POLYPHOSPHATE ACCUMULATION BY MARINE BACTERIA

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CERTIFICATE

This is to certify that the work presented in the thesis entitled "POLYPHOSPHATE ACCUMULATION BY MARINE BACTERIA " is based on the original research done by DASAN E.V. under my guidance and supervision at the Department of Biotechnology and no part there of has been included in any other thesis for the award of any degree.

M. Chandrasekaran

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1.INTRODUCTION

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. It's function in energy transduction stores energy as high energy phosphate bonds in ATP, CP, other nucleiotide 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 poly,P 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 booms 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 lastly 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 / 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 phospho-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 N.A in prebiotic evolution. It is probably evident from its presence in volcanic condensates and deep oceanic steam vents. In spite its occurence 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 for 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 Phosphoenol 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).

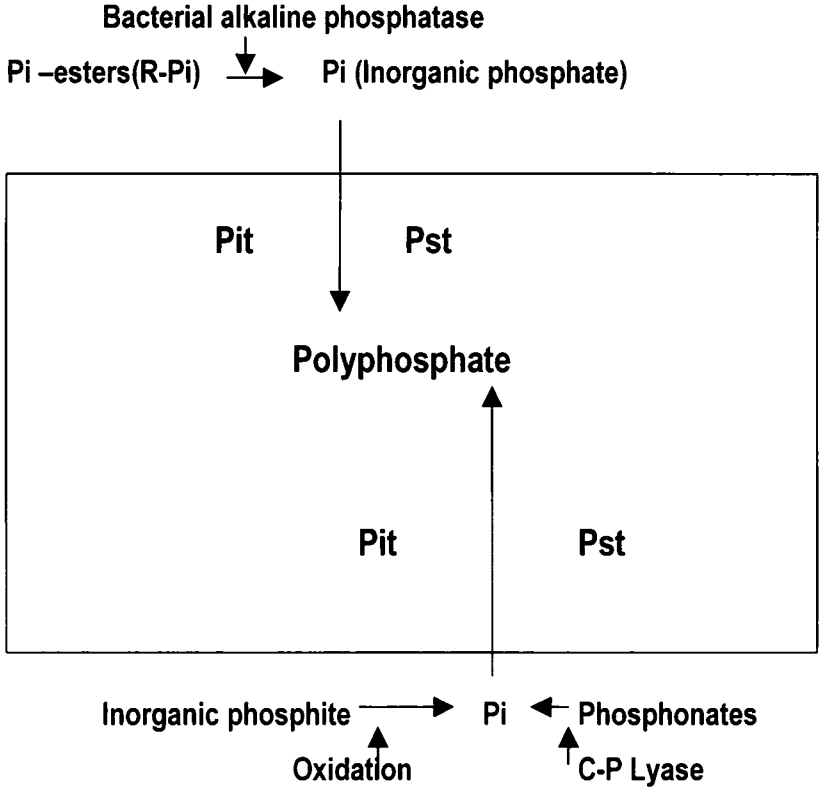
Genetic improvement of bacteria to remove Pi from waste waters have been tested, using *E.coli* as the test organism, and the ability of *E.coli* MV 1184 to accumulate polyP was enhanced by modifying genetic regulation and increasing the dosage of the gene encoding PPK, Acetate kinase (ACK) and the **Pst**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



The Pst system is a periplasmic protein dependent transporter and belongs to the super family 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 0.1mM 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 PO_4 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

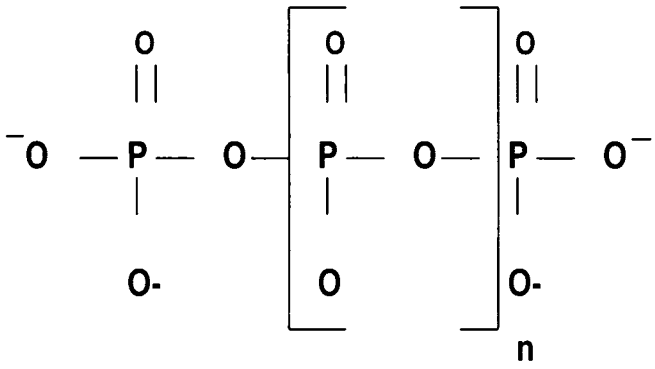
pyrophosphatase enzymes. Ca^{2+} replace Mg^{2+} from the inorganic pyrophosphatase of *E.coli* inhibiting the enzyme activation and catalysis. Ca^{2+} is a powerful inhibitor of all known pyrophosphatases (Avaeva, *et al., 2000*).

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).



Structure of inorganic polyphosphate chain

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 nueleoside, 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. cereviseae* (PPX 1). Two more enzymes available for polyP analysis are polyP glucokinase and polyPphosphotransferase 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. ceviseae*.

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.

$$nATP \xrightleftharpoons{PPK} poly\ (P)n + nADP.$$

PPK purified to homogeneity from *E.coli* catalyzes the readily reversible transfer of the terminal (▯) 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}