1.1 Preface

Phosphate (Pi) is one among the most important essential residues in maintenance and inheritance of life, with far diverse physiological roles as structural, functional, and energy transduction. In nature, Pi is often a growth-limiting factor, being an essential constituent in all types of living organisms. It is a ubiquitous residue present in most biomolecules ranging from PPi to DNA. Many organisms have evolved complex regulatory systems to assimilate Pi efficiently and accumulate a Pi reserve. It is present in the phospholipids, which make the cellular membranes and the various intracellular compartments. Its function in energy transduction stores energy as high energy phosphate bonds in ATP, CP, other nucleotide biophosphate, high energy phosphorylated molecules like Phosphoenol pyruvate (PEP), polyphosphate (polyP), and it might be much more than what is known to date. Almost all the known biochemical processes for energy metabolism, synthesis and break down of carbohydrates, fats, proteins, nucleic acids and polyP need Pi residue as the essential moiety. It is involved in the various extra and intracellular signal transduction, excitation, action potential generation and propagation in all excitable tissues, nerves and muscular tissue. High-energy phosphate bond is the key in actin myosin interaction executing the muscle contraction, movements of the cells and organs ranging from fibroblasts, smooth muscles, heart etc.

Phosphate accumulation in wastewaters containing run off of fertilizers and industrial discharges is a global problem that results in algal blooms in bays, lakes, and waterways. The outbreak of algal blooms is one of the environmental concerns resulting from eutrophication in lakes and other surface waters (Ohtake et al., 1985). Algal blooms degrade water quality by producing an offensive odour and taste. The nuisance growth of algae renders boating and fishing difficult and discourages swimming. Excessive growth of algae consumes dissolved oxygen when aerobic bacteria leading to mass mortality of fish and other aquatic organisms decompose them. Algal toxin is also a serious problem in drinking water supplies.

Pi is found to be the limiting factor for algal growth in nature and it's removal from wastewater can effectively control the algal eutrophication of surface waters (Hammond, 1971). Activated sludge process used for wastewater treatment is very effective in removing organic pollutants but this removes Pi relatively poorly. To make activated sludge more effective in
removing Pi, it appears essential to enable sludge microorganisms to take up and store Pi in excess of their requirement for growth (Ohtake, et al., 1985). The uses of chemical methods like lime, alum or ferric chloride to remove Pi is expensive and inefficient. Currently biological phosphorus removal processes utilizing anaerobic and aerobic conditions have been adopted for sewage and wastewater treatment. Under aerobic conditions the activated sludge microbes accumulated excess Pi in the cell and it was released during the anaerobic phase. This principle is utilized in activated sludge process for Pi removal from wastewater, in which the Pi is effectively removed along with the sludge microbes (Ohtake, et al., 1985). In this process aerobically activated bacteria take up the phosphate and convert to polyphosphate (polyP), which is then removed along with the bacteria as a sludge.

Activated sludge with good phosphorus removal capacity shows clear phosphate release under anaerobic conditions and phosphate uptake under aerobic conditions (Cemeau et al., 1986). This release and uptake of phosphate corresponded stochiometrically to the change in polyP content of activated sludge (Cemeau et al., 1986).

Currently available methods for removing phosphates from wastewater are based primarily on polyP accumulation by the activated sludge bacteria (Ohtake, et al., 1985). However, because of the complexity of the sludge microbes and the limited knowledge about their polyP metabolism, the process operates essentially by the "black box" principle with less predictability and stability. These processes require the sludge microorganisms to be subjected to alternating aerobic and anaerobic cycles. Pi uptake by the sludge microbes takes place under aerobic conditions while Pi release under the anaerobic phase. The conventional wastewater treatment systems could be induced to accumulate phosphate significantly in excess to the requirement for the normal bacterial growth. This process is called enhanced biological phosphorus removal (EBPR) (Toerien, et al., 1990).

The characteristic feature of EBPR plant is the alternating anaerobic and aerobic phases where the influent wastewater and the return sludge are mixed together at the beginning of the plant with an anaerobic zone (no aeration) and an aerobic zone (aerated) at the end of the plant. Presence of anaerobic zone was found to be essential in order to obtain significant phosphate removal (Davelaar, et al., 1978). Phosphate is efficiently removed during the aerated zone after its release during the anaerobic phase, and the (EBPR) plant obtains almost complete removal of phosphate from the wastewater, in addition to the carbon and nitrogen removal. The microbial flora
of the activated sludge was found to be complex and the Pi removal mechanism remains less understood. The acidogenic bacteria (catalyzing degradation of complex substrates to acetate), nitrifying organisms (catalyzing oxidation of ammonium to nitrite and nitrate), denitrifying organisms (catalyzing conversion of nitrate to nitrogen) and the strictly aerobic bacteria have been studied (Toerien, et al., 1990). *Acinetobacter* sp. is reported to be important in phosphate removal of EBPR (Fuhs and Chen, 1975), and EBPR process is now widely used to remove excess phosphate form wastewater. Understanding how the energy state of the cell and the environmental phosphate levels affect polyP metabolism is essential for further improvement in efficiency and predictability of the system (Keasling, et al., 2000).

The economy of phosphate removal processes will be enhanced to be profitable if it is combined with fertilizer industries like ammonium phosphate, and ammonium polyphosphate industries which has rapidly grown over the last 10 years to meet the growing demand of ammonium phosphate in world wide agriculture. Basically, there is only one commercial method for producing ammonium phosphate and that is by the reaction of ammonia with phosphoric acid although many variations have been added in the process techniques and objective in the recent times. The fertilizer ammonium phosphate contains either orthophosphate or polyphosphates or mixture of both, containing a variety of polymeric forms mixed with some orthophosphate. The stream efficiencies of fertilizer plants are normally lower than the average in the chemical industry, because of the problems of corrosion, handling melts, slurries and particles. The microbiological processes can open newer ways of manufacturing large-scale phosphate and polyP containing fertilizers in harmony with the environment and maintaining environmental sanitation.

