PHOSPHATASES FROM BACTERIA ISOLATED FROM THE ARABIAN SEA AND COCHIN ESTUARY

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CERTIFICATE

This is to certify that the thesis entitled **PHOSPHATASES FROM BACTERIA ISOLATED FROM THE ARABIAN SEA AND COCHIN ESTUARY** *is an authentic record of the research work carried out by* Mr. Sreevalsam Gopinath, *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of* **Doctor of Philosophy** *in Microbiology of the Cochin University of Science and Technology and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

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Abbreviations

P	:	Phosphorus
P_i	:	Inorganic phosphate
PME	:	Phospho monoesters
DOP	:	Dissolved organic phosphorus
N	:	Nitrogen
PMEase	:	Phospho monoesterase
p-NPP	:	*p*-Nitrophenyl phosphate
MUP	:	4-methyl umbelliferyl phosphate
EDTA	:	Ethylene diamine tetra acetate
$\mu g\ l^{-1}$	:	Microgram per litre
K_m	:	Michaelis Menten constant
EUS	:	Epizootic ulcerative syndrome
sp	:	Single species
ASW	:	Artificial Sea Water
g	:	Gram
ml	:	Milli litre
M	:	Moles
mM	:	Milli moles
^0C	:	Degree Celsius
Fig.	:	Figure
Enz.	:	Enzyme
Abs.	:	Absorbance
Rev/min	:	Revolutions per minute
rpm	:	Revolutions per minute
Conc.	:	Concentration
Ext	:	Extract
ft	:	Feet
et al.	:	Co-authors
nm	:	Nanometer
*p*NP	:	*p*- nitrophenol
*P*NPP	:	*p*-nitrophenyl phosphate
ATP	:	Adenosine tri phosphate
ANOVA	:	Analysis of variance
ppm	:	Parts per million
AcPase	:	Acid phosphatase
AlPase	:	Alkaline phosphatase
AcP	:	Acid phosphatase
AlP	:	Alkaline phosphatase

CONTENTS


Chapter 1

INTRODUCTION

The bio-geochemical cycle of phosphorus is substantially influenzed by bacteria, actinomycetes, algae, fungi and zooplankton (Jansson *et al.*, 1988) more than the macrophytes and larger animals in the aquatic environment. Phosphorus (P) is an essential element for the growth and reproduction of bacteria and plays a very significant role in many aspects of cell metabolism. Most of the essential cellular components, like nucleic acids, lipids and sugars, are phosphorylated. The phosphate equilibrium in bacteria is regulated by the phosphate input from the surrounding medium. Potentially, bacteria can be limited by phosphorus or other nutrients in environments where

organic carbon is plentiful (Benner *et al.*, 1988). There is a long - standing debate as to whether it is phosphorus (P) or nitrogen (N) that constrains primary production in marine environments. Over geological time scales phosphorus is the critical nutrient in marine environments because the microorganisms tide over nitrogen limitation by extracting atmospheric nitrogen by nitrogen fixation (Redfield, 1958).

Some environments are abundant in inorganic phosphate (P_i) but, natural waters are often P_i - limited (Corner and Davies 1971). However, increasing evidence has been reported for the role of phosphate as a regulator of primary production in a diversity of marine systems, both coastal and open-ocean environments. Data obtained using advanced techniques for measuring *in situ* physiological characteristics, have suggested that P stress occurs in microbial populations in marine environments (Tanoue *et al.*, 1995; Scanlam *et al.*, 1997).

Organic phosphomonoesters (PME) in lakes and coastal waters may constitute between 10 and 70% of the dissolved organic phosphorus pool (Taft *et* al., 1977; Kobori and Taga, 1979; Veldhuis *et al.*, 1987). PME consist of molecules that cannot directly enter the cells, because of their high molecular weights and large size. Hence they are not directly utilized by living organisms but have to be

hydrolyzed by microbial extracellular enzyme action outside the microbial cell (Wynne, 1977).

Microbes play a significant role in the transformation of phosphorus. Organophosphorus compounds are decomposed and mineralized by many enzymatic complexes produced especially by heterotrophic bacteria, algae and zooplankton. Amongst these "Phosphomonoesterases" (EC3.1.3), also known as "phosphatases" are most important.

Phosphatases are designated either "acid" or "alkaline" phosphatases according to their pH optima.

Acid phosphatase (EC 3.1.3.2)

(Orthophosphoric monoester phosphohydrolase, acid optimum)

Alkaline phosphatase (EC 3.1.3.1)

(Orthophosphoric monoester phosphohydrolase, alkaline optimum)

Phosphatases are nonspecific enzymes, often induced via *de novo* synthesis when the concentration of the P_i drops below some threshold level. These enzymes catalyze the hydrolysis of phosphomonoesters (PME), a fraction of the dissolved organic phosphorus (DOP) pool. Once released from the organic component, the free phosphate is taken up by the organisms. As PME are cleaved

into orthophosphate and organic moiety, these enzymes are believed to have an essential function in the nutrient dynamics of lakes (Jansson *et al.*, 1988).

Vembanad Lake is the largest among the extensive system of backwaters in the south west coast of India. Cochin backwaters, situated within the geographical coordination of 9^0 40' and 10^0 12' of Northern latitude and 76^0 10' and 76^0 30' of Eastern longitude is located at the tip of the Northern Vembanad Lake. This tropical positive estuarine system has its northern boundary at Azheekode and southern boundary at Thannirmukham bund. The Lake has a length of 80 km and the width ranges between 500 and 4000m (Shetty, 1965). Considerable variations in depth occur (2-13m) in different regions. The estuary has permanent connections with the Arabian Sea through a channel at Cochin gut and another at Azheekode. Water from two major rivers viz., Periyar and Muvattupuzha, drain into this estuary.

Large inputs from industrial units, sewage works and agricultural runoffs influenze the concentrations of P and other nutrients in the estuary (Anirudhan *et al.*, 1987; Lakshmanan et al., 1987). Studies by Lakshmanan *et al.* (1987) showed that many regions of the estuary does not contain any measurable amounts of phosphates, while <40$\mu g^-at.l^{-1}$ of PO_4^{3-} were detected in some parts.

According to Menon *et al.* (2000) there is a close correlation between the P cycle and primary production in the estuary. Salinity and the N cycle were found to be completely unconnected with the productivity rhythm.

Anthropogenic activities have made an adverse impact on the potential of the estuary that used to support high levels of bioproductivity and biodiversity. Contrary to the popular belief that a typically stratified estuary is less vulnerable to pollution than lagoons or unstratified or mixed estuaries, as pollutants flush rapidly, Cochin estuary is vulnerable to a build-up of contaminants and receives contaminated freshwater inputs and discharges of effluents and partially treated sewage from many points. As a result, Cochin estuary is widely regarded as one of the polluted estuaries in India. The nutrients and pollutants introduced into the estuary to a great extent control the distribution and abundance of biota in the estuary.

Synthetic agrochemicals widely used to increase the production of food and to ensure protection from epidemic diseases as well as from obnoxious plants and animals, that find their way into the aquatic systems through sewage and land runoff cause serious ecological problems. The principal concern about the pesticides arises from their toxicity, persistence and propensity to undergo

bioaccumulation. Though organophosphorus pesticides undergo biodegradation relatively easier than the organochlorines, they may persist sufficiently long in the marine environment to cause either acute or sublethal effects. Bacteria and fungi capable of producing extracellular phosphatases, the enzymes involved in the degradation of organophosphorus compounds, has been found to utilize various organophosphorus pesticides, suggesting their ability to degrade the pesticides. Employment of phosphatase producing microorganisms is now being envisaged as a potential bioremediation tool to combat pollution.

Though extensive studies have been reported on acid and alkaline phosphatases from various organisms inhabiting estuarine and marine habitats, attempts to understand the important characters of the enzymes, the ability of the microorganisms to utilize organophosphorus compounds as P_i source and also their possible role in pollution abatement has been meagre. This existing scenario inspired the layout of the present study.

The objectives of the investigation undertaken can be briefed as follows:

- To isolate and identify the microbial strains from Cochin backwaters and near shore areas and select the most potent acid and alkaline phosphatase producing strains.

- To understand the culture conditions required for optimal growth and phosphatase production by the bacteria.

- To analyze the various factors that regulates the synthesis of acid and alkaline phosphatase.

- To study the characteristics of the acid and alkaline phosphatases.

- To investigate the ability of the phosphatase producing strains to utilize organophosphorus compounds as phosphorus source and degrade organophosphorus pesticides.

The thesis is presented in 8 chapters. The first chapter gives a brief introduction to the subject. The second chapter presents the review of literature, to give an overview of the history and present status of research on phosphatases around the world. The third chapter deals with isolation, identification and screening of bacteria for phosphatase

production. The fourth chapter describes the effect of culture conditions on growth and phosphatase production. In the fifth chapter regulation of phosphatase synthesis is discussed. The sixth chapter deals with the characteristics of acid and alkaline phosphatases. Studies on utilization of organophosphorus compounds and organophosphorus pesticides as phosphorus sources comprise the seventh chapter. The major findings of the thesis are summarized in chapter eight. This chapter is followed by the list of literature consulted.


Chapter 2

REVIEW OF LITERATURE

There has been considerable data available over the time scales to depict that microbial populations are capable of utilizing dissolved organic phosphorous (DOP) compounds as a source of phosphorous (Chu, 1946; Harvey, 1953; Johannes, 1964; Taft *et al.*, 1977; Berman, 1988; Nausch and Nausch, 2000).

The possible ecological importance of phosphatase enzyme in releasing orthophosphate for phytoplankton growth was first suggested by the work of Steiner (1938). Steiner opined that repeated incorporation of molecules of phosphorus by epilimnetic phytoplankton

would not be possible without the active participation of phosphatases. He showed in his filtration experiments that phosphatases were excreted by zooplankton, and the evidence of enzyme activity was demonstrated by the cleavage of organophosphorus compounds. Pioneering investigations on phosphatases were carried out by Overbeck (1961) and his colleagues in lakes and artificial ponds and in marine waters by Goldschmidt (1959).

Since then phosphatase activities in natural waters have been the focus of much research attention (Reichardt *et al.*, 1967; Berman, 1969; Perry, 1972; Hino, 1988). The original rationale for this interest was the assumption that the level of phosphatase activity measured in natural water samples could be used as an indicator of the nutritional status of aquatic organisms in respect to phosphorous (Berman, 1970) and also a measure of lake trophy (Chrost & Krumbeck, 1986). Phosphatases are now used as markers for phosphate stress (Dyhrman and Palenik, 1999). Although in many microorganisms, phosphatase is induced in situations where orthophosphate availability is low, it has been difficult to define absolute levels of this activity, which would be a characteristic indicator for phosphorous limitation or sufficiency in natural waters (Healey, 1978). Nevertheless phosphatases continue to be measured and when used with appropriate caution, can be useful indicators of

phosphorous cycling and availability to microorganisms in aquatic ecosystems (Karl and Craven, 1980; Pettersson, 1980; Gage and Gorham, 1985; Huber and Kidby, 1985; Hino, 1988).

Reasonably high concentrations of PME and phosphomonoesterase (PMEase) enzyme activities occur together naturally, often at times when phosphate concentration is low (Francko & Heath, 1979). The concentrations of dissolved organic matter, as such, in aquatic environments that are directly utilizable however, are vanishing low (Jorgensen, 1987; Chróst *et al.*, 1986), thereby limiting the rate of growth and metabolism of heterotrophic bacteria. Studies have indicated that bacteria can contribute significantly to the total microplankton biomass (Chróst *et al.*, 1986; Chróst, 1990) and that the bacterial secondary production is comparable to phytoplankton primary production.

Consistent with the view that PME may be an important source of phosphorous for phytoplankton growth, a bloom of *Aphanizomenon flos-aquae* followed appearance of phosphatase activity associated with the algal trichomes and subsequent disappearance of detectable PME (Heath & Cooke, 1975) in a mesotrophic lake. This and other studies have supported the widely held view that microorganisms may synthesize PMEase as an adaptive

response to Pi- limitation, and that it benefits from this by recovering Pi from PME in sufficient quantities to support growth.

Phosphatase activities were noted to increase as the cellular phosphate content decreased (Abd-Alla, 1994c). Much of the cellular and extracellular phosphatase activities were realized when cellular phosphate contents decreased to or below 0.115% of cell protein. The production of phosphatases in response to phosphate limitation has been reported in both prokaryotic and eukaryotic organisms (Reid and Watson 1971; Tarafdar and Chhonkar 1979; Tarafdar *et al.* 1988).

Phosphatase activity can be used as an indicator of phosphorus nutritional status of the system (Berman, 1970; Perry, 1972). Several studies (Wynne, 1977; Elgavish *et al.*, 1982) have shown that an internal phosphate pool regulates the synthesis of repressible phosphatase, i.e., when the pool is filled, synthesis of enzyme is shut down and derepression occurs after the depletion of the pool (Patni *et al.*, 1977; Hassan and Pratt, 1977). There is also a chance of storage of phosphate as polyphosphate body in the cell and the dependence of alkaline phosphatase activity on the level of stored polyphosphates was suggested (Taft and Taylor, 1976). The phosphatase activity starts when the level of polyphosphates falls below the optimum required level (Rhee, 1973). Synthesis of

phosphatase may be repressed by PO_4^{3-} in bacteria and microalgae (Siuda and Chróst, 1987).

In contrast with this view, a wide-ranging study reported that phosphatase activity was not a good indicator of phosphorous limitation (Pick, 1987). Daughtrey *et al.*, (1973) observed that mineralization occurs rapidly even at sites with adequate phosphate. Again no relationships between phosphatase activity and inorganic nutrient levels could be observed in interstitial sediments of Porto Novo (Ayyakkannu and Chandramohan, 1979), in Tokyo Bay (Taga and Kobrori, 1978), and in two isolates of marine bacteria (Healy and Hendzel, 1976).

Barik and Purushothaman (1998) reported a strain of *Bacillus* sp. which produces two types of alkaline phosphatase, one subjected to repression-derepression effect and a constitutive one which is independent of orthophosphate concentration. This nature of enzyme was also suggested in the case of *Escherichia coli* and *Staphylococcus aureus* (Kuo and Blumenthal, 1961) and in *Pseudomonas* sp. and *Alteromonas haloplanktis* (Hassan and Pratt, 1977). Phosphatases are non-specific (Garen and Levinthal, 1960) and although it is repressed by orthophosphate in many bacteria, it is constitutive in others (Kuo and Blumenthal, 1961). *Bacillus* sp. RK11,

produced phosphatase extracellularly even in the presence of high concentrations of phosphate (Kelly, 1975).

Although doubts have been expressed concerning the ecological significance of phosphatases in nature (Rigler, 1961), Jansson *et al.* (1988) concluded that phosphatases efficiently hydrolyze naturally occurring organophosphates, and that the phosphorus turnover in biota is dependant on this process. The high levels of acid and alkaline phosphatases observed in the vegetative cells of *Myxococcus coralloides* D (González *et al.*, 1987, 1989, 1994a, b) favour the view that the phosphatases have a nutritional role in the vegetative cells, providing orthophosphate from the phosphorylated metabolites found in the medium. Thingstad *et al.* (1999) observed that the growth rates of heterotrophic bacteria are P_i limited in the Northwest Mediterranean during summer.

Extensive studies have been reported on phosphatases from various land and aquatic animals, rhizosphere and soil microorganisms, phytoplankton etc (Heath & Cooke, 1975; Seargeant and Stinson, 1979; McComb *et al.*, 1980; Moss, 1982; Hernandez *et al.*, 1994, 1996).

Microorganisms known to produce phosphatases include *Escherichia coli* (Garen and Levinthal, 1960; Torriani, 1968a,b;

Lazzaroni and Portalier, 1981; Coleman, 1987; Golovan *et al.*, 2000), *Micrococcus sodonensis* (Glew and Heath, 1971), *Bacillus subtilis* (Glen and Mandelstam, 1971;Ghosh and Ghosh, 1972; Ichikawa and Freese, 1974; Ghosh *et al.* 1977), *Neurospora crassa* (Davis and Lees, 1973), *Bacillus licheniformis* (Nicholas and Hulett, 1977; Hydrean *et al.* 1977), *Aspergillus niger* (Rokosu and Uadia, 1980), *Bacillus subtilis* and *Bacillus licheniformis* (Rothstin *et al*, 1982), *Vibrio cholerae* (Roy *et al.*, 1982), *Capnocytophaga sp.* (Poirier and Holt, 1983), *Myxococcus coralloides* D (González *et al.*, 1994a, b) and *Citrobacter koseri* and *Micrococcus varians* I (Sharma *et al.*, 1995).

Novick *et al.*, (1981) hypothesized that acid phosphatases play an important role in the biosynthesis of cell wall in yeasts. González *et al.*, (1994b) conducted partial purification and studied the biochemical properties of acid and alkaline phosphatases from *Myxococcus coralloides* D.

Generally, two analytical techniques have been used to determine phosphatase activity. The classical spectrophotometric assay (Reichardt *et al.*, 1967) based on the enzyme mediated hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) and fluorescent methods that employ substrates such as 3-O-methylfluorescein phosphate (Perry, 1972) or 4-methylumbelliferyl phosphate (MUP) (Pettersson and Jansson, 1978). For studies in natural waters where

enzyme activities are low, the fluorimetric approach has been widely used. For most of the other studies, variations of the *p*-nitrophenyl phosphate spectrophotometric assay are used (Berman, 1970; Wynne, 1977, 1981). However, Berman *et al.* (1990) pointed out that neither of these substrates (*p*-NPP, MUP, etc.) are normally found in aquatic ecosystems and therefore the phosphatase measurements using these 'unnatural' substrates may not reflect *in situ* activities with natural phosphate esters.

The alkaline phosphatase of *E.coli* has been extensively studied by Torriani (1960, 1968a, b) who reported that the enzyme is repressed by orthophosphate (Torriani, 1960; Rao and Torriani, 1988). It has been shown for phosphatases from several bacteria that the rate of enzyme production is subjected to regulation either by a specific inducer or by specific repressor. The maximum rate of enzyme synthesis was attained when inducer was added to the growth medium or when the repressor was removed (Torriani, 1960). Interestingly, alkaline phosphatase is produced during sporulation in *B. subtilis*, in spite of the presence of Pi concentrations that completely repress the activity in vegetative cells (Ichikawa and Freese, 1974).

The inverse relationship between phosphatase production and external phosphate has been described (Veldhius *et al.*, 1987) and may reflect an inverse relationship between phosphatase synthesis

and internal phosphate, especially when the external phosphate has a long-term effect (Wynne, 1981). Studies have demonstrated that phosphatases are controlled by several compartments involved in phosphorus metabolism, such as internal phosphorus (Gage and Gorham, 1985).

The release of extracellular alkaline phosphatase help the organisms hydrolyze the organic phosphate in their natural habitat and to use the inorganic phosphate released. These could be used for removal of phosphate contents of polluted water (Sharma *et al.*, 1995). Phosphatase activities of soil microorganisms play an important role in the degradation of complex phosphorus compounds and are thus believed to have an important role in the degradation of organophosphorus pesticides (Sethunathan and Yoshida, 1973; Siddaramappa *et al.* 1973).

Plants absorb only inorganic phosphorus (Rendig and Taylor, 1989). Pi supply can be the limiting factor for plant growth (Bhat and Nye, 1974). Organic P can constitute 4 to 90% of the total soil P (Cosgrov, 1967). Therefore phosphorus mineralization is an important soil process because it results in release of inorganic phosphorus to the soil solution (Alexander, 1977). Mineralization is mediated by soil microorganisms (Irving and Cosgrove, 1971, 1974) and almost half of the microorganisms present in the soil and on plant roots possess the

ability to mineralize organic phosphorus through the action of phosphatases (Cosgrove, 1967; Tarafdar *et al.*, 1988). Abd-Alla, (1994c) studied the ability of rhizobia and bradyrhizobia to solubilize rock phosphate and reported that *Rhizobium leguminosarum* plays an important role in the release of available phosphorus from organic phosphorus sources through the production of phosphatases which can be activated by a range of cations.

Abd-Alla, (1994a) reported the ability of *Rhizobium leguminosarum* to survive and utilize glucose-1-phosphate, ATP and β-glycerophosphate and attributed this to acid and alkaline phosphatase activities in supplying available phosphorus for rhizobial growth. The organism, however, failed to utilize 4-nitrophenyl phosphate. The enhancement of alkaline phosphatase activity in *Citrobacter koseri* and *Micrococcus varians* I in the presence of Na-β-glycerophosphate was observed by Sharma *et al.* (1995).

Alkaline phosphatase producing *Escherichia coli* could utilize poly phosphates, a linear poly-P100, as sources of Pi (Rao and Torriani, 1988). It was observed that commercial poly phosphates (poly-P_is) with chain lengths ranging from 5 to 100 residues, released free P_i by spontaneous hydrolysis. However poly-P_i did not support growth of the alkaline phosphatase negative mutant. They concluded that their results imply that alkaline phosphatase activity was required

for the degradation of poly-P100. In order to show that high-molecular weight poly-P_is are substrates of alkaline phosphatase, a chemically synthesized high-molecular weight (chain length of ca. 200) poly-$^{32}P_i$ was incubated with a purified preparation of alkaline phosphatase from *E. coli*. The poly-P_i was hydrolyzed by the enzyme at a constant rate.

Divalent metal ions, heavy metal ions and other monovalent ions act as inhibitors or cofactors of phosphatases (González *et al.*, 1994b). These facts have been described in several bacterial phosphatases (Cheng *et al.*, 1970; Ghosh *et al.*, 1977; Bock and Kowalsky, 1978). Of the divalent metals, Mn^{2+} is a metal specifically required for the production of secondary metabolites of many *Bacillus* species and is essential for sporulation in some bacilli (Charney *et al.*, 1952; Curran and Evans, 1954). Mounter *et al.* (1955) observed that enzyme activity was stimulated by Mn^{2+} while Co^{2+} was generally inhibitory in *Escherichia coli, Pseudomonas fluorescens* and *Streptococus faecalis*. Mg^{2+} and Cu^{2+} inhibited phosphatase activity in *Aerobacter aerogenes* (Mounter and Tuck, 1956). Day *et al.* (1968) in his reports on Zn-containing alkaline phosphatases of *E. coli* opined that the presence of metal ions in the reaction mixture may exert their effect on enzyme activity by affecting the rate of enzyme-substrate combinations. Sodium fluoride considerably inhibits acid phosphatase from bacteria (Hollander, 1971), but has little effect on the alkaline

form. The release of extracellular alkaline phosphatase in *Micrococcus sodonensis* (Glew and Heath, 1971) is totally dependant on the presence of Mg^{2+} and is the result of a selective permeation process.

Bacillus sp. RK11, an alkalophilic isolate from soil reported by Kelly (1975), produces extracellular alkaline phosphatase. In the absence of Mn^{2+} in a complex medium, no alkaline phosphatase production or sporulation by the organism was detected. Alkaline phosphatase activity was stimulated by magnesium, which binds to an effector site on each subunit that is different from the site for zinc (Linden *et al.*, 1977).

In the investigations on phosphatases from *Candida utilis*, Fernandez *et al.* (1981) observed that only Mg^{2+} ions activated alkaline phosphatase. Studies by Crofton (1982) show that alkaline phosphatase is a dimeric molecule and is composed of two subunits. Each subunit contains a tightly bound atom of zinc, which is essential for the structural integrity of the enzyme, and a second, less tightly bound zinc atom, which is involved in the catalytic process (Crofton, 1982). However, Coleman (1987) found that many alkaline phosphatases, including that of *E. coli*, are associated with Zn^{2+}. However Fe^{2+} and Mn^{2+} activated acid phosphatase as compared to alkaline phosphatase.

1971) in which Ca^{2+} was required for the expression of enzyme activity. Zn^{2+} was required in less concentration in *C. koseri*. Mg^{2+} ions enhance phosphatase activity significantly, where as Cu^{2+} and Hg^{2+} inhibit the enzyme activity (Chen *et al.*, 1996). They suggested that the inhibition by Hg^{2+} was of an uncompetitive type.

EDTA and $HgCl_2$ completely inhibited the phosphatase activity in *Citrobacter koseri* and *Micrococcus varians* I studied by Sharma *et al.* (1995). This suggests that alkaline phosphatases from both the bacteria were metal dependant. Acid phosphatase from *Streptococcus equisimilis* studied by Malke (1998) was functional in the presence of EDTA. Methanol, ethanol and ethylene glycol are a few of the other substances known to inhibit the enzyme activity to various extents (Chen *et al.*, 1996). Naphthalene inhibits alkaline phosphatase activity, but increases acid phosphatase activity (Elumalai *et al.*, 1996).

Chróst and Overbeck (1987) reported that the specific activity of alkaline phosphatase decreased when the ambient P_i concentrations were higher than 15 µg l^{-1}. It is not quite correct to conclude that phosphatase synthesis is derepressed or activated directly by low P_i concentrations. The mechanism of phosphatase derepression is regulated by the intracellular phosphate pool in microbial cells. P_i was a strong competitive inhibitor of phosphatase in

microalgae (Chróst and Overbeck, 1987), but inhibition of phosphatase synthesis in bacteria was only slight (Chróst *et al.*, 1986).

Hernandez *et al.* (1995) found that the temperature optima of alkaline phosphatase production for the two algal species were in the range of 25-30^{0}C. He suggested from the data obtained that the temperature in the field could limit phosphatase production.

The optimum pH levels for microbial phosphatases are unlikely to be encountered in their natural environments (Hernandez *et al.*, 1995). A possible explanation for the weak relationship between the pH optimum of phosphatase activity and the typical pH of the natural environment from which the organisms are isolated is given by Islam and Whitton (1992). These, on the basis of the study by Fedde and Whyne (1990) on human fibroblasts, suggest that the substrate concentration used in the assays can induce higher pH optimum than if the assays were performed at ambient concentration.

The effect of salinity on phosphatase activity is partly due to the increase of ionic strength in the assay medium (Wilson *et al.*, 1964). Nevertheless the results obtained by Hernandez *et al.* (1995) and Mahasneh, *et al.* (1990) showed that this effect is attributable not only to the ionic strength itself, but that there was also a specific effect of particular ions, such as Na^+ and Mg^{2+}. There may be a pronounced

effect of both Na^+ and Cl^- on permeability and phosphate uptake by these organisms (Ullrich-Eberius and Yinghol, 1974; Cembella *et al.*, 1984 a, b).

Studies on phosphatases from marine organisms and marine habitats have been conducted by several workers (Thomson and Mc Leod, 1974; Avilova, 1985; Chan and Dean, 1987; Goldman *et al.*, 1990; Hernandez, 1996). Hernandez, *et al.* (1994) determined the alkaline phosphatase activity in 44 species of marine macrophytes collected along the Southern coast of Spain and observed that alkaline phosphatase hydrolyzes the external PME, utilizing them as additional sources of phosphorous. After the reports on the role of phosphate as a factor limiting algal growth in a diversity of marine environments, particularly coastal waters (Sakshaug & Olsen, 1986; Veldhius *et al.*, 1987; Wheeler and Björnsäter, 1992.), there has been a consequent increasing interest in the study of alkaline phosphatase activity in benthic micro algae from coastal and inshore waters (Atkinson, 1987; Laponite and O'Connel, 1989; Weich and Graneli, 1989; Hernandez, *et al.*, 1993).

Berman (1988) in his experiments found that the seven organophosphorus compounds which were tested could all sustain reasonable rates of growth and enzyme yields in the four bacterial species studied. There were, however, definite species differences in

response to the various phosphorus sources (Berman, 1988). In a study on Lake Memphramagog, Currie and Kalff (1984a) concluded that the algal populations preferentially utilized DOP released from bacteria, while the latter always showed higher affinities and more uptake of orthophosphate. There seem to be little doubt that bacteria are usually more effective in taking up P_i than algae (Rhee, 1973; Currie and Kalff, 1984b; Currie *et al.*, 1986). Results obtained by Berman (1988) showed that the addition of organophosphorus sources appeared to reduce the dependency of microbiota on P_i as a P source. He observed that the bacteria were capable of exploiting organophosphorus sources even in the presence of P_i. Berman (1988) concluded that in many aquatic systems DOP compounds could have the potential to supply a substantial part of microbial and planktonic P nutrition irrespective of which fraction of microbiota utilizes organophosphorus sources through phosphatase activities.

Studies on micro algae and bacteria have demonstrated that phosphatase activity is common in the outer surface of the cells (Kuenzler and Perras, 1965; Cembella, *et al.*, 1984a, b; Islam and Whitton, 1992). In some cases, low activity has been detected in cell organelles (Aaronson and Patni, 1976). Other studies have confirmed the existence of extracellular phosphatase activity (Grainger, *et al.*, 1989; Lubian, *et al.*, 1992). However it is difficult to distinguish whether

the enzyme is actually excreted or whether the activity is a result of cell degradation (Cembella, *et al.*, 1984a, b).

The effect of temperature, pH, substrate specificity etc. on phosphatases has been studied by several workers. In his studies on acid and alkaline phosphatases from *Myxococcus coralloides* D, González *et al.* (1994b) observed that optimum pH for catalytic activity for acid phosphatase was 4.5, with high activity between pH 3.5 and 5.5, the alkaline phosphatase showed high activity between pH 7.0 and 8.5, with maximum activity at 8.1. Acid phosphatase was stable between pH values of 3.0 to 9.0, while alkaline phosphatase was relatively stable from pH 6 to 9, but less stable below and above this range.

Acid and alkaline phosphatases from *Myxococcus coralloides* D (González *et al.*, 1994b) had temperature optima of 43^{0}C and 37^{0}C respectively. Alkaline phosphatase from *Myxococcus coralloides* D (González *et al.*, 1994b) was found to be more heat sensitive than acid phosphatase. Alkaline phosphatase from *Escherichia coli* reported by Reid and Watson (1971) was more heat stable in comparison.

Whitton *et al.* (1990a) inferred that the effect of temperature on alkaline phosphatase was pH dependant. The optimum temperature

was 32^0C at pH 7.0 and at pH 10.3 the optimum was 25^0C. However the pH optimum observed was 7.0.

Alkaline phosphatase is often a derepressible enzyme only synthesized when PO_4 in the cell or in the cell's environment is limiting, but where the cell is still able to grow, i.e., there are sufficient carbon, nitrogen and other essential elements are available (Torriani, 1968a; Flint and Hopton, 1976). Inorganic phosphate limitation results in the synthesis of a battery of enzymes including phosphatases and the proteins involved in the rapid uptake of PO_4 from the environment (Filloux *et al.*, 1988). The enzyme activity of bacterial cells may have an important function in the survival of bacteria under different stress conditions, like the absence PO_4. The ability to synthesize phosphatases may give a cell a competitive advantage (Ozkanca and Flint, 1996). Phosphatase activity has been studied in natural environments where these enzymes play a key role in the mineralization of PO_4 compounds and hence the maintenance of the ecological equilibrium (Jorgensen, 1976; Siuda, 1984). The enzyme may be involved in the survival of bacteria under adverse conditions possible through its action as a scavenger of inorganic PO_4 (Ozkanca and Flint, 1996). The effects of changing environmental conditions on the health of microbial cells and its phosphatase activity have been studied only briefly (Matavulj & Flint, 1987). Phosphatase activity has

been linked to the survival of bacteria in PO_4-depleted environments. Gauthier *et al.* (1990) have shown that some enzyme activities including phosphatases increased in *E. coli* cells in nutrient-free seawater, ascribed to nutrient starvation and derepression of enzyme activity. They have suggested that long term survival of *E. coli* in sea water is dependant on the ability of the cell to synthesize phosphatases and accumulate K^+ and glutamate ions (Gauthier *et al.*, 1991).

Chen *et al.* (1996) observed that the optimum temperature for the hydrolysis of *p*NPP by alkaline phosphatase was 47^0C and the pH optimum was 8.2. At pH 8.3 and temperature 37 ^{0}C the Michaelis constant (K_m) was 8.0×10^{-4} mol litre^{-1}. Alkaline phosphatase studied by Hsiao (1965) had an optimum pH of 10.5 and temperature in the range 25-30^{0}C. He obtained a K_m value of 2.179×10^{-6} of *p*NPP litre^{-1} at pH 10.5 and temperature 25 ^{0}C.

Two prominent groups of common synthetic pesticides viz., organophosphates and organochlorines are encountered in the Cochin estuary. The pesticides regularly used in this region are Dimecron, Monocrotophos, Nuvacron, Thymet, Henosan and Fernoxan (Sujatha *et al.* 1993). Sujatha *et al.* (1999) elucidated the distribution profile of the common pesticide species encountered in the aquatic environment around Greater Cochin. Their studies reported significant loadings of

pesticides in the estuary, apparently from the agricultural and industrial discharges and the large amount of urban run off/municipal sludge that drain in. The outbreak of Epizootic Ulcerative Syndrome (EUS) in murrels, eels, mullets, pearl spot, barbs, glassy perchlets, half and full breaks seen in fields of Kuttanad and at the 'Kol' lands in Trichur is suspected to be due to the indiscriminate use of pesticides in this region (Kurup, 1992).


Chapter 3

SCREENING AND SELECTION OF PHOSPHATASE PRODUCING BACTERIA

The bacteria are distributed widely in the aquatic environment. Morphologically, most aquatic bacteria have their equivalents among the basic types of terrestrial bacteria. Systematically, aquatic bacteria are not a homogenous group. Their representatives are found in almost all orders of the class of bacteria. A sharp separation of soil bacteria and aquatic bacteria is not easily possible, since inland waters, particularly flowing waters, and the shores are constantly exposed to contamination from the soil. On the other hand, open sea allows the development of an autochthonous marine flora. Most of the marine bacteria are halophilic. Besides these, there are also in the

marine habitat other bacteria which are merely halotolerant found mainly near the coasts and in estuaries (Rheinheimer, 1968). Terrestrial bacteria also may be present in coastal waters depending on the opportunities for contamination from the land.

Growth in most aquatic environments is limited by the availability of essential nutrients. Genuine aquatic bacteria are distinguished by their ability to utilize very small concentrations of nutrients. In aquatic systems depleted of one or more essential nutrients, their replenishment takes place by biodegradation of organic materials brought about by extra cellular enzymes secreted by microorganisms. Phosphatase producing bacteria play a significant role in aquatic environments where inorganic phosphorus is not sufficient. The enzyme cleaves the organophosphorus compounds and makes the inorganic phosphorus moiety available for uptake by organisms.

Bacteria were isolated from water samples collected from four sites in Cochin estuary and two sites in Arabian Sea and identified upto genera. The isolates were screened for phosphatase production and the acid and alkaline phosphatases produced were quantitatively determined. The most potent strains were selected for further studies.

