity of marine bacteria isolated from long island Sound was highly stable even though there was variation in Genera obtained.

In the present study ureolytic activity was exhibited by only 51.04% of isolates from Valappu, while only 43.9% of the isolates from Cherai exhibited ureolytic activity parallel to the present study. Chandrika (1999) recorded 44.4% of the total flora isolates from Cochin backwaters.

$$CO(NH_2)_2 + H_2O \longrightarrow CO_2 + 2NH_3$$

The ecological advantage of spore formers is their strong ureolytic activity. In an enrichment medium wide variety or chemo heterotrophlis can grow in pure culture on this medium. While incorporating these flora in the preparation of probiotic the pH has to be taken care of as high concentration of free NH_3 can make the medium alkaline.

Many microorganisms possess the enzyme urease, the catalyse responsible for hydrolysing urea. Urease is a constitutive enzyme in other it is an inducible enzyme. Ammonium carbonate is an intermediate compound in the urea hydrolysis. *Bacillus, micrococcus, pseudomonas, klebsiella, pseudococcus, clostridium* are the flora actively hydrolising urease. 32.69% of the flora are capable of hydrolysing urea from sediment. (Table 25 and 26)

In the studies of Austin, (1988) *B.maximus* isolate did not degrade urea at all. Voges Proskauer reaction was also negative. While in the present study, 70.07% of B.subtilis, 43.75% of *B.pumilus*, 50% of *B.alvei* and *B.polymyxa* and 40% of B.licheniformis and all strains of B.cereus degraded urea at Valappu while in Cherai only lesser no: of strains degraded urea besides B.subtilis 61.1%, 54.54% of *B.pumilus*, 66.6% of *B.licheniformis* and 50% of *B.polymyxa*. Studies of Chandrika (1999) reported that 95% of the isolate showed positive Voges Proskanes reaction showing the ability to produce acetoin. Tyrosine decomposition

was reported only in 33.15% of isolates from Cherai while 50.27% isolates from Valappu station decomposed tyrosine.

The procedure for identification of lactobacillus was exemplified by **Bergy's manual of determinative bacteriology** (1976). (Table 30 and 31). All the strains of lactobacillus from Cherai and Valappu shows gram-positive reaction with long slender rods which were non motile and without the enzyme catalase. Temperature tolerance at 15°C was exhibited by 91.7% of lactobacillus strain from Valappu and 80.6% of strains from Cherai while 45°C was tolerated by 95% of *L.acidophilus* and 86.15% of *L.fermentum only from both the stations.*

Nitrate was reduced to nitrite by 25% of *L.buchnerii* from Cherai station while 36.67% of lactobacillus strains from Valappu station reduced nitrated. Glucose fermentation was found in almost all strains of lactobacillus isolated from both Cherai and Valappu. As many complex compounds must be transformed to simpler forms prior to use by the organisms.

$C_6H_{12}O_6 \rightarrow 2C_3H_4O_3 + 4H$

Glucose Pyruvic acid

B.amylase are not common in micro-organisms. The maltose and low molecular weight oligo saccharides that are converted to glucose by

mediation of the enzyme α-glucosidase so that starch is transformed ultimately to glucose. The simple sugars are water soluble and penetrate the cells to be used as energy sources for growth and protoplasmic synthesis.

Mannose is essentially the only sugar which are present in some marine plants apart from galactose. The Mannose : Glucose ratio in the environment will be always 2:1. In the present study 75.9% of the strains from Cherai and 70% of strains from Valappu fermented mannose. 66.5% of strains from Valappu and 72.3% of strains from Cherai showed cellobiose fermenting capacity. Fructose and galactose, lactose and maltose was fermented by almost all the strains of lactobacillus from both Cherai and Valappu. While rhamnose and xylase fermentation was weaker with most of the strains.