Microorganisms remove Pi from environment through phosphate uptake mechanism, which involves a biphasic pumping system in bacteria. The two pumping systems (i) Phosphate Inorganic Transport system (*Pit*), constitutively expressed in the cell (Wanner, 1996), is a less specific system and active at high Pi concentrations in their environment. Under these conditions, Pi is stored in the form of polyP reserve for future survival under Pi starvation. *Pit* is a mono component membrane channel. (ii) Phosphate specific transport system (*Pst*) - This becomes active when Pi level in the medium lowers, under conditions of Pi limitation, and serves as the major scavenger of Pi. *Pst* is a four-component membrane channel formed of the sub units, *PstS*, *PstA*, *PstB*, *PstC*, which are coded by the respective four genes. These four structural genes along with a regulatory site (*phoU*) together constitute an operon called *pho regulon* which regulates the *Pst* system.
Many organisms have evolved complex regulatory systems to assimilate Pi efficiently and accumulate polyP as a Pi reserve. The only known pathway for biosynthesis of polyP is from ATP by polyphosphate kinase (PPK). PolyP turnover is mediated by PPK, Exo I endopolyphosphatase, polyP glucokinase, polyP fructokinase, polyP adenylate kinase and polyP AMP phosphotransferase enzymes, indicating it’s physiological importance.

PolyP plays a critical role in several environmental and biotechnological problems. Understanding how environmental conditions affect native polyP metabolism and manipulation of polyP metabolism through genetic and metabolic engineering can ultimately lead to newer and cost effective processes to remove contaminants especially phosphate and heavy metals from the waste water, reassuring environmental health and sanitary conditions.

Biologically synthesized polyP is a linear polymer of a few tens to many hundreds of inorganic orthophosphoric acid (Pi) residues linked by high-energy phospo-anhydride bonds (Kulaev, 1975). PolyP has been detected in abundance in all the living forms ranging from the prokaryotes to mammals, plants, in the volcanic condensates, and deep oceanic steam vents, indicating that it can be formed spontaneously by simple condensation of orthophosphoric acids under high temperature. PolyP is present in the mammalian cells and sub-cellular organelles like mitochondria, lysosomes, while relatively higher in nuclei. PolyP is more abundant in microbes than in plants and animals (Kornberg, 1995).

In bacteria polyP accumulation occurs under conditions of nutritional imbalance unfavorable for growth (Harold, 1966). It has been shown that many bacteria exhibit rapid and extensive poly P accumulation, called polyP over plus, when Pi is added to cells previously subjected to Pi starvation stress (Harold, 1966). However, the mechanism underlying polyP accumulation is not clearly known.

PolyP is now thought to be as ubiquitous and more ancient than Nucleic acids (NA) and likely a prominent precursor of NA in prebiotic evolution. It is probably evident from its presence in volcanic condensates and deep oceanic steam vents. In spite its occurrence in every living organisms ranging from bacteria, fungi, protozoa, plants and animals including mammals (Kulaev, 1979), polyP has been ignored and dismissed as a "molecular fossil" (Kornberg, 1995). RNA preceded DNA and proteins in evolution, while PolyP might have appeared before any of these organic polymers, as ubiquitous and more ancient than nucleic acids, likely as a prominent precursor in prebiotic evolution.
Physiological roles implicated to polyP are many while the exact role of polyP is yet unknown. However, it is believed to have several roles: (i) as a source of energy due to ready conversion to ATP as well as other nucleotide triphosphates by PPK, (ii) as a cellular Pi reservoir, (iii) as a substitute for ATP in kinase reactions, (iv) as cellular chelator for metals - Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), (v) as a buffer against alkaline stress, (vi) as a regulator for transcription, (vii) in developing competence and forming the channel for DNA entry during transformation.

**Poly P has several applications such as:**

(i) as an ATP (NTPs) regenerating system in biochemical and industrial use. The cost of ATP for use as an enzymatic phosphorylating agent on an industrial scale is prohibitive as is the cost of agents, such as creatine P; and Phosphoenerol pyruvate, that might be used in an enzymatic ATP-regenerating system, polyP has been used in their place (Butler, 1977). (ii) As an antibacterial agent used in all processed meat, poultry, and fish products. In its use in virtually all processed meat poultry and fish products, polyP also serves as an antibacterial agent. (iii) As a safe additive to meat it enhances water binding, emulsification, colour retention, and antioxidant capacity. (iv) It is used in cheese, tooth paste, and drinking water. (v) Inhibitors of PPK might be effective broad-spectrum antimicrobial tools especially against antibiotic resistant bacteria. (vi) In depollution of phosphate from the environment. (vii) As a component of chemical fertilizers for slow and prolonged Pi release, have osmotic and pH advantage in the soil. (viii) As Insulating fibers. A calcium polyphosphate fiber has been synthesized with all the properties of asbestos and could be a safe substitute (Griffith, 1992). PolyP can be potentially employed to generate ATP using PPK in industrial processes, as a component of chemical fertilizers and its other economic market values might be of great help for promoting the use of engineered microbes for removing Pi from wastewaters.