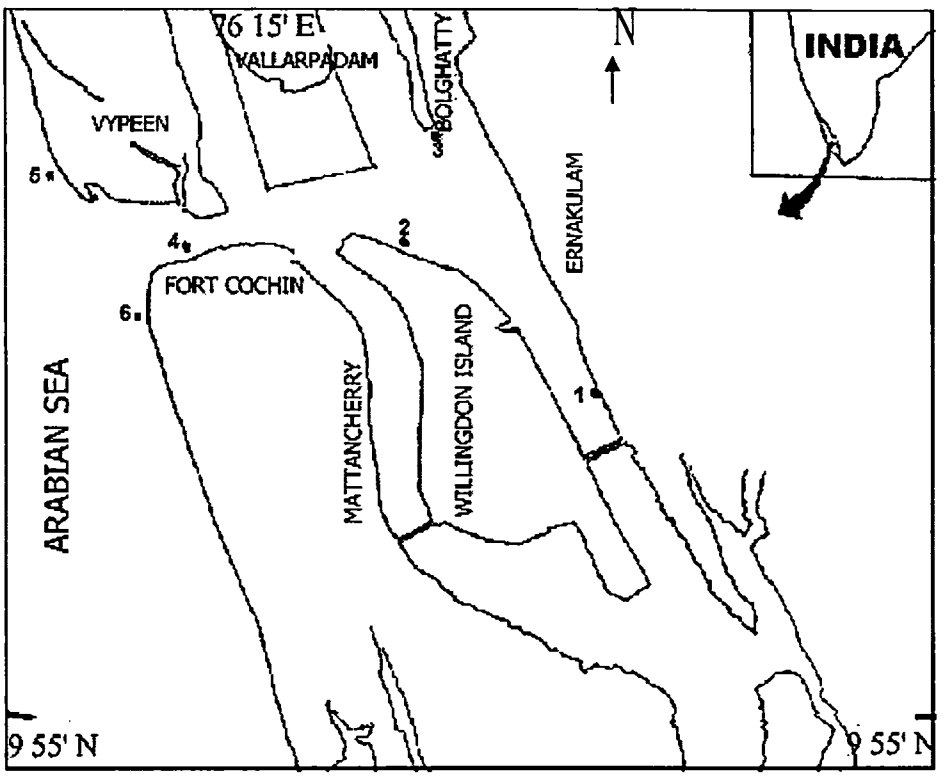
3.1 METHODOLOGY

3.1.1 Sampling area

The area of sampling was confined to four stations in the Cochin harbour region of the Vembanad Lake and two stations in the Arabian Sea adjacent to the Cochin barmouth. The station locations are indicated in Fig. 3.1. The stations were fixed so as to represent a cross section of the central region of the estuary. The two sampling sites in the Arabian Sea were in the coastal waters near Vypeen and Fort Cochin, where the impacts of anthropogenic activities are at the maximum than any open ocean site.

3.1.2 Collection of samples and isolation of bacteria

Water samples were collected in sterile bottles from the selected stations in Cochin backwaters and Arabian Sea, from 1- 2 ft. below the surface. Serial dilutions of the samples were prepared using 50% sterile seawater for backwater samples and 100% seawater for samples collected from Arabian Sea and pour plated with Zobell's agar 2216e medium. The plates were incubated at 28^0C for 24 - 48 hours. The colonies that developed were sub-cultured on to nutrient agar slants. These cultures were then streaked on nutrient agar plates and



Station 1: Marine Science Jetty

Station 2: Willington Island

Station 3: Bolghatty

Station 4: Barmouth

Station 5: Vypeen

Station 6: Fort Cochin

Fig. 3.1 Map of Cochin estuary showing locations of stations.

the separated colonies were isolated in pure culture and maintained in nutrient agar slants with periodic sub culturing.

3.1.3 Identification of cultures

Identification of the isolated cultures up to generic level was done based on gram staining, spore staining, morphological, physiological and biochemical examinations employing the schemes prepared from the descriptions in Bergey's manual of determinative bacteriology (Buchnan and Gibbons, 1974) (Table 3.1 and Fig. 3.2).

3.1.4 Screening for phosphatase production

The isolated cultures were screened for phosphatase production by the method of Baird - Parker (1966). Basal nutrient agar plates containing 1ml of 1% solution of Phenolphthalein di phosphate were spot inoculated with the isolated bacterial cultures and incubated till sufficient growth was observed. These plates were then exposed to NH_3 vapours by inverting it over a petridish containing NH_3 solution. Pink colouration of cultures indicated the presence of the phosphatase enzyme. Assays for the quantitative determination of acid and alkaline phosphatase were carried out with the isolates, which exhibited elevated levels of phosphatase production illustrated by deep pink colouration.

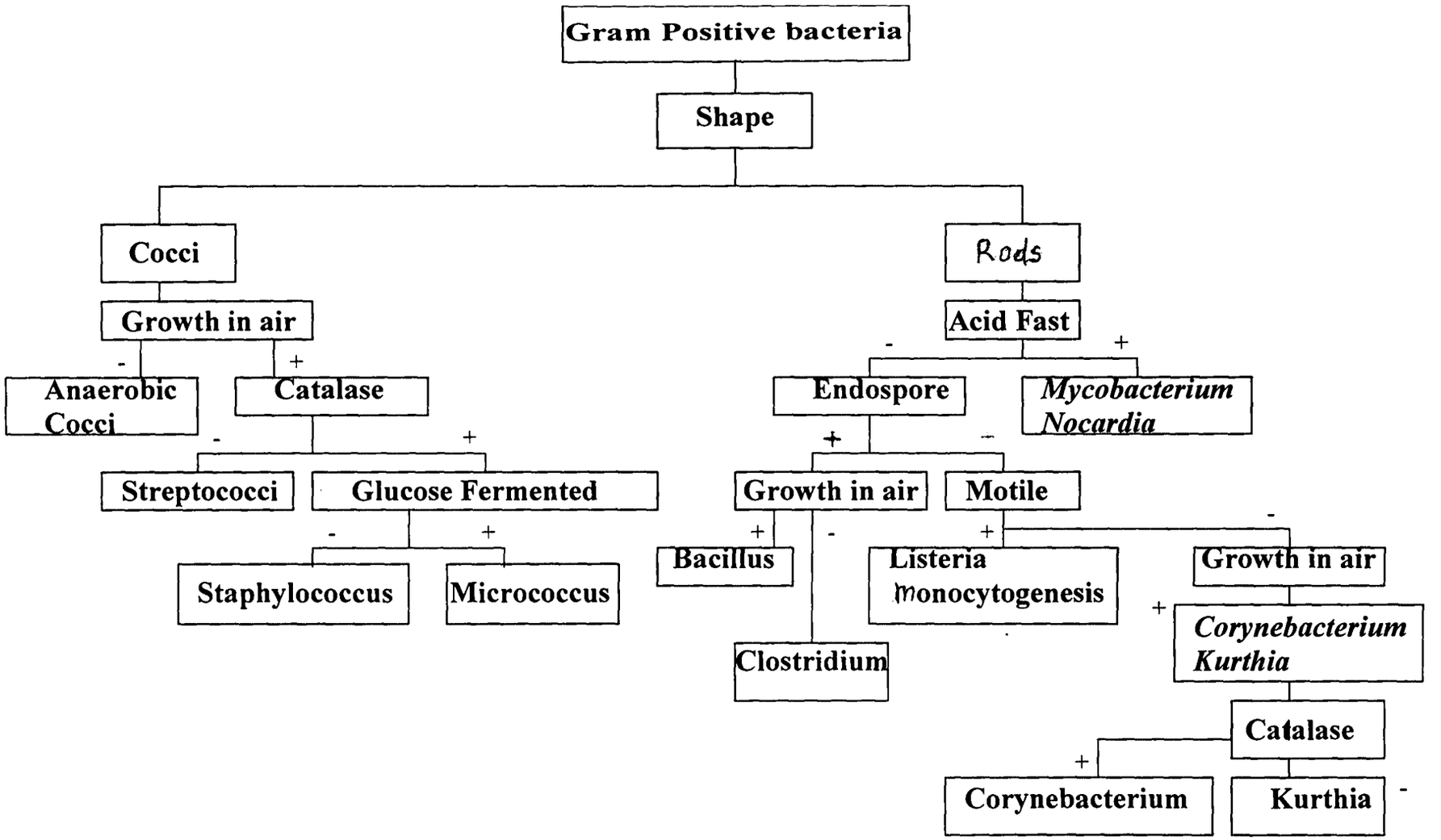


Fig. 3.3. Scheme for identification of Gram Positive Bacteria

Key to some gram negative rods that grow on nutrient agar

Species	HL TEST (MOF)	Oxidase	Arginine hydrolysis	Gelatin liquefaction	Growth on Mac Conkey	Mannitol Motility
Achromobacter	OX	+	-	+	+	-
Acinetobacter anitratus	OX	-	-	-	+	-
A. lwofii	Alk / none	-	-	-	+	-
A. mallei	OX	-	+	+	-	-
A. parapertussis	Alk	-	-	-	+	-
Aeromonas	F	+	+	+	+	V
Alcaligenes bronchiseptica	Alk	+	-	-	+	+
A. faecalis	Alk	+	-	+	+	+
Chromobacterium lividum	OX	+	-	+	V	+
C. violacium	F	+	+	+	V	+
Enterobacteria	F	-	V	V	+	V
Flavobacterium	OX	+	-	+	V	-
Moraxella	Alk	+	-	V	V	-
Pseudomonas	OX or none	+	+	V	+	+
Pateurella	F	+	-	-	-	+
Vibrio	F	+	-	+	+	+
Yersinia	F	-	-	-	+	-

OX = Oxidative; F = Fermentative; Alk = Alkaline reaction; V = Strains/ species vary.

Table 3.1. Key for identifying gram negative bacteria

3.1.5 Quantitative determination of acid and alkaline phosphatase

3.1.5.1 Inoculum preparation

The strains which were found to be highly potent phosphatase producers by the Baird-Parker's test were inoculated into nutrient agar slants and incubated for 24 hours. Cells were harvested using small aliquots of sterile liquid medium and a volume adequate to obtain an absorbance (Abs.) of 0.02 at 600nm for the total medium was added to 100ml of the broth. This was treated as the Abs. at 0 hours of incubation. The absorbance was measured using Hitachi 200-20 UV-Visible spectrophotometer.

3.1.5.2 Preparation of the enzyme

The method of Sakata *et al.* (1977) was followed. 100 ml nutrient broth in a 250ml conical flask was inoculated with the selected culture and incubated at 28^0C. The samples were drawn to determine cell growth and the remaining cell suspensions were centrifuged at 12000g for 20 minutes at 4^0C and the cell free supernatant fluid (as crude enzyme) were assayed for phosphatase activity.

3.1.5.3 Assay of the enzyme

Acid and alkaline phosphatases were assayed according to Reichardt *et al.*, (1967), using *p*-nitrophenyl phosphate (*p*NPP), a

colourless substrate that produces a colorimetric end-product *p*-nitrophenol (*p*NP). The buffer–substrate mixture for the assay was prepared by dissolving 0.203g of *p*NPP in 100 ml of citrate (pH 4.8) and glycine (pH 9.5) buffers for acid and alkaline phosphatase respectively. The assay mixture was incubated at 37^0C for 30 minutes. The absorbance was measured at 408 nm using a spectrophotometer. Standard graphs were plotted by adding serial dilutions of *p*-nitrophenol to the corresponding buffer solutions. Enzyme activity was determined by calculating the amount of *p*- NP released.

One enzyme unit is defined as the amount of enzyme catalyzing the liberation of 1 μg of *p*-nitrophenol per ml per minute (Galabova, *et al.*, 1993).

Two of the most potent strains, a *Streptococcus* sp. producing acid phosphatase maximally and a *Flavobacterium* that returned highest alkaline phosphatase yields were selected for further studies and tests according to Bergey's manual of determinative bacteriology (Buchnan and Gibbons, 1974) were carried out for their species identification. The scheme for the identification of *Flavobacterium* up to species level is shown in Fig. 3.3.

3.1.6 Measurement of growth

Bacterial growth was determined by measuring the absorbance of the culture fluid spectrophotometrically at 600nm and was expressed in units of absorbance (Abs.).

3.2 RESULTS

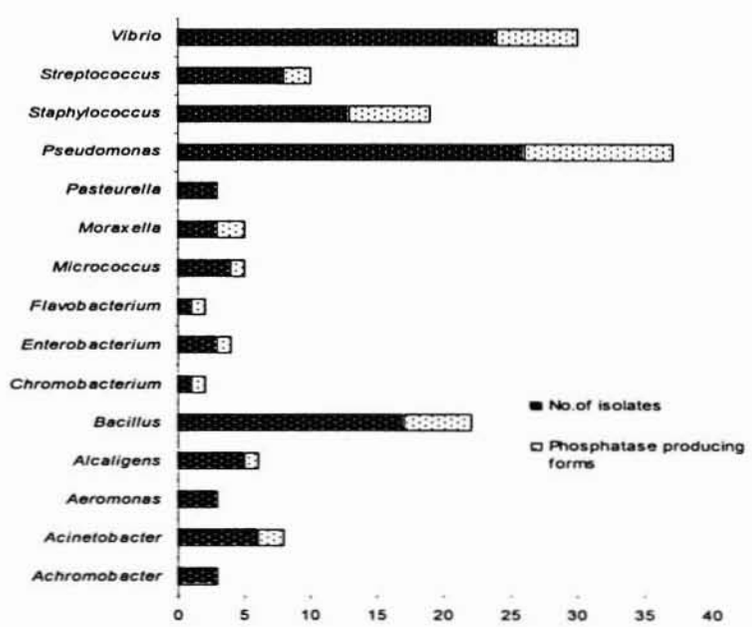
A total of 120 bacterial strains were isolated from water samples collected from Cochin estuary and Arabian Sea and identified up to genera (Table: 3.2 & Fig.3.4). In general Gram-negative bacteria were dominant (68%) than Gram-positive bacteria (32%). The predominant genera isolated were *Pseudomonas, Vibrio, Bacillus and Staphylococcus.*

Of the 120 isolates 39 (33%) were observed to produce phosphatase (Table: 3.2 & Fig.3.4). The genera in which maximum number of isolates showed phosphatase activity were *Pseudomonas, Vibrio, Staphylococcus* and *Bacillus.*

Table 3.2. Generic distribution of isolates and phosphatase producing forms.

Genus	No. of isolates	Phosphatase producing forms
Achromobacter	3	0
Acinetobacter	6	2
Aeromonas	3	0
Alcaligens	5	1
Bacillus	17	5
Chromobacterium	1	1
Enterobacterium	3	1
Flavobacterium	1	1
Micrococcus	4	1
Moraxella	3	2
Pasteurella	3	0
Pseudomonas	26	11
Staphylococcus	13	6
Streptococcus	8	2
Vibrio	24	6
Total	120	39

Fig. 3.4. Generic distribution of isolates and phosphatase producing forms



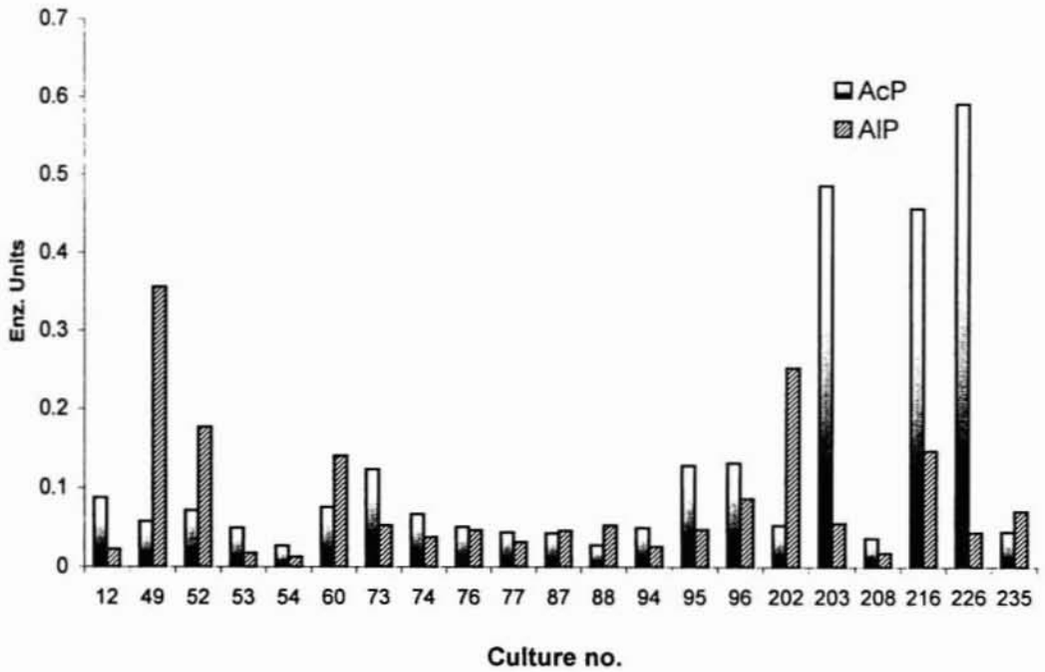
3.2.1 Quantitative determination of acid and alkaline phosphatase

The results of the enzyme assays conducted after 48 hours of incubation at 28^0C are presented in Table 3.3 and Fig. 3.5.

Table 3.3. Quantitative determination of acid phosphatase and alkaline phosphatase

Genus	Culture no.	Acid phosphatase	Alkaline phosphatase
Pseudomonas	12	0.09	0.02
Micrococcus	49	0.06	0.36
Micrococcus	52	0.07	0.18
Streptococcus	53	1.05	0.02
Staphylococcus	54	0.03	0.01
Flavobacterium	60	0.08	1.14
Bacillus	73	0.12	0.05
Bacillus	74	0.07	0.74
Chromobacterium	76	0.05	0.05
Streptococcus	77	0.04	0.03
Vibrio	87	0.04	0.05
Streptococcus	88	0.03	0.05
Staphylococcus	94	0.05	0.03
Bacillus	95	0.13	0.05
Vibrio	96	0.43	0.09
Streptococcus	202	0.05	0.25
Streptococcus	203	1.29	0.06
Moraxella	208	0.04	0.02
Streptococcus	216	0.46	0.15
Vibrio	226	0.59	0.04
Pseudomonas	235	0.05	1.07

Fig. 3.5. Quantitative determination of acid phosphatase and alkaline phosphatase



Maximum acid phosphatase was observed in *Streptococcus* sp. and alkaline phosphatase in *Flavobacterium* sp. These isolates were used for further studies.

3.2.2 Identification of the selected strains

The attempt to identify the species of the acid phosphatase producing *Streptococcus* following the description in Bergey's manual of determinative bacteriology (Buchnan and Gibbons, 1974) did not yield the desired results. Detailed studies required to verify whether this isolate is a novel bacterium, not yet described, could not be

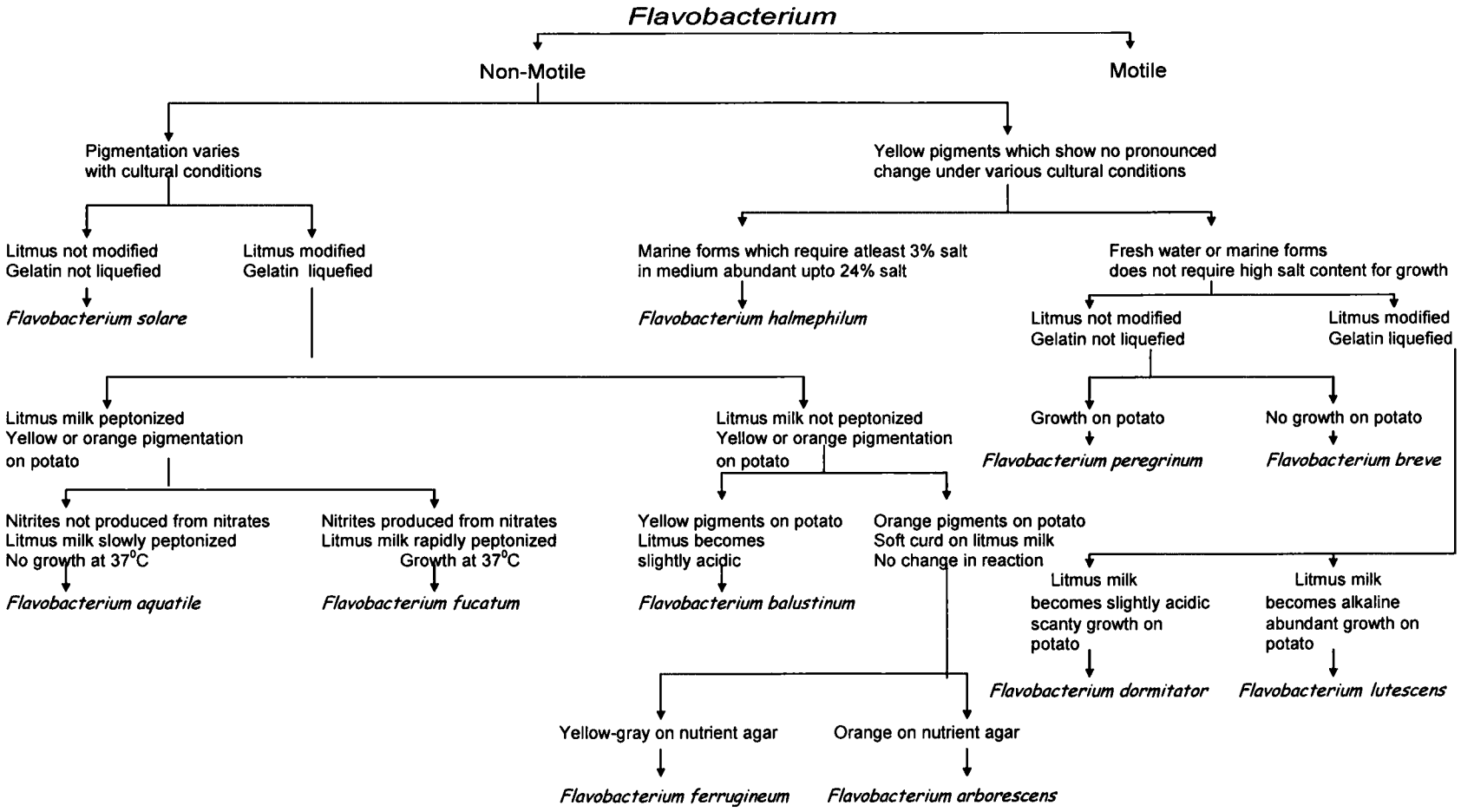
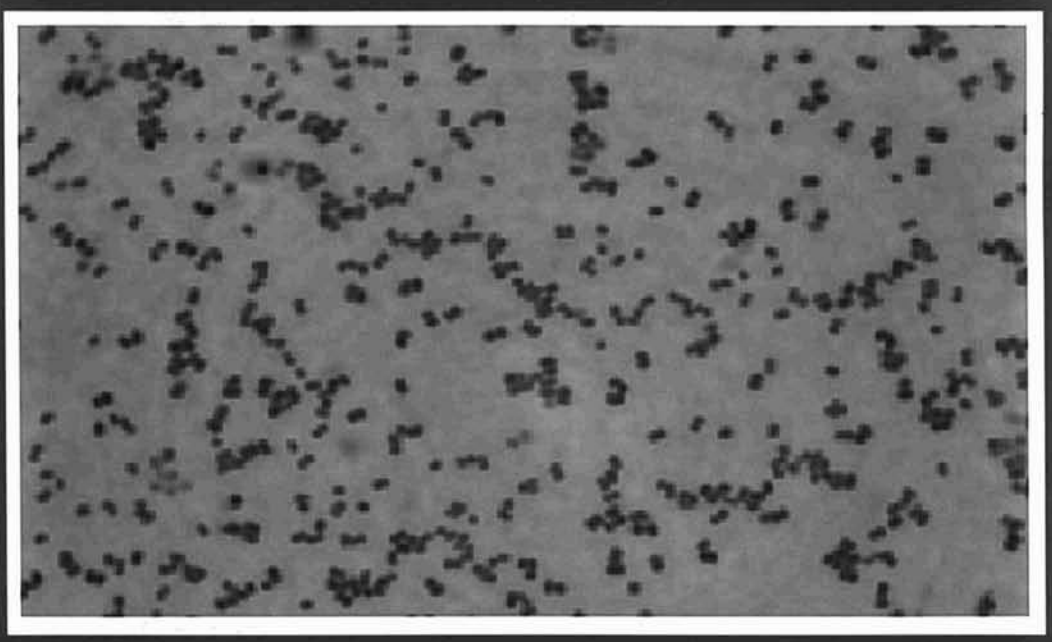
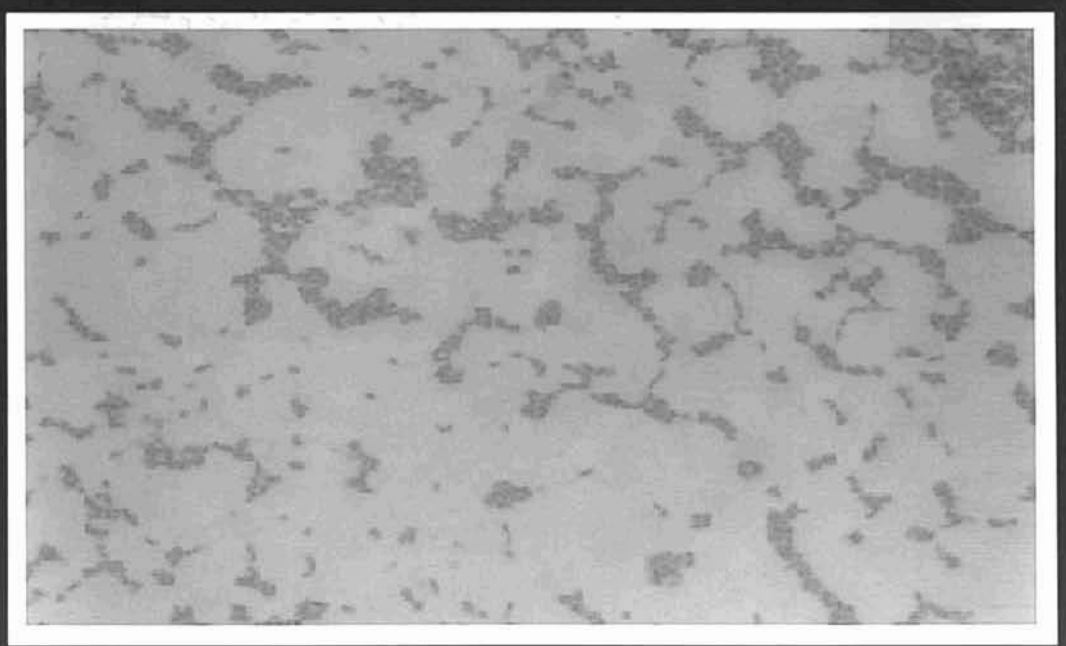


Fig. 3.2 Key for identification of *Flavobacterium.*

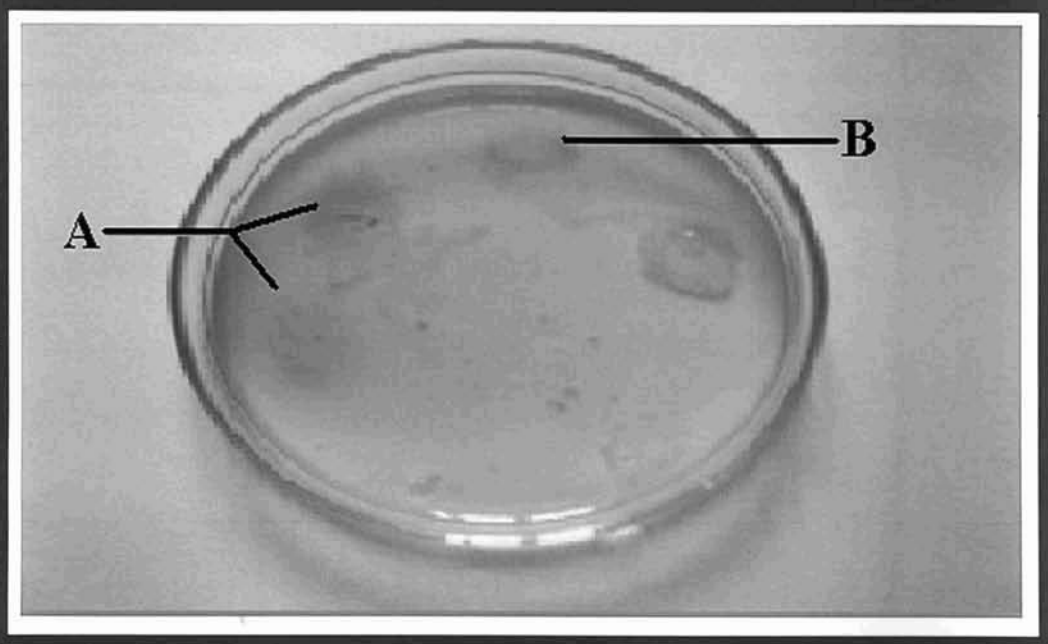


A. *Streptococcus* sp.

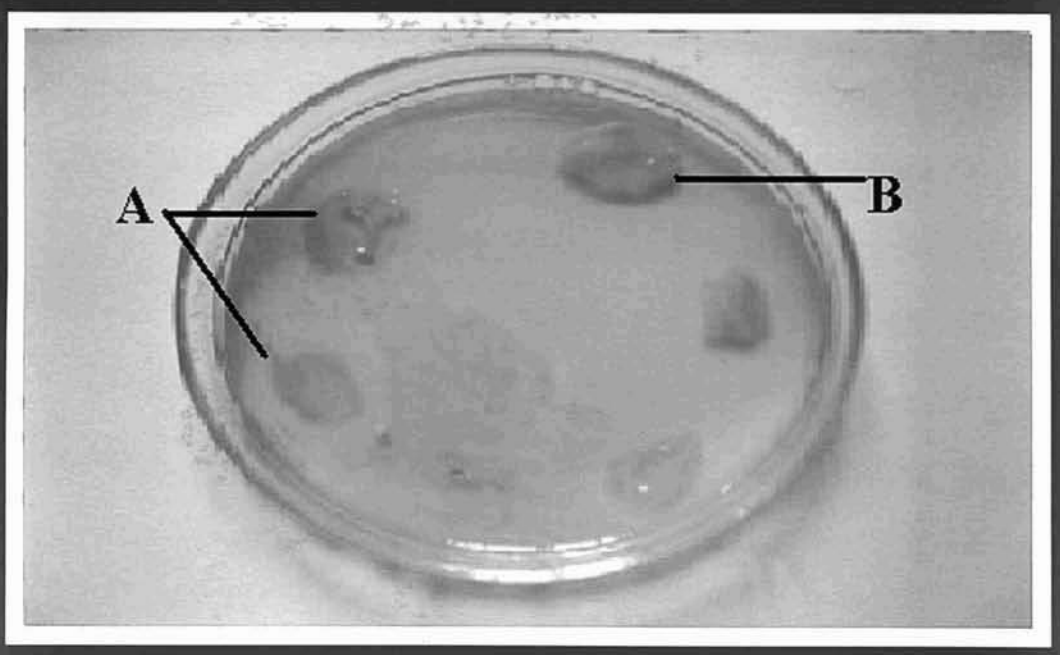


B. *Flavobacterium breve*

Plate: 1. Gram stained preparation of selected bacterial strains



A. Phosphatase producing *Streptococcus* sp.
B. Non-phosphatase producing bacteria



A. Phosphatase producing *Flavobacterium breve*
B. Non-phosphatase producing bacteria

Plate: 2. Baird-Parker test
Screening of phosphatase producing bacteria

incorporated within the constrains of this study. The yellow pigmented, non-motile, *Flavobacterium* was found not to require high salt content for growth. The bacteria did not modify litmus or gelatin nor did it grow on potato. Based on these characters the alkaline phosphatase producing microbe was identified as *Flavobacterium breve*.

3.3 DISCUSSION

In the present study, the predominant genera isolated from the Cochin estuary and the Arabian Sea were *Pseudomonas, Vibrio, Bacillus* and *Staphylococcus.* Gram negatives were found to be more predominant forms than the gram positive bacteria. Earlier studies conducted in this region yielded similar results (Philip, 1987; Chandrika and Ramachandran, 1994; Amar, 2001). Philip (1987) reported the dominance of gram negative forms and found *Vibrio* and *Pseudomonas* to be the predominant genera obtained from Cochin backwaters. She also cited considerable abundance of *Bacillus*. The occurrence and seasonal distribution of aerobic heterotrophic bacteria in water and sediment and their ecophysiology and biochemical characters were studied in Cochin backwaters by Chandrika and Ramchandran (1994). The composition of microflora isolated from

Cochin estuary by Amar (2001) showed domination by *Bacillus*, followed by *Coryneforms*, *Vibrio*, *Streptococcus*, *Pseudomonas* and *Acinetobacter*.

In this study 33% of the bacteria tested showed the ability to produce phosphatases. Bacteria, capable of elaborating hydrolytic enzymes, from the Cochin estuary has drawn the attention of a few earlier scientists. Philip (1987) carried out studies on the proteolytic properties of bacteria and their association with fish and shell fish. The production of other enzymes such as amylase, lipase and gelatinase were also studied. The presence of extracellular amylase in three *Vibrio* sp. isolated from the Cochin estuary and its characterization formed the research interests of Saramma (1992). Chitoclastic and proteolytic bacteria from Cochin backwaters were isolated and studied by Amar (2001).

The important factors which determine the activity of various groups of bacteria in an environment are the availability of organic matter susceptible to degradation and the source of organic matter (Itturiga and Hoppe, 1977). Anthropogenic activities have made an adverse impact on the potential of the Cochin estuary that used to support high levels of bioproductivity and biodiversity. Cochin estuary is vulnerable to a build-up of contaminants and receives contaminated freshwater inputs and discharges of effluents and partially treated

sewage from many points. As a result, Cochin estuary is now widely regarded as one of the polluted estuaries in India (Menon *et al.*, 2000). The nutrients and pollutants introduced into the estuary to a great extent control the distribution and abundance of biota in the estuary. Cochin estuary receives large inputs from industrial units, sewage works and agricultural runoffs. (Anirudhan *et al.*, 1987; Lakshmanan *et al.*, 1987). They largely influenze the concentrations of P and other nutrients in the estuary. Menon *et al.* (2000) ascertained a close correlation between the P cycle and primary production in the estuary while, salinity and the N cycle are completely unconnected with the productivity rhythm. The enzyme activity of bacterial cells may have an important function in the survival of bacteria under different stress conditions, like the depletion of PO_4. The ability to synthesize extracellular enzymes may give those bacteria a competitive advantage (Ozkanca and Flint, 1996) in stressed environments.

Among the bacteria isolated the *Streptococcus* sp. was found to be the best acid phosphatase producing microbe and the maximum alkaline phosphatase production was observed in *Flavobacterium breve*. Cochin backwaters were found to harbour a good number of phosphatase producing bacteria, which can be believed to have a major role in the decomposition and transformation of organophosphorus compounds in the estuary.


Chapter 4

EFFECT OF CULTURE CONDITIONS ON GROWTH AND PHOSPHATASE PRODUCTION

The rate of growth and metabolism of bacteria depend very much on the composition of the medium and the prevalent environmental conditions. Various bacterial strains differ in their nutritional requirements and cultural conditions for optimal growth and enzyme production. In nature, many species do not always develop best within their optimal range, as they may be inhibited by competition with other organisms. This is also the reason why some inhabitants of extreme conditions show, in pure culture, entirely different optima than that of their natural environment. The growth of microorganisms is affected by a great variety of physical and chemical factors, of which,

some are particularly important. They influenze not only the size and composition of microbial populations, but also the morphology and physiology of individual bacteria.