Mannitol was fermented by 81.25% of L.plantarum and 92.8% of L.casei from Cherai and Valappu. Ribose was fermented by 61.8% of strains from Valappu and 79.2% of strains from Cherai while xylose and rehalose fermentation was weaker in all the strains. In 1947, Rosenfeld and Zobell carried out a detailed study of antibiotic producing marine microorganisms. Although they did not attempt an isolation of specific antibiotic produced by marine bacteria. It was evident from their work that various aspect of microorganisms, indigenous to the sea, released anti-

microbial substance. Since then, there have been several reports of bacteria with inhibitory effects isolated from seawater; the main purpose of these mostly have been to characterise the specific antagonistic compounds or bactereocins produced. Only recently have bacteria been isolated from other marine habitats.

Although the microbiology of the intestinal tracts of marine and fresh water fish has been investigated by many researchers, few studies have addressed the production of inhibitory components by these bacteria based on the proteolytic activity, enzyme potential, alkali tolerance, temperature tolerance and halotolerance. The highly potential species of *Bacillus subtilis* and *Lactobacillus acidophilus* was selected for the study. (Table 37 and 38)

In the present study, growth and survival of *Penaeus monodon* juveniles were greater treated with probiotic bacteria (*Bacillus subtilis* and *lactobacillus acidophilus*) were greater when compared to the control. Significant difference were found between the treatment control tanks. The bacillus subtilis shrimps showed higher growth and survival rate than lactobacillus treated shrimps.

Parallel to the present study (Rengipipat et. al., 1998, a,b) reported that growth and survival difference between bacillus – treated and non

treated *P.monodon* were more pronounced when Rengipipat et. al., 2000 reported that no significant difference was obtained with all treatments.

In the present study the better growth rate may be due to the effect of probionts on nutritional profile and also on their ability to colonise the intestine and improve their feeding efficiency by producing some microbial enzymes or artemia were reared exclusively on a diet of bacteria while B.plicatilis has also grown with bacteria (Yasuda and Tata, 1980; Gatesoupe et. al., 1989). (Fig 18 and 19)

Intriago and Jones (1993) reported best growth of artemia to pre-adult stage on a mixture of flexibactis and algae and concluded that bacteria acted not only as food, but also aided digestion of algae through the presence of exo enzyme. Recently their apparatus have been used to improve shrimp health and yields. First, use of specific disease-resistant shrimp (SPF) second, vaccination or immuno-stimulation of shrimp to promote immune response. Lastly probiotic used to stimulate immunity and to exclude pathogen. In addition, stress reduction is used to improve disease susceptible to less virulent pathogen. In these cases, the pathogens are often considered secondary infections (Song et. al., 1993) since shrimps possess a non-specific immune response (Anderson, 1992), vaccination or immuno stimulation may provide only short term protection against specific pathogen (Sing and Song, 1996, Sing et. al., 1996).

Effective probiotic treatment on the other hand may provide broader-spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in shrimp gut. At variance is the study of Gildberg et. al., (1998) who observed highest mortality in fish given the diet containing lactic acid bacteria, where as no significant difference was observed between fish given feed supplemented with cod muscle protein and hydrolysed cod muscle protein. Besides the production of anti-microbial substance, a great variety of mechanism have been proposed for the action of probiotics (Monter and Pugh, 1993). Eg. Competition for adhesion receptor in the intestine, competition for nutrients and immuno-stimulation. Further investigation on these lines throw more insight into the actual mechanism of probiont action. (Fig 20 and 21)

The present study showed the presence of viable *Bacillus subtilis* and *Lactobacillus acidophilus* in intestine and faeces of shrimp fed with these probionts Competitive exclusion most likely occurred in this case. Water quality is not affected by probiotic feed additive. The water quality is maintained throughout the experiment (Rengpipat et. al., 1998 a) but shrimp health. Prophylactic functions can be enhanced (Austin et. al., 1992, 1995; Rengpipat et. a;., 1998 a, b; Phianphak et. al., 1999). At the same time, probiont will proliferate in rearing water thus providing a better

environment for shrimp by reducing the level of certain pathogens in the culture water (Moriarty, 1998). (Fig 22 and 23) *Bacillus subtilis*, a *saprophylic* strain appears harmless to shrimp survival during normal culture (Rengipipat et. al., 1998 a). This suggests that probiotic treatment is an effective alternative for enhancing shrimp health.