Several strains have been isolated from the activated sludge with high polyP accumulation ranging from 4-10% dry weight, as in *Acinetobacter* (Fuhs and Chen, 1975) and *Arthrobacter globiformis* (Shoda et al., 1980) However, in spite of their high polyP, none of the above strains exhibited the characteristic aerobic Pi uptake and anaerobic Pi release properties of the activated sludge with acceptable Pi removal (Nakamura et al., 1991) isolated *Microlunatus phosphovorus* from the activated sludge which accumulated polyphosphate under aerobic conditions and released it under anaerobic conditions, with Pi accumulation of 10-20mg/g cell. Many microbes accumulate excess Pi as polyP under unfavourable growth condition such as low pH, anaerobiosis, sulfur starvation (Harold, 1966). Some bacteria take up Pi far in excess of their requirements for growth
and accumulate polyP after being subjected to Pi starvation by the predominance of PPK action during this condition (Harold 1963, Ohtake et al., 1985).

*Pseudomonas putida* HAS 29, isolated from a bench scale activated sludge system designed for enhanced biological Pi removal, showed the characteristic property of activated sludge, taking up Pi and accumulation as polyP under aerobic condition and release of Pi and polyP breakdown under anaerobic conditions (Ohtake et al., 1999).

Genetic improvement of bacteria to remove Pi from waste waters have been tested, using *E.coli* as the test organism, and the ability of *E.coli* MV 1184 to accumulate polyP was enhanced by modifying genetic regulation and increasing the dosage of the gene encoding PPK, Acetate kinase (ACK) and the *Pst* system (Vieira and Messing, 1987).

Accumulation of phosphate is a constant process in the marine environment resulting from the death and decay of the inhabitant plants and animals as well as from the inflowing waters carrying wastes from the land. Marine bacteria are thus exposed to higher Pi concentration in the environment and have become part of the active phosphate cycle of the marine ecosystem. Thus they are expected to have relatively better Pi uptake and polyP accumulation, besides tolerance to osmotic and toxic stress, for probable exploitation in the wastewater treatment. In this context of the need for new and better strains for phosphate removal from waste water and potential uses of polyP, an attempt was made in the present study to screen potential polyP accumulating bacteria.
1.2 Review of Literature

**Phosphate uptake**

Pi transport into bacterial cells is accomplished by several parallel transport systems (Wanner, 1996). Two of the better characterized Pi transport systems are the constitutively expressed, low affinity Pit system and the carefully regulated high affinity Pst system. The Pit system is a simple component transporter belonging to the group of systems which are energized by the proton motive force (Elvin et al., 1986).

![Diagram of phosphate uptake systems](image)

The Pst system is a periplasmic protein dependent transporter and belongs to the superfamily of ABC (ATP-binding cassette) transporters (Higgins, 1992). The Pst system is induced by Pi limitation and serves as a major scavenger of Pi residues under conditions of Pi limitation.

Bacteria use inorganic Pi as the preferred source of phosphate and its uptake is by a biphasic Pi transport system where one mechanism will function at a higher phosphate level and other operate at the Pi levels lower in their environment. When in excess, Pi is taken up by the Phosphate inorganic transport system (Pit) which is made constitutively in the cell (Wanner, 1996),
and under this condition bacteria can store Pi in the form of polyphosphate as a reserve for Pi and other functions (Kornberg, 1995). Since polyP can serve as a Pi source for the biosynthesis of nucleic acids, phospholipids and other biochemical molecules under the conditions of Pi starvation (Harold, 1963), its accumulation is likely to be a protective mechanism for survival during Pi starvation. Bacteria have evolved a much more complex system to survive during Pi starvation conditions. Under conditions of Pi limitation the phosphate specific transport (Pst) system is turned on, and this system serves as a major scavenger of Pi residue. When inorganic Pi is not available bacteria use other forms of phosphate like organophosphates (phosphate esters) inorganic phosphite (Pt) and phosphonates (Pn) as alternatives. Most organophosphates are not transportable as such and the phosphate has to be released from the source before being taken up by a process which involve hydrolytic cleavage catalyzed by a variety of enzymes secreted by the bacteria including alkaline phosphatase (Bap) which is made at very high levels under condition's of Pi starvation (Wanner, 1996).

Phosphonates are large class of organophosphorous molecules with direct carbon phosphorous (C-P) bonds unlike the (-C-O-P-) ester bond between carbon and phosphate. Hence, utilization of these compounds for Pi require cleavage of the C-P bond by the enzyme C-P lyase. However, Pt seems to be enzymatically oxidized to Pi before being used as a phosphate source, which is also induced by Pi starvation stress(Wanner, 1996).

Studies with Acinetobacter sp. have showed that the largest quantity of Pi was removed within the first hour during the lag phase compared to 24 hrs uptake (Muyima and Cloete, 1995) and the results also indicated that Pi was released slowly between 2 and 8 hrs and removed significantly after 24 hrs. Excess Pi removal was reported to occur mainly under aerobic conditions, while Pi uptake and release processes were reversible (Ohake, et al., 1985). In the activated sludge process, when the aerobic phase of the cycle exceeds 4 hrs, a slow release of Pi occurs even during the aerobic phase (Osborn and Nicholls, 1978). However, the intracellular phosphate accumulations vary according to the environmental factor (Kulaev and Vagabov, 1983).

The phenomenon of luxury uptake, polyP over plus and polyP accumulation, and release have been demonstrated in many bacteria. When, E.coli cells accumulated excessive levels of polyP, they released it into the medium, probably as a mechanism by which a further increase in cellular polyP is limited. This release was first observed during Pi uptake experiment with E.coli MV 1184 strain (Kato et al., 1993a). Rate of polyP release was essentially equivalent to that of Pi uptake after the cells accumulated excessive levels of it and stopped when the Pi in the medium
was removed completely and resumed on addition of Pi, to the culture. PolyP release was stopped when the Pi uptake was inhibited by 0.1 mM carbonyl cyanide m – chlorophenyl hydrazone (Hardoyo et al., 1994).