To culture microorganisms, it is necessary to establish a suitable environment in which a particular microbe can survive and reproduce best. By understanding the specific requirements of a microbial species, it is possible to establish conditions *in vitro* to support the optimal growth and enzyme production of that organism. Influence of some of the important factors like pH, NaCl concentration, temperature of incubation etc. on growth and phosphatase production by the two selected species of bacteria were studied in the present instance.

4.1 METHODOLOGY

4.1.1 Organisms

Two strains, a *Streptococcus* sp. isolated from the Arabian Sea in which maximum acid phosphatase and *Flavobacterium breve* isolated from Cochin estuary in which maximum alkaline phosphatase production were observed were selected for further studies.

4.1.2 Growth medium

Nutrient broth and a mineral medium containing artificial seawater base (ASW) of Mac Leod (1968) and 0.2% sucrose as carbon source were used.

4.1.2.1 Medium composition

Nutrient broth

Peptone	5 g
Beef extract	3 g
NaCl	15 g
pH	7.2
Water	1000 ml

Mineral medium

Composition of Artificial seawater base (ASW)

NaCl	23.4 g l^{-1}
$MgSO_4 . 7H_2O$	24.6 g l^{-1}
KCl	1.5 g l^{-1}
$CaCl_2 . 2H_2O$	2.9 g l^{-1}

Salts were dissolved separately and combined.

.....contd.

Basal Medium

Tris (hydroxymethyl)amino methane (adjusted to pH 7.5 with HCl)	6.1 g l^{-1}
NH_4Cl	1.0 g l^{-1}
$K_2HPO_4.3H_2O$	75 mg l^{-1}
$FeSO_4.7H_2O$	28 mg l^{-1}
Sucrose	2 g l^{-1}
Yeast extract	100mg l^{-1}

Basal medium was mixed with half strength ASW.

4.1.3 Effect of period of incubation

4.1.3.1 Still culture

The incubation period required for maximal growth and enzyme production was determined by inoculating the cultures in liquid media. Growth and enzyme production were monitored at four hour intervals up to a period of 48 hours.

4.1.3.2 Shaker culture

The cultures were inoculated in liquid media and incubated on an orbital shaker (100 rpm) at room temperature. Growth and

enzyme production were measured at four hour intervals up to 48 hours.

4.1.4 Phase of phosphatase production

To determine the phase of enzyme production, cultures were inoculated in nutrient broth, incubated on a shaker and the growth and enzyme production were determined at regular intervals.

4.1.5 Effect of physico-chemical factors on growth and phosphatase production

4.1.5.1 Effect of pH

The effect of pH on growth and enzyme production was analyzed by inoculating the organisms in nutrient broth having varying pH (pH ranging from 3 to 10). The cultures were incubated at 28^0C for 24 hours and growth and enzyme production were measured.

4.1.5.2 Effect of NaCl concentration

Effect of NaCl concentration on growth and enzyme production was studied by inoculating the cultures in nutrient broth having varying NaCl concentrations (0.25g - 3.0g / 100 ml). Growth and enzyme production were determined after 24 hours of incubation at 28^0C.

4.1.5.3 Effect of temperature

Effect of temperature on growth and enzyme production was determined by inoculating the bacterial cultures in nutrient broth and incubating them at different temperatures (15^0C - 50^0C). Growth and enzyme production were determined after 24 hours of incubation at 28^0C.

4.1.5.4 Effect of various salts

The effect of various ions on growth and phosphatase production was studied by adding individually varying concentrations of the salts, sodium nitrate, sodium fluoride, sodium bicarbonate, sodium phosphate and potassium chloride as source of ions to nutrient broth and inoculating the media with the cultures. Growth and enzyme production were detected after 24 hours of incubation at 28^0C.

4.1.5.5 Effect of carbon source

Effect of source of carbon on growth and enzyme production was determined by inoculating the cultures in mineral medium containing different concentrations of glucose, sucrose, maltose or mannitol as carbon sources. Growth and enzyme production were determined after incubation for 24 hours.

4.1.5.6 Effect of nitrogen source

Effect of nitrogen source on growth and enzyme production was determined by inoculating the cultures in mineral medium containing one of the nitrogen sources at 1% concentration. The nitrogen sources used were peptone, beef extract, yeast extract, malt extract, tryptone, ammonium chloride, ammonium sulfate, ammonium nitrate and sodium nitrate. The cultures were incubated at 28^0C for 24 hours and growth and enzyme production were determined.

4.1.6 Statistical analysis

The data obtained were subject to regression analysis and ANOVA (two-factor without replication).

4.2 RESULTS

4.2.1 Effect of period of incubation on growth and phosphatase production

The effect of period of incubation on growth and phosphatase production by *Streptococcus* sp. and *Flavobacterium breve* were studied in both still and shaker cultures.

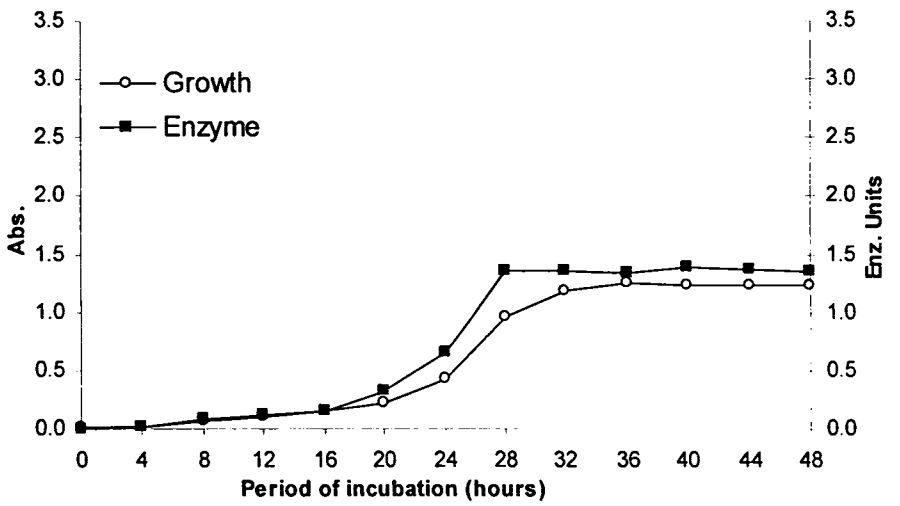
4.2.1.1 Still culture

In still culture, an incubation period of 32 hours was found to be necessary for maximal growth of *Streptococcus* sp. (Table 4.1 and Fig. 4.1). Acid phosphatase production began after eight hours. In 28 hours an acid phosphatase production of 1.35 Enz. Units was attained and more or less same enzyme activity was maintained till 48 hours. Growth and enzyme production showed highly significant relation to incubation period at 0.1% level.

Table 4.1 Effect of period of incubation (still culture) on growth and acid phosphatase production

Period of incubation (Hours)	Growth	Enzyme
0	0.01	0.00
4	0.02	0.03
8	0.07	0.08
12	0.10	0.11
16	0.15	0.16
20	0.22	0.33
24	0.42	0.64
28	0.97	1.35
32	1.18	1.36
36	1.25	1.34
40	1.24	1.40
44	1.24	1.37
48	1.23	1.36

Fig. 4.1 Effect of period of incubation (still culture) on growth and acid phosphatase production by *Streptococcus* sp.

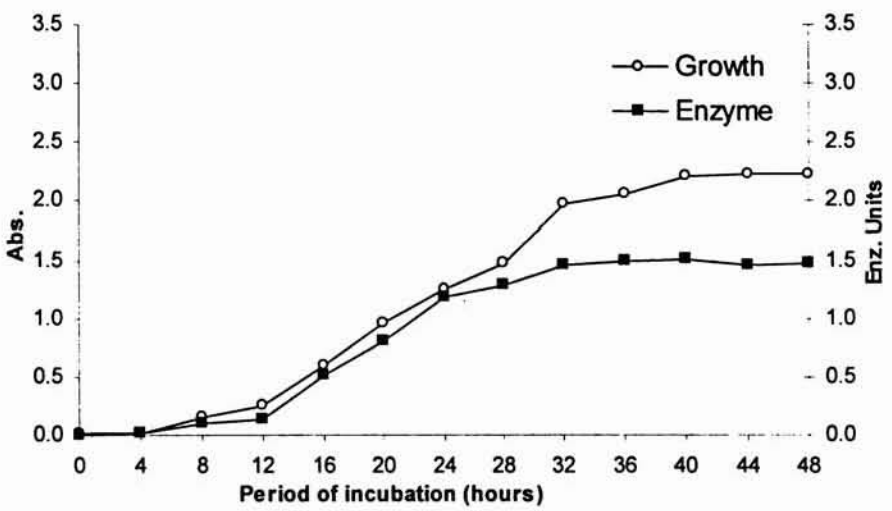


Effect of period of incubation on growth and alkaline phosphatase production by *Flavobacterium breve* is presented in Table: 4.2 and Fig. 4.2. In still culture, it required an incubation period of 32 hours to attain maximum growth (2.0-2.1 Abs.). The enzyme production began after 8 hours and reached its optimum range of 1.2 to 1.5 Enz. Units at 24 hours. The enzyme was stable in the medium and there was no noticeable change on further incubation. The incubation period was found to have a significant effect ($p<0.001$) on growth and alkaline phosphatase production.

Table 4.2 Effect of period of incubation (Still culture) on growth and alkaline phosphatase production by *Flavobacterium breve*

Period of incubation (Hours)	Growth	Enzyme
0	0.0	0.0
4	0.0	0.0
8	0.2	0.1
12	0.3	0.1
16	0.6	0.5
20	1.0	0.8
24	1.2	1.2
28	1.5	1.3
32	2.0	1.5
36	2.1	1.5
40	2.2	1.5
44	2.2	1.5
48	2.2	1.5

Fig. 4.2 Effect of period of incubation (Still culture) on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.1.2 Shaker culture

On incubation on an orbital shaker (100 rpm), the bacteria exhibited better growth and enzyme production as compared to the still culture experiments. After 32 hours of incubation *Streptococcus* sp. showed maximum growth (2.43Abs.) and acid phosphatase production peaked with 3.06 Enz. units in 28 hours (Table 4.3 & Fig. 4.3).

As depicted in Table 4.4 and Fig. 4.4, maximal growth of 3.32 (Abs.) was obtained for *Flavobacterium breve* in 32 hours and the optimum alkaline phosphatase production amounted to 2.25 enzyme units in 36 hours. Shaking was found to have a highly significant ($p < 0.001$) effect on growth and phosphatase production by both the organisms.

Table 4.3 Effect of shaking on growth and acid phosphatase production by *Streptococcus* sp.

Period of incubation (Hours)	Growth	Enzyme
0	0.01	0.00
4	0.07	0.00
8	0.51	0.61
12	0.95	1.25
16	1.44	1.82
20	1.99	2.26
24	2.22	2.76
28	2.28	3.06
32	2.43	2.93
36	2.34	2.88
40	2.28	2.88
44	2.19	2.71
48	2.22	2.72

Fig. 4.3 Effect of shaking on growth and acid phosphatase production by *Streptococcus* sp.

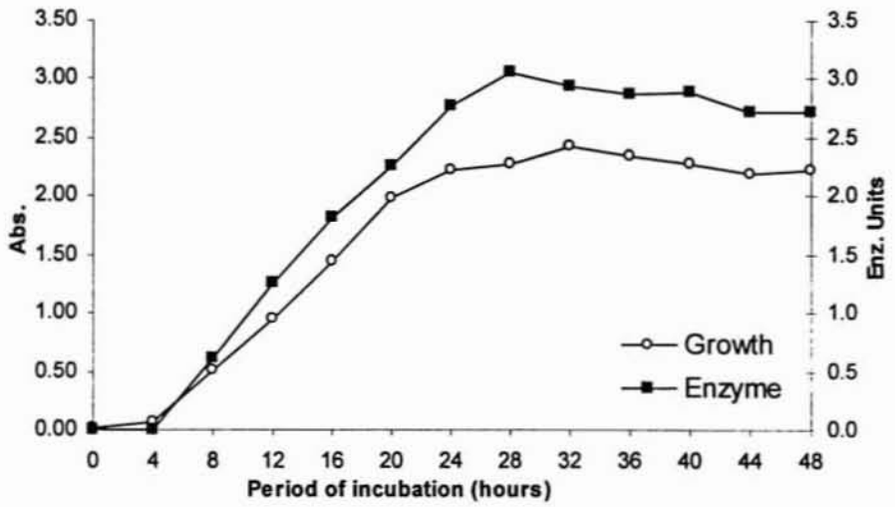
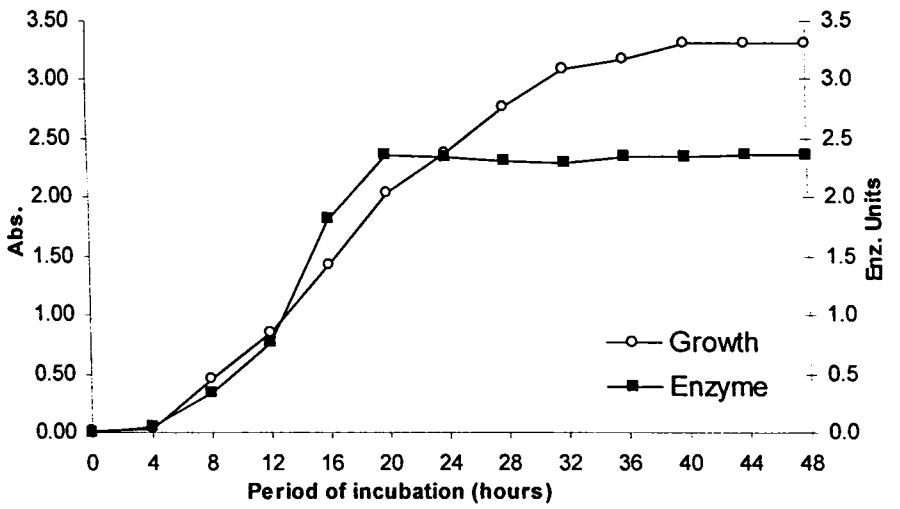


Table 4.4 Effect of shaking on growth and alkaline phosphatase production by *Flavobacterium breve*

Period of incubation (hours)	Growth	Enzyme
0	0.01	0.00
4	0.03	0.04
8	0.46	0.34
12	0.85	0.76
16	1.43	1.82
20	2.05	2.37
24	2.39	2.35
28	2.76	2.32
32	3.09	2.30
36	3.17	2.35
40	3.32	2.35
44	3.31	2.36
48	3.32	2.36

Fig. 4.4 Effect of shaking on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.2 Growth phase of phosphatase production

Results of experiments to determine the growth phase at which phosphatase production occurs are shown in Table 4.3 and Fig. 4.3. The *Streptococcus* sp. spent about 4 hours in the lag phase, the next 12 hours in the logarithmic phase and then entered the stationary phase. The enzyme production started at about 8 hours, when the cells were in their early exponential phase of growth and enzyme production continued till the cells entered the stationary phase. There was no further increase in enzyme production when the cells were in the stationary phase.

The growth curve plotted for *Flavobacterium breve* also showed almost similar results (Table 4.4 and Fig. 4.4). The lag phase was observed for a duration of 4 hours and the exponential phase extended for the next 24 hours, followed by the stationary phase. The enzyme production was detected during the early logarithmic phase from about 8 hours of incubation and continued till the cells entered the stationary phase.

4.2.3 Effect of pH

Maximum growth and acid phosphatase production by *Streptococcus* sp. were found to be at pH 7 (Table 4.5 and Fig. 4.5). Considerable growth and enzyme production were also observed at pH 8. However, there was a sharp decline in growth and enzyme production in the acidic range and above pH 9.

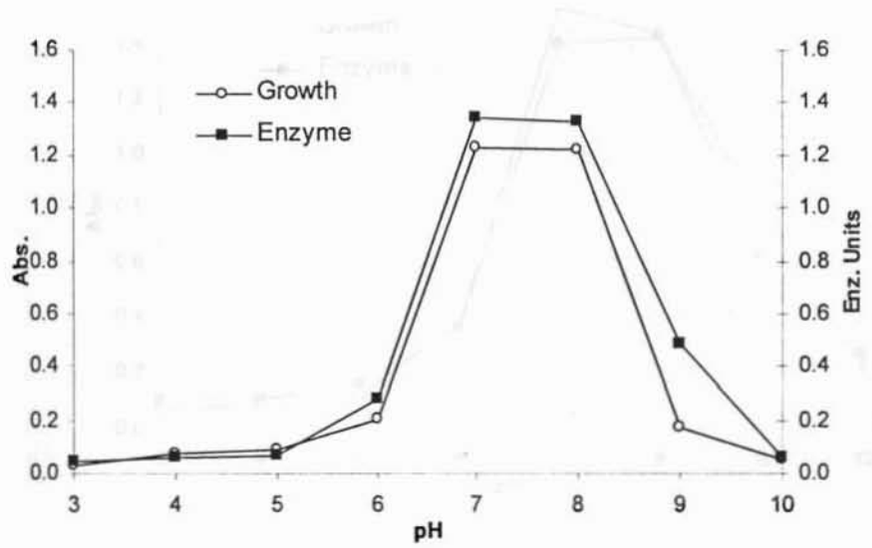
Table 4.5 Effect of pH on growth and acid phosphatase production by *Streptococcus* sp.

pH	Growth	Enzyme
3	0.03	0.04
4	0.08	0.06
5	0.09	0.07
6	0.20	0.28
7	1.23	1.34
8	1.22	1.33
9	0.17	0.49
10	0.05	0.06

pH 8 was found to be most favourable for growth and alkaline phosphatase production by *Flavobacterium breve*, (Table 4.6 and Fig. 4.6) though pH 7 also supported appreciable growth. However, the enzyme production was found to be retarded as the pH of the medium was lowered (acidic range) or increased to pH 9 and above.

The statistical analysis of these results indicate that the growth and phosphatase production by the *Streptococcus* sp. and *Flavobacterium breve* were significantly ($p<1\%$) dependant on pH.

Fig. 4.5 Effect of pH on growth and acid phosphatase production by *Streptococcus* sp.



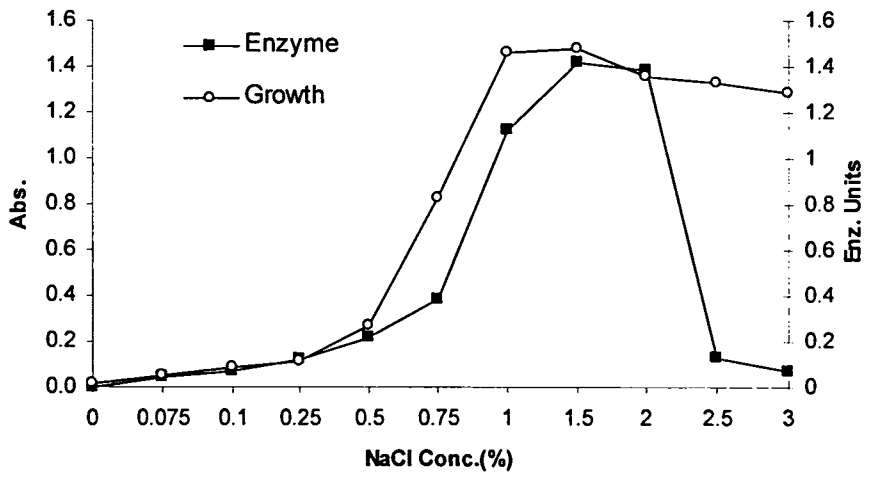
4.2.4 Effect of NaCl concentration

When the organisms were grown in media having different levels of NaCl concentration to study the effect of NaCl concentration on growth and enzyme production, optimum NaCl concentration required for growth and acid phosphatase production by *Streptococcus* sp. was found to be 1.5% (Table 4.7 and Fig. 4.7). Sodium chloride was found to be essential for the growth of the organism. Higher concentrations of NaCl sustained growth, but not enzyme production. NaCl concentration above 2% was found to inhibit acid phosphatase synthesis. The variations in NaCl concentrations were found to have a significant (1% level) effect on growth and acid phosphatase production.

Table 4.7 Effect of NaCl concentrations on growth and acid phosphatase production by *Streptococcus* sp.

NaCl (%)	Growth	Enzyme
0.00	0.02	0.00
0.075	0.05	0.04
0.10	0.09	0.07
0.25	0.11	0.12
0.50	0.27	0.22
0.75	0.83	0.38
1.00	1.46	1.12
1.50	1.48	1.42
2.00	1.36	1.38
2.50	1.33	0.13
3.00	1.29	0.07

Fig. 4.7 Effect of NaCl concentration on growth and acid phosphatase production by *Streptococcus* sp.

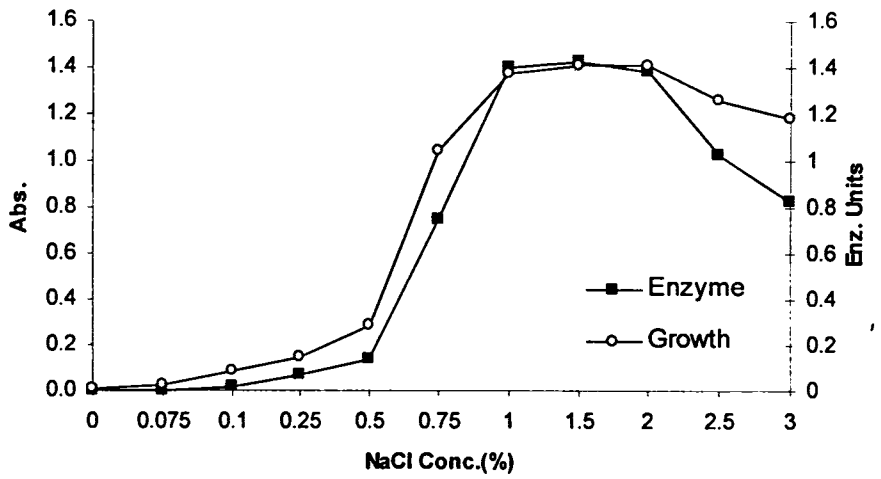


Flavobacterium breve showed changes in the rate of growth and enzyme production when exposed to different concentrations of NaCl (Table 4.8 and Fig. 4.8). The organism attained optimum growth and alkaline phosphatase production at a NaCl concentration of 1.5%. A slight decrease in growth and alkaline phosphatase production was noticed as the NaCl concentration was increased to 3%. The effect of NaCl on growth and alkaline phosphatase production was found to be significant at 1% and 5% levels respectively.

Table 4.8 Effect of NaCl concentration on growth and alkaline phosphatase production by *Flavobacterium breve*.

NaCl (%)	Growth	Enzyme
0.00	0.01	0.00
0.075	0.03	0.00
0.10	0.09	0.02
0.25	0.15	0.07
0.50	0.29	0.14
0.75	1.04	0.75
1.00	1.37	1.40
1.50	1.41	1.43
2.00	1.41	1.38
2.50	1.26	1.03
3.00	1.18	0.83

Fig. 4.8 Effect of NaCl concentration on growth and alkaline phosphatase production by *Flavobacterium breve*.



4.2.5 Effect of temperature on growth and enzyme production

In the present study the optimum temperature for growth and acid phosphatase production by *Streptococcus* sp. was observed to be

25°C and 30°C respectively (Table 4.9 and Fig. 4.9). There was a significant decline in the enzyme production at incubation temperatures of 35°C and above. However, temperatures ranging from15°C to 40°C was found to be suitable for the growth of *Streptococcus* sp.

Table 4.9 Effect of temperature on growth and acid phosphatase production by *Streptococcus* sp.

Temp (°C)	Growth	Enzyme
5	0.04	0.01
10	0.13	0.09
15	0.72	0.48
20	1.08	0.99
25	1.33	1.06
30	1.26	1.07
35	0.72	0.37
40	0.46	0.17
45	0.19	0.04
50	0.04	0.03
55	0.02	0.00
60	0.01	0.00

It is clear from the Table 4.10 and Fig.4.10 that the temperatures ranging from 15°C to 35°C were favourable for the growth of *Flavobacterium breve*, the optimum being 25°C. The organism responded with peak enzyme production at the same temperature. Considerable decrease in alkaline phosphatase production was encountered when the incubation temperature was increased to 35°C and above.

Fig. 4.9 Effect of temperature on growth and acid phosphatase production by *Streptococcus* sp.

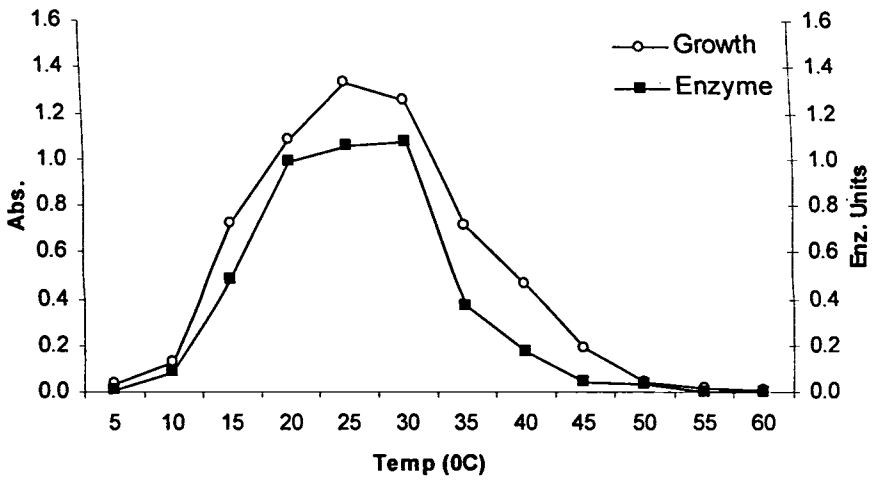
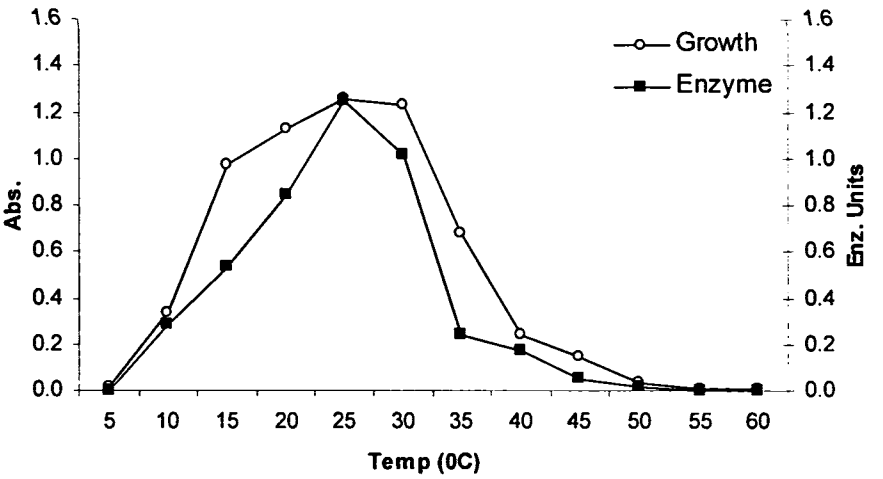


Table 4.10 Effect of temperature of incubation on growth and alkaline phosphatase production by *Flavobacterium breve*

Temp (°C)	Growth	Enzyme
5	0.02	0.00
10	0.33	0.29
15	0.97	0.53
20	1.12	0.84
25	1.26	1.25
30	1.23	1.01
35	0.68	0.24
40	0.24	0.17
45	0.14	0.06
50	0.04	0.02
55	0.01	0.00
60	0.01	0.00

Fig. 4.10 Effect of temperature of incubation on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.6 Effect of various carbon sources

Among the various carbon supplements, the medium containing 0.5% maltose yielded maximum growth of *Streptococcus* sp. (Table 4.11 and Fig. 4.11). However, it did not support acid phosphatase production. The use of sucrose as the carbon source too yielded good growth. The maximum enzyme production was observed when the carbon source used was 0.2% sucrose (Table 4.12 and Fig. 4.12). Statistical analysis revealed that the variations in carbon sources did not have significant effect on growth, while acid phosphatase production was found to be significantly ($p<0.05$) higher with the use of

sucrose as carbon source. Maltose yielded significantly ($p<0.05$) lower amounts of phosphatase than the other carbon sources.

Table 4.11 Effect of various carbon sources on growth of *Streptococcus* sp.

Conc. (%)	Glucose	Sucrose	Mannitol	Maltose
0.05	1.22	1.27	1.22	1.28
0.10	1.22	1.39	1.24	1.22
0.20	1.22	1.41	1.29	1.64
0.50	1.22	1.36	1.23	1.71

Fig. 4.11 Effect of various carbon sources on growth of *Streptococcus* sp.

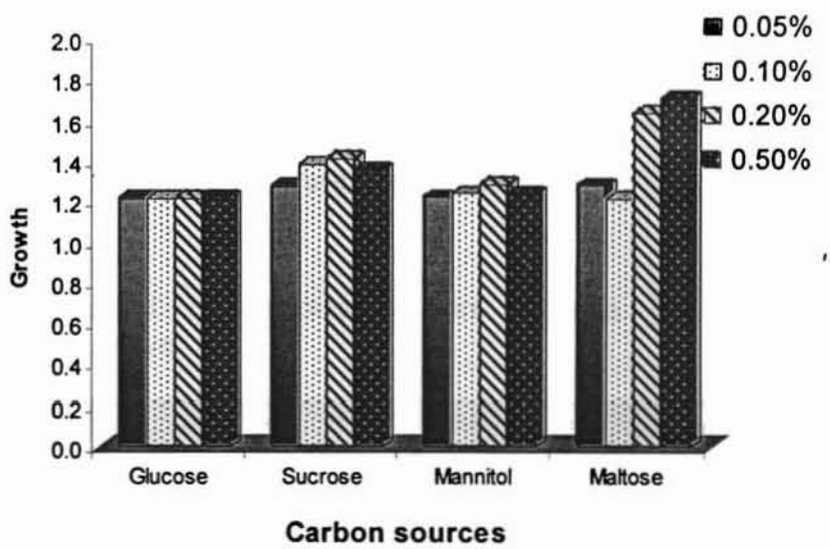


Table 4.12 Effect of various carbon sources on acid phosphatase production by *Streptococcus* sp.

Conc. (%)	Glucose	Sucrose	Mannitol	Maltose
0.05	1.13	1.46	1.28	1.08
0.10	1.14	1.60	1.31	1.03
0.20	1.13	1.95	1.42	1.06
0.50	1.14	1.71	1.32	1.11

Fig. 4.12 Effect of various carbon sources on acid phosphatase production by *Streptococcus* sp.

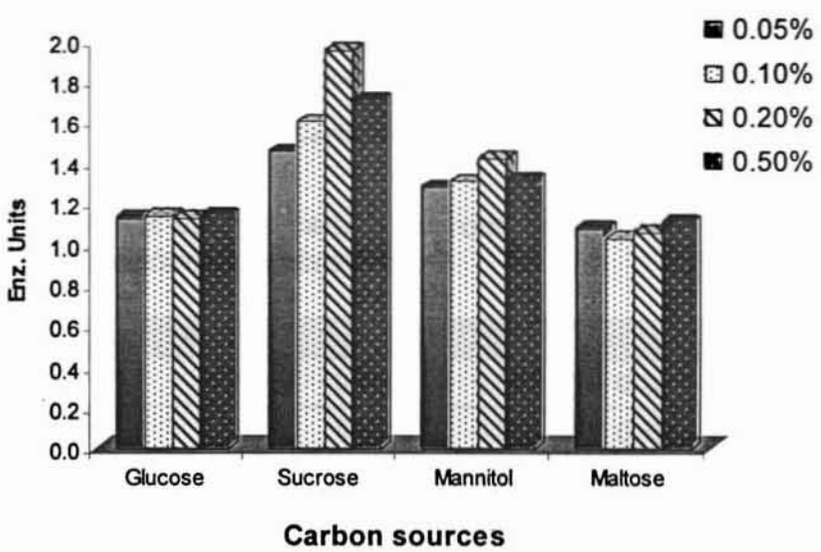


Table 4.13 Effect of various carbon sources on growth of *Flavobacterium breve*.

Conc. (%)	Glucose	Sucrose	Mannitol	Maltose
0.05	0.86	1.01	0.52	0.28
0.10	1.05	1.10	0.91	0.43
0.20	1.06	1.11	1.14	0.71
0.50	1.04	0.88	1.11	0.92

Fig. 4.13 Effect of various carbon sources on growth of *Flavobacterium breve*

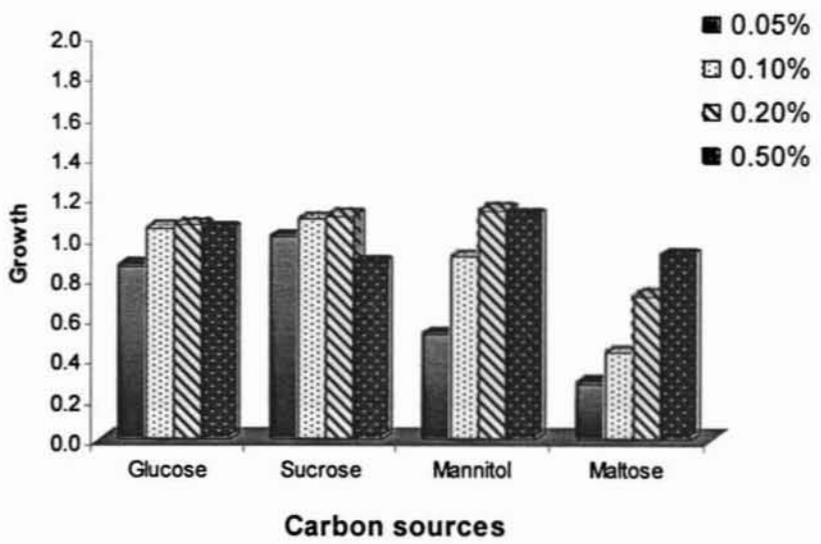
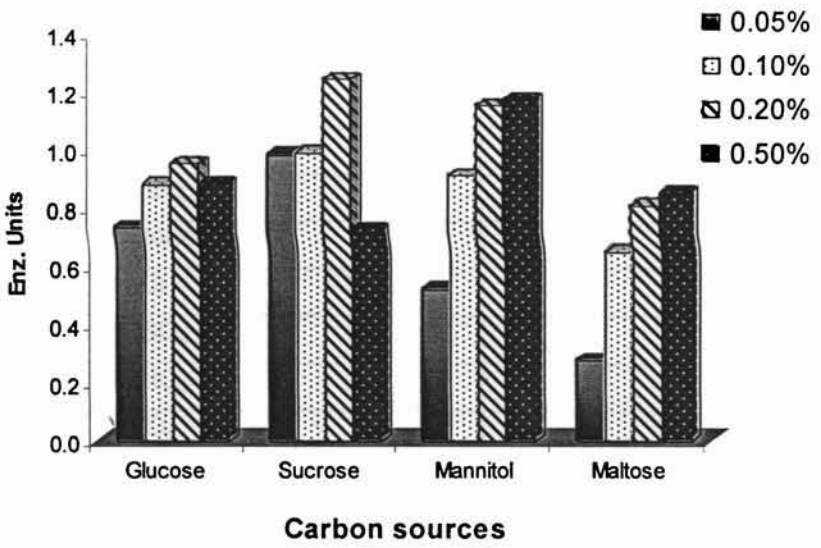


Table 4.14 Effect of various carbon sources on alkaline phosphatase production by *Flavobacterium breve*

Conc. (%)	Glucose	Sucrose	Mannitol	Maltose
0.05	0.73	0.98	0.52	0.28
0.10	0.88	0.99	0.91	0.65
0.20	0.95	1.24	1.15	0.81
0.50	0.88	0.72	1.17	0.85

It was observed that the *Flavobacterium breve* preferred 0.2% mannitol to other carbon supplements for optimal growth (Table 4.22 and Fig. 4.22). Sucrose with a concentration of 0.2% gave the maximal enzyme output. However, no significant change in growth or alkaline phosphatase production was observed with variations in carbon sources.