The research on 'probiotics' has formed the focal point of the present study as well as representing the development of knowledge required by serious and responsible fish farmers. The global fish farming industry is characterised by dynamism and change. There is a constant stream of new knowledge and the result of research are published all the time. New species are being used in production and a combination of indigenous new Bacillus species B.subtilis - enzyme potential, *B.polymyxa* – pH tolerance, alkali tolerant *B.licheniformis* - thermo tolerant and halotolerant showed all the 4 Bacillus isolated in the present study can be used in probiotic production as unique production methods are seeing the light of the day.

VI. Summary

1. The studies on the "Role of Bacillus and Lactobacillus from Marine Environment for Sustainable Aquaculture Practices" was conducted during April 2001 to March 2002 in order to study ecophysiology and biodiversity of Bacillus and Lactobacillus in the marine environment and their role as potential probiotics in culture of *Penaeus monodon* juveniles. Viable isolates of *Bacillus and Lactobacillus* was selected for the probiotic study.

2. Data on physico-chemical parameters were also collected understand their influence on the occurrence and distribution of *Bacillus and Lactobacillus.*

3. The present observation suggests that aquaculture ponds always have a high load of bacterial flora and the apparent fluctuation in Bacillus and Lactobacillus count reflected complex nutritional and physico-chemical variations within the ecological niches.

4. Adaptation was largely restricted to within certain salinity ranges (3-24ppt) characteristic for the particular ecological group. The general drop in salinity to near fresh water condition observed during monsoon is due to dilution by the fresh water influx while the difference in surface

and bottom salinity is caused by the tidal influence creating a two layered flow.

5. The bacterial parameters were influenced by physico-chemical and biological factors. Total bacillus Count of water from Cherai showed significantly positive correlation with dissolved oxygen and phosphate at 5% level. While total bacterial count was negatively correlated to nitrate to 5% level.

6. The total count in shrimp intestine showed no correlation between environmental parameters and total bacterial count.

7. Total bacillus count in water sample showed negative correlation with temperature at 5% level.

8. The intensity of Bacillus in sediment was positively correlated with ammonia at 5% level. While no correlation existed between total bacillus count and environmental parameters in the shrimp intestine.

9. Lactobacillus in water was negatively correlated with pH at 5% level while in sediment and shrimp intestine total lactobacillus count showed no correlation with environmental parameters.

10. At valappu the total bacterial count in water showed a positive correlation at 1% level with nitrate and dissolved oxygen and negative correlation was observed with salinity at 5% level.

11. Total plate count in sediment showed no correlation with any of the environmental parameters. While in shrimp intestine positive correlation existed with oxygen at 1% level and nitrate at 5% level. Salinity was negatively correlated at 5%level.

12. The bacillus count in water was influenced by temperature at 5% level. While Lactobacillus in water showed no correlation with the environmental parameters.

13. Bacillus count in sediment showed significant correlation with phosphate at 5% level and Lactobacillus in sediment was influenced by oxygen at 5% level.

14. The intestinal bacillus was significantly correlated with nitrate and nitrite at 5% level, while total intestinal lactobacillus was influenced by phosphate at 5% level.

15. In the present study percentage composition of 14 bacillus species isolated were as follows : *B.subtilis* (20.18%), *B.sphaericus* (11%),

B.pumilus (12.38%), B,megaterium (17.88%), B.firmus (10.09%), B.circulans (6.88%), B.alvei (3.66%), B.macerans (1.37%), B.cereus mycoides (0.45%).