In *E. coli* PPK preferentially attaches to the outer membrane even though ATP is the substrate of the enzyme (Akiyama et al., 1992). This location of PPK and the lack of a leader sequence to translocate it to the outer membrane suggest that the enzyme may be present in Bayer patches, described as fusions of inner and outer membranes communicating directly between the cell exterior and interior compartments (Bayer, 1968).

*Klebsiella aerogenes*, which is closely related to *E. coli*, exhibits extensive polyP accumulation and there are two patterns for the process (Harold, 1966). When growth and nucleic acid synthesis are blocked by depriving the organism of sulfate, Pi uptake from the medium continues, resulting in polyP accumulation, and this phenomenon is called "luxury uptake" (Fuhs and Chen, 1975). This process is now known to occur in many bacteria, including those isolated from the activated sludge samples, when the growth is arrested by lack of a nutrient other than Pi. On the other hand, addition of Pi to *Klebsiella aerogenes* cells previously subjected to Pi starvation induces rapid and extensive accumulation of polyP and upon resumption of growth and nucleic acid synthesis, the polyP is gradually degraded by conversion to nucleic acids. This pattern of polyP synthesis is called **polyP overplus**. Potassium, Mg, and a source of energy are likely required for polyP overplus in *K. aerogenes* (Harold, 1966).

In *K. aerogenes* PPK activity increased in response to Pi starvation and decreased upon addition of Pi, but PPX activity did not increase during Pi starvation. This is in contrast to the response in *E. coli* where PPK and PPX levels were quite similar even under conditions of Pi limitation. The difference between PPK and PPX activities is likely responsible for polyP overplus in *K. aerogenes* (Ohtake, et al., 1999). The genetically modified *Klebsiella* (ATCC 9621) bearing multicopy own *ppk* removed approximately 80% of the Pi from the medium while normal strain removed only 50% with in the first 2 hrs. It accumulated 0.9µM polyP per mg protein and as Pi 20% of its dry weight. However, multicopy *ppk-ppx* did not improve Pi uptake. The results suggest the potential of genetic improvement of *K. aerogenes* for enhanced polyP accumulation (Ohtake, et al., 1999).

With the cloning of the gene encoding PPK it has become possible to genetically engineer polyP accumulation in bacteria which are needed to improve the maximal Pi uptake efficiency of the
useful strains that dominate the flora of activated sludge especially *Acinetobacter* strains (Kornberg, 1995). PPK has been cloned in *E.coli* showing enhanced rate and extent of Pi removal from the medium, which when coupled with cloning of *pst* gene for Pi specific transport Pi uptake and polyP accumulation reached as high as 38-48% of the dry weight of the cell (Hardoyo et al., 1994).

Understanding the fundamentals regarding the biochemical mechanisms and genetics of bacterial Pi transport and metabolism is essential for improving their abilities to remove Pi from waste waters as well as their perspective application in other areas (Ohtake, et al., 1996). To date the available informations on *ppk* is limited to that from *E.coli* and *Klebsiella aerogenes*. Moreover, understanding PO₄ metabolism can add to the basic knowledge of cell biology, the normal and abnormal, and genetic improvement of the bacterial polyP accumulation will serve as the first step to make sludge microorganisms more effective in Pi removal from waste waters. There is a growing interest in the role of polyP accumulation in biological Pi removal from waste waters (Hardoyo et al., 1994).

Genetic improvement of bacterial polyP accumulation may serve as the first step to make sludge microorganisms more effective in removing Pi from wastewaters. Pi uptake studies using the engineered and wild strain *E.coli* showed increasing the dosage of *ppk* gene alone doubled the Pi uptake and Pi content of *E.coli* (Kato et al., 1993 a). Introduction of *ppk* and acetate kinase (*ack*) genes into *E.coli* (MV1184) resulted in much more improvement than *ack* alone, attaining almost 90% Pi removal within 4 hrs growth.

Efficiency of *E.coli* MV 1184 (Vieira and Messing, 1987) to accumulate polyP was enhanced by modifying genetic regulation and increasing the dosage of the gene encoding PPK, Acetate kinase (ACK) and the *Pst* system. Kinetic analysis suggested Pi transport across the cell membrane as the possible rate-limiting step for polyP, accumulation in *E.coli*. When recombinant *E.coli* accumulated high levels of polyP, they released phosphate compounds into the medium (Hardoyo et al., 1994). PolyP release might be probably a mechanism by which a further increase in cellular polyP is limited and the rate of this polyP release was found to be dependent on that of Pi uptake. However, no polyP release was observed after the complete removal of Pi from the medium by the cells and resumed polyP release soon after the addition of Pi into the medium.
Effect of Environmental factors on phosphate uptake and cellular metabolism

The uptake and storage of phosphate was influenced by the external pH in mycorrhizal roots of pine and the fungus of *Suillus bovinus* (Thomas and Anke, 1997). External pH in the range of 3.5-8.5 influenced the Pi metabolism in mycorrhizal roots and the fungus in pure culture used the accumulated Pi as mobile polyP, while the internal pH was found to be constant. The Pi uptake rate and polyP accumulation responded differently to external pH. In all cases, maximal Pi uptake occurred at an external pH close to 5.5 and at pH 8.5, both the roots and the fungus showed a distinct lag in Pi uptake, which was reversed when the external pH was lowered to 7.5.

An irreversible effect on Pi uptake was also observed as a consequence of variation in external pH. In the upper range of external pH, Pi uptake and storage was strongly inhibited as a consequence of insolubility of phosphate at pH 8.5, as at this pH uptake was not possible and polyP concentration was maximum at pH 7.5. An external pH above 9 may cause increase in internal pH (Torimitsu *et al.*, 1984).