Fig. 4.14 Effect of various carbon sources on alkaline phosphatase production by *Flavobacterium breve*



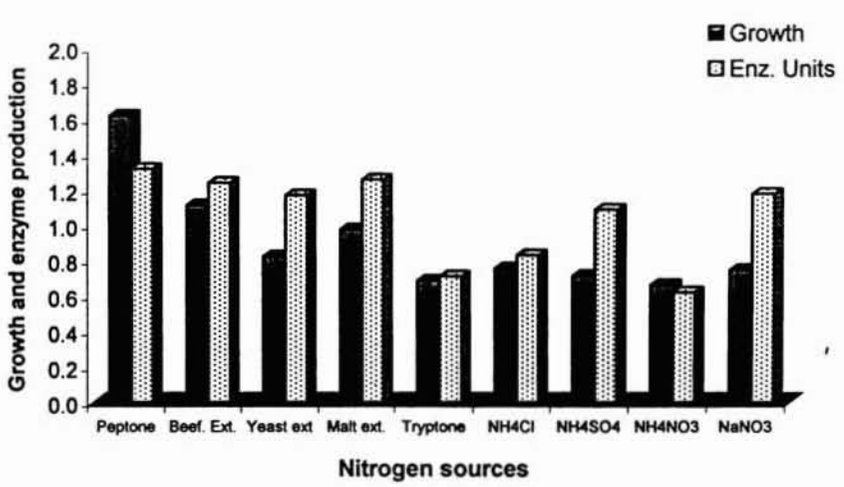
4.2.7 Effect of various nitrogen sources:

Maximum growth and acid phosphatase production by *Streptococcus* sp. was obtained when the nitrogen source used was peptone (Table 4.15 and Fig. 4.15). Pronounced growth was also observed when beef extract and malt extract were added to ,the mineral medium. Growth was significantly ($p < 0.05$) higher when peptone was the nitrogen source used followed by malt extract and beef extract. Significantly ($p < 0.05$) lower growth was obtained with NH_4NO_3 and Tryptone. Acid phosphatase production was not significantly affected by variations in nitrogen sources.

Table 4.15 Effect of various nitrogen sources on growth and acid phosphatase production by *Streptococcus* sp.

N sources	Growth	Enzyme
Peptone	1.62	1.32
Beef ext.	1.11	1.24
Yeast ext	0.82	1.17
Malt ext.	0.98	1.26
Tryptone	0.69	0.71
NH_4Cl	0.76	0.83
NH_4SO_4	0.71	1.09
NH_4NO_3	0.66	0.62
$NaNO_3$	0.75	1.18

Fig.4.15 Effect of various nitrogen sources on growth and acid phosphatase production by *Streptococcus* sp.

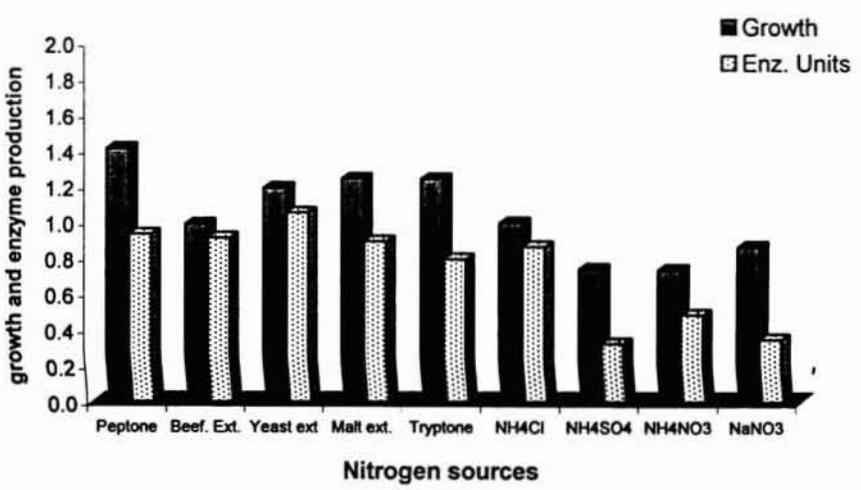


In the case of *Flavobacterium breve* peptone was found to be the most preferred source of nitrogen for maximal growth. All the nitrogen sources supplied supported growth (Table 4.16 and Fig. 4.16). Yeast extract induced maximum enzyme production by the organism.

Table 4.16 Effect of various nitrogen sources on growth and alkaline phosphatase production by *Flavobacterium breve*

N sources	Growth	Enzyme
Peptone	1.41	0.93
Beef ext.	0.99	0.91
Yeast ext.	1.19	1.05
Malt ext.	1.24	0.89
Tryptone	1.24	0.79
NH_4Cl	0.99	0.86
NH_4SO_4	0.74	0.32
NH_4NO_3	0.73	0.48
$NaNO_3$	0.86	0.34

Fig. 4.16 Effect of various nitrogen sources on growth and alkaline phosphatase production by *Flavobacterium breve*



Though significantly ($p<0.1\%$) increased growth was obtained by adding some of the nitrogen sources, there was no significant difference between them. There was also no significant

($p<0.01\%$) difference between the alkaline phosphatase yields with various nitrogen sources.

4.2.8 Effect of various salts

The results shown in Tables 4.17 to 4.31 and Figs. 4.17 to 4.31 indicate that the ionic requirements for growth and enzyme production by the *Streptococcus sp.* and *Flavobacterium breve* are not very specific. Growth and enzyme production by these bacteria were inhibited by several salts

4.2.8.1 Sodium fluoride

The *Streptococcus* sp. was able to grow (1.26 Abs.) and synthesize acid phosphatase (0.3 enzyme units) in presence of very low sodium fluoride concentrations *i.e.*, up to 0.075 M (Table 4.17 & Fig. 4.17).

Table 4.17 Effect of NaF on growth and acid phosphatase production by *Streptococcus* sp.

NaF (M)	Growth	Enzyme
0.05	1.03	0.24
0.075	1.26	0.30
0.10	1.08	0.28
0.25	0.45	0.09
0.50	0.03	0.01
1.00	0.02	0.00
1.50	0.02	0.00

There was a steep fall in growth and enzyme production with further increase in NaF concentrations culminating in total inhibition in the presence of 0.5 M NaF (Table 4.18 & Fig. 4.18). *Flavobacterium breve* was found to succumb to even low levels of NaF in the growth media.

Fig. 4.17 Effect of NaF on growth and acid phosphatase production by *Streptococcus* sp.

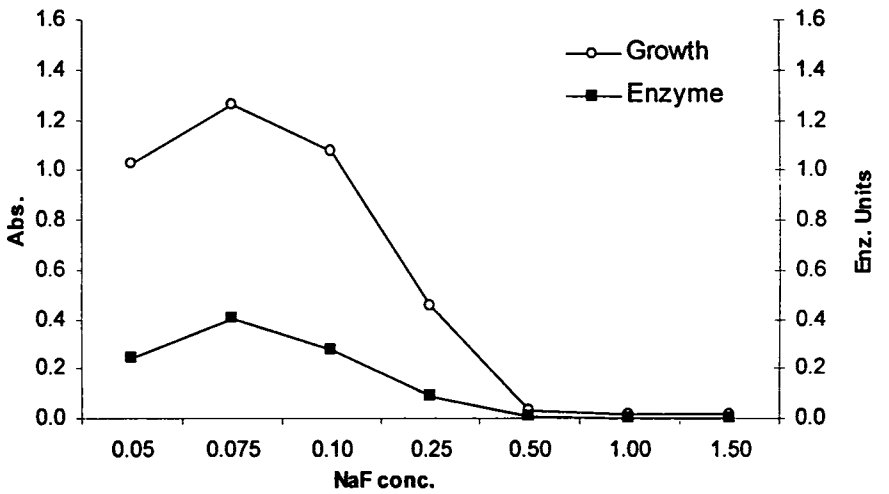
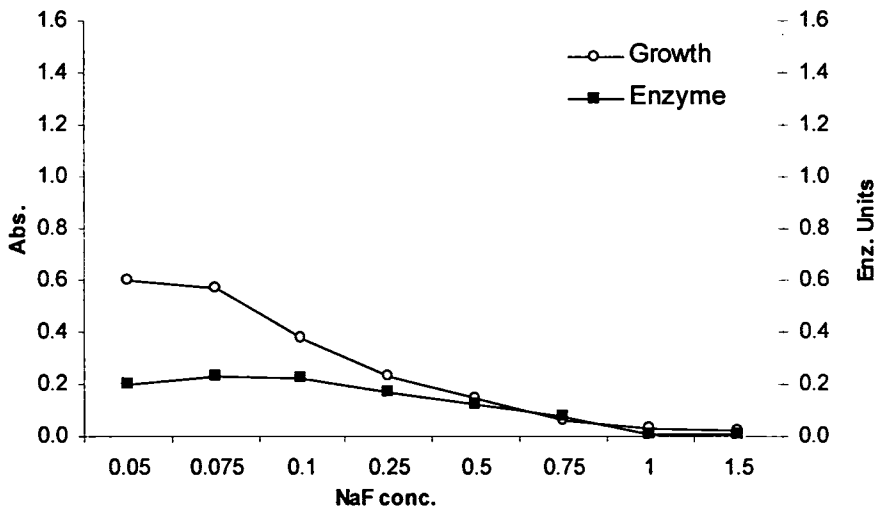


Table 4.18 Effect of NaF on growth and alkaline phosphatase production by *Flavobacterium breve*

NaF (M)	Growth	Enzyme
0.05	0.6	0.20
0.075	0.57	0.23
0.1	0.38	0.22
0.25	0.23	0.17
0.5	0.15	0.12
0.75	0.06	0.08
1.0	0.03	0.01
1.5	0.02	0.01

Fig. 4.18 Effect of NaF on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.8.2 Sodium sulphate

Streptococcus sp. could grow and produce acid phosphatase in presence of sodium sulphate up to a concentration of 0.5 M after which the inhibitory effect of the salt was found to increase with concentration (Table 4.19 & Fig. 4.19). Identical results (Table 4.20 & Fig. 4.20) were encountered with *Flavobacterium breve* in which Na_2SO_4 was found to support growth and enzyme production up to 0.25 M and a regular decline with further increase in salt concentration was noticed.

Table 4.19 Effect of Na_2SO_4 on growth and acid phosphatase production by *Streptococcus* sp.

Na_2SO_4 (M)	Growth	Enzyme
0.05	0.98	0.74
0.075	1.47	0.90
0.10	1.46	0.99
0.25	1.20	1.01
0.50	0.97	1.09
0.75	0.52	0.19
1.00	0.11	0.05
1.50	0.06	0.03

Fig. 4.19 Effect of Na_2SO_4 on growth and acid phosphatase production by *Streptococcus* sp.

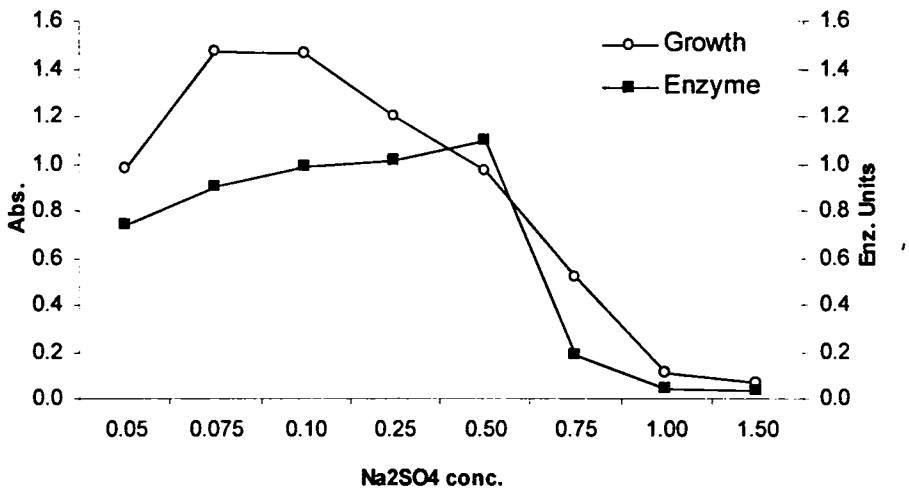
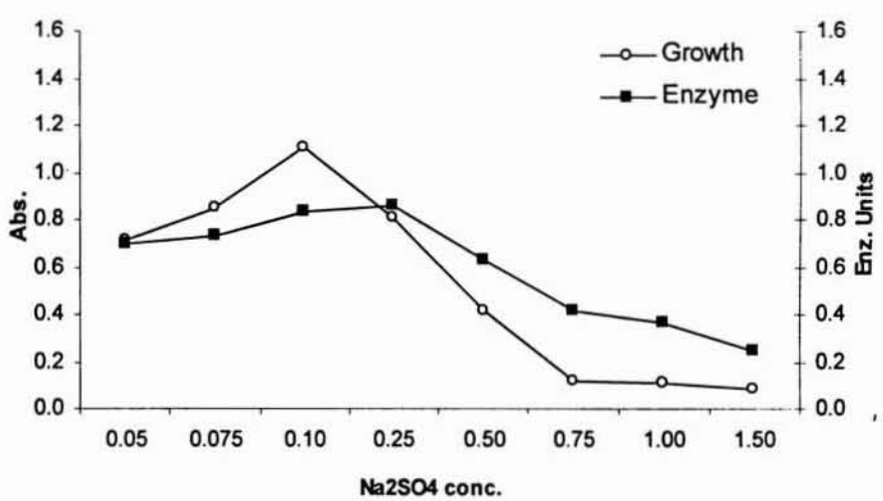


Table 4.20 Effect of Na_2SO_4 on growth and alkaline phosphatase production by *Flavobacterium breve*

Na_2SO_4 (M)	Growth	Enzyme
0.05	0.71	0.70
0.075	0.85	0.74
0.1	1.10	0.83
0.25	0.81	0.86
0.5	0.42	0.63
0.75	0.12	0.42
1	0.11	0.37
1.5	0.09	0.25

Fig. 4.20 Effect of Na_2SO_4 on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.8.3 Sodium bicarbonate

$NaHCO_3$ totally inhibited the growth and enzyme production in *Streptococcus* sp. (Table 4.21 & Fig. 4.21) whereas, *Flavobacterium*

breve was found to grow and produce low quantities of alkaline phosphatase in the presence of low levels of the salt in the medium (Table 4.22 & Fig. 4.22). Increase in the salt levels led to a sharp reduction in growth and enzyme production leading to total inhibition.

Table 4.21 Effect of $NaHCO_3$ on growth and acid phosphatase production by *Streptococcus* sp.

$NaHCO_3$(M)	Growth	Enzyme
0.05	0.09	0.06
0.075	0.07	0.06
0.10	0.05	0.05
0.25	0.04	0.04
0.50	0.03	0.00
0.75	0.01	0.00
1.00	0.00	0.00

Fig. 4.21 Effect of $NaHCO_3$ on growth and acid phosphatase production by *Streptococcus* sp.

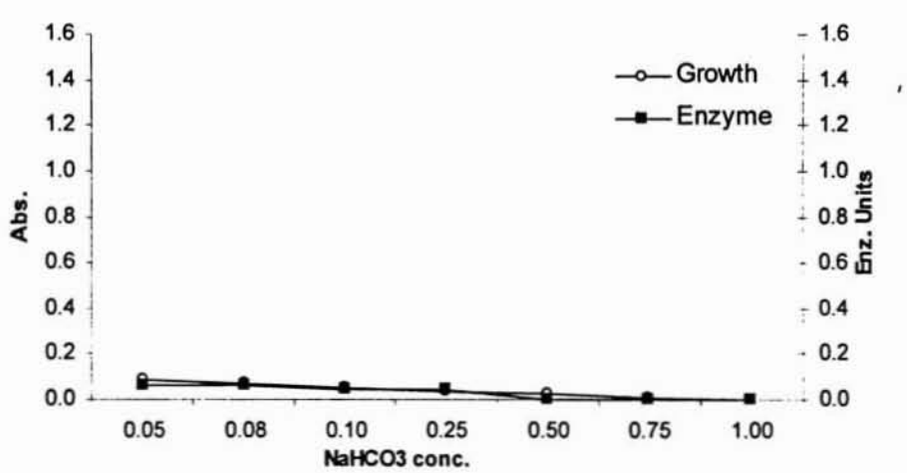
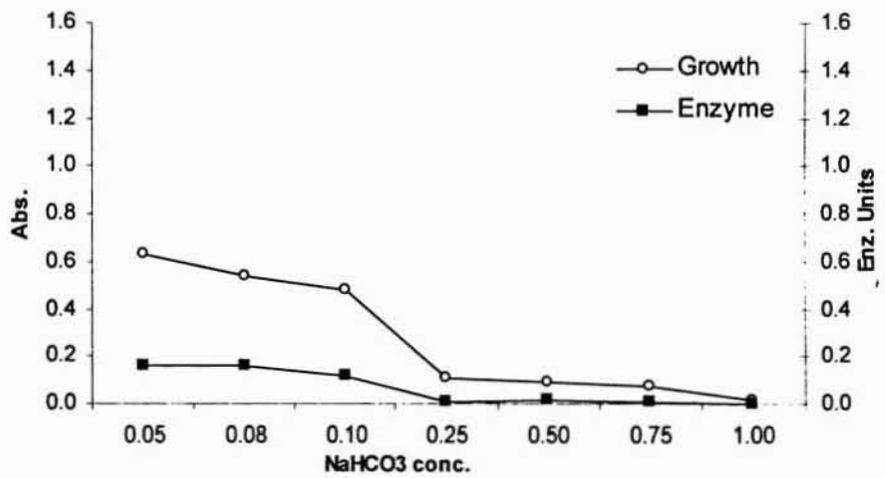


Table 4.22 Effect of $NaHCO_3$ on growth and alkaline phosphatase production by *Flavobacterium breve*

$NaHCO_3$ (M)	Growth	Enzyme
0.05	0.67	0.28
0.075	0.63	0.16
0.1	0.54	0.16
0.25	0.48	0.12
0.5	0.11	0.01
0.75	0.09	0.02
1	0.08	0.01
1.5	0.02	0.00

Fig. 4.22 Effect of $NaHCO_3$ on growth and alkaline phosphatase production by *Flavobacterium breve*



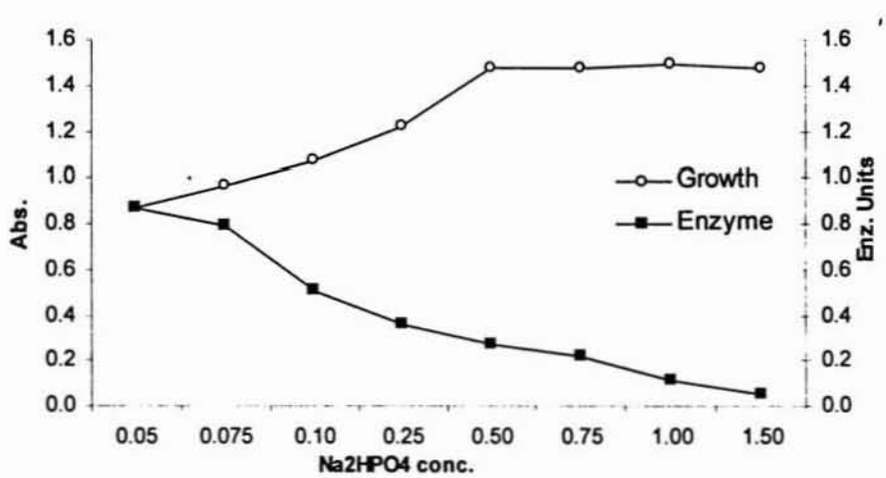
4.2.8.4 *di* Sodium hydrogen phosphate

The results presented in Tables 4.23 & 4.24 and Figs. 4.23 & 4.24 reveal identical responses elicited from *Streptococcus* sp. and *Flavobacterium breve* on addition of Na_2HPO_4

Table 4.23 Effect of Na_2HPO_4 on growth and acid phosphatase production by *Streptococcus* sp.

Na_2HPO_4 (M)	Growth	Enzyme
0.05	0.87	0.57
0.075	0.96	0.59
0.10	1.08	0.51
0.25	1.22	0.36
0.50	1.48	0.27
0.75	1.47	0.22
1.00	1.50	0.11
1.50	1.48	0.05

Fig. 4.23 Effect of Na_2HPO_4 on growth and acid phosphatase production by *Streptococcus* sp.

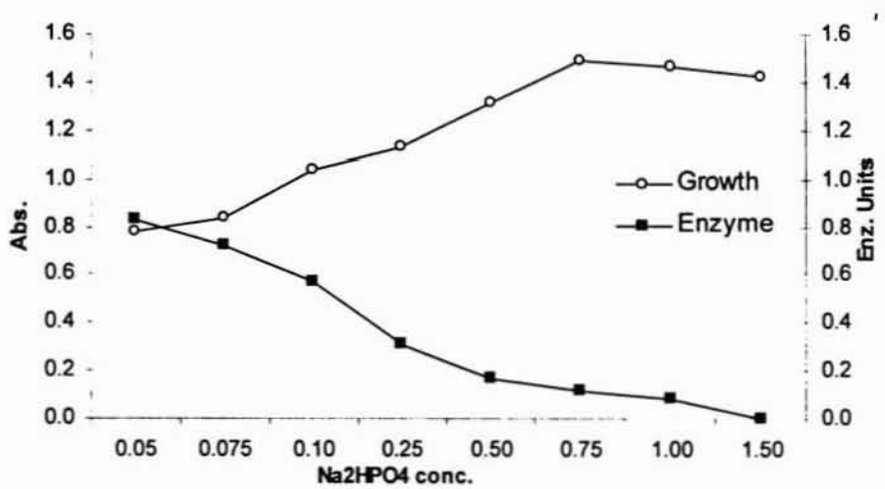


Addition of the salt produced an enhancement to a certain extent in both the bacteria after which steady growth was observed. An inverse relationship between orthophosphate concentration and phosphatase production was evident in the case of both the phosphatase producing bacteria.

Table 4.24 Effect of Na_2HPO_4 on growth and alkaline phosphatase production by *Flavobacterium breve*

Na_2HPO_4 (M)	Growth	Enzyme
0.05	0.78	0.83
0.075	0.84	0.72
0.1	1.04	0.57
0.25	1.13	0.31
0.5	1.32	0.17
0.75	1.49	0.12
1.0	1.46	0.08
1.5	1.43	0

Fig. 4.24 Effect of Na_2HPO_4on growth and alkaline phosphatase production by *Flavobacterium breve*



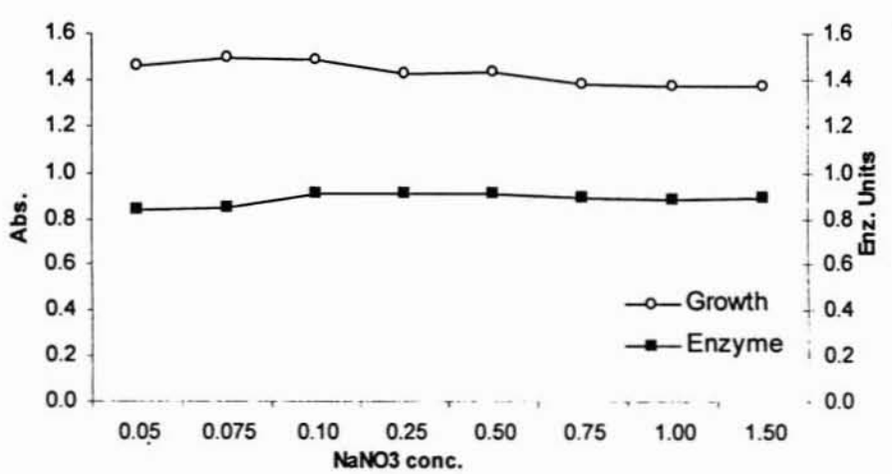
4.2.8.5 Sodium nitrate

$NaNO_3$ seems to have no pronounced effect on growth and phosphatase production by either bacteria (Tables 4.25 & 4.26 and Figs. 4.25 & 4.26).

Table 4.25 Effect of $NaNO_3$ on growth and acid phosphatase production by *Streptococcus* sp.

$NaNO_3$ (M)	Growth	Enzyme
0.05	1.46	0.84
0.075	1.49	0.85
0.10	1.48	0.91
0.25	1.42	0.91
0.50	1.44	0.91
0.75	1.38	0.89
1.00	1.37	0.88
1.50	1.38	0.89

Fig. 4.25 Effect of $NaNO_3$ on growth and acid phosphatase production by *Streptococcus* sp.

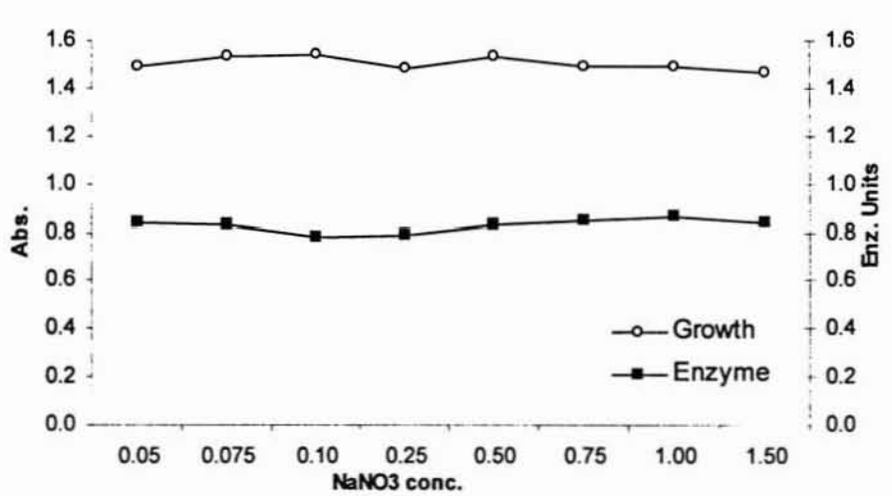


The *Streptococcus* sp. and *Flavobacterium breve* were found to grow and synthesize phosphatases consistently, apparently uninfluenced, at all concentrations of $NaNO_3$.

Table 4.26 Effect of $NaNO_3$ on growth and alkaline phosphatase production by *Flavobacterium breve*

$NaNO_3$ (M)	Growth	Enzyme
0.05	1.49	0.84
0.075	1.53	0.83
0.1	1.54	0.78
0.25	1.48	0.79
0.5	1.53	0.83
0.75	1.49	0.85
1	1.49	0.86
1.5	1.47	0.84

Fig. 4.26 Effect of $NaNO_3$ on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.8.6 Potassium chloride

Streptococcus sp. showed varied patterns of growth and enzyme production with the increase in KCl concentration (Table 4.27 & Fig. 4.27). No significant effect on growth of the organism was observed. On the other hand, the acid phosphatase synthesis, sustained initially, was found to recede inversely proportionate to the KCl concentration.

Table 4.27 Effect of KCl on growth and acid phosphatase production by *Streptococcus* sp.

KCl (M)	Growth	Enzyme
0.05	1.37	0.88
0.075	1.50	0.86
0.10	1.50	0.91
0.25	1.48	0.90
0.50	1.46	0.85
0.75	1.48	0.54
1.00	1.52	0.41
1.50	1.46	0.24

KCl was found to influenze both growth and enzyme production in *Flavobacterium breve* (Table 4.28 & Fig 4.28). After demonstrating consistency at low levels of KCl, the growth of the organism showed a sharp decline from 0.75 M KCl concentration. The enzyme production showed a gradual recession with the increase in KCl concentration.

Fig. 4.27 Effect of KCl on growth and acid phosphatase production by *Streptococcus* sp.

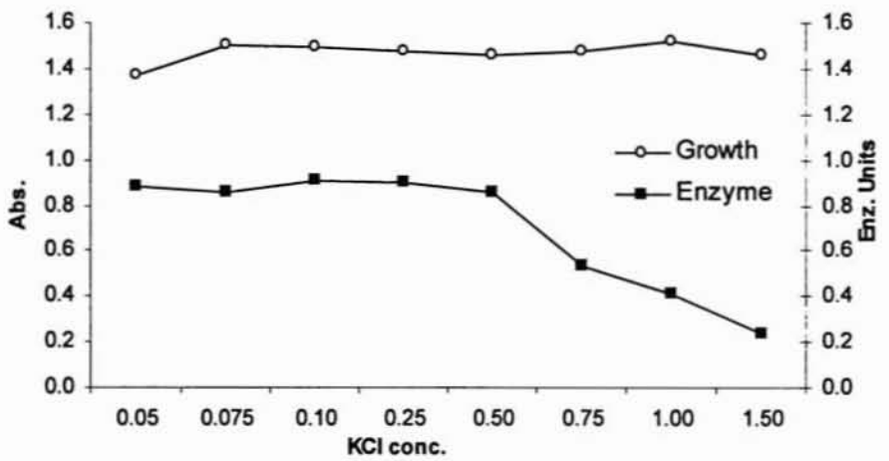
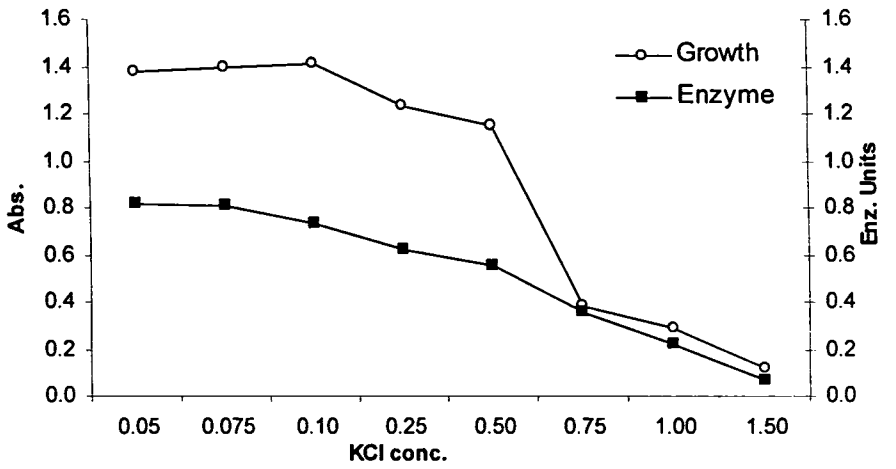


Table 4.28 Effect of KCl on growth and alkaline phosphatase production by *Flavobacterium breve*

KCl (M)	Growth	Enzyme
0.05	1.38	0.82
0.075	1.39	0.78
0.1	1.41	0. 73
0.25	1.24	0.62
0.5	1.15	0.49
0.75	0.39	0.36
1	0.29	0.22
1.5	0.12	0.07

Fig. 4.28 Effect of KCl on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.8.7 Magnesium chloride

$MgCl_2$ elicited varied responses from the two bacteria. The *Streptococcus* sp. was unfazed by the presence of $MgCl_2$ in the medium (Table 4.29 & Fig. 4.29). Steady growth and acid phosphatase production was observed at all concentrations of the salt. *Flavobacterium breve* exhibited even growth, while enhancement of alkaline phosphatase synthesis proportionate to the increase in $MgCl_2$ levels up to 0.5 M was observed (Table 4.30 & Fig. 4.30). Further increase in the salt concentrations did not affect the yield of the enzyme from the organism.

Table 4.29 Effect of $MgCl_2$ on growth and acid phosphatase production by *Streptococcus* sp.

$MgCl_2$ (M)	Growth	Enzyme
0.05	1.12	0.79
0.075	1.08	0.77
0.10	1.06	0.69
0.25	1.11	0.75
0.50	1.17	0.80
0.75	1.15	0.81
1.00	1.09	0.83
1.50	1.11	0.79

Fig. 4.29 Effect of $MgCl_2$ on growth and acid phosphatase production by *Streptococcus* sp.

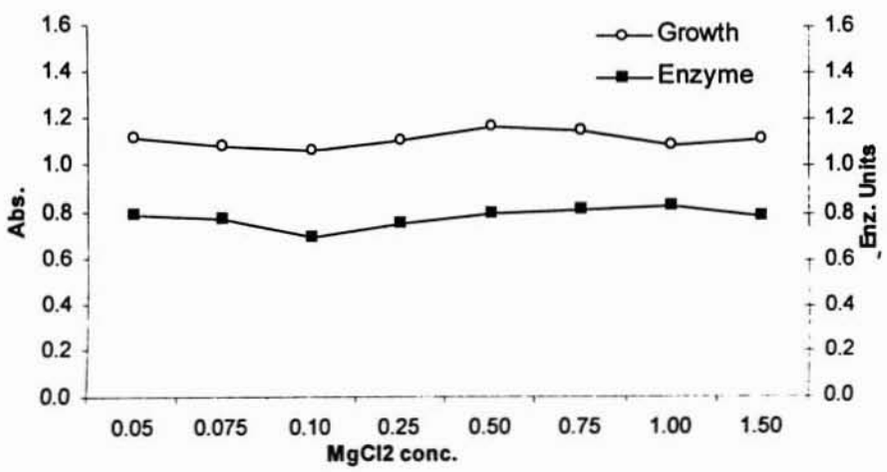
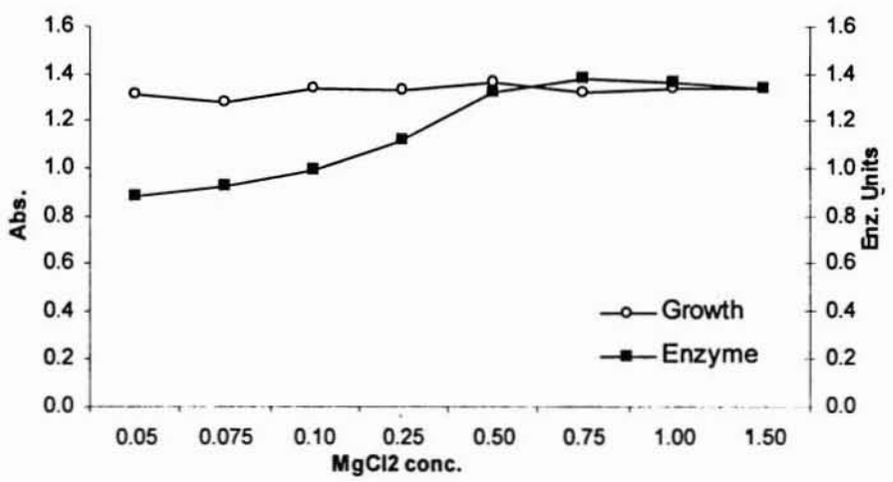


Table 4.30 Effect of $MgCl_2$ on growth and alkaline phosphatase production by *Flavobacterium breve*

$MgCl_2$ (M)	Growth	Enzyme
0.05	1.31	0.88
0.075	1.28	0.92
0.1	1.34	0.99
0.25	1.33	1.12
0.5	1.36	1.32
0.75	1.32	1.38
1	1.34	1.36
1.5	1.34	1.34

Fig. 4.30 Effect of $MgCl_2$ on growth and alkaline phosphatase production by *Flavobacterium breve*



4.2.8.8 Calcium chloride and Manganese chloride

The presence of high levels of $CaCl_2$ in the medium was found to have an inhibitory action on the growth and enzyme production by the *Streptococcus* sp. (Table 4.31 & Fig. 4.31). The organism was able to survive and synthesize acid phosphatase at low concentrations but, as the concentration of the salt increased, the growth and enzyme production decreased gradually.