16. Based on the classification it was found that Bacillus subtilis predominated from cherai and Valappu about 44 strains were isolated from both the stations.

17. High proteolytic activity, enzyme potential, alkali tolerance, halotolerance and temperature tolerance were exhibited by 4 species mainly *bacillus subtilis, bacillus licheniformis, B.megeterium and B.polymyxa.*

18. Percentage composition of 8 lactobacillus species isolated were *L.acidophilus (22.28%), L.plantarum (20.48%), L.fermentum (16.86%), L.brevis (11.44%), L.curvatus, (10.24%), L.casei (6.62%), L.butchnerii (6.02%), L.cornyformis (3.61%), and L.coprophilus (1.20%).*

19. *L.acidophilus and L.plantarum* predominated in the two stations Cherai and Valappu.

20. *L.acidophilus, L.plantarum, L.fermentum, L.casei* exhibited high enzyme potential, fermentation and tolerance capacity.

21. Diversity increase at Valappu showed a high dominance index during post monsoon and species richness index was also recorded high during the post monsoon season. While diversity index and evenness index was high during pre-monsoon period. This variation may be probably due to various biological and physico-chemical environmental factors.

22. Species concentration factors, measured by Simpson's index was very low in perennial ponds on comparing the various seasons for species evenness index high values were obtained in Pokkali pond, i.e., in Cherai, the dominance index and species richness index represents high values in post monsoon season.

23. Bacillus subtilis and Lactobacillus acidophilus were selected for probiotic preparation for *Peneaus monodon* juveniles, based on their potential characters.

24. There was a high increase in growth rate in Bacillus and Llactobacillus fed *P.monodon* when compared to the control fed group when comparing the growth rate between Bacillus and Lactobacillus fed animals, Bacillus treatment group showed highest growth rate.

25. The survival rate was also higher in the experimental group when compared to the control group. 86.6% for bacillus treated group and 85% of lactobacillus group when compared to 81.6% only.

26. The length-weight relationship also increased for the treatment group when compared with the control group during the 90 days treatment.

27. The total bacterial count of the gut micro flora in treatment waters revealed localisation of bacillus subtilis and lactobacillus acidophilus throughout the gut of the experimental animal when compared to the control.

28. The incidence of the vibrio count in the treatment tank decreased by the influence of bacillus and lactobacillus while in the control tank vibrio count was stagnant. This shows that the phenomenon of competitive exclusion by the probiotic species has worked out here.

29. The viable species of bacillus and lactobacillus from the marine environment can be used as probiotic. Commercial probiotic can be avoided and the cost of production can be reduced and the indigenous flora as probiotics in fish farming shows signs of success. There is an urgent need for further studies to determine the best methods of administration and dosage.

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Biofilm Formation by *Bacillus subtilis* on Different Substrates and its Significance in the Production of Probiotics and 'DMS' Preparations.

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Biofilm formation by *Bacillus subtilis* was studied by using different substrates to know the efficiency of enzyme and biomass production. *Bacillus subtilis* isolated from aquaculture farm sediments were inoculated with glucose peptone broth (GPP-broth) and GPP-broth plus Triton & Tween 80, which are non-ionic synthetic surfactant. Biofilm formation was tested with biomass and alpha-amylase & protease activity. The results showed that biofilm formation was more on starchy substrates in the presence of 1% Tween 80, the surfactant showing higher nutrient availability and higher production of alpha-amylase and alkaline protease. Apart from their use in processes such as starch hydrolysis for sugar syrups, baking and brewing, they are also the most important enzymes in aquaculture probiotic and detritus management systems ("DMS").

To explore new types of alkaline proteases from *Bacillus* sp. which are able to withstand high temperature, salinity and high pH, the present investigation deals with production, purification and characterization of a new stable alkaline protease from an isolate of *Bacillus* species.

The isolated alkaline protease and amylase producing bacteria were further characterized using various techniques and ultimately identified as *Bacillus subtilis*.