Three mechanisms have been proposed, which maintain this constant internal pH. They include (i) a purely passive inflow and outflow (ii) an active mechanism driven by Na+/K+ pump (iii) an energy consumptive, Na- coupled Cl – HCO$_3$ exchange (Roos and Boron, 1981). A passive mechanism cannot keep internal pH constant for longer periods of 3-4 hrs. An ATP requiring active mechanism must be involved and in higher plants one of these mechanisms is metabolically controlled using malate dehydrogenase / phosphoenolpyruvate carboxylase (Davies, 1973).

In addition, intracellular pH is maintained by proton transport (Serrano, 1984) and co-transport of anions (Hager *et al.*, 1981, 1984) by a H$^+$ATPase (Rea and Poole, 1986) and a pyrophosphatase (Hager *et al.*, 1986). In roots a pool of energy is commonly represented by sugar phosphates. It has been postulated for mycorrhizal fungi that vacuolar polyphosphate could be an energy source. Non mycorrhizal roots did not show polyP at any pH (Harley *et al.*, 1954, Martin *et al.*, 1985).

At pH 8.5 there was no detectable Pi uptake, which may be due to an increase in insoluble CaHPO$_4$ complex in the medium decreasing the available phosphate with an increase in pH, cycle 6.5 → 8.5 → 6.5, while, returning back to pH6.5 led to increase in available Pi, uptake, and polyP (Thomas and Anke, 1997). Several divalent cations like Mg$^{2+}$, CO$_2^-$, Mn$^{2+}$, Zn$^{2+}$ stimulate the yeast mitochondrial soluble polyphosphatase (Lichko, *et al.*, 2000). Mg$^{2+}$ is also a prosthetic group in
pyrophosphatase enzymes. Ca\(^{2+}\) replace Mg\(^{2+}\) from the inorganic pyrophosphatase of *E.coli* inhibiting the enzyme activation and catalysis. Ca\(^{2+}\) is a powerful inhibitor of all known pyrophosphatases (Avaeva, *et al.*, 2000).

Studies on *S. cerevisiae* have showed that the length of the polyphosphate chain is dependent on the orthophosphate content in the culture medium. When grown in a complete medium during the early hours of growth yeast accumulated low molecular mass chains initially followed by elongation of the chains to high molecular mass polymers, later. After 7 hrs of phosphate starvation the yeast used the phosphate reserve in the form of polyphosphate in the various cell compartments almost completely to support their vitality, which was evidenced by considerable shortening of the polyP chains during starvation. On a complete medium, there was initially active synthesis of short chains. During the early logarithmic phase the degree of polymerization declined drastically and the high polymer polyP chains were detected at the late stationary phase, when the synthesis was stopped (Vagabov, *et al.*, 2000).

Based on a comparative analysis of cellular Pi and polyP in 8 strains of the photosynthetic bacteria (4 each from seawater and fresh water), one marine *Chromatium* Sp. strain was selected and the effect of NaCl and seawater on intracellular Pi and polyP was studied. Intracellular phosphate and polyP content increased up to 280mM NaCl and 40-50% seawater was found to be optimum (Hiroaki *et al.*, 1997).

**Poly phosphate (PolyP)**

Polyphosphate was observed as the metachromatic granules in yeast for the first time by Liebermann (1888). However, it was only after the studies conducted by Wiame (1947), Ebel (1948), Kornberg *et al.*, (1956), Belozersky (1958), Lohmann (1958) and others during the late 1940 and 1950s this biological molecule received due attention of the scientific community. The metachromatic granules were used as a diagnostic feature of medically important bacteria like *Corynebacterium diphtheriae* during the last two centuries and it was thought to be nucleic acid particles. Decades later, Wiame (1947), while viewing under an electron microscope, observed these granules to disintegrate and volatalize by the beam of electrons unlike the nuclear materials and later recognized them as a polymer of inorganic orthophosphoric acid residues. Later, an enzyme in *E.coli* that formed the polymer of inorganic phosphate from ATP and readily converted the polymer back to ATP was found and identified as polyphosphate kinase (PPK) (Kornberg *et al.*, 1956).
PolyP arises from Pi by simple dehydration and condensation at elevated temperatures which is evident in the volcanic condensates and deep oceanic steam vents (Yamagata, et al., 1991). The anhydride bond energy and Pi of polyP are possible sources for nucleoside triphosphates which form the building blocks of RNA and DNA (Waehneldt and Fox, 1967, Kulaev and Skryabin, 1974). Mixed carboxylic – phosphate anhydrides provide a route to chemical polypeptide synthesis starting with amino acid and polyP (Harada and Fox, 1965). Among the species of phosphates special mention should be made for the simplest member, pyrophosphate (PPi) which was believed solely as a metabolic product in the various biosynthetic reactions and hydrolyzed by the potent enzyme inorganic pyrophosphatase to drive these pathways (Kornberg, 1957). Later studies showed PPi to be a substitute for ATP (Wood, 1985). A role for PPi as well as for the long chain polyP in prebiotic events leading to the evolution of ATP deserves attention (Kornberg, 1995).

PolyP accumulation is also prominent in Archaebacteria and may be the substrates for enzymatic attack by nucleoside, mono, di or triphosphates. A systematic search among these ancient organisms might uncover enzymes that carry out such salvage reactions in the biosynthesis of nucleiotides, co-enzymes and other factors.

Despite the prominence of polyP in many organisms such as in the vacuolar deposits of yeast cells, which may represent 10-20% of cellular dry weights, this molecule remains least attended. Studies of Harold (1966), Kulaev (1987) Wood (1988) and few others disclosed the
ubiquity of polyP and identified a few related enzyme activities. Almost 99% of the yeast cellular polyP is seen in their vacuoles.