Table 4.31 Effect of $CaCl_2$ on growth and acid phosphatase production by *Streptococcus* sp.

$CaCl_2$ (M)	Growth	Enzyme
0.05	1.32	0.81
0.075	1.24	0.74
0.10	1.17	0.58
0.25	1.03	0.42
0.50	0.80	0.31
0.75	0.77	0.22
1.00	0.76	0.14
1.50	0.57	0.12

$CaCl_2$ and $MnCl_2$ had identical effects on growth and enzyme production by *Flavobacterium breve* (Tables 4.32 & 4.34 and Figs. 3.32 & 4.34). The salts had apparently no effect on the growth of the organism while, alkaline phosphatase synthesis showed an initial escalation proportionate to the increase in salt concentration and was found to remain unaltered further on.

Fig. 4.31 Effect of $CaCl_2$ on growth and acid phosphatase production by *Streptococcus* sp.

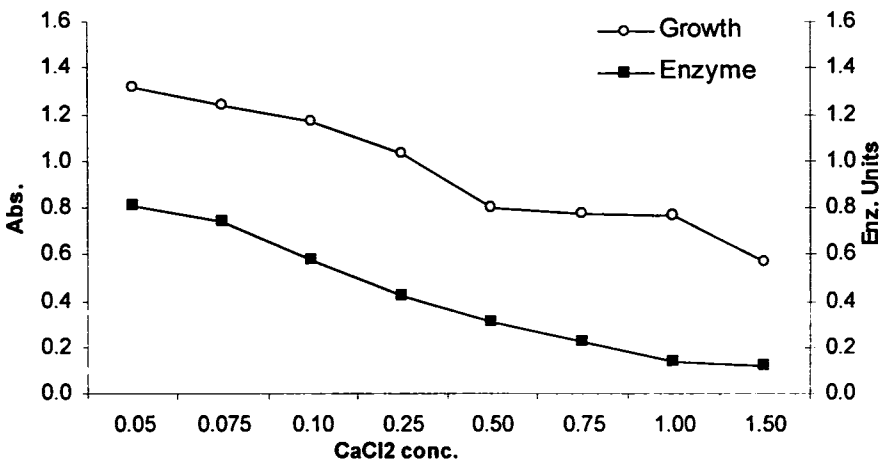
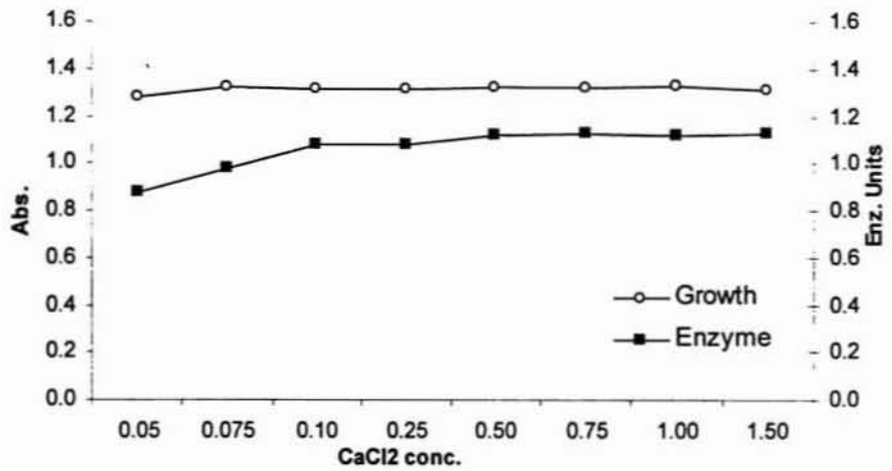


Table 4.32 Effect of $CaCl_2$ on growth and alkaline phosphatase production by *Flavobacterium breve*

$CaCl_2$ (M)	Growth	Enzyme
0.05	1.28	0.88
0.075	1.33	0.98
0.1	1.31	1.08
0.25	1.31	1.08
0.5	1.32	1.12
0.75	1.32	1.13
1	1.33	1.12
1.5	1.31	1.13

Fig. 4.32 Effect of $CaCl_2$ on growth and alkaline phosphatase production by *Flavobacterium breve*



Streptococcus sp. showed no deviations in growth and acid phosphatase production in the presence of $MnCl_2$ in the medium (Table 4.33 & Fig 4.33).

Table 4.33 Effect of $MnCl_2$ on growth and acid phosphatase production by *Streptococcus* sp.

$MnCl_2$ (M)	Growth	Enzyme
0.05	1.38	0.86
0.075	1.39	0.87
0.1	1.41	0.87
0.25	1.42	0.89
0.5	1.39	0.89
0.75	1.39	0.92
1.0	1.40	0.92
1.5	1.41	0.92

Fig. 4.33 Effect of $MnCl_2$ on growth and acid phosphatase production by *Streptococcus* sp.

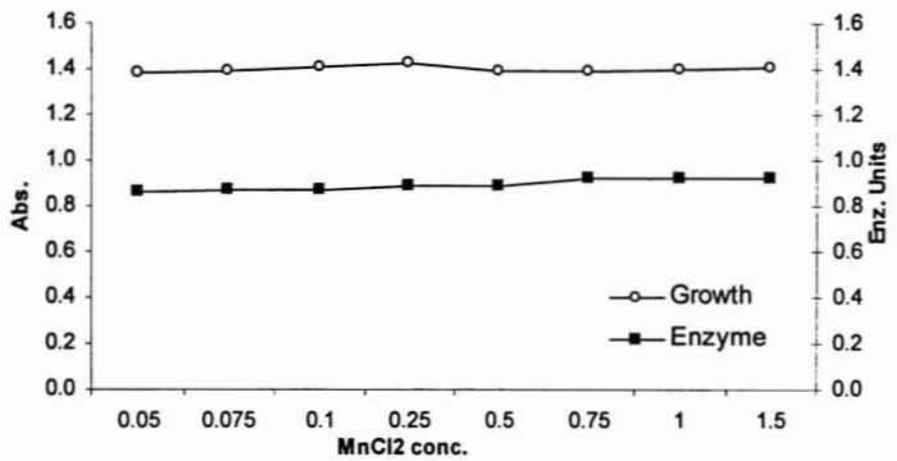
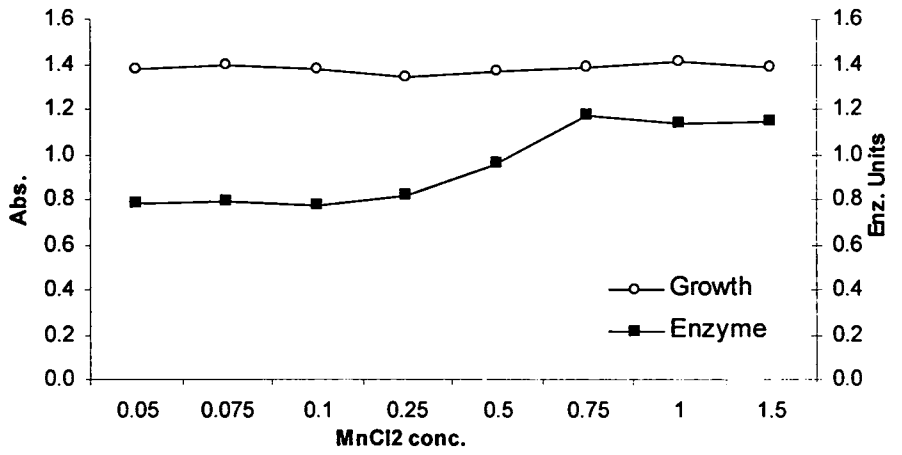


Table 4.34 Effect of $MnCl_2$ on growth and alkaline phosphatase production by *Flavobacterium breve*

$MnCl_2$ (M)	Growth	Enzyme
0.05	1.38	0.78
0.075	1.39	0.79
0.1	1.38	0.77
0.25	1.34	0.82
0.5	1.37	0.96
0.75	1.39	1.17
1	1.41	1.13
1.5	1.39	1.15

Fig. 4.34 Effect of $MnCl_2$ on growth and alkaline phosphatase production by *Flavobacterium breve*



The growth of *Streptococcus* sp. was significantly affected by NaF ($p< 0.05$), Na_2SO_4 ($p< 0.001$), $NaHCO_4$ ($p< 0.01$), Na_2HPO_4 ($p< 0.05$), and $CaCl_2$ (0.001). $NaNO_3$, KCl, $MgCl_2$ and $MnCl_2$ were found to have no significant effect on the growth while, the ions of all the salts except $NaNO_3$ and $MgCl_2$ were found to have significant effect on acid phosphatase production by the organism. The effect of Na_2SO_4, $NaHCO_4$, Na_2HPO_4, $CaCl_2$ and $MnCl_2$ were significant at 1% levels, NaF at 5% and KCl at 0.01% level.

$NaNO_3$, $MgCl_2$, $CaCl_2$ and $MnCl_2$ had no significant effect on the growth of *Flavobacterium breve.* KCl ($p< 0.001$), Na_2SO_4 ($p< 0.001$), Na_2HPO_4 ($p< 0.001$) and $NaHCO_3$ ($p< 0.05$) were found to significantly influenze the growth of the organism. Alkaline phosphatase production was found to be affected significantly at 0.1%

levels by NaF, Na_2CO_3, and KCl, at 1% level by Na_2HPO_4 and $MnCl_2$ and at 5% levels by $NaHCO_3$ and $MgCl_2$.

4.3 DISCUSSION

The nutritional requirements of different organisms vary widely. Bacteria grow well in environments where nutrients required for their growth and reproduction are sufficiently available. However, in aquatic ecosystems, most species do not always encounter the optimal conditions in which they develop best. A variety of physical and chemical factors affect the growth of microorganisms. They may face nutrient depletion or competition from other inhabitants and at times, will be hampered by the presence of inhibitors in the environment. In natural ecosystems, most nutrients are not usually available for bacterial metabolism in their simple inorganic form, but are present as complex organic polymeric substrates which cannot be utilized directly by the organism. This causes nutrient starvation, which in turn induces the synthesis of enzymes, such as phosphatases, by the bacteria, for the transformation involving step-wise depolymerization and hydrolysis of the organic substrates (Wynne, 1977).

Although there are numerous factors of different kinds which act on bacteria, some of these are particularly important. They influenze not only the size and composition of microbial populations, but also the morphology and physiology of individual bacteria.

Under laboratory conditions, bacteria in pure culture may exhibit an entirely different optimum than one might have expected from their natural environment. The incubation temperature, pH values, salt concentration and other chemicals influenze the metabolism and reproduction of bacteria. The synthesis of enzymes may be either promoted or inhibited by deviations in levels of these factors above or below the optimum.

4.3.1 Effect of period of incubation on growth and phosphatase production

The incubation period required for maximal growth and enzyme production depends on the bacterial species and the type of culture. Optimum incubation periods for production of the enzyme α-amylase by *Bacillus licheniformis* in still and shaker culture were 32 hours and 20 to 25 hours respectively (Ghosh and Chandra, 1984). In the present study, *Streptococcus* sp. and *Flavobacterium breve* were found to grow well and produce extracellular enzymes under laboratory conditions. The *Streptococcus* sp. attained optimal growth and enzyme production after 32 hours of incubation in still culture . The

growth recorded was 1.25 Abs. and the enzyme synthesis was initiated after 8 hours of incubation. In 32 hours, the growth recorded was 1.25 Abs. and the bacteria yielded 1.35 Enz. Units of acid phosphatase. Higher yields of growth and acid phosphatase production were obtained on shaking. A growth of 2.43 Abs. and an enzyme production of 3.06 Enz. Units were recorded in 32 and 28 hours respectively. The enzyme synthesis was initiated in 8 hours of incubation. Similar results were obtained from the *Flavobacterium breve*. The maximum still culture yields were 2.2 Abs. and 1.5 Enz. Units, while the shaker culture realized a maximum growth of 3.32 Abs. and 2.37 Enz. Units of alkaline phosphatase.

The results obtained indicate, in general, an increase in growth and enzyme production by both the bacteria in shaker culture.. However, the incubation time required to attain maximum yields were not significantly altered. It can be inferred from these findings that shaking augments the total yield and not essentially decrease the generation time. The early initiation of enzyme synthesis and increase in phosphatase yield could be attributed to the increase in bacterial metabolism as a result of shaking.

4.3.2 Growth phase of phosphatase production

In many bacteria, enzyme secretion takes place at the logarithmic phase of growth. This was found true in the production of various enzymes by *Bacillus stearothermophilus* (Davis *et al.*, 1980), *Bacillus licheniformis* (Rothstin *et al.*, 1982; Priest and Thirunavukkarasu, 1985), *Clostridium isolate* (Madi *et al.*, 1987), *Pseudomonas saccharophila* (Kadam and Fogarthy, 1984), *Vibrio gazogenes* and *V. parahaemolyticus* and *Vibrio* sp. (Saramma, 1992). *Streptomyces aureofaciens* showed maximal production of cell-bound amylase in the logarithmic phase and extracellular amylase was produced maximally in the stationary phase of growth (Naohira *et al.*, 1985).

In the present investigation also the phosphatase synthesis was initiated in the early exponential or logarithmic phase of growth in the *Streptococcus* sp. and *Flavobacterium breve* and continued till the cultures entered the stationary phase. There was no further increase in enzyme activity after that. It may be inferred that phosphatase secretion by *Streptococcus* sp. and *Flavobacterium breve* is growth associated. The phosphatase produced by these bacteria showed considerable stability also as they retained their maximum activity throughout the stationary phase.

4.3.3 The effect of pH on growth and phosphatase production

pH is a very important factor affecting growth and metabolism of bacteria. Most bacteria can grow and produce enzymes only within the pH range from 4 to 9 (Thimann, 1964). The optimum for most aquatic bacteria is between pH 7 and 8.5 (Weiss, 1973). This corresponds to the pH range of most of the water bodies. Both the bacteria under study were found to grow appreciably and produce phosphatases in this pH range. *Streptococcus* sp. was found to grow best and synthesize phosphatase at pH 7 while, pH 8 was favoured by *Flavobacterium breve* for optimum yields. Acidic pH was found to be more harmful than alkaline pH for growth and enzyme production by both the bacteria.

4.3.4 Effect of NaCl on growth and phosphatase production

The salinity-nutrient changes in the Cochin estuary is evidently influenced by the influx of freshwater and intrusion of seawater (Anirudhan *et al.*, 1987). During monsoon, the entire estuary attains near-freshwater conditions, except at bottom near the barmouth. In October, with the withdrawal of monsoon, a gradual increase in salinity to the range from 10 to 20 ppt occurs (Lakshmanan *et al.*, 1982; Anirudhan *et al.*, 1987). This may account for the moderately halophilic nature of the *Streptococcus* sp. and

Flavobacterium breve, being isolated from the water samples collected at this time of the year. Rheinheimer, (1968) has pointed out that the salt tolerant forms are observed at regions with freshwater influx and also near the coast. In the present study the organism showed good growth and enzyme production at 1% to 2% NaCl concentration. There was considerable reduction in enzyme production when the NaCl concentration exceeded 2%. Therefore, the two organisms used in the present study may not be obligate halophiles.

4.3.5 Effect of temperature on growth and phosphatase production

All bacteria exhibit a characteristic minimum, optimum, and maximum growth temperature. In the present study higher temperatures were apparently more harmful than lower temperatures to both the organisms. The optimum temperature for growth and phosphatase production for the *Streptococcus* sp. and *Flavobacterium breve* were found to be in the range from 20^0C to 30^0C emphasizing the mesophilic nature of the bacteria. This is also in concordance with the surface water temperatures encountered in Cochin estuary during September-October (Lakshmanan *et al.*, 1982).

4.3.6 Effect of sources of carbon on growth and phosphatase production

The composition and concentration of media greatly affect the growth and production of enzymes by bacteria. One of the most

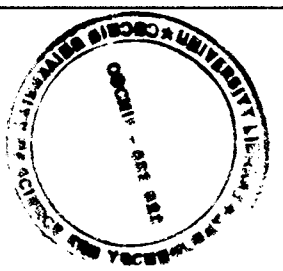
important requirements for microbial growth is a source of carbon. Carbon is the structural backbone of living matter, being the essential component of the organic compounds that make up a living cell.

Vibrio alginolyticus and *Vibrio* sp, isolated from the Cochin estuary, showed maximal growth in glucose media whereas, *V. parahaemolyticus*, isolated from the same region, expressed maximum growth in starch medium (Saramma, 1992). Maltose was found to be the best carbon source for amylase production by all the three strains.

In the present study, *Streptococcus* sp. had maximum growth in maltose while, mannitol was found to be the most suitable carbon source for *Flavobacterium breve*. Both the bacteria expressed optimal phosphatase yields in sucrose. Therefore it may be construed that the various carbon sources influenze the growth and metabolism of bacteria. This study also showed the ability of these aquatic bacteria to grow and produce enzymes using different carbon sources.

4.3.7 Effect of source of nitrogen on growth and phosphatase production

Considerable amount of nitrogen are required for protein synthesis, primarily to synthesize amino acids and also for the formation of nucleic acids. Therefore, the source of nitrogen also influenze growth and enzyme secretion in bacteria. In the present study, *Streptococcus* sp. was found to prefer peptone to the other eight



nitrogen sources used, while *Flavobacterium breve* grew best in peptone and yielded maximum alkaline phosphatase, when yeast extract was the nitrogen source used. Saramma (1992) reported yeast extract as the nitrogen source of choice for *Vibrio alginolyticus* and ammonium chloride for *V. parahaemolyticus* and *Vibrio* sp. isolated from the Cochin estuary. These results on comparison denote that the nitrogen sources required by various bacteria vary with the species. In general both these bacteria showed the ability to utilize different nitrogen sources for growth and enzyme production.

4.3.8 Effect of various salts on growth and phosphatase production

Growth and phosphatase production by bacteria are affected by the presence of various salts in the medium either positively or negatively.

Bacillus sp. RK11, an alkalophilic isolate from soil (Kelly 1975), produced extracellular alkaline phosphatase only in the presence of Mn^{2+} in a complex medium. No alkaline phosphatase production or sporulation by the organism was detected in the absence of Mn^{2+}. No other divalent metal could be substituted for Mn^{2+} in enzyme production or sporulation (Kelly *et al.*, 1984). Of the divalent metals, Mn^{2+} is a metal specifically required for the production of secondary metabolites of many *Bacillus* species and is essential for

sporulation in some bacilli (Charney *et al.*, 1952; Curran and Evans, 1954). In the present study, Mn^{2+} was found to augment alkaline phosphatase synthesis by *Flavobacterium breve*, while, the acid phosphatase secretion by *Streptococcus* sp. was unaltered by the ion. However, Mn^{2+} was not found to be vital for enzyme synthesis.

The release of extracellular alkaline phosphatase in *Micrococcus sodonensis* (Glew and Heath, 1971) is totally dependant on the presence of Mg^{2+} and is the result of a selective permeation process. In the present study Mg^{2+} was found to augment alkaline phosphatase synthesis considerably, but the enzyme production was not found to be totally dependant on any particular ion. Sodium fluoride and sodium sulphate at higher concentrations inhibited both acid and alkaline phosphatase production whereas, sodium bicarbonate inhibited growth and enzyme production even at lower concentrations. Sodium phosphate was found to enhance growth of these bacteria but, enzyme production was reduced. However, sodium nitrate neither enhances nor inhibits growth and enzyme production. Potassium chloride, at lower concentration enhanced growth but at high concentration it was inhibitory. Magnesium chloride enhanced growth and enzyme production by *Flavobacterium breve* whereas, there was no effect on *Streptococcus* sp. Calcium chloride was found to reduce acid phosphatase production by *Streptococcus*, but it did not affect

growth and alkaline phosphatase production by the *Flavobacterium breve*. It may therefore be concluded from the results obtained in this study that the ionic requirement is not very specific for all bacteria and the important function of these ions is to maintain the osmotic balance between the cells and their environment.

The results affirm that the cultural conditions required for growth and enzyme synthesis varies with the type and species of bacteria.


Chapter 5

REGULATION OF PHOSPHATASE SYNTHESIS

The synthesis of extracellular enzymes in most aquatic bacteria is regulated by the nutritional status of the environment. The production of virtually all extracellular enzymes is repressed when readily utilizable nutrient sources are available. This mode of regulation is called catabolite repression.

Synthesis of extracellular enzymes may be inducible, partially constitutive or totally constitutive depending on the microorganism and the enzyme involved. Since the substrates for extracellular enzymes are often too large to enter the cell, it is

generally assumed that the inducing molecule is a product of the enzyme activity rather than the substrate itself. It has been proposed that the enzyme is secreted at a low constitutive rate. If the substrate is present, then the low molecular weight product accumulates to a certain level, enters the cell, and serves as an inducer (Priest, 1984). When environmental conditions inhibit an enzyme activity, the induction of its synthesis will not occur because the product of catalysis will not be generated. It is also well documented that synthesis of many enzymes in aquatic microorganisms is repressed by the end product that is derived from the substrate and accumulates in the cell or in the surrounding environment. Only the synthesis of extracellular enzymes becomes derepressed once the concentration of the readily utilizable substrates in the surrounding environment falls below a critically low level.

In the present study, experiments were carried out to examine whether any induction or repression in phosphatase synthesis occurs in the bacteria selected.

5.1 METHODOLOGY

5.1.1 Effect of various organophosphorus compounds on phosphatase production.

Effect of *p*-nitrophenyl phosphate, sodium-β-glycerophosphate and triethyl phosphate on phosphatase synthesis was studied by inoculating the cultures in mineral medium containing 0.5 % each of these compounds individually as additional sources of P. The culture fluid was centrifuged and assayed for phosphatases after 24 hours.

5.1.2 Effect of orthophosphate enrichment on phosphatase production.

Various concentrations (0-5ppm) of orthophosphate were added to cultures in nutrient broth to study the effect of orthophosphate enrichment on growth and phosphatase production. Measurements of growth and enzyme activity were conducted after 24 hours.

5.1.3 Effect of chloramphenicol on phosphatase production.

The antibiotic chloramphenicol (5μg ml^{-1}) was added to cells actively secreting phosphatase to study the effect of chloramphenicol on phosphatase production. Enzymes were assayed at one hour intervals.

5.1.4 Effect of actinomycin D on phosphatase production.

Effect of antibiotic actinomycin D on phosphatase production was studied by adding 5µg ml^{-1} of actinomycin D to cells actively secreting phosphatase. Enzymes were assayed every hour.

5.2 RESULTS

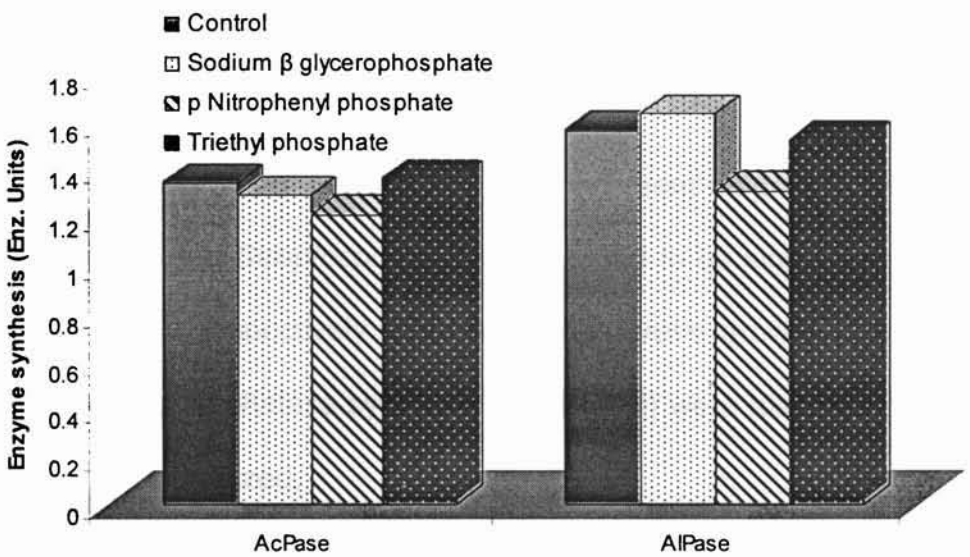
5.2.1 Effect of various organophosphorus compounds on phosphatase secretion

The addition of sodium-β-glycerophosphate, *p*-nitrophenyl phosphate and triethyl phosphate to the culture medium did not produce any pronounced effect on acid and alkaline phosphatase secretion by the two selected strains (Table 5.1 and Fig. 5.1). Statistical analyses reveal that there was no significant effect on enzyme synthesis on addition of organophosphorus compounds.

Table 5.1 Effect of various organophosphorus compounds on acid phosphatase and alkaline phosphatase secretion.

P source	AcP(Enz.Units)	AlP(Enz.Units)
Control	1.34	1.57
Sodium β glycerophosphate	1.29	1.64
p- Nitrophenyl phosphate	1.21	1.31
Triethyl phosphate	1.36	1.52

Fig. 5.1 Effect of various organophosphorus compounds on acid phosphatase and alkaline phosphatase secretion.



5.2.2 Effect of orthophosphate enrichment on phosphatase secretion

Orthophosphate enrichment inhibited acid and alkaline phosphatase production by the two selected strains (Tables 5.2 & 5.3 and Figs. 5.2 & 5.3). However orthophosphate enrichment was found to support growth.

Table 5.2 Effect of orthophosphate enrichment on acid phosphatase secretion.

Conc.(ppm)	Growth	Enzyme
0	1.15	1.34
1	1.22	1.10
2	1.34	0.61
3	1.48	0.27
4	1.64	0.09
5	1.42	0.02

The effect of orthophosphate enrichment on growth and acid production by the *Streptococcus* sp. was found to be significant at 5% and 1% levels respectively. *Flavobacterium breve* growth (1% level) and alkaline phosphatase synthesis (0.1% level) were also found to be significantly influenzed.

Fig. 5.2 Effect of orthophosphate enrichment on acid phosphatase secretion.

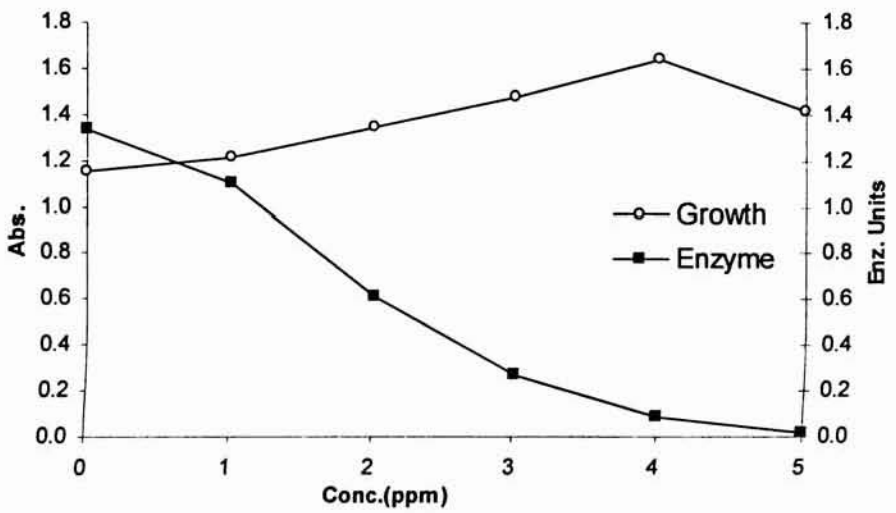
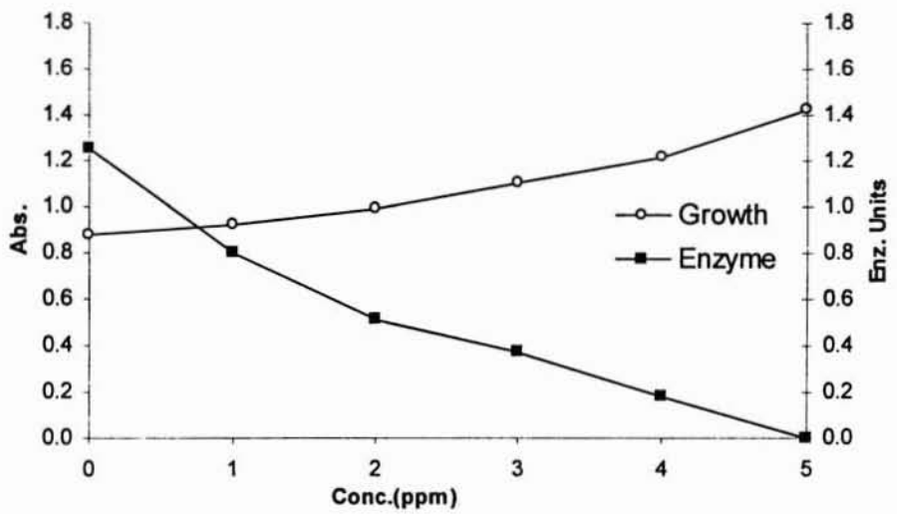


Table 5.3 Effect of orthophosphate enrichment on alkaline phosphatase secretion

Conc.(ppm)	Growth	Enzyme
0	0.87	1.25
1	0.93	0.80
2	0.99	0.51
3	1.10	0.37
4	1.22	0.18
5	1.43	0.00

Fig. 5.3 Effect of orthophosphate enrichment on alkaline phosphatase secretion



Effect of chloramphenicol on phosphatase secretion

The addition of chloramphenicol to cells actively secreting phosphatases brought about an immediate inhibition of both acid and alkaline phosphatase syntheses (Tables 5.4 & 5.5 and Figs. 5.4 & 5.5).

Table 5.4 Effect of chloramphenicol on acid phosphatase secretion

Period of incubation (Hours)	Enz. Units	
	Control	With Chloramphenicol
0	0	0.00
1	0	0.00
2	0	0.00
3	0.03	0.03
4	0.11	0.11
5	0.33	0.33
6	0.64	0.34
7	0.78	0.38
8	0.92	0.38
9	1.12	0.38
10	1.25	0.38
11	1.36	0.39
12	1.36	0.39

Fig. 5.4 Effect of chloramphenicol on acid phosphatase secretion

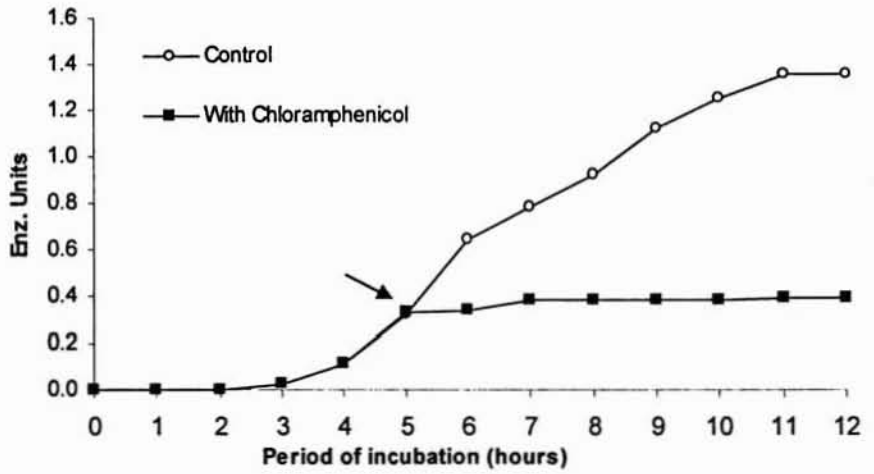
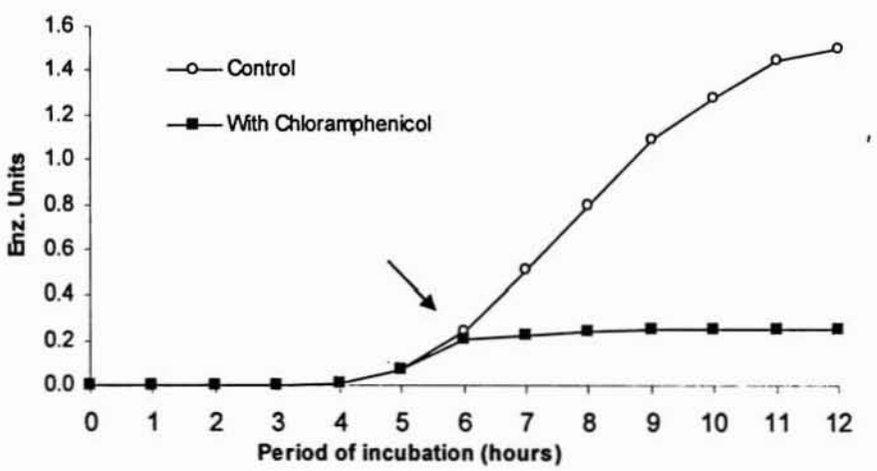


Table 5.5 Effect of chloramphenicol on alkaline phosphatase secretion

Period of incubation(Hours)	Enz. Units	
	Control	With Chloramphenicol
0	0	0
1	0	0
2	0	0
3	0	0
4	0.01	0.01
5	0.07	0.07
6	0.24	0.21
7	0.51	0.22
8	0.80	0.24
9	1.09	0.25
10	1.28	0.25
11	1.45	0.25
12	1.50	0.25

Fig.5.5 Effect of chloramphenicol on alkaline phosphatase secretion



5.2.3 Effect of Actinomycin D on acid phosphatase secretion

On addition of actinomycin D, cells continued phosphatase secretion for a short period and then the secretion was stalled (Tables 5.6 &5.7 and figs. 5.6 & 5.7).

