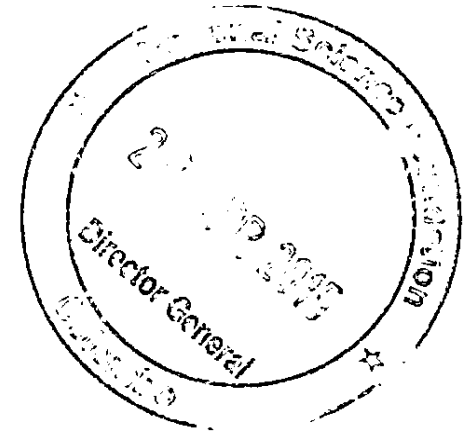


Final Report



FR1700

Contract Number: RG/2010/BT/03

Title of the Project: Isolation and Typing of Efficient Rock Phosphate Solubilizing Bacteria for use as Biofertilizers

FR 1700

Section 1

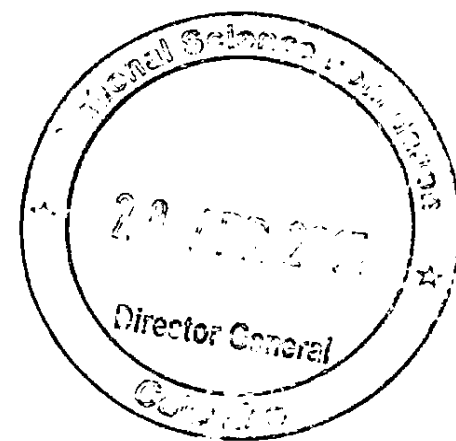
Information regarding Project/Project Personnel

- i) **Contract Number:** RG/2008/BT/01
- ii) **Title of the Project:** Isolation and Typing of Efficient Rock Phosphate Solubilizing Bacteria for use as Biofertilizers
- iii) **Principal Investigator:** Dr. A.A.L. Ratnatilleke
- iv) **Co-Investigators:** Nil.
- v) **Institute where Research is being carried out:** Department of Chemistry,
University of Kelaniya
- vi) **Date of award:** ...15.../...10... / ... 2008.....
(First installment was released on 06 /06 / 2010)
- vii) **Date of completion of project:** ...15.../ 01 /...2013.....
- viii) **Total allocation of funds:** Rs...1,695,741.66 #
- ix) **Total spent:** Rs...1,681,928.64 #
- x) **Number of Research Students employed:** two
- xi) **Post graduate degree completed with dates:** *M.Sc* thesis in progress.
- xii) **Number of Technical Assistants and/or laborers employed and period of service:**
Nil.

Subjected to confirmation by the Accounts branch, University of Kelaniya

Section 2

Executive Summary of the project



The objective of this study was to isolate and characterize efficient phosphate solubilizing bacteria (PSB) from Eppawala rock phosphate (Rock-P) deposit, Sri Lanka, and to evaluate their ability to solubilize Rock-P and to promote early growth of rice. Twenty PSB strains were isolated from Eppawala rock phosphate deposit. Evaluation of their ability to solubilize CaHPO_4 , AlPO_4 and FePO_4 and Rock-P on modified Pikovskaya agar and liquid media, revealed six efficient Rock-P solubilizers and were chosen for further studies. The strain EP-11 released, the highest amount of soluble P, 118 ppm in liquid medium containing Rock-P (1% (w/v), <0.037 mm). P Solubilization activity of all six strains occurred with significant drop in culture pH. Characterization of six strains by 16S rRNA-RFLP genotyping revealed that they belong to three RFLP genotypes. The isolate EP-11 remarkably improved the soil available P, rice plant height, biomass and P uptake of traditional rice variety Suduheenati and modern variety BG352 grown in pots- cultures supplemented with Rock-P as the sole P fertilizer. The most prominent effect on growth of rice seedlings was observed on Suduheenati variety where soil P, plant shoot length, dry weight, and plant P content were increased by 226%, 80%, 300%, and 70%, with respect to uninoculated control. Whereas with Bg 352 those values remained 99%, 48%, 150% and 27% respectively. The present results demonstrate that capability of this strain to convert insoluble Rock-P into plant available forms and therefore hold a great potential for development as biofertilizer to enhance soil fertility and promote plant growth.

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(Section 2)

Section 3

Report in Detail

i. Introduction

1.1 Need for fertilizer

Sri Lanka is basically an agricultural country. The economic stability of Sri Lanka is mainly dependent on the agricultural yield. Modern agriculture is evolving with much greater emphasis on the efficiency of the production process than in the past years with an aim of increasing production and productivity. The process has gathered momentum with the development of high yielding varieties in response to the challenge of feeding rapidly increasing human population. Although, the technology has already made an impact, the crop yields in most developing countries still continue to be low relative to their genetic potential. A major factor contributing to this gap is the heavy dependence on industrially manufactured chemical fertilizers.

1.2 Chemical Phosphorus Fertilizers Application

Many soil used for agriculture in Sri Lanka are infertile with low level of essential plant nutrients, especially nitrogen (N), phosphorus (P). Deficiency of P is so extreme in many soils that without adequate application of this element, investment in other agricultural technologies such as use of N fertilizer and legume for biological nitrogen fixation may not be effective to increase crop productivity. Presently the major fraction of total P inputs for the crops, in Sri Lanka is provided by application of water soluble super phosphate fertilizer. In addition, intensification of agricultural production in Sri Lanka necessitates the addition of phosphate not only to increase crop production but also to improve soil phosphatic status in order to avoid further soil degradation. However, lack of domestic P fertilizer industry and limited availability of foreign exchange for fertilizer imports have made it more expensive and hence less affordable by local farmers.

1.3 Importance of Phosphorus for Plants

Phosphorous is an important plant nutrient, next only to nitrogen and classed along with nitrogen and potassium as a major plant nutrient element. It is associated with several important role in plant metabolisms such as cell division, development, photosynthesis, N_2 fixation, energy transfer (ATP), signal transduction, macromolecular biosynthesis (DNA and RNA), respiration and regulation of metabolic pathways (Tandon 1987). The plant obtains their P requirements from soil solution as orthophosphate anions, predominantly $H_2PO_4^{4-}$ and HPO_4^{2-} ions.

1.3.1 The Phosphorus Cycle