Several enzymes have been purified and used for studies on polyP metabolism (Ahn and Kornberg, 1990, Akiyama, et al 1993, Wurst and Kornberg, 1994). They include PPK and exo polyphosphatase from *E.coli* (PPX2) and exo poly phosphatase from *S. cerevisiae* (PPX 1). Two more enzymes available for polyP analysis are polyP glucokinase and polyP phosphotransferase which attack the terminal residue of polyP with glucose (Hsieh et al 1993) and AMP respectively (Bonting et al; 1991). PolyP is more abundant in microbes than in higher forms and the diversity of accumulation range from its undetectable level in *E.coli* to as high as 20% dry weight in *S. cerevisiae*.

Although the presence of polyP had been noted widely in prokaryotes, fungi and algae, the distribution and abundance of polyP in more complex eukaryotic forms remained uncertain. The very low levels in animal cells and sub-cellular compartments left polyP obscure till recently due to lack of definitive and sensitive methods to analyse and study its metabolic and functional role (Gabel and Thomas, 1971). Recent exploratory studies with improved enzymatic assay methods have confirmed presence of polyP in a wide variety of cells in cultures and animal tissues. The concentration of which generally range from 10-100μM as Pi equivalents and in sizes of 100-1000 residues (Kumble and Kornberg, 1995). Among the sub cellular organelles, polyP has been identified in lysosomes (Pisoni and Lindley, 1991) and in mitochondria (Kornberg, 1995) and is relatively enriched in nuclei (Kumble and Kornberg 1995).

*Biosynthesis of polyP*

Biologically synthesized polyP is a linear polymer of few tens to many hundreds of orthophosphoric acid residues linked by high-energy phosphoanhydride bonds. Polyphosphate kinase (PPK) enzyme synthesise polyP from ATP with a more favoured reverse reaction.

\[
\text{nATP} \xrightarrow{\text{PPK}} \text{poly (P)n} + \text{nADP.}
\]

PPK purified to homogeneity from *E.coli* catalyzes the readily reversible transfer of the terminal (i) phosphate of ATP to polyP. The enzyme is a homotetramer of 80 K.D. subunits. With ADP in excess PPK converts nearly 90% of the polyP to ATP which was identified by using 14
CADP and $^{32}$P – polyP. The only known pathway for this synthesis of polyP is from ATP PPK (Ahn and Kornberg, 1990).

The Gene encoding this kinase, *ppk*, is part of an operon immediately upstream to the gene for exopolyphosphatase (PPX), *ppx*, thus along with a regulatory site constituting *ppk/ppx* operon (Akiyama et al., 1992). PPX of *E.coli*, a dimer of 58 KD subunits, hydrolyzes the terminal residues of polyP to Pi recessively and nearly completely with a strong preference for long chain polyP (Akiyama et al., 1993).

A third enzyme exopolyphosphatase isolated from *Saccharomyces cerevisiae* (*ScPPX1*) is the most powerful of them, releasing as high as 30,000 Pi residues per min/ enzyme molecule at 37°C (Wurst and Kornberg, 1994). It acts with 40 times the specific activity of *E.coli* PPX and exhibit a far broader size range for polyP from 3-1000 residues. Cloning the gene for this enzyme in *E.coli* have enabled over production of the enzyme. It is suggested that this enzyme may be used to remove the polyP contaminating any DNA preparations besides use as an analytical reagent for polyP (Rodriguez, 1993). The gene encoding PPK (*ppk*) has been cloned, sequenced, knocked out and even over expressed in *E.coli* (Akiyama et al, 1992). It is located at 53.4 min of *E. coli* linkage map. The open reading frame encodes a sequence of 687 amino acids (mass of 80.278 KD), and the enzyme is a homotetramer.

PPK from *Klebsiella aerogenes* have been cloned and the nucleotide sequence analysis of *ppk* of *Klebsiella aerogenes* showed that the PPK protein shared 93% amino acid residues with *E.coli* PPK protein (Kato et al., 1993a). PPK- polymerizes the terminal Pi of ATP into polyP in a freely reversible reaction (Kornberg, 1995). Utilization and degradation of polyp is catalyzed by exopolyphosphatase (PPX) and several polyP specific kinases including, polyP glucokinase and polyP fructokinase (Kulaev, 1975). Both PPK and PPX seem to be made constitutively in *E.coli* and polyP accumulation is not induced by Pi starvation stress in *E.coli*, but as a result of stringent response (Kuroda and Korenberg, 1997a).

To date the genes encoding PPK have been cloned and sequenced from a group of bacteria, including *E.coli* (Akiyama et al., 1992). *K. aerogenes* (Kato et al., 1993b) *Neisseria meningitidis* (Tinsley and Gotschlich, 1995), *Acinetobacter calcoaceticus* (Geissdorfer et al., 1995), and *Pseudomonas putida* HAS 29 (Chandrasekaran, 1998). *Pseudomonas putida* PPK gene codes
polypeptide chain of 741 amino acid, which showed only 32% homology with amino acid sequence of *E.coli* PPK, while 53% with *A. calcoaceticus* PPK. (Ohtake et al., 1999).

Polyphosphate plays an important regulatory role in the virulence of pathogens, gene knock out mutants of *P. aeruginosa* lacking PPK lack motility, quorum sensing, biofilm formation and as a result are turned avirulent (Kornberg, 2000).

The ATP regenerating system consisting of ADK, PPK, and polyP was shown to be promising for practical utilization of polyP as ATP substitute (Kornberg 1995). In *E.coli* highest amount of high polymer polyP occurs only in the end of latent and beginning of logarithmic growth phase. When the culture passes to exponential growth, the level of intracellular polyP dramatically decreases. Thus polyP accumulation precedes active growth and it is utilized by the growing cells (Nesmeyanova, 2000). Several enzymes are known to utilize polyP confirming the diversity of polyP functions in cells, and they include polyphosphatase, polyP mannokinase and polyP glucokinase (Kornberg, 1995).