Table 5.6 Effect of Actinomycin D on acid phosphatase secretion

Period of incubation (Hours)	Enz. Units	
	Control	With Actinomycin D
0	0	0
1	0	0
2	0	0
3	0.03	0
4	0.11	0.11
5	0.33	0.32
6	0.64	0.45
7	0.78	0.53
8	0.92	0.53
9	1.12	0.55
10	1.25	0.55
11	1.36	0.55
12	1.36	0.55

Fig. 5.6 Effect of Actinomycin D on acid phosphatase secretion

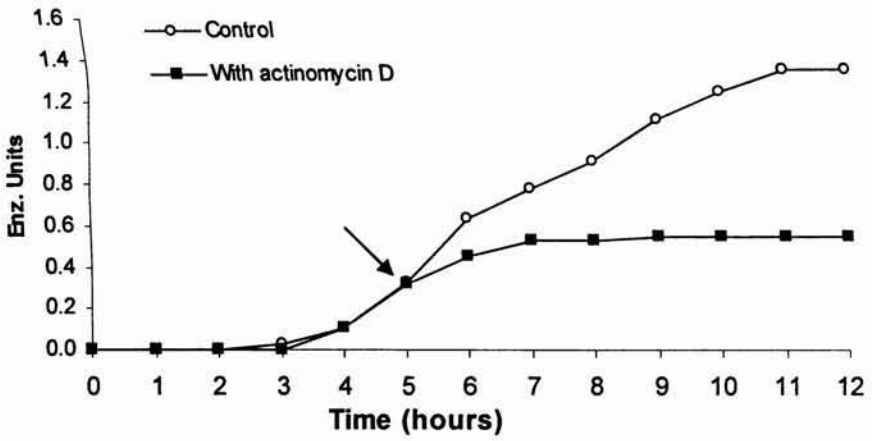
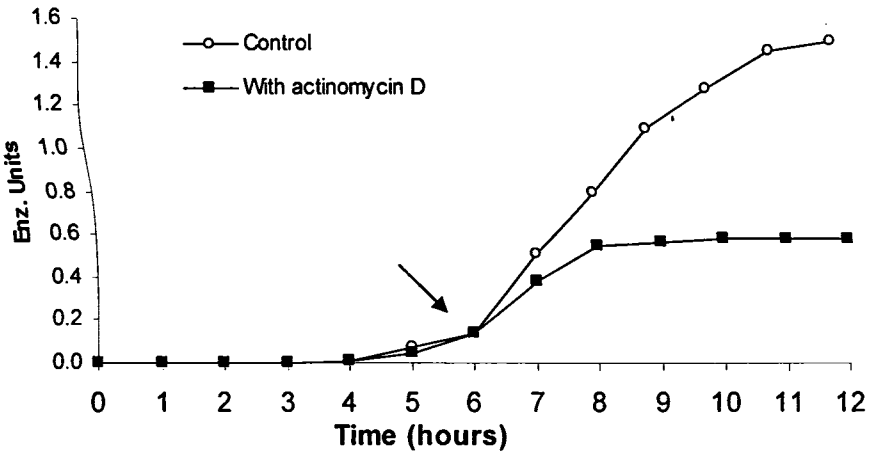


Table 5.7 Effect of Actinomycin D on alkaline phosphatase secretion

Period of incubation (Hours)	Enz. Units	
	Control	With Actinomycin D
0	0	0
1	0	0
2	0	0
3	0	0
4	0.01	0.01
5	0.07	0.05
6	0.14	0.14
7	0.51	0.38
8	0.80	0.55
9	1.09	0.56
10	1.28	0.58
11	1.45	0.58
12	1.5	0.58

Fig. 5.7 Effect of Actinomycin D on alkaline phosphatase secretion



5.3 DISCUSSION

5.3.1 Effect of various organophosphorus compounds on phosphatase secretion

Phosphatases are capable of hydrolyzing a wide range of organophosphorus compounds (Li *et al.*, 1998). *Rhizobium leguminosarum* was able to utilize glucose-6-phosphate, ATP and Na-β-glycerophosphate (Abd-Alla, 1994b). Sharma *et al.* (1995) observed that the presence of Na-β-glycerophosphate enhanced alkaline phosphatase secretion in *Citrobacter koseri* and *Micrococcus varians* I. Hernandez *et al.* (1996) studied the effect of glucose-6-phosphate on various bacteria and algae and opined that bacteria were more efficient than the algae in utilizing the P source supplied. However, the addition of glucose-6-phosphate did not enhance the enzyme synthesis. In the

common Cyanobacterium *Synechococcus,* both P_i-limitation and organic-P sources induced phosphatase production in parallel with enhanced phosphate uptake capacity (Ray *et al.,* 1991; Aiba *et al.,* 1993; Scanlan *et al.,* 1993; Donald *et al.,* 1997).

Enzyme stimulation and repression by substrates and end-products are usually assumed to be the main regulators of enzyme synthesis (Chróst, 1990). In that case, the presence and alteration of different types of substrates may influenze the production of phosphatases (Nausch, 2000; Nausch and Nausch, 2000). Results obtained by Berman (1988) showed that the addition of organophosphorus sources appeared to reduce the dependency of microorganisms on P_i as a P source. He observed that bacteria were capable of exploiting organophosphorus sources even in the presence of P_i. Berman (1988) concluded that in many aquatic systems organophosphorus sources (DOP compounds) could have the potential to supply a substantial part of P nutrition through phosphatase activities. Whitton *et al.* (1990a, b) studied the effect of a range of phosphorus sources including, *p*-NPP, Na-β-glycerophosphate, Glucose-1-phosphate and phytic acid on phosphatase production in *Nostoc commune* UTEX 584. With the exception of phytic acid, all P sources sustained growth. Phosphatase

production was detected only after the cellular phosphate content had decreased to 0.75% dry weight.

In the present study there was no evident enhancement of phosphatase production when organophosphorus sources were supplemented in the growth medium. These results, along with the findings of other researchers, suggest that though the biosynthesis of the enzyme is switched on when P_i supply is low, the incidence of organophosphorus substrates not necessarily enhances the phosphatase synthesis. It may be concluded that it is the cellular phosphate and not the presence of degradable substrates that monitors the enzyme synthesis.

5.3.2 Effect of orthophosphate enrichment on phosphatase production

Three sources of orthophosphate available to aquatic microorganisms are i.) P_i initially present in water, ii.) P_i in intra cellular pools and iii.) enzymatically released P_i. During periods of P_i depletion bacterial and phytoplankton communities produce phosphatases to cater their P_i needs by cleaving the inorganic P from the organic part of the dissolved organic P (DOP) (Vrba *et al.*, 1993, Jamet *et al.*, 1995). When sufficient P_i concentration is present in the environment, synthesis of the enzyme is shut down and derepression occurs when

further depletion of the P_i occurs (Patni *et al.*, 1977; Hassan and Pratt, 1977).

The presence of repressible phosphatase has been reported by several workers. Phosphatases repressed by P_i were reported by Torriani (1960) from *E. coli*. Sharma *et al.* (1995) observed that the presence of inorganic phosphates such a Na_2HPO_4, NaH_2PO_4 and di ammonium hydrogen phosphate inhibited alkaline phosphatase secretion in *Citrobacter koseri* and *Micrococcus varians* I. Barik and Purushothaman (1998) found that the phosphatases produced by *Pseudomonas* sp. and *Bacillus* sp. were repressible. Current results obtained are in accordance with these findings. Both acid and alkaline phosphatases were repressed by orthophosphate enrichment.

The present results are corroborated by the results obtained by other workers like Siuda and Chróst (1987) who opined that syntheses of phosphatases may be repressed by PO_4^{3-} in bacteria and microalgae and Chróst and Overbeck (1987) who reported that the specific activity of alkaline phosphatase decreased when the ambient P_i concentrations were higher than 15 µg l^{-1}. It is not quite correct to conclude that phosphatase synthesis is derepressed or activated directly by low P_i concentrations. The mechanism of phosphatase derepression is regulated by the intracellular phosphate pool in microbial cells. P_i was a strong competitive inhibitor of phosphatase in

microalgae (Chróst and Overbeck, 1987), but inhibition of phosphatase synthesis in bacteria was only slight (Chróst *et al.*, 1986). Ozkanca and Flint (1996) reported the survival of *E. coli* in lake water microcosms supplemented with high PO_4 in which the synthesis of phosphatase would be repressed and in low PO_4 medium where synthesis of the enzyme would be derepressed.

An inverse relationship between phosphatase production and external phosphate, especially when the external phosphate has a long-term effect, has been described by Wynne (1981) and Veldhius *et al.* (1987). Studies have demonstrated that phosphatase synthesis is controlled by several compartments involved in phosphorus metabolism, such as internal phosphorus (Gage and Gorham, 1985).

5.3.3 Effect of chloramphenicol and actinomycin D

Phosphatase synthesis is influenzed by various factors. It has been shown that for several bacteria, the rate of phosphatase production is subject to regulation either by a specific inducer or by specific repressor (Torriani, 1960). Chloramphenicol inhibits peptidyl transferase, a ribozyme in 50S ribosomal subunit of prokaryotes and blicks translation. At lower concentrations it does not affect RNA synthesis. Chloramphenicol was added to culture medium when the cells were actively engaged in the synthesis of phosphatases to find

out whether the synthesis is a *de novo* process. It was found that both acid and alkaline phosphatase synthesis were rapidly and completely inhibited. This confirms that the secretion of acid and alkaline phosphatases was the result of *de novo* synthesis.

The antibiotic, actinomycin D, binds to guanine in DNA, distorting the DNA and thus blocking transcription. Actinomycin D was added to the growth medium when the cells had started phosphatase secretion. The enzyme secretion could be sustained only for a short period. The exact cause of this transient continuation of phosphatase synthesis is unknown, but, it is in accordance with the findings of Coleman and Elliott (1965) who observed a stimulation of the synthesis of extracellular ribonuclease by a *Bacillus* sp. after addition of actinomycin D to cells actively secreting the enzyme.

Two hypotheses have been proposed which are consistent with the present data. Both *et al.* (1972) postulated that the mRNA pool for extracellular enzymes in *Bacillus amyloliquefaciens* resulted from a positive imbalance of transcription over translation. An alternate hypothesis was proposed by O'Connor *et al.* (1978) who suggested that there were two forms of mRNA for the extracellular protease produced by *Bacillus amyloliquefaciens*, a short-lived form, immediately available for translation and a relatively stable, non-translatable form. They suggested that in the presence of inhibitors of

transcription, the stable form was converted into the translatable species which would account for the continued production of the enzyme in the absence of transcription. The existence of mRNA pools specific for extracellular protease was first reported by Both (1972). Observations similar to those obtained with acid and alkaline phosphatases in the present study were also made with protease secretion by *Vibrio alginolyticus* (Reid *et al.,* 1980), amylase and protease secretion by *Vibrio gazogenes* (Ratcliffe *et al.,* 1982) amylase production by *Alteromonas rubra* (Gavrilovic *et al.*, 1982) and amylase production by three *Vibrio* sp. (Saramma, 1992).


Chapter 6

CHARACTERISTICS OF PHOSPHATASES

The importance assigned to the non-specific organophosphorus hydrolyzing enzymes, acid and alkaline phosphatases, is due to its broad spectrum of applications. Their relevance ranges from the use of acid phosphatase as a diagnostic tool to detect liver disorders in human beings, considered to be at the zenith of evolution, to its role as an indicator of phosphate starvation in the forms of life which survived the primary turmoil to live through the various phases of evolution of life on earth - the prokaryotes.

Phosphatases are recognized for their faculty to hydrolyze a wide range of phosphomonoesters. These enzymes are known to play a major role in nutrient recycling in aquatic ecosystems, particularly when phosphates available for living organisms are in growth limiting supply. Phosphatases interest the molecular biologists due to their ability to catalyze the removal of 5' residues from DNA, RNA and ribonucleoside triphosphates and deoxyribonucleoside triphosphates. Phosphatases hold relevance in various fields including agriculture, pollution, diagnostic and therapeutic applications. Generally, phosphatases are being extracted from calf intestine and *E. coli* for commercial purposes. Solovyov (1998) claims the alkaline phosphatase isolated from marine microorganisms, *Alteromonas macleodii* and *Deleya marina*, to be more promising than the conventional alkaline phosphatases due to their comparatively advantageous characteristics, especially with respect to their higher specific activities.

The characteristics of an enzyme determine its effectiveness. To identify the applications of an enzyme it is essential to be aware of these properties. With this objective, an insight into the properties of the acid and alkaline phosphatases from *Streptococcus* sp. and *Flavobacterium breve* is envisaged.

6.1 METHODOLOGY

6.1.1 Partial purification of the enzymes

Partial purification of acid and alkaline phosphatases was done by ammonium sulphate precipitation followed by dialysis. To the chilled cell free culture fluid, solid ammonium sulphate was added with gentle stirring to 30% saturation and incubated at 4^0C for two hours. It was then centrifuged and the precipitate was discarded. More ammonium sulphate was added to 70% saturation. The precipitate was collected by centrifugation after two hours incubation at 4^0C and dissolved in 0.2M phosphate buffer (pH 7). This solution was dialyzed overnight against the same buffer at 4^0C. This dialyzed enzyme was used for further studies.

6.1.2 Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

6.1.3 Effect of pH on enzyme activity and stability

The effect of pH on phosphatase activity was tested using different buffers ranging from pH 3 to pH 10.6 in the reaction mixture. The enzyme activity was measured after incubation for 30 minutes at

37^{0}C. Stability of the enzymes at various pH values was tested by incubating the dialyzed enzyme solutions in different buffers in the pH range from 3 to 10 for two hours. After incubation, the residual enzyme activity was assayed at pH 4.8 and 9.5 for acid phosphatase and alkaline phosphatase respectively.

6.1.4 Effect of temperature on enzyme activity and stability

Effect of temperature on enzyme activity was determined by incubating the reaction mixture at 5^{0}C intervals of temperatures from 5 to 60^{0}C for 30 minutes and the enzyme activity was measured. In addition, acid and alkaline phosphatase activities were also measured at 37^{0}C, the temperature prescribed in the standard method for enzyme assays. To study the effect of temperature on enzyme stability the dialyzed enzyme was incubated at different temperatures (5^{0}C to 60^{0}C) for two hours and the residual activity was assayed at 37^{0}C.

6.1.5 Effect of substrate concentration on the activity of the enzymes

Effect of substrate concentration on phosphatase activity was tested by varying the concentration of *p*-nitrophenyl phosphate in the reaction mixture from 0.5 mg to 2.5 mg ml^{-1}. After incubation at 37^{0}C, the enzyme activity was determined by measuring the *p*-

nitrophenol formed. Lineweaver- Burk plot was constructed by plotting the inverse of initial velocity against the inverse of substrate concentration. From the plot, the K_m was calculated.

6.1.6 Effect of various ions on phosphatase activity

Effect of various ions on enzyme activity was determined by adding various concentrations of different salts to the reaction mixture. pH was adjusted to 4.8 for acid phosphatase and 9.5 for alkaline phosphatase and enzyme activity was assayed after 30 minutes incubation.

6.2 RESULTS

6.2.1 Partial purification of phosphatases

Acid and alkaline phosphatases from the culture fluids were partially purified by ammonium sulphate precipitation followed by dialysis. Results are presented in Table 6.1.

Acid phosphatase from the *Streptococcus* sp. could be purified 3.64 fold with 62.09% yield and specific activity 73.57 units mg protein^{-1}. Alkaline phosphatase from *Flavobacterium breve* was purified 3.73 fold with 63.74% yield and specific activity 89.23 units mg protein^{-1}.

Purification step	Volume (ml)	Total Activity (Enz Units)	Total Protein (mg)	Specific Activity (Enz Units/mg protein)	Yield (%)	Purification (fold)
Acid Phosphatase						
Culture extract	100	166	8.2	20.23	100	1
$(NH_4)_2SO_4$ precipitation and dialysis	5	103	1.4	73.57	62.09	3.64
Alkaline Phosphatase						
Culture extract	100	182	7.6	23.95	100	1
$(NH_4)_2SO_4$ precipitation and dialysis	5	116	1.3	89.23	63.74	3.73

Table 6.1. Partial purification of acid and alkaline phosphatases

6.2.2 Effect of pH on phosphatase activity and stability

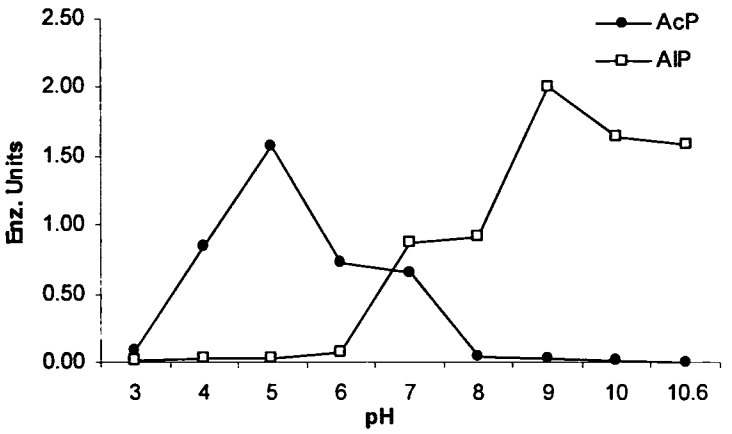
Effect of pH on enzyme activity was measured by assaying the activity in different buffers (pH 3 to 10). Results are given in Table 6.2 and Fig. 6.1. Optimum acid phosphatase activity was at pH 4.8. Considerable activity was observed in the pH range from 4 to 7. pH 3 and alkaline pH were found to inhibit acid phosphatase activity. Alkaline phosphatase activity peaked at pH 9. In general, the results obtained indicate that pH 7 and above sustained alkaline phosphatase activity. Acidic pH was found to be detrimental to alkaline phosphatase activity.

Table 6.2 Effect of pH on phosphatase activity

pH	AcP	AlP
3	0.08	0.01
4	0.32	0.02
5	1.06	0.03
6	0.73	0.07
7	0.65	0.87
8	0.05	0.91
9	0.02	2.01
10	0.01	1.64
10.6	0.00	1.59

Acid and alkaline phosphatase activities were found to be significantly influenced by the variations in pH ($p < 0.05$ and $p < 0.001$ respectively).

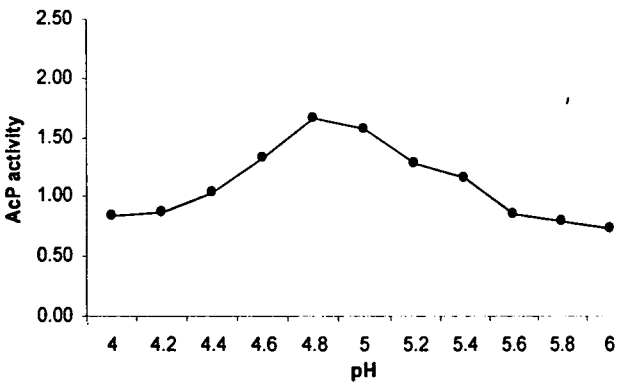
Fig. 6.1 Effect of pH on phosphatase activity



The results obtained for acid phosphatase activity in the optimal pH range (pH 4 – 6) assayed at 0.2 unit intervals are given in Table 6.3 and Fig. 6.2. Peak enzyme activity was obtained at pH 4.8.

Table 6.3 : Fig. 6.2 The effect of pH on acid phosphatase activity

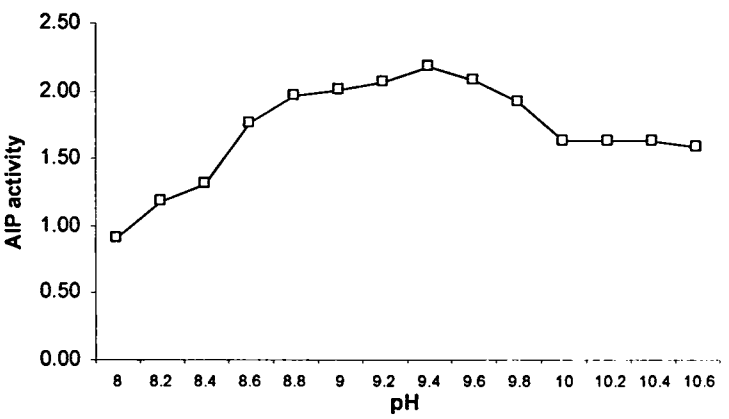
pH	AcP
4	0.87
4.2	0.87
4.4	1.04
4.6	1.32
4.8	1.66
5	1.57
5.2	1.28
5.4	1.16
5.6	0.86
5.8	0.79
6	0.73



Maximum alkaline phosphatase activity within the optimum pH range of the enzyme was obtained at pH 9.4. Results are presented in Table 6.4 and Fig. 6.3.

Table 6.4 : Fig. 6.3 The effect of pH on acid phosphatase activity

pH	AIP
8	0.91
8.2	1.18
8.4	1.32
8.6	1.76
8.8	1.96
9	2.01
9.2	2.06
9.4	2.18
9.6	2.08
9.8	1.92
10	1.64
10.2	1.64
10.4	1.63
10.6	1.59



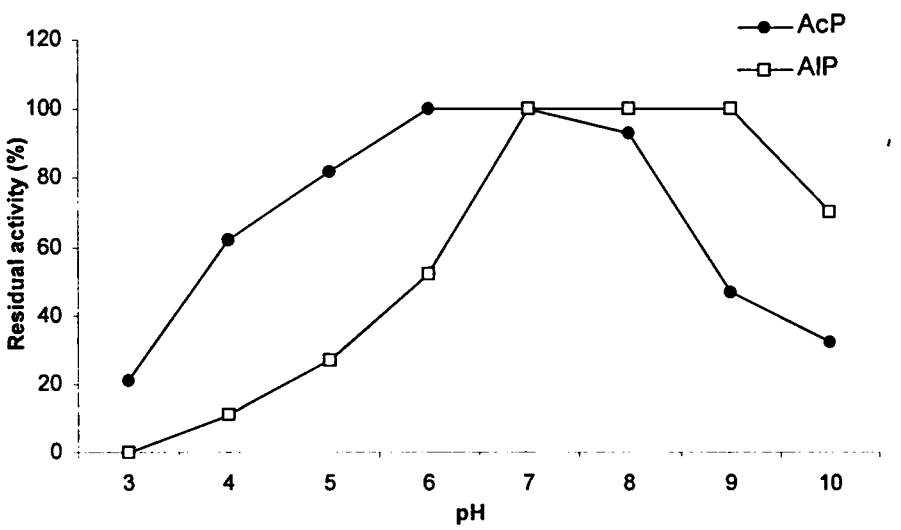
The enzymes were incubated for two hours in buffers having different pH (pH 3 to10.6) to examine the effect of pH on stability of phosphatases. Enzyme activities were then assayed at pH 4.8 and 9.5 for acid phosphatase and alkaline phosphatase respectively. Results are presented in Table 6.5 and Fig. 6.4.

Table 6.5 Effect of pH on phosphatase stability

pH	Residual activity (%)	
	ACP	AIP
3	21	0
4	62	11
5	82	27
6	100	52
7	100	100
8	93	100
9	47	100
10	32	70

Acid phosphatase from the *Streptococcus* sp. was stable at a pH range from 5 to 8. The enzyme showed maximum stability at pH 6 and 7 and retained 93% activity at pH 8 and 82% activity at pH 5.

Fig. 6.4 Effect of pH on phosphatase stability



Alkaline phosphatase was very stable at pH 7 to 9. It retained 70% activity at pH 10 and only 52% activity at pH 6. The enzyme was completely inactivated at pH 3 while 11% activity was retained at pH 4 and 27% at pH 5.

6.2.3 Effect of temperature on phosphatase activity and stability

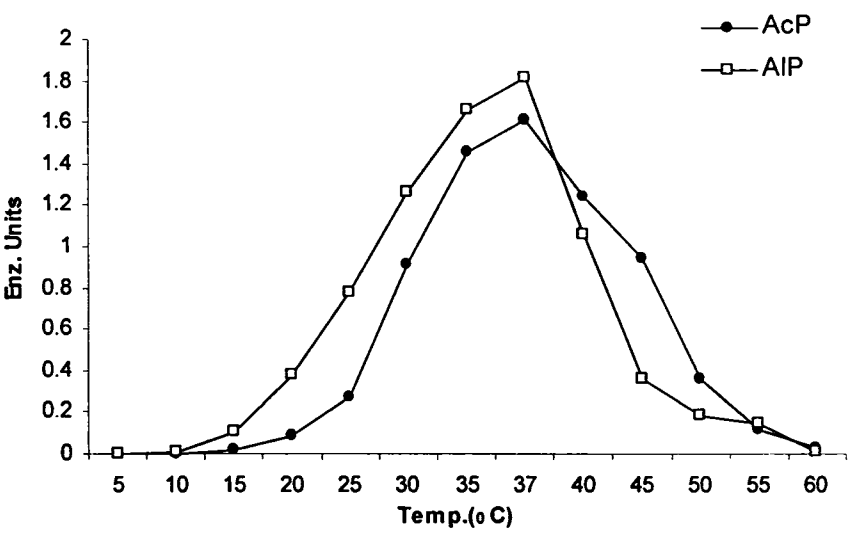
Acid phosphatase favoured a temperature in the range from 30^{0}C to 45^{0}C for optimal activity, while maximum alkaline phosphatase activity was in the range 25^{0}C to 40^{0}C (Table 6.6 and Fig. 6.5).

Table 6.6 Effect of temperature on phosphatase activity

Temp (^{0}C)	AcP	AlP
5	0	0
10	0	0.01
15	0.02	0.11
20	0.09	0.38
25	0.27	0.78
30	0.91	1.26
35	1.46	1.66
37	1.61	1.82
40	1.24	1.06
45	0.94	0.36
50	0.36	0.18
55	0.12	0.15
60	0.03	0.01

Both the enzymes attained their peak activities when incubated at 37^{0}C. Considerable reduction in enzyme activity was observed at lower (<20^{0}C) and higher (>50^{0}C) temperatures. The values obtained were significant at 0.1% level for acid phosphatase and 1% level for alkaline phosphatase.

Fig. 6.5 Effect of temperature on phosphatase activity



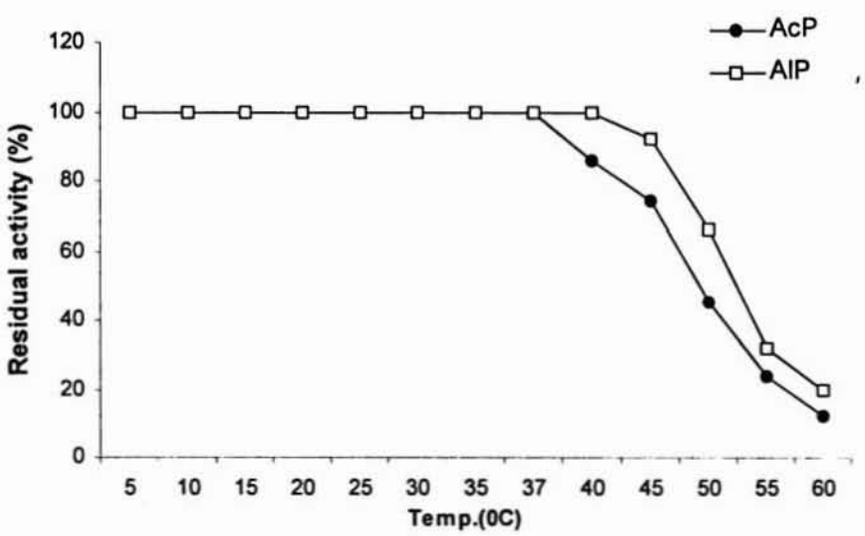
The effect of temperature on enzyme stability was monitored by incubating the dialyzed enzyme at different temperatures (5^{0}C - 60^{0}C) for two hours and assaying the residual activity at 37^{0}C. Acid and alkaline phosphatases showed 100% stability up to 40^{0}C and 45^{0}C respectively (Table 6.7 and Fig 6.6). There was a gradual decrease in activity as the temperature was raised. Acid phosphatase showed 86% stability at 40^{0}C, 45% at 50^{0}C % 12% at 60^{0}C. The

residual activity of alkaline phosphatase was 66% at 50^{0}C & 20% at 60^{0}C.

Table 6.7 Effect of temperature on phosphatase stability

Temp (^{0}C)	Residual activity (%)	
	AcP	AlP
5	100	100
10	100	100
15	100	100
20	100	100
25	100	100
30	100	100
35	100	100
37	100	100
40	86	100
45	74	92
50	45	66
55	24	32
60	12	20

Fig. 6.6 Effect of temperature on phosphatase stability



6.2.4 Effect of substrate concentration on phosphatase activity

A substrate concentration of 2 mg ml^{-1} yielded optimum acid and alkaline phosphatase activity (Table 6.8 and 6.9).

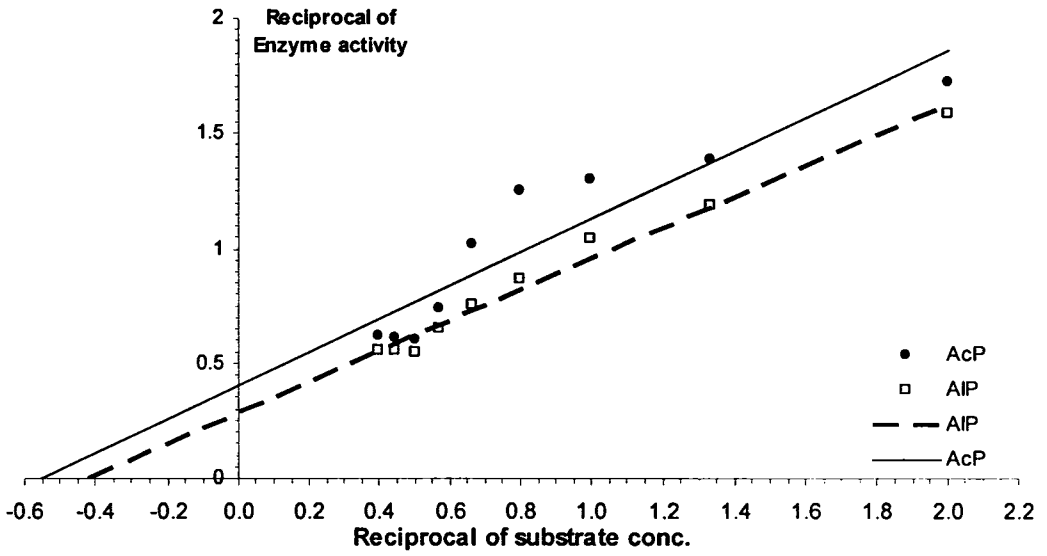
Table 6.8 Effect of substrate concentration on phosphatase activity

p NPP (mg ml^{-1})	AcP	AlP
0	0	0
0.50	0.33	0.36
0.75	0.58	0.63
1.00	0.77	0.96
1.25	0.8	1.15
1.50	0.98	1.32
1.75	1.36	1.53
2.00	1.66	1.82
2.25	1.64	1.8
2.50	1.62	1.81

Table 6.9 Effect of substrate concentration on phosphatase activity

		AcP		AlP	
S	1/S	V	1/V	V	1/V
0.5	2.00	0.58	1.72	0.63	1.59
0.75	1.33	0.72	1.39	0.84	1.19
1	1.00	0.77	1.30	0.96	1.04
1.25	0.80	0.8	1.25	1.15	0.87
1.5	0.67	0.98	1.02	1.32	0.76
1.75	0.57	1.36	0.74	1.53	0.65
2	0.50	1.66	0.60	1.82	0.55
2.25	0.44	1.64	0.61	1.8	0.56
2.5	0.40	1.62	0.62	1.81	0.55

Fig. 6.7 Lineweaver-Burk Plot.



The K_m values for both the enzymes were calculated from the Lineweaver-Burk plot (Fig. 6.7). The values were 4.9 x 10^{-3} Ml^{-1} for acid phosphatase and 6.3 x 10^{-3} M l^{-1} for alkaline phosphatase.

6.2.5 Effect of various ions on phosphatase activity

6.2.5.1 Effect of sodium (Na+) and chloride (Cl^-) ions

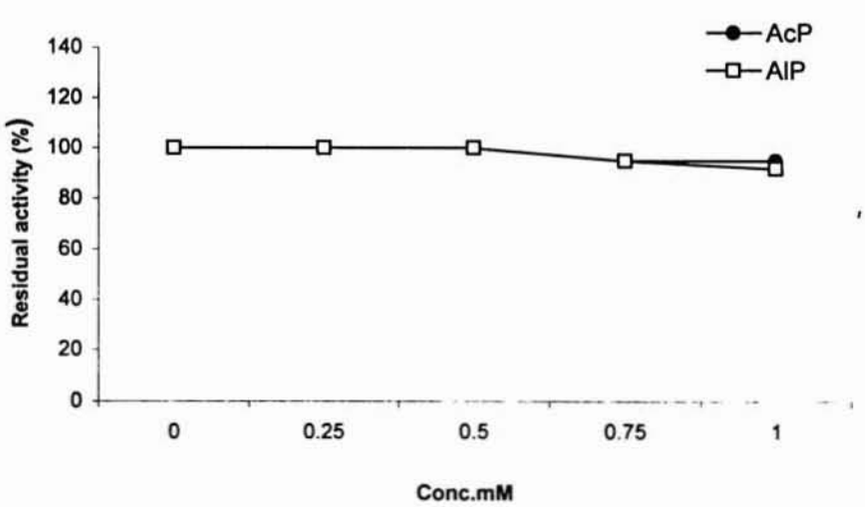
Effect of sodium chloride on enzyme activity was determined by adding different concentrations of NaCl (5mM to 40mM) to the assay medium containing the dialyzed enzyme solution. Results are given in Table 6.10 and Fig. 6.8. NaCl was found to have no significant effect on enzyme activity. The enzyme activity remained,

more or less, the same at different concentrations of NaCl, in the case of both acid and alkaline phosphatase.

Table 6.10 Effect of sodium (Na+) and chloride (Cl^-) ions

NaCl conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	100	100
10	100	100
15	95	95
20	95	92
25	93	90
30	92	90
35	90	90
40	90	90

Fig. 6.8 Effect of sodium (Na^+) and chloride (Cl^-) ions



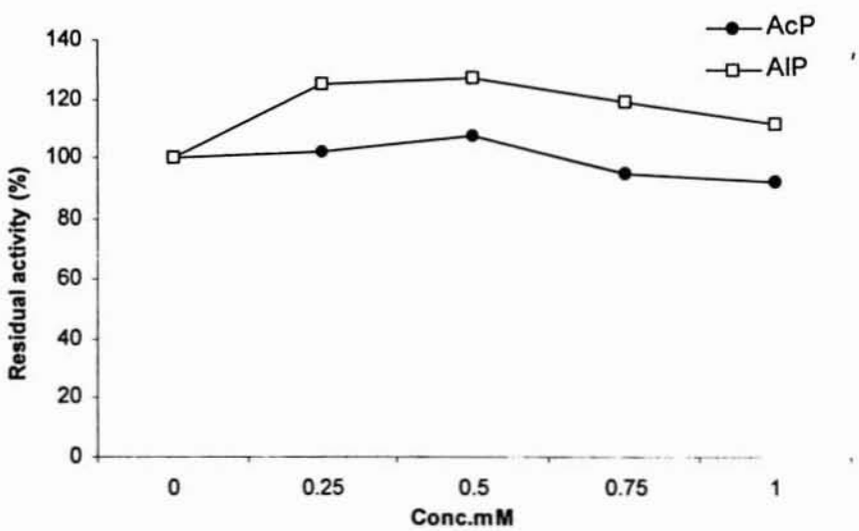
6.2.5.2 Effect of Calcium (Ca^{2+})

Presence of calcium was found to augment phosphatase activities up to 10mM (Table 6.11 and Fig. 6.9). Lower concentrations had an enhancing effect on acid and alkaline phosphatase activity, while on increasing concentration a slight reduction in phosphatase activity was observed.