PolyP is involved in the mineralization process in bone tissue. Osteoblasts contain higher concentration of polyP and exopolyphosphatase which can release the phosphate required for calcium precipitation during the enzyme process and both respond to modulators of the osteoblasts (Leyhausen, et al 1998) PPK is required for adaptation at the onset of amino acid starvation. The adaptation to amino acid starvation is mediated by the network of stringent response and polyP metabolism. PolyP accumulation accompanied by increased levels of stringent factors in response to amino acid starvation was observed in *E.coli* (Kuroda and Ohtake, 2000)

### Applications of Polyphosphate

**ATP substitute and Energy Source**

Polyphosphate kinase (PPK) converts polyP to ATP by catalyzing an ADP attack on the termini of the polyP chain. An aggregate of polyP seen associated with the membrane bound PPK could generate large amount of ATP at that spot. Another source of ATP is from AMP attack an polyP by AMP phospho transferase forming ADP.

AMP phosphotransferase has been purified from *Acinetobacter* (Bonting et al., 1991), in *E.coli* and *Myxococcus xanthus* (Kornberg 1995). In yeast the usual cellular ATP level is 5-10 mm while the massive vacuolar polyP deposits exceeds 200mm and in Myxobacteria cell in stationary phase the granular aggregate can reach upto 50mM. It can also replace ATP in phosphorylation of
glucose by polyP glucokinase which use either ATP or polyP as the donor, a more phylogenetically ancient species showed a preference for polyP over ATP (Hsieh et al., 1993). The ATP regenerating system consisting of ADK, PPK, and polyP was shown to be promising for practical utilization of polyP as ATP substitute (Kornberg, 1995).

Thus in view of its energy equivalence to ATP, polyP qualifies as an ATP substitute in all its kinase roles involving a variety of acceptors and for active transport of nutrients and metabolites across the membranes, indicating the diversity of physiological functions of polyP.

Reservoir for Pi

A stable level of cellular Pi, essential for normal cellular metabolism and growth is insured by a cellular Pi reservoir into which polyP contribute by the action of exopolypophosphatase action (Kornberg,1995). Cellular polyP as an aggregate, complexed with multivalent counter ion, enjoys a clear osmotic advantage over free orthophosphoric acid, which is highly acidic (Kornberg 1995).

Chelator of metal ions:

PolyP, being a poly anion, is a strong chelator of metal ions. *Lactobacillus plantarum* lacking super oxide dismutase that removes super oxide free radicals has an extra ordinary high level of Mn$^{2+}$ (30mM) chelated to 60mm polyP (Archibald and Fridovich, 1982). In yeast the regulation of cellular Ca$^{2+}$ by vacuolar Ca$^{2+}$ depends on its binding to polyP, which acts as Ca$^{2+}$ sink within the vacuole lumen (Dunn, et al,1994). Chelation of Ca$^{2+}$ and Mg$^{2+}$ essential for the cell wall structure in Gram +ve bacteria might be the mechanism for antibacterial action of polyP. Chelation of other metals like Zn, Cu, Fe, Cd may either reduce their toxicity or affect their functions(Lee et al., 1994).

Buffer against alkali ions

As in yeast, algae accumulate polyP in vacuoles. The halotolerant green algae *Dunaliella salina*, deposit polyP as high as 1 M in Pi equivalents and when stressed at alkaline pH, amines enter the algal vacuoles and are neutralized by the protons released by the enzymatic hydrolysis of polyP, thus providing a high capacity buffering system protecting the cellular cytoplasmic pH. This algae is cultivated as a food source in large out door ponds (Pick and Weiss, 1991).

Channel for DNA entry

Though the transformation of competent *E.coli* for genetic engineering is a commonly used technique and despite the wide use of Ca$^{2+}$ recipe to induce competence, the mechanism by which
the highly charged DNA penetrates the lipid bilayer cell membrane remains least understood. The discovery of polyhydroxy butyrate (PHB) complex formation with Ca\(^{2+}\) and polyP in the competent cell membrane was a significant advance (Reusch and Sadoff, 1988). In a proposed model, the Ca\(^{2+}\) is bounded by ion dipoles to the carbonyl ester groups of PHB and by ionic interactions with polyP. This complex might produce profound preferred physical changes in the competent cell membranes, increased rigidity at ambient temperatures and biphasic melting (Reusch and Sadoff, 1988). Whether and how these alterations facilitate DNA entry remains unclear although PPK mutant \(E. coli\) lacking long chain polyP develop short chain (60 residues) polyP during development of competence (Kornberg, 1995).

**Regulator for stress and survival**

PolyP readily interacts with basic proteins (eg. Histones) and with basic domains of non histone nuclear proteins (Offenbacher and Kline, 1984). Such interactions can affect gene functions in positive or negative ways. Its presence in several sizes and complex forms in various cellular compartment and location, and fluctuation in response to nutritional and other parameters are suggestive of functions in the network of responses to stress and the many signals that govern stages in cell cycle and development. It might have roles in the multiple metabolic adjustments during the stationary phase of the cell cycle, “life after the log” in \(E. coli\) in a dynamic interval in which many genes are induced to cope with the environmental stress to ensure survival (Siegele and Kolter, 1992).

PPK mutants lacking long chain polyP showed no phenotypic changes in the exponential phase of growth, while the stationary phase exhibited striking differences and deficiencies (Crooke et al., 1994). The mutant survived less well, less resistant to heat, oxidants and osmotic challenges, and shifts to a small colony phenotype, all of which are suggestive of an adaptive phenotypic change (Harris et al., 1994). Thus polyP may enter in the cascade of events that prepare cells for coping with “life in the slow lane” (Kornberg, 1995). \(E. coli\) accumulated polyP in response to a nutritional down shift from a rich to a minimum medium (Ault-Rich et al., 1998; Rao et al., 1998).