Table 6.11 Effect of Calcium (Ca^{2+})

$CaCl_2$ Conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	102	125
10	108	127
15	95	119
20	92	112

Fig. 6.9 Effect of Calcium (Ca^{2+})



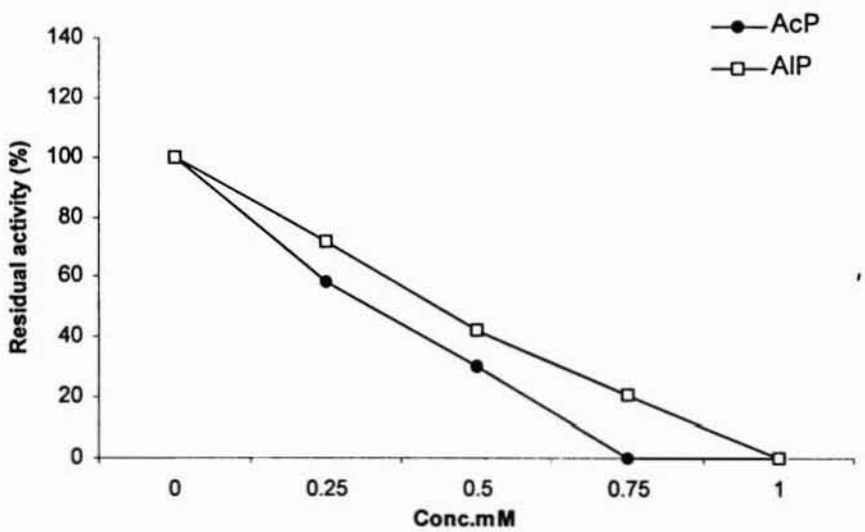
6.2.5.3 Effect of Copper (Cu^{2+})

Cuprous chloride was found to be inhibitory to phosphatase action (Table 6.12 and Fig. 6.10).

Table 6.12 Effect of Copper (Cu^{2+})

$CuCl_2$Conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	58	72
10	30	42
15	0	21
20	0	0

Fig. 6.10 Effect of Copper (Cu^{2+})



Cuprous chloride, at 0.5mM concentration, reduced acid phosphatase activity to 58% and alkaline phosphatase activity to 72%. An increase in $CuCl_2$ concentration to 10mM caused a decrease in acid and alkaline phosphatase activities to 30% and 40% respectively. Acid phosphatase activity was totally inhibited at 15mM and alkaline phosphatase at 20mM concentration.

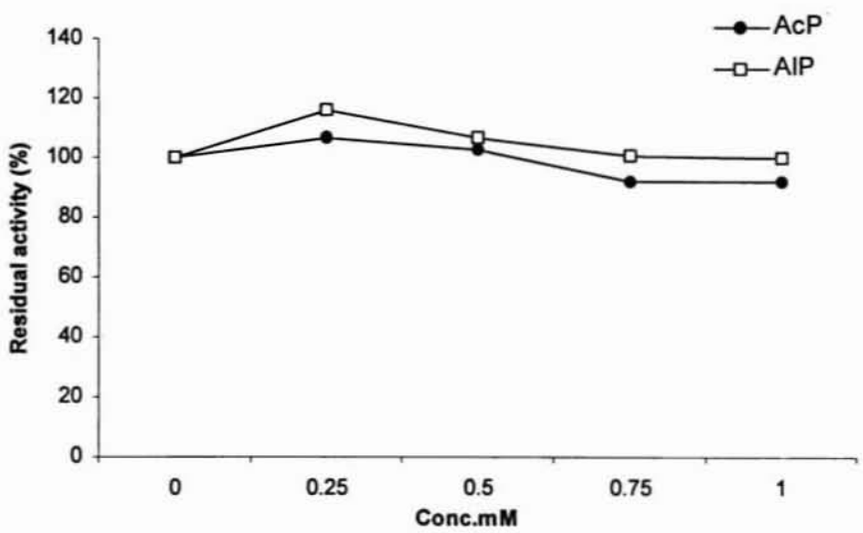
6.2.5.4 Effect of Cobalt (Co^{2+})

There was an increase in phosphatase activities in the presence of Cobalt chloride (Table 6.13 and Fig. 6.11). Lower concentrations (up to 10mM) enhanced acid phosphatase activity. High alkaline phosphatase activity was observed at all concentrations used.

Table 6.13 Effect of Cobalt (Co^{2+})

$CoCl_2$ conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	107	116
10	103	107
15	92	101
20	92	100

Fig. 6.11 Effect of Cobalt (Co^{2+})



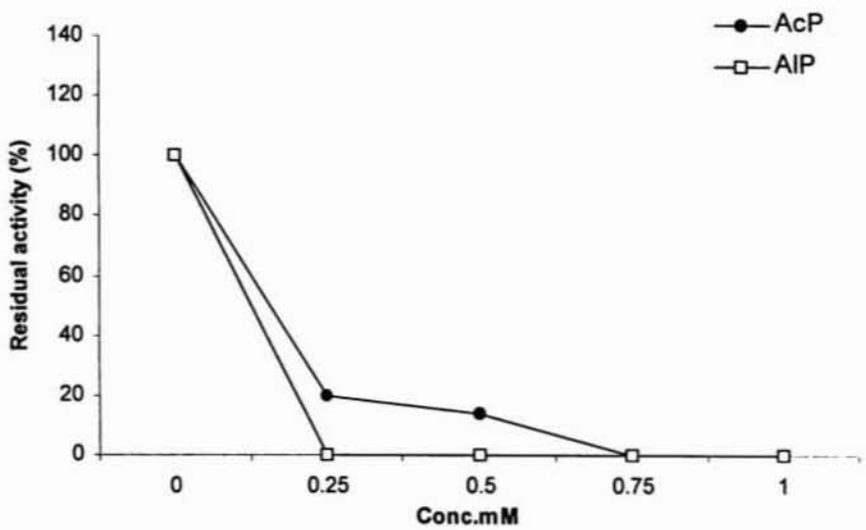
6.2.5.5 Effect of Ferric ion (Fe^{3+})

Ferric chloride was found to be inhibitory to the activity of phosphatases (Table 6.14 and Fig. 6.12). At 5mM concentration alkaline phosphatase lost its activity whereas, acid phosphatase retained about 14% activity. Further increase in ferric chloride concentration to 15mM caused complete inhibition of acid phosphatase activity.

Table 6.14 Effect of Ferric ion (Fe^{3+})

$FeCl_3$ conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	20	0
10	14	0
15	0	0
20	0	0

Fig. 6.12 Effect of Ferric ion (Fe^{3+})



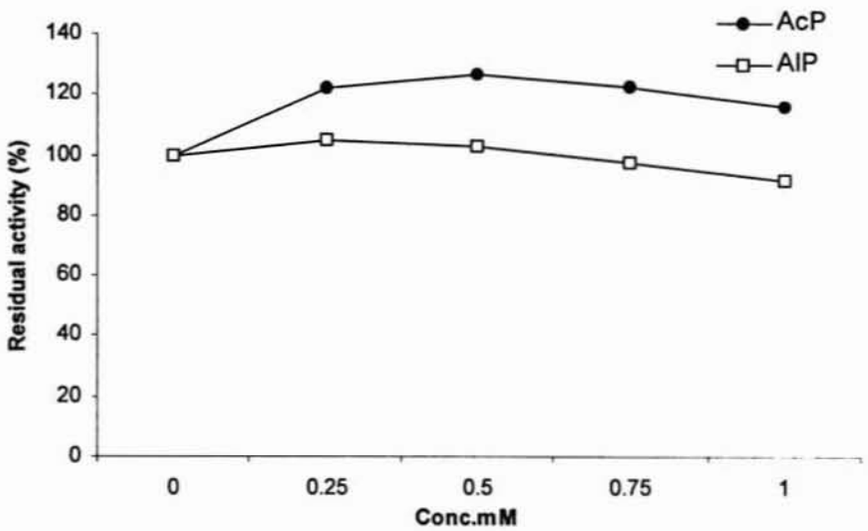
6.2.5.6 Effect of Manganese (Mn^{2+})

Manganese chloride was found to increase phosphatase activity (Table 6.15 and Fig. 6.13). The enhancement was more pronounced on acid phosphatase with maximum residual activity of 127% at 10mM $MnCl_2$ conc., than alkaline phosphatase, which showed 105% residual activity at 5mM $MnCl_2$ conc.

Table 6.15 Effect of Manganese (Mn^{2+})

$MnCl_2$ Conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	122	105
10	127	103
15	123	98
20	116	92

Fig. 6.13 Effect of Manganese (Mn^{2+})



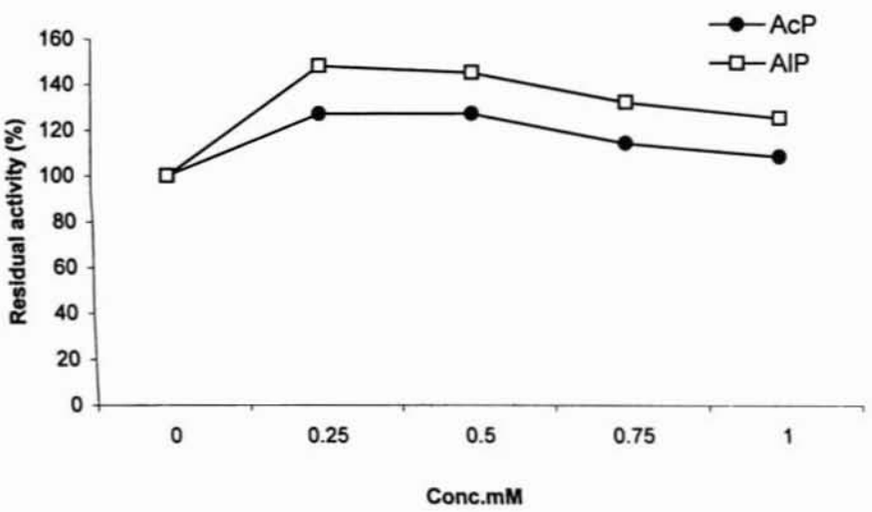
6.2.5.7 Effect of Magnesium (Mg^{2+})

There was a marked increase in phosphatase activities in the presence of magnesium chloride. Results are given in Table 6.16 and Fig. 6.14. Alkaline phosphatase activity was enhanced by 148% by a $MgCl_2$ concentration of 5mM. High acid phosphatase activities (127% at 5 & 10mM conc.) were also detected.

Table 6.16 Effect of Magnesium (Mg^{2+})

$MgCl_2$ Conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	127	148
10	127	145
15	114	132
20	108	125

Fig. 6.14 Effect of Magnesium (Mg^{2+})



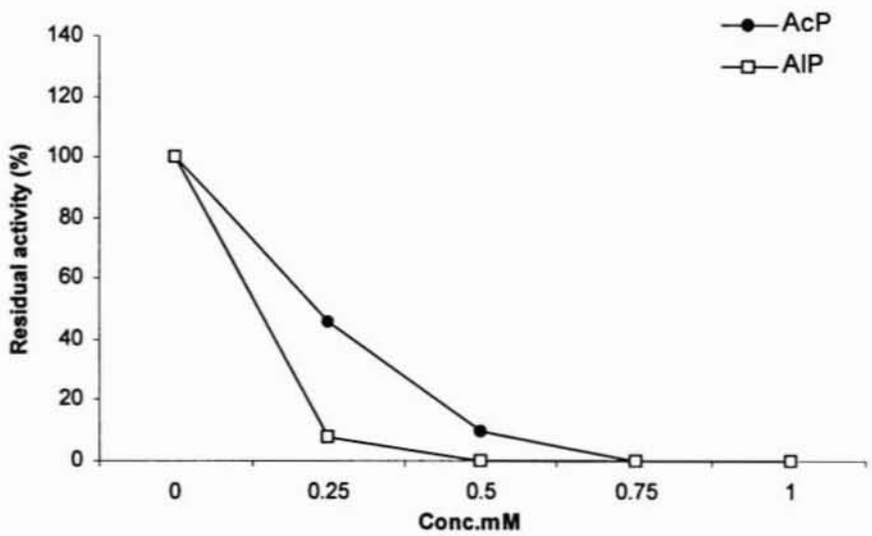
6.2.5.8 Effect of Mercuric ion (Hg^{2+})

Phosphatase activities decreased drastically at a mercuric chloride concentration of 5mM (Table 6.17 and Fig. 6.15). There was a fall in acid phosphatase activity by 46% while alkaline phosphatase activity was reduced to 8%. Total inhibition of alkaline phosphatase activity occurred at 10mM $HgCl_2$ concentration and acid phosphatase was totally inhibited at 15mM $HgCl_2$.

Table 6.17 Effect of Mercuric ion (Hg^{2+})

$HgCl_2$ Conc.(mM)	Residual Activity	
	AcP	AIP
0	100	100
5	46	8
10	10	0
15	0	0
20	0	0

Fig. 6.15 Effect of Mercuric ion (Hg^{2+})



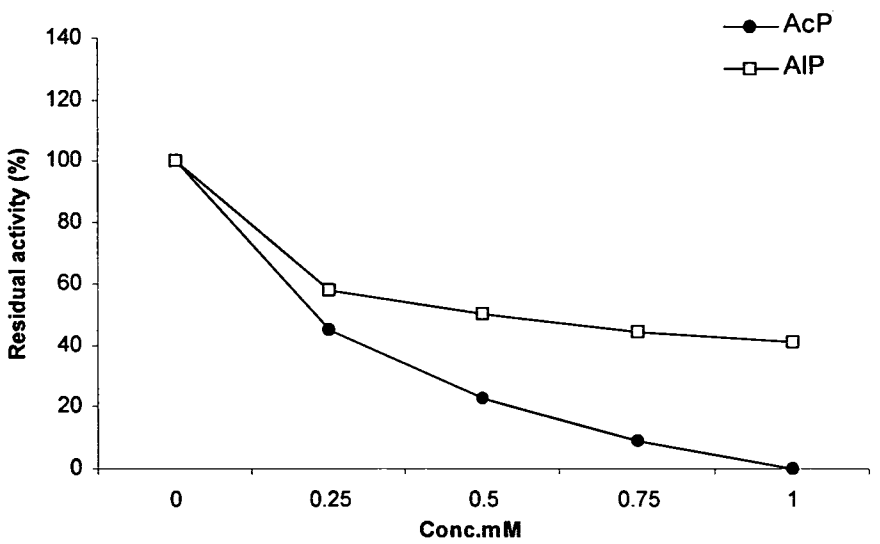
6.2.5.9 Effect of Potassium (K^+)

Potassium chloride was found to be inhibitory to the activity of phosphatases (Table 6.18 and Fig. 6.16). 5mM KCl reduced the acid phosphatase activity to 45% and alkaline phosphatase activity to 58%. At 20mM concentration acid phosphatase activity was completely stalled whereas, alkaline phosphatase retained 41% activity.

Table 6.18 Effect of Potassium (K^+)

KCl Conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	45	58
10	23	50
15	9	44
20	0	41

Fig. 6.16 Effect of Potassium (K^+)



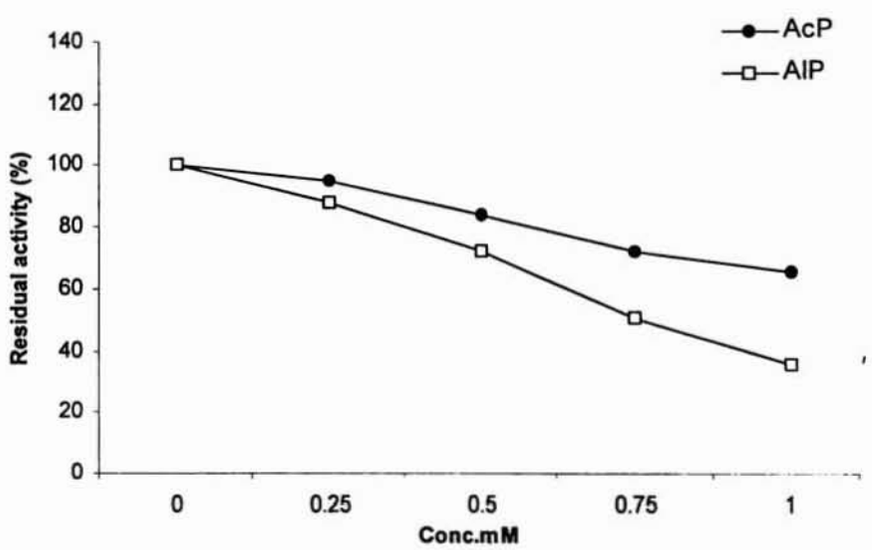
6.2.5.10 Effect of Zinc (Zn^{2+})

There was a reduction in phosphatase activities in the presence of zinc chloride (Table 6.19 and Fig. 6.17). A gradual decrease in activities occurred with increase in $ZnCl_2$ concentration. 95% acid phosphatase activity detected at 5mM concentration was reduced to 66% at 20mM concentration. An increase in $ZnCl_2$ concentration from 5mM to 20mM brought down alkaline phosphatase activity from 88% to 36%.

Table 6.19 Effect of Zinc (Zn^{2+})

$ZnCl_2$ Conc. (mM)	Residual Activity	
	AcP	AlP
0	100	100
5	95	88
10	84	72
15	72	51
20	66	36

Fig. 6.17 Effect of Zinc (Zn^{2+})



6.3 DISCUSSION

6.3.1 Partial purification of phosphatases

Partial purification of acid phosphatase from *Streptococcus* sp. resulted in 62.09% yield while, 63.74% yield was obtained for the alkaline phosphatase from *Flavobacterium breve*. The specific activity obtained for acid phosphatase was 73.57 units mg protein^{-1} and 89.23 units mg protein^{-1} for alkaline phosphatase. The results obtained are comparable to the acid and alkaline phosphatases of other microorganisms. Poirier and Holt (1983) partially purified acid and alkaline phosphatases from *Capnocytophaga ochracea* and recorded 50% yield for acid phosphatase with a specific activity of 78.02 units mg protein^{-1} and 64% yield and 72.91 units mg protein^{-1} for alkaline phosphatase. Gonzalez *et al.* (1994b) in their experiments on phosphatases from *Myxococcus coralloides* D obtained 47.4% yield and specific activity 137.7 units mg protein^{-1} for acid phosphatase and 48.8% yield and specific activity 108.3 units mg protein^{-1}. Day and Ingram (1973) recorded 80% yield and specific activity 50 units mg protein^{-1} on partial purification of *Pseudomonas aeruginosa* alkaline phosphatase. *E. coli* alkaline phosphatase was partially purified by Torriani (1968) to obtain 95% yield and 40-46 μM min^{-1} mg protein^{-1}.

The two phosphatases partially purified from *Citrobacter* sp. by Jeong and Makaskie (1999) also showed similar properties.

6.3.2 Effect of pH on acid and alkaline phosphatase activity and stability

Enzyme activities are markedly influenzed by the hydrogen ion concentration of the medium. Each enzyme has an optimum pH at which the rate of the reaction it catalyzes is at its maximum. Small deviations in pH from the optimum value lead to decreased activity due to changes in the ionization of groups at the active site of the enzyme. Larger deviations in pH lead to the denaturation of the enzyme protein itself, due to interference with the many weak noncovalent bonds maintaining its three-dimensional structure.

Acid phosphatases produced by many microorganisms have pH optima in the range of 4 to 6. They include acid phosphatases from *Aspergillus fumigatus* (Wyss *et al.* 1998), *Bacillus* subtilis (Kerovuo *et al.*, 1998), *Candida lipolytica* (Galabova *et al.*, 1993) *Capnocytophaga ochracea* (Poirier and Holt, 1983), *Citrobacter* sp. (Jeong and Macaskie, 1999), *E. coli* (Rao and Torriani, 1988), and *Penicillium chrysogenum* (Haas *et al.*, 1992). Acid phosphatase from *Myxococcus coralloides D* (Gonzalez *et al.*, 1994a, b) had optimum activity at pH 4.5. The enzyme showed high activity between pH 3.5 and pH 5.5. Malke (1998) reported an acid phosphatase from

Streptococcus equisimilis, which exhibited activity in a pH range from 4.5 to 6.4 with an optimum at pH 5. In the present study, the optimum pH for acid phosphatase activity was found to be 4.8. In consonance with other reports, a pH range from 4 to 6 was conducive to the activity of the acid phosphatase from the *Streptococcus* sp. The enzyme was inactivated at pH 3 and alkaline pH. However, the pH optima of acid phosphatase from some microorganisms show variation from this pH range. Acid phosphatases from *Aspergillus niger* having an optimum pH of 2.5 were reported by Piddington *et al.* (1993) and Wyss *et al.* (1998). Wyss *et al.* (1998) further added that the enzyme showed virtually no activity at pH 5 and above.

The alkaline phosphatase from *Myxococcus coralloides D* showed high activity between pH 7.0 and 8.5, with maximum activity at 8.1 (Gonzalez *et al.*, 1994b). pH 9 was best suited for the activity of *Micrococcus sodonensis* alkaline phosphatase (Glew and Heath, 1971). *Pseudomonas aeruginosa* studied by Day and Ingram (1973) produced an alkaline phosphatase that showed broad activity profile between pH 8 and 10.5, which reached a maximum at pH 10.5. Identical results were obtained in the present study with *Flavobacterium breve* alkaline phosphatase, which demonstrated high activity between pH 8 and 10.6 with the peak activity at pH 10.4. Microorganisms known to produce alkaline phosphatase include

Capnocytophaga ochracea (Poirier and Holt, 1983), *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Huang *et al.*, 1998). The alkaline phosphatase activity of *Micrococcus varians* I and *Citrobacter koseri* was found to be maximum at pH 10.5 and 10 respectively (Sharma *et al.*, 1995).

Acid phosphatase was highly stable at pH 6 and 7, while alkaline phosphatase was most stable at pH range 7 to 9. *Myxococcus coralloides* D acid phosphatase was reported to be stable between pH values 3 to 9, whereas, alkaline phosphatase produced by the same bacteria was relatively stable from pH 6 to 9 (Gonzalez *et al.*, 1994b). Maximum stability in the alkaline phosphatases of both *Micrococcus varians* I and *Citrobacter koseri* was seen at pH 9.5 (Sharma *et al.*, 1995).

The optimum pH levels for microbial phosphatases are unlikely to be encountered in their natural environments (Hernandez *et al.*, 1995). Therefore, the bacteria which possess an enzyme active over a wide range of pH will be better equipped to survive pH fluctuations especially in aquatic environments contaminated by inflow of industrial and domestic wastes. *Flavobacterium breve* which produces an alkaline phosphatase that was found to be active at a wide pH range from 7 to 10.6 capable of hydrolyzing organophosphorus compounds in the marine environment where the

pH is slightly alkaline should be considered to be capable of hydrolyzing organophosphorus in the marine environment where the pH is slightly alkaline and the *Streptococcus* sp. producing acid phosphatase, active in acidic pH, need to be contemplated as a promising prospect in aquatic ecosystems turned acidic by inflows of domestic and industrial wastes.

6.3.3 Effect of temperature on phosphatase activity and stability

Increase in temperature accelerates the velocity of an enzyme catalyzed reaction until a maximum is reached, after which the velocity decreases and finally results in the denaturation of the enzyme. This optimum, maximum and minimum temperatures are characteristic of each enzyme and the greatest activity is displayed at the optimum temperature. In the present study, highest acid and alkaline phosphatase activities were encountered at 37^0C. In *Myxococcus coralloides* D (Gonzalez *et al.*, 1994b), 43^0C was found to be optimal for the activity of the acid form and 37^0C for the alkaline one. Alkaline phosphatase produced by the *Bacillus* sp. RK11 studied by Kelly *et al.* (1984) attained optimum activity at 30^0C. Alkaline phosphatase from *Pseudomonas aeruginosa* exhibited an optimum temperature of 41^0C (Day and Ingram, 1973).

Data on heat stability of the enzymes obtained from this study showed that the enzyme is resistant to temperature variations normally encountered in the aquatic environments. Acid and alkaline phosphatases showed 100% stability up to 40^0C and 45^0C respectively. Acid phosphatase showed 86% stability at 40^0C, 45% at 50^0C and 12% at 60^0C. The residual activity of alkaline phosphatase was 66% at 50^0C and 20% at 60^0C. Acid phosphatase obtained from *Myxococcus coralloides* D retained 30 %, 25 % and 20 % activity at 60^0C, 70^0C and 80^0C respectively, when assayed after 30 minutes incubation (Gonzalez *et al.* 1994). Alkaline phosphatase from the same organism had 25% activity at 60^0C, 5% at 70^0C and was totally inactivated at 80^0C. However, Sharma *et al.* (1995) reported that the effect of temperature on the stability of alkaline phosphatase showed an irregular pattern. Wyss *et al.*, (1998) observed that acid phosphatase from *Aspergillus niger* was inactivated only at temperatures above 60^0C to 65^0C, while that of *A. fumigatus* lost its activity at temperatures of above 55^0C.

6.3.4 Effect of substrate concentration

Studies of extracellular enzyme activities are used to describe the degradation of organic matter in aquatic ecosystems (Hollibaugh and Azam, 1983; Azam and Cho, 1987; Billen, 1991; Boetius and Lochte, 1994, 1996; Karner *et al.*, 1995; Martinez *et al.*,

1996; Nausch and Nausch, 2000; Stihl *et al.*, 2001). They are determined by the addition of artificial substrates (Darrah and Harris, 1986; Chróst, 1991a, b; Hoppe, 1983; Shand and Smith, 1997).

In the present study the acid phosphatase produced by the *Streptococcus* sp.had a K_m value of 4.9 x 10^{-3} M l^{-1} and alkaline phosphatase from *Flavobacterium breve* showed a K_m of 6.3x10^{-3}M l^{-1}

Phosphatases produced by different bacterial species show variation in their K_m values. *Myxococcus coralloides* D is known to have a K_m value of 1.5 x 10^{-3} mol l^{-1} for acid phosphatase and 5.0 x 10^{-3} mol l^{-1} for alkaline phosphatase (Gonzalez *et al.,* 1994b). A K_m of 6.6 x 10^{-5} mol l^{-1} for *p*- nitrophenyl phosphate has been reported for *Pseudomonas aeruginosa* alkaline phosphatase by Day and Ingram (1973). Similar results are also reported for *M. sodonensis* (Glew and Heath, 1971) and *E. coli* (Csopak et al., 1972).

6.3.5 Effect of various ions

Divalent metal ions, heavy metal ions and other monovalent ions act as inhibitors or cofactors of phosphatases in several bacteria (Cheng *et al.*, 1970; Ghosh *et al.*, 1977; Bock and Kowalsky, 1978; González *et al.*, 1994b).

Metal ions in the reaction mixture may exert their effect on enzyme activity by affecting the rate of enzyme-substrate combination (Day *et al.*, 1968). Mg^{2+} ions were found to be an activator of phosphatases from many microorganisms (Linden *et al.*, 1977; Fernandez *et al.,* 1981; González *et al.*, 1994b; Chen *et al.*, 1996). Studies on the effect of divalent cations on alkaline phosphatase activity by Sharma *et al.* (1995) reveal that Mg^{2+} enhanced enzyme activity in *Citrobacter koseri* and *Micrococcus varians* I whereas, Ca^{2+} was found to be effective only in *Citrobacter koseri.* In *Micrococcus varians* I it retarded the activity. This is, however, in contrast to the results reported for *Micrococcus sodonensis* (Glew and Health, 1971) in which Ca^{2+} was required for the expression of enzyme activity. In the present investigation Mg^{2+} was found to have considerable enhancing effect on both the phosphatases while, the effect of Ca^{2+} was more pronounced in alkaline phosphatase.

The results obtained by Mahasneh *et al.* (1990) and Hernandez *et al.,* (1995) showed that in the marine environments specific ions, such as Na^{+} and Mg^{2+} exert varied effects on phosphatase activities. There may be a pronounced effect of both Na^{+} and Cl^{-} on permeability and phosphate uptake by these organisms (Ullrich-Eberius and Yinghol, 1974; Cembella *et al.*, 1984a, b). Whitton *et al.* (1990b) reported that Ca^{2+} increased phosphatase

activity markedly, but Na^+ and K^+ had a negligible effect. Gauthier *et al.* (1991) suggested that long-term survival of *E. coli* in sea water is dependant on the ability of the cell to synthesize phosphatases and K^+ ions were essential for its activity. The results obtained in this study are not completely consistent with these findings. Na^+ did not exhibit any influence as the phosphatase activities remained, more or less, the same at different concentrations of NaCl, but the addition of K^+ caused distinct reduction in the activities of acid and alkaline phosphatases.

Zn^{2+} is found to influenze the activity of alkaline phosphatase in many ways. Studies by Crofton (1982) show that alkaline phosphatase is a dimeric molecule and is composed of two subunits. Each subunit contains a tightly bound atom of zinc, which is essential for the structural integrity of the enzyme, and a second, less tightly bound zinc atom, which is involved in the catalytic process. Mg^{2+} ions exert an allosteric effect on the enzyme to stimulate dephosphorylation process. Zn^{2+} can also bind to the Mg^{2+} site with greater affinity than Mg^{2+} itself, resulting in loss of activity (Chróst, 1991a). Zn^{2+} was required in less concentration in *C. koseri.* Coleman (1987) found that Zn^{2+} is associated with many alkaline phosphatases, including that of *E. coli.* Whitton *et al.* (1990b) reported the inhibition of phosphatase activity by zinc and suggested that the

inhibitory effects of Zn^{2+} are reversible. In the present investigation a gradual decrease in the phosphatase activities was observed with increase in $ZnCl_2$ concentration.

Mounter *et al.* (1955) observed that enzyme activity was stimulated by Mn^{2+} while Co^{2+} was generally inhibitory in *Escherichia coli, Pseudomonas fluorescens* and *Streptococus faecalis*. Studies by Whitton *et al.* (1990b) revealed that Fe^{3+} and Zn^{2+} inhibited phosphatase activity in cyanobacteria. Abd-Alla, (1994a) in his studies on *Rhizobium leguminosarum* has reported the influenze of various cations on acid and alkaline phosphatase activities. Ca^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} promoted both acid and alkaline phosphatases. Ca^{2+}, Mg^{2+} and Co^{2+} enhanced alkaline phosphatase activity more than acid phosphatase. However, Fe^{2+} and Mn^{2+} activated acid phosphatase as compared to alkaline phosphatase. The activity of the enzyme was severely inhibited by Cu^{2+}, Al^{3+}, Fe^{3+} and Zn^{2+}. Chen *et al.*, (1996) found that Cu^{2+} and Hg^{2+} inhibited the enzyme activity and suggested that the inhibition by Hg^{2+} was of an uncompetitive type. $HgCl_2$ completely inhibited the phosphatase activity in *Citrobacter koseri* and *Micrococcus varians* I (Sharma *et al.*, 1995). The activity of the enzyme was severely inhibited by Zn^{2+}. Mg^{2+} and Cu^{2+} inhibited phosphatase activity in *Aerobacter aerogenes* (Mounter and Tuck, 1956).

The results obtained in this study are, more or less, consistent with these findings. Mg^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+} were found to have enhancing effect on acid and alkaline phosphatase activities, while, those inhibitory were Cu^{2+}, Fe^{3+}, Hg^{2+}, K^{+} and Zn^{2+}. Though ions exercise their effect on the activity of phosphatase, it can be concluded that their influenze on the acid and alkaline forms vary and also show discrepancy from one organism to the other.


Chapter 7

ORGANOPHOSPHORUS UTILIZATION AND DEGRADATION OF PESTICIDES

The organophosphorus compounds present in aquatic environments consist of molecules that cannot directly enter the cells, because of their high molecular weights and large size. Hence they are not directly utilized by living organisms but have to be hydrolyzed by the action of microbial extracellular enzymes (Wynne, 1977). Inorganic phosphate is abundant in some environments but natural waters are often P_i - limited (Corner and Davies, 1971). This often results in the synthesis of a battery of enzymes, including phosphatases by bacteria (Filloux *et al.*, 1988). Phosphatases are known to hydrolyze organophosphorus compounds into P_i and the corresponding organic moiety. The present investigation envisages

studies to scrutinize the phosphatase producing *Streptococcus* sp. and *Flavobacterium breve* under conditions of P_i stress and the ability of these microorganisms to degrade organophosphorus pesticides. The organophosphorus sources used were *p*-nitrophenyl phosphate, sodium β glycerophosphate and triethyl phosphate. The pesticides used were:

i. Nuvacron:
   (O, O-Dimethyl-O (2-methyl-carbonyl-1-methyl vinyl) -phosphate)
   (36% Monocrotophos)

ii. Dimecron:
   (2-chloro-2-diethylcarbonyl-1-methylvinyl-dimethyl phosphate
   (85% Phosphamidon)

iii. Rogor 30E:
   (O, O-dimethyl-S-(N-Methylcarbomyl methyl)phosphorodithioate
   (30% Dimethoate)

iv. Metacid 50:
   (O, O-dimethyl O-(*p*-nitrophenyl) Phosphorothioate
   (50% Methyl Parathion)

7.1 METHODOLOGY

7.1.1 Utilization of organophosphorus compounds as sources of P_i

To study the ability of phosphatase producing bacteria to utilize organophosphorus compounds as sources of P_i, 0.5% of *p*-nitrophenyl phosphate, sodium-β-glycerophosphate and triethyl phosphate were added individually to 250ml flasks containing 100ml MacLeod's artificial sea water (ASW) base mineral medium devoid of other P sources and were inoculated with phosphatase producing *Streptococcus* sp. and *Flavobacterium breve* and two non phosphatase producing forms (Non Pase I & II), an *Aeromonas* sp. and one *Staphylococcus* sp. One set of uninoculated flasks were retained as control to detect any release of P_i by self-degradation of the organophosphorus compounds added. Mineral medium without any P sources, inoculated with each of the bacteria under study was also maintained and checked for growth and P_i after incubation. Initial P_i concentration was measured at 0 hour of incubation. Growth, phosphatase activity and P_i concentration were measured after 24 hours incubation at 28^0C. Ascorbic acid method (Eaton *et al.*, 1999) was followed for P_i determination and growth was determined by measuring the absorbance at 600nm.

7.1.2 Degradation of organophosphorus pesticides

1% of the commonly used organophosphorus pesticides, Dimecron, Nuvacron, Rogor and Metacid, were added individually to 250ml flasks containing 100ml MacLeod's artificial sea water (ASW) base mineral medium devoid of other P sources to detect the ability of the phosphatase producing bacteria, *Streptococcus* sp. and *Flavobacterium breve*, to degrade the pesticides. The two phosphatase producing and two non phosphatase producing bacteria were inoculated and incubated at 28^0C for 24 hours. Growth, phosphatase activity and P_i concentration were then determined. Initial P_i concentration was measured at 0 hour of incubation. Each bacterium was also inoculated in mineral medium without any P sources and checked for growth and P_i after incubation. One set of uninoculated flasks with each pesticide were maintained under the same incubation conditions as control to detect any release of P_i by self-degradation of the organophosphorus pesticides added.