\(E. coli\) mutants lacking PPK and PPX failed to accumulate polyP and exhibited an extended lag phase of growth recovery during the nutritional down shift and the lag was abolished by the introduction of normal PPK gene. This phenomenon was attributed to an impaired adaptation to amino acid starvation, because supplementation of amino acid to the mutant \(E. coli\) abolished the extended lag and resumed it on removal of amino acid from the medium. The levels of ppGpp
remained high for a longer time in the mutant compared to the transient increase in ppGpp in the wild type, when exposed to nutrient down shift, all indicating ppk as important for the rapid recovery from amino acid starvation (Kuroda and Kornberg 1997b, Kuroda et al., 1999). In E. coli amino acid starvation induced enzymes for amino acid synthesis, which is necessary for cells to adapt to starvation (Winkler, 1996). Upon starvation and during the stationary phase pppGpp and ppGpp, the stringent factors, elevations preceeded polyP increase and induced over 40 enzymes for intracellular protein digestion to generate amino acid required to synthesize essential enzymes for adaptation under down shifted conditions (Yen, 1980).

PPK mutants, defective in polyP failed to increase protein degradation and starvation specific enzyme synthesis leading to sustained increase in ppGpp level, and extended growth lag (Kuroda et al., 1999). ppGpp is important for the accumulation of polyP and plays a direct or indirect role in protein degradation while ppk-ppx mutants could not mediate it even in the presence of elevated ppGpp, indicating that polyP could be the direct or indirect mediator of protein degradation (Kuroda et al., 1999). Only little is known about the degradation pathway that is activated in response to nutritional deprivation while PolyP accumulation take place when cells are not actively multiplying i.e., during the lag and stationary phase (Harold, 1963; Lawson and Tonhazy, 1980).

_Regulator of Development_

During the vegetative growth in _M. xanthis_ there is 10-fold increase in polyP and polyP – AMP phosphotransferase activity. PolyP may be an energy source for fruiting bodies, and for deposition in spores when present at concentrations as high as 50mM. During the stationary phase stress response increase in ppGpp precedes polyP formation and mutants that fail to synthesise ppGpp also fail to increase their polyP levels. ppGpp seems to have a regulatory role in polyP formation (Kornberg, 1995). Developmental changes in microbes like- fruiting body and spore formation in _Myxobacteria_ sp, sporulation in bacteria (eg: _Bacillus_ sp.) and fungi, and heterocyst formation in cyanobacteria (eg: _Anaba_ sp.), occur in response to starvation of one or another nutrients and stress, and the stationary phase polyP may be involved in these cellular adjustments to deprivation.
Component of Cell capsule

In Neisseria sp almost half of the total cellular polyP occur loosely attached to the cell surface and polyP appears as a component of the cell capsule, and whether this can contribute to pathogenesis of these bacteria is yet to be understood (Tinsley, et al, 1993).

Bioremediation

Many studies have observed an apparent relationship between polyP and increased resistance to heavy metals. It has been proposed that cells use polyP to detoxify heavy metals once they have entered the cell (Pettersson, et al, 1985). These observations indicate potential application of polyP accumulating microbes towards bioremediation of heavy metal contamination in wastewater. Genetically engineered P. aeruginosa expressing a plasmid encoded inducible ppk accumulated large quantities of polyP and removed uranyl from solution, forming uranyl phosphate complex deposited on the cell surface. PolyP has been found to be distributed in the periplasmic area and bacterial cell surface in large amount. Metal phosphates are highly insoluble and will precipitate on cell surface (Montgomery, et al, 1995).

Marine environment remains almost unexplored with respect to polyP accumulating bacteria except for a few reports. Seawater is rich in cations and these have been reported to increase Pi uptake and stimulation of polyP formation in a variety of microorganisms (Healey, 1982, Van Groenestijn et al., 1988). In the marine Chromatium sp. studied polyP / Pi ratio was found to be 2.1 with intracellular Pi content of 0.865 μM/mg dry weight. The marine Chromatium sp. strain was evaluated for the effect of NaCl and sea water on intracellular Pi and polyP. Intracellular phosphate and polyP content increased up to 280mM NaCl and 40-50% seawater was found to be optimum (Hiroaki et al., 1997), while E.coli recorded 1.1 and 0.645 respectively (Kato et al., 1993a).

The seawater contains all the elements of the periodic table and it is relatively rich in Pi, Ca, Mg, and Na compared to fresh water, the cations are reported to increase Pi uptake and PolyP accumulation. Moreover accumulation of phosphate is a constant process in the marine environment resulting from the death and decay of the inhabitant plants and animals as well as from the in flowing waters carrying wastes from the land. Marine bacteria are thus exposed to higher Pi concentration in the environment and have become part of the active phosphate cycle of the marine ecosystem. Thus they are expected to have relatively better Pi uptake and polyP accumulation, besides tolerance to osmotic and toxic stress, for probable exploitation in the wastewater treatment.
1.3. Scope of the Present Study

There is absolute dearth of knowledge on phosphate uptake and polyphosphate accumulation by marine microorganisms and there is immense scope for probable industrial application of marine polyphosphate accumulating bacteria in future. Hence, the present study was carried out with the following major objectives.

- Screening of polyphosphate accumulating bacteria from the sea water and sediments of coastal environments of peninsular South India, both from east of Tamilnadu and west coast of Kerala
- Selection of potential strains that could show impressive phosphate uptake pattern, and polyP accumulation during the course of growth
- Impact of various environmental and nutritional factors on the rate Pi uptake and polyP accumulation