7.2 RESULTS

7.2.1 Utilization of organophosphorus compounds as sources of P_i

p-nitrophenyl phosphate, sodium-β-glycerophosphate and triethyl phosphate were supplied as sole sources of P to MacLeod's artificial sea water base minimal medium (ASW) devoid of any inorganic phosphates to examine the phosphate releasing capabilities of the *Streptococcus* sp. and *Flavobacterium breve*. The enzyme assays conducted after 24 hours incubation indicates that phosphate stress induced phosphatase production in both the bacteria (Table 7.1 & Fig. 7.1).

Table 7.1 Effect of organophosphorus compounds on phosphatase production

P sources	Phosphatase production (Enz. Units)	
	Streptococcus	*Flavobacterium*
p-NPP	0.427	0.453
Na-β-glyc.	0.423	0.473
Triethyl phosphate	0.453	0.438
ASW(without P)	0.032	0.05

The three organophosphorus compounds supplied as sole sources of phosphorus were found to support growth of the two phosphatase producing bacteria. P sources which the *Streptococcus* sp. favoured were Sodium β glycerophosphate > *p* nitrophenyl phosphate > Triethyl phosphate, while *p*-nitrophenyl phosphate > Sodium-β-glycerophosphate > Triethyl

phosphate were the P sources of choice observed for *Flavobacterium breve*. The alkaline phosphatase producing bacterium showed better adaptability under the provided growth-limiting conditions. No growth was observed in media inoculated with the two non phosphatase producing bacteria (Non Pase I & II) and in media without any P sources.

Fig. 7.1 Effect of organophosphorus compounds on phosphatase production

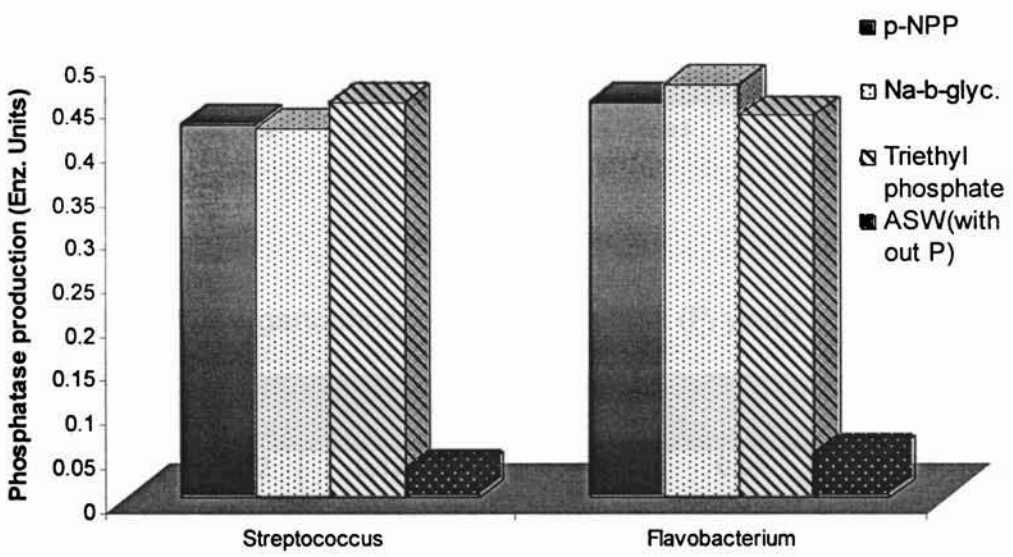


Table 7.2 Effect of organophosphorus compounds on growth

P sources	Growth (Abs.)			
	Streptococcus	*Flavobacterium*	Non Pase I	Non Pase II
p-NPP	0.194	0.753	0.032	0.026
Na-β-glyc.	0.247	0.56	0.024	0.021
Triethylphosphate	0.112	0.159	0.01	0.02
ASW (without P)	0.027	0.089	0.01	0.01

Fig. 7.2 Effect of organophosphorus compounds on growth

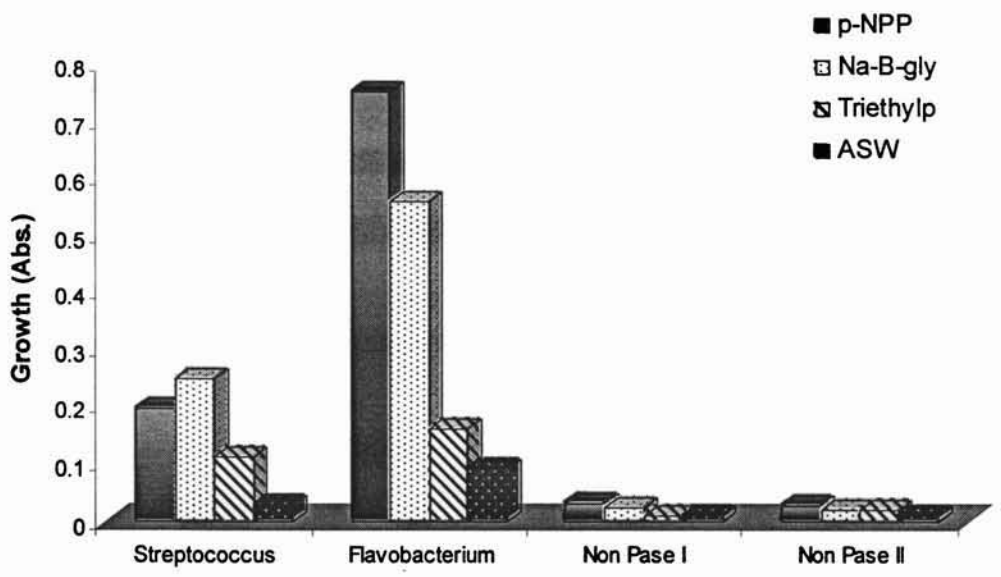


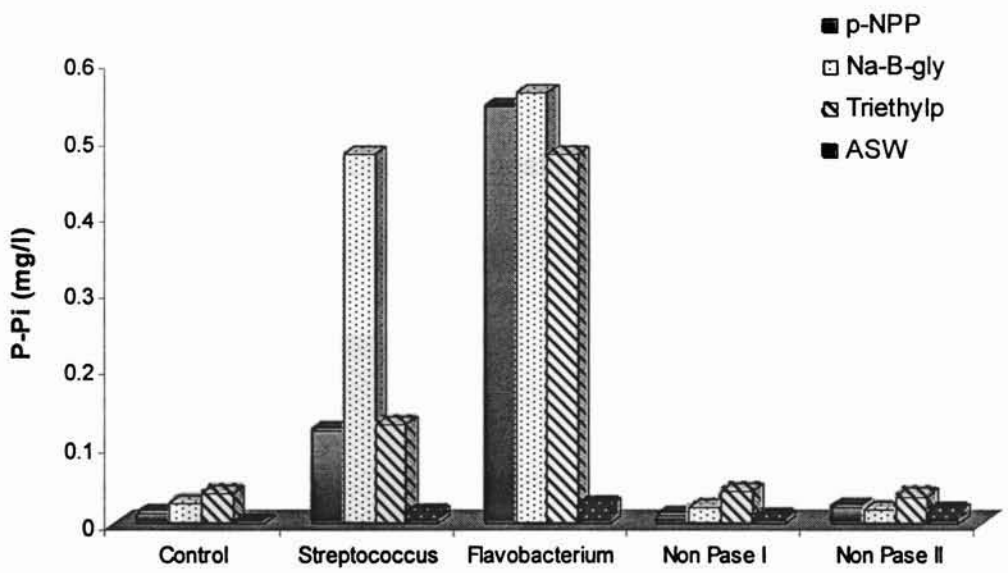
Table 7.3 Effect of organophosphorus compounds (P_i yield)

P sources	$P\text{-}P_i$ (mg l^{-1})				
	Control	*Streptococcus*	*Flavobacterium*	Non Pase I	Non Pase II
p-NPP	0.012	0.121	0.544	0.01	0.021
Na-β-gly	0.027	0.479	0.56	0.021	0.018
Triethylp	0.038	0.128	0.48	0.042	0.035
ASW (without P)	0	0.012	0.022	0.008	0.016

Media inoculated with phosphatase producing cultures realized appreciable P_i yields (Table 7.3 & Fig. 7.3). High yields of P_i were attained from all the three organophosphorus sources when inoculated with *Flavobacterium breve*. The acid phosphatase producing bacterium was found to favour sodium-β–glycerophosphate than the other two organophosphorus

sources used. No P_i yields were obtained in media inoculated with the two non phosphatase producing bacteria (Non Pase I & II) and in media without any P sources.

Fig. 7.3 Effect of organophosphorus compounds (P_i yield)



7.2.2 Degradation of organophosphorus pesticides

The organophosphorus pesticides were supplied as sole sources of P to Mc Leod's artificial sea water base mineral medium devoid of any inorganic phosphates to examine the phosphate releasing capabilities of *Streptococcus* sp. and *Flavobacterium breve*. The enzyme assays conducted after 24 hours incubation show that the phosphate stress induced phosphatase production in both the organisms (Table 7.4 & Fig. 7.4).

Table 7.4 Effect of organophosphorus pesticides on phosphatase production

P sources	Phosphatase production (Enz. Units)	
	Streptococcus	*Flavobacterium*
Nuvacron	0.462	0.487
Dimecron	0.451	0.453
Rogor	0.445	0.458
Metacid	0.431	0.493
ASW(without P)	0.028	0.045

Fig. 7.4 Effect of organophosphorus pesticides on phosphatase production

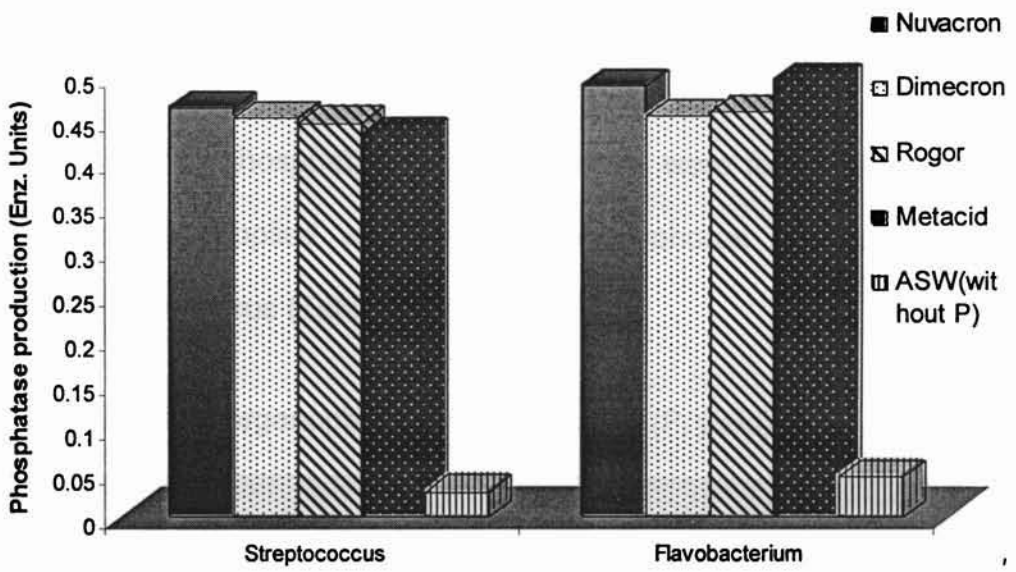
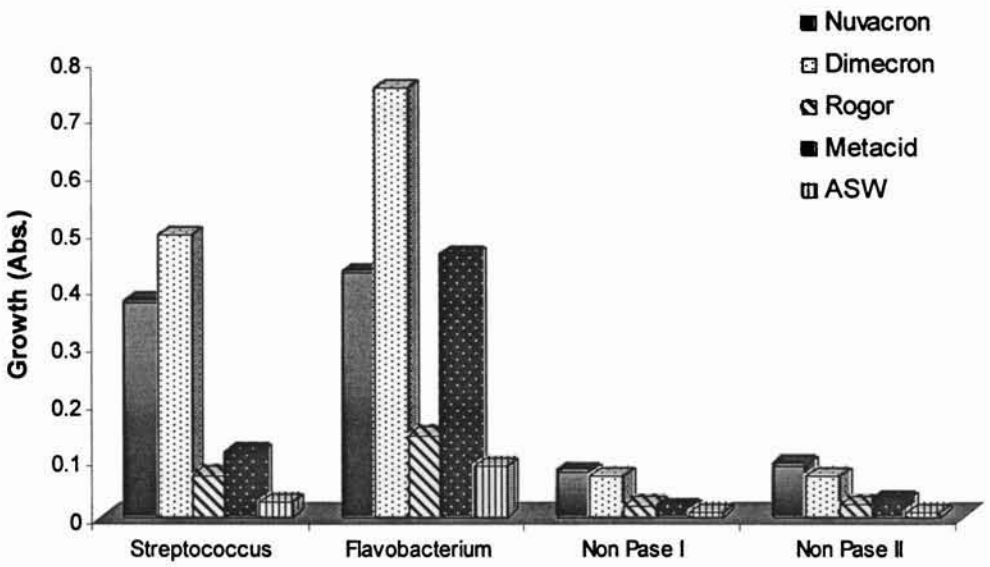


Table 7.5 Effect of organophosphorus pesticides on growth

P sources	Growth (Abs.)			
	Streptococcus	*Flavobacterium*	Non Pase I	Non Pase II
Nuvacron	0.377	0.427	0.08	0.091
Dimecron	0.494	0.753	0.073	0.071
Rogor	0.074	0.144	0.021	0.024
Metacid	0.112	0.159	0.017	0.03
ASW(without P)	0.027	0.089	0.01	0.01

Fig. 7.5 Effect of organophosphorus pesticides on growth



The two phosphatase producing forms were found to survive and grow in media supplied with the organophosphorus pesticides as sole sources of phosphorus (Table 7.5 &Fig. 7.5).

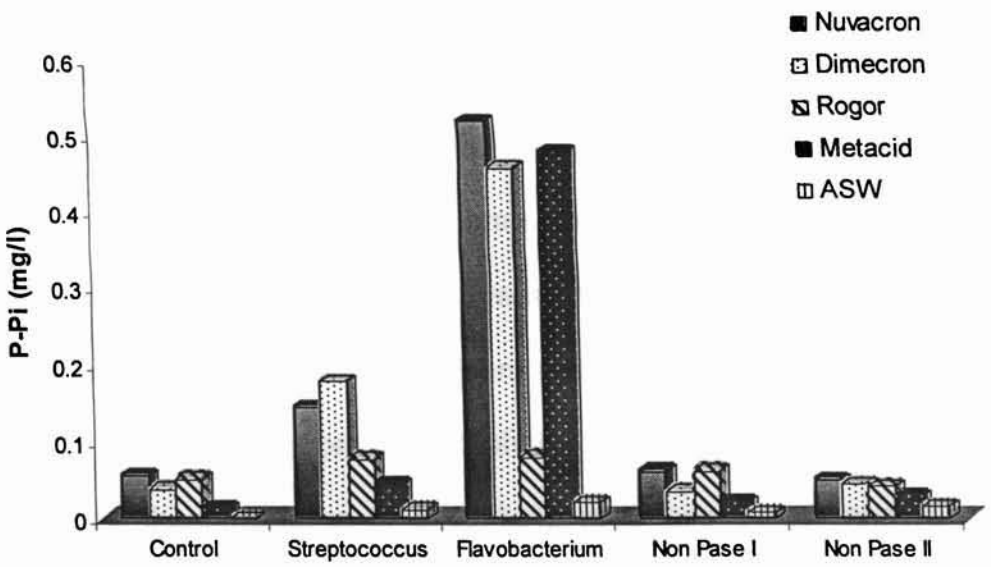
Table 7.6 Effect of organophosphorus pesticides (P_i yield)

P sources	P-P_i				
	Control	*Streptococcus*	*Flavobacterium*	Non Pase I	Non Pase II
Nuvacron	0.055	0.144	0.521	0.062	0.05
Dimecron	0.038	0.179	0.46	0.036	0.045
Rogor	0.051	0.078	0.08	0.062	0.042
Metacid	0.014	0.045	0.482	0.021	0.028
ASW(without P)	0	0.012	0.022	0.008	0.016

Maximum growth was observed in *Flavobacterium breve* inoculated in the medium supplied with dimecron. Both the bacteria were found to prefer the pesticides Dimecron > Nuvacron > Metacid > Rogor. No growth was

observed in media inoculated with the two non phosphatase producing bacteria (Non Pase I & II) and in media without any P sources.

Fig. 7.6 Effect of organophosphorus pesticides (P_i yield)



Flavobacterium breve cultures returned good P_i yields in media incorporated with the pesticides nuvacron, dimecron and metacid (Table 7.6 & Fig. 7.6). The best yields of P_i among the *Streptococcus* sp. inoculated media were attained from those supplied with dimecron and nuvacron. Low P_i values were observed in media containing Rogor for the alkaline phosphatase producing form and both rogor and metacid for the acid phosphatase producer. No P_i yields were obtained in media inoculated with the two non phosphatase producing bacteria (Non Pase I & II) and in media without any P sources.

7.3 DISCUSSION

The enzyme activity of bacterial cells may have an important role in the survival of bacteria under different stress conditions. Phosphatase activity has been linked to the survival of bacteria in PO_4-depleted environments (Ozkanka and Flint, 1996). *Streptococcus* sp and *Flavobacterium breve* were challenged with P_i stress to corroborate their aptitude to thrive under P limiting conditions. Both the bacteria were found to respond positively by synthesizing phosphatases. The present results confirm the ability of these bacteria to utilize various organophosphorus compounds as sole P sources by triggering the production of phosphatases. While growth and P_i yields were realized in media inoculated with the acid and alkaline phosphatase producers, neither was observed in media with the two non phosphatase producing bacteria (Non Pase I & II) or in media without any P sources. This indicates that the P_i yields obtained were the products of hydrolysis of the supplied organophosphorus compounds by the phosphatases produced by the bacteria. The alkaline phosphatase was found to be more efficient than the acid form, as evident from the respective P_i yields. This may be attributed to the pH of the medium (7.5) being more favourable for alkaline phosphatase activity. Similar experiments conducted by Gauthier *et al.* (1990) have shown that alkaline phosphatase activities increased in *E. coli* cells in nutrient–free sea water, ascribed to nutrient starvation and derepression of

enzyme activity. They suggested that the long-term survival of *E. coli* in sea water is dependant on the ability of the cell to synthesize alkaline phosphatase (Gauthier *et al.*, 1991). The production of phosphatases in response to phosphate limitation has been reported in both prokaryotic and eukaryotic organisms (Reid and Wilson 1971; Tarafdar and Chhonkar 1979; Tarafdar *et al.* 1988). Phosphatase activities were noted to increase as the cellular phosphate content decreased (Abd-Alla 1994a). Much of the cellular and extracellular phosphatase activities were realized when cellular phosphate contents decreased to or below 0.115% of cell protein.

The Cochin estuary is a water body of major port activities in the country. Salinity-nutrient changes and distribution in the Cochin backwaters is largely affected by the intrusion of sea water and fresh water inflow in addition to the urban drainage, agricultural and industrial discharges (Anirudhan *et al.*, 1987). Several man-made changes in this ecological system during the past two decades have resulted in large changes in the hydrography of Cochin backwaters. Sujatha *et al.* (1993) have identified pesticide "hot spots", regions highly contaminated with high concentrations, in the estuary. The principal concern about the pesticides arises from their toxicity, persistence and strong propensity to undergo bioaccumulation (Safe, 1982). Two prominent groups of common synthetic pesticides viz., organophosphates and organochlorines are encountered in the Cochin estuary. The pesticides regularly used in this region include Dimecron, Malathion, Monocrotophos, and Nuvacron. Sujatha

et al. (1993, 1995, 1999) elucidated the distribution profile of the common pesticide species encountered in the aquatic environment around Greater Cochin. Their studies reported significant loads of pesticides in the estuary, apparently from the agricultural and industrial discharges and the large amount of urban run off/municipal sludge that drain in. A maximum malathion concentration of 13.013μgl^{-1} was recorded during premonsoon and the minimum value recorded was 1.373 μgl^{-1} during the postmonsoon season. The outbreak of Epizootic Ulcerative Syndrome (EUS) in murrels, eels, mullets, pearl spot, barbs, glassy perchlets, half and full breaks seen in fields of Kuttanad and at the 'Kol' lands in Trichur is suspected to be due to the indiscriminate use of pesticides in this region (Kurup, 1992).

The sources of pesticides transported to Cochin estuary could be from a single source of contamination (point sources) like the effluents from a pesticide manufacturing plant that was responsible for the high concentration of malathion, methyl parathion and endosulfan in regions of the estuary surrounding the industrial concern (Sujatha *et al.*, 1995) or sources, widespread and diffuse in nature (nonpoint sources), such as agricultural runoffs and urban sewage. Sujatha *et al.* (1999) attributed the high pesticide levels observed in certain areas of the estuary to agricultural run offs and soil erosion, by which pesticides in sediments are transported to the aquatic environment. Organophosphorus pesticides are labile to biodegradation. Phosphatase producing microorganisms are believed to have an important

role in the degradation of organophosphorus pesticides. Abd-Alla (1994b) used organophosphorus pesticides like Diethyl (4-nitrophenyl) phosphorothionate, Dimethyl (4-nitrophenyl) phosphorothionate and Dimethyl (4-nitrophenyl) phosphinothionate as substrates for phosphatases from *Rhizobium* and *Bradyrhizobium* strains. He associated the degradation of these pesticides with the action of phosphatases.

Similar results were obtained in the present study. P_i starvation was found to induce acid and alkaline phosphatase production by the *Streptococcus* sp and *Flavobacterium breve* respectively. The inorganic phosphate yield obtained from the culture media could be considered the product of degradation of the pesticides, since the media were devoid of any P source other than the added pesticide. The lack of significant amounts of P_i in the uninoculated media point to the fact that self-degradation of the pesticides has not occurred. Therefore involvement of the phosphatase enzyme in bringing about the release of P_i is apparent. From the observation that the P_i yields were drastically higher with the alkaline phosphatase producing bacterium it was evident that alkaline phosphatase was the most efficient of the phosphatases. This could be due to the pH of the medium being in the range favourable to the alkaline form. However, no inorganic phosphate yields could be observed in the medium supplemented with the insecticide rogor in spite of the presence of phosphatase enzyme. With the available data the exact reason for this deviant observation could not be

drawn, but it may be assumed that the particular pesticide was not prone to enzyme catalysis and hence, survived degradation.

Urbanization, industrialization and harbour development activities have brought about considerable changes in the water quality of the Cochin backwaters in recent years. Accidental spillage and seepage during tanker operations leading to frequent leakages of crude oil and petroleum products (Sen Gupta, 1991, 1992) and discharge of ballast waters into the estuary often result in high concentrations of petroleum hydrocarbons (PHC) in the estuary. In a close monitoring of the distribution of PHC of surface and sub-surface waters of Cochin estuary for about two years, Menon and Menon (2001) opined that tanker and fishing vessels add large quantities of PHC into the water and sediment. It was also noted that the estuarine circulation, the oscillatory nature of the tidal currents and the dissolved and the suspended organic load of the water cause the distribution of PHC to all regions of the estuary. An oil spill occurred in Cochin harbour on 15 March 1996 (Anon, 1996). Water analysis on 17 March 1996 showed the presence of 14.1 mg/l oil in subsurface waters at the monitoring station and about 99.4% mortality was noticed among the settled larvae of the polychaete *Hydroides elegans* (Paul *et al.*, 1998). Death was due to both smothering by the heavier fractions of the slick as well as the toxic effect of petroleum hydrocarbons in the medium. Strategies for incorporating microbial utilization

of petroleum hydrocarbons from such polluted ecosystems need to be developed (Atlas, 1991).

Biodegradation of petroleum in the natural ecosystem is complex and depends on the nature of microflora and environmental factors (Atlas, 1991; Leahy and Colwell, 1990). Information on the microbial degradation of crude oil in the marine environment is available from studies of coastal waters in cold and temperate regions (Leahy and Colwell, 1990; Prince, 1993) and tropical marine environment (Bhosle and Mavinkurve, 1986; David *et al.*, 1995; De Souza *et al.*, 1996). Evidence has been presented suggesting that supplementation of certain ecosystems with nitrogen and phosphorus would increase the degradation of hydrocarbons (Atlas, 1991; Rosenberg *et al.*, 1992). Subsequent studies showed that addition of oleophilic/agricultural fertilizers simulated biodegradation of crude oil (Lee and Levy, 1989; Lee *et al.*, 1995). In theory, approximately 150mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1g of hydrocarbon to cell material (De Souza *et al.*, 1996). Bioremediation may be described as a process of encouraging the natural process of biodegradation to clean up spills of all kinds (Prince, 1993). After the successful study carried out during the Exxon *Valdez* accident (Atlas and Bartha, 1992), strategies based on nutrient enrichment have generally been reported as perhaps the most environmentally sound and least intrusive of all the promising new technologies. Although soluble nutrients can be used successfully (Pritchard

and Costa, 1991), they would be either washed away or may eventually result in undesired eutrophication. Excessive application of these fertilizers has been shown to cause several detrimental effects including oxygen depletion (Lee *et al.*, 1993). Hence, the best bioremedial measure would be to use microbial cultures that can serve the dual purpose of nutrient augmentation (such as replenishment of phosphorus) and removal of oil (De Souza *et al.*, 1996). Van Hamme *et al.* (2000) found that effective oil degradation could be attained with a mixed-bacterial inoculum dominated by the following six genera: *Acinetobacter*, *Alcaligenes*, *Ochrobactrum*, *Pseudomonas/Flavimonas*, *Stenotrophomonas* and *Yersinia*. He suggested that the efficiency could be achieved because the organisms were not under nutrient stress. The nutrient sufficiency was attributed to the action of phosphatases produced by one or more bacteria in the inoculum used. However, the dynamics of the mixed bacterial cultures were not detailed by the researcher. It is well known that phosphorus is a limiting factor in the biodegradation of hydrocarbons (De Souza *et al.*, 1996). It can be ascertained from the findings of Van Hamme *et al.* (2000) that the presence of phosphate solubilizing bacteria could serve as a remedy to the P_i starvation of the hydrocarbon degraders. From the results obtained in the present study it can be established that the phosphatase producing bacterium, *Flavobacterium breve*, isolated from the Cochin estuary, is efficient in solubilizing organophosphorus compounds and releasing inorganic phosphates under conditions of P_i stress. Sharma *et al.* (1995)

stated that the phosphatase producing microbes could be used for removal of phosphate contents of polluted water and replenishment of P. It can be envisaged that the concurrent introduction of this phosphatase producing microbe should increase the efficiency of the oil degrading bacteria, since this approach should augment the availability of phosphorus for the biodegradation of hydrocarbons. This claims considerable bearing in light of the fact that Cochin estuary is inadequate in the requirements of readily available phosphorus (Anirudhan *et al.*, 1987). With detailed studies on this application, the utilization of this relatively unexplored microbial activity could serve as an excellent bioremediation technique to combat oil pollution in aquatic ecosystems such as the Cochin estuary.


Chapter 8

SUMMARY

The Cochin estuary is subject to constant changes in physicochemical factors. The characteristics of this estuary are influenced by the two major rivers flowing into it, while the estuary itself is prone to strong tidal currents. The combined effect of these phenomena gives rise to seasonal and tidal fluctuations of hydrographical conditions. The salinity-nutrient levels are also subject to fluctuations. This can cause variations in the microbial flora of this water body. The estuary receives contaminated freshwater from various domestic and industrial outlets which hamper the quality of water considerably. The microorganisms which thrive in such

environments are those which possess the machinery to combat these unfavourable conditions. This is aided by a battery of enzymes, the synthesis of which is instigated by environmental stress. Phosphatases, belonging to the class of enzymes which hydrolyze organophosphorus substrates, are often synthesized on induction under conditions of inorganic phosphate limitation. The phosphate levels in the Cochin estuary are reported to be below detectable limits in most of the regions. Organophosphorus pesticides were also detected in the estuarine waters in concentrations hazardous to aquatic life. In this scenario, investigations on native bacteria capable of producing phosphatases hold remarkable relevance, more than mere academic interest. In the wake of these demands, the present study was undertaken to understand the capabilities of bacteria, existing naturally, to produce phosphatases and their ability to utilize organophosphorus compounds and degrade organophosphorus pesticides.

A total of 120 bacterial strains were isolated from Cochin estuary and near shore areas, identified up to genera and screened for phosphatase activity. Among the population dominated by gram negative forms (68%), predominant genera were *Pseudomonas, Vibrio, Bacillus* and *Staphylococcus.* Of these 39 isolates (33%) were found to produce phosphatases. Maximum acid phosphatase production was obtained from a *Streptococcus* sp. and *Flavobacterium breve* yielded

highest alkaline phosphatase. These strains were selected for further studies.

In order to extract maximum yields of enzyme from the organisms *in vitro*, the cultural conditions for optimal growth and enzyme production by *Streptococcus* sp. and *Flavobacterium breve* were studied. The organisms produced phosphatases in their exponential phase of growth. Shaking was found to considerably enhance growth and enzyme production by the two selected strains. A neutral or slightly alkaline pH was preferred by the organisms. Acid phosphatase production was hindered by high salt concentrations, while, in *Flavobacterium breve* alkaline phosphatase production remained unaltered at 3% NaCl. This also indicates that these organisms may not be obligate halophiles. *Streptococcus* sp. and *Flavobacterium breve* were observed to grow and produce phosphatase optimally at temperatures in the range of 20^0C to 30^0C, emphasizing the mesophilic nature of the bacteria. Sucrose was the carbon source of choice for both organisms, while the various nitrogen sources enhanced growth. *Streptococcus* sp. was found to prefer peptone for maximum growth. *Flavobacterium breve* yielded maximum growth in the presence of peptone and alkaline phosphatase in yeast extract. However, no significant difference could be detected between the nitrogen sources used. Acid phosphatase production was not influenzed by variations in nitrogen sources. In addition to carbon and

nitrogen, growth and phosphatase production by bacteria were dependant on various ions in the media. The results obtained in the present study have shown that the ionic requirements, in general, are not very specific.

In natural systems various factors regulate enzyme synthesis by microorganisms. Some of the aspects that are known to influenze phosphatase production were studied. The addition of organophosphorus compounds was not found to enhance the synthesis of phosphatase in the *Streptococcus* sp. and *Flavobacterium breve* grown in phosphorus containing media. It can be inferred that it is the cellular phosphate levels and not the presence of degradable substrates that control the production of phosphatases. Acid and alkaline phosphatases produced by these bacteria were found to be subject to catabolite repression. Higher levels of orthophosphate completely repressed phosphatase synthesis.

Chloramphenicol was found to interfere with acid and alkaline phosphatase production. Rapid inhibition of phosphatase synthesis suggests that the synthesis is a *de novo* process. When actinomycin D was added to the cultures actively secreting phosphatases, there was a transient continuation of phosphatase synthesis and inhibition followed. This indicates the presence of a pool of mRNA specific for phosphatases.

The characteristics of phosphatases vary with the type of enzyme, the source organism, the ambient environmental conditions and a multitude of other factors. To recognize the relevance of phosphatases in various fields it is important to understand the characteristics of these enzymes, since the efficiency of an enzyme is imparted by its properties.

In the present investigation, the phosphatases could be partially purified by ammonium sulphate precipitation, followed by dialysis. 62.09% yield of acid phosphatase was obtained from the *Streptococcus* sp. with a specific activity of 73.57 Enz. Units mg protein^{-1}. The alkaline phosphatase yield from *Flavobacterium breve* was 63.74% and the specific activity was found to be 89.23 Enz. Units mg protein^{-1}.

Acid phosphatase activity was maximum at pH 4.8 while, peak alkaline phosphatase activity was obtained at pH 9.5. The acid phosphatase was found to be stable at a near-neutral pH range and the alkaline form was highly stable at pH 7 to 9. Both the enzymes showed optimum activity at 37^{0}C and were considerably stable up to 45^{0}C. Their stability decreased gradually, with increase in temperature suggesting that these phosphatases are not very thermostable.

A substrate concentration of 2 mg ml^{-1} organophosphate yielded optimum acid and alkaline phosphatase activity. The K_m values obtained in this study

were 4.9 x 10^{-3} M l^{-1} for acid phosphatase and 6.3 x 10^{-3} M l^{-1} for alkaline phosphatase.

Sodium chloride was not found to be essential for the activity of the two phosphatases. The addition of several ions had identical effects on acid and alkaline phosphatase activities, though the magnitudes of the effect varied with the ion and between the two phosphatases. Calcium, cobalt, manganese and magnesium enhanced the phosphatase activities, while those found to be inhibitory for the enzyme activities were copper, iron, mercury potassium and zinc.

The *Streptococcus* sp. and *Flavobacterium breve* were found to grow and produce phosphatases in the presence of the organophosphorus compounds and the pesticides incorporated as sole sources of phosphorus. Considerable amounts of P-P_i were released in the media after 24 hours of incubation. This can be attributed to the hydrolyzing capabilities of high levels of phosphatases observed.

The Cochin estuary, considered to be one of the polluted estuaries in India, receives contaminants of diverse nature from various portals. The major threat to estuarine life arises from the presence of petroleum hydrocarbons in alarming amounts in water and sediments. The growth of the hydrocarbon solubilizing bacteria, considered to be the solace for aquatic systems tampered by oil spills, are known to be limited by P requirements. The ability of bacteria to

degrade hydrocarbons without P limitation, can be augmented when inoculated as a mixed bacterial culture, containing phosphatase producers. The presently investigated bacteria, due to their ability to synthesize copious amounts of phosphatases, which are active and stable at ambient temperatures and pH generally encountered in natural waters, can be considered to be adequate aids in assisting oil degradation.

The awareness of the presence of organophosphorus compounds in the Cochin backwaters invites bioremedial measures to eliminate the harmful contaminants. The fact that phosphatase producing bacteria efficiently degrade organophosphorus pesticides was reestablished in this study. This property of the bacteria in the estuary might be playing a significant role in preventing the pesticide levels in the estuary from increasing beyond the present tolerable limits.

Against this background, findings that phosphatase synthesizing bacteria capable of removing the toxic contaminants, such as petroleum hydrocarbons and pesticides, exist naturally is appeasing and invites measures to augment its efficiency in pollution abatement. This investigation forms the preliminary endeavor towards resolving a few serious threats having disastrous implications faced by the Cochin estuary. Investigations on the efficiency of these bacteria, especially under the demanding environmental conditions, including

competition from other species which the bacteria will encounter in the dynamic estuarine waters are further required to project these bacteria as efficient bioremediation tools. This investigation bestows us hopes for a better tomorrow.

- The findings of chapter 3 has been presented at the "International Conference On Microbial Biotechnology, Trade and Public Policy",
  Osmania University, Hyderabad, July 15-17, 2000.
  "Phosphatases from halophilic bacteria"
  Gopinath.S. and Saramma.A.V.
  (Web Publication). microbiologyou.com
- Chapter 4 (in part) has been presented at the International conference "New Horizons in Biotechnology-2001"
  RRL, Trivandrum. April 18-21, 2001.
  "Cultural conditions for the growth and acid phosphatase production by the marine bacterium *Streptococcus* sp."
  Gopinath.S. and Saramma.A.V.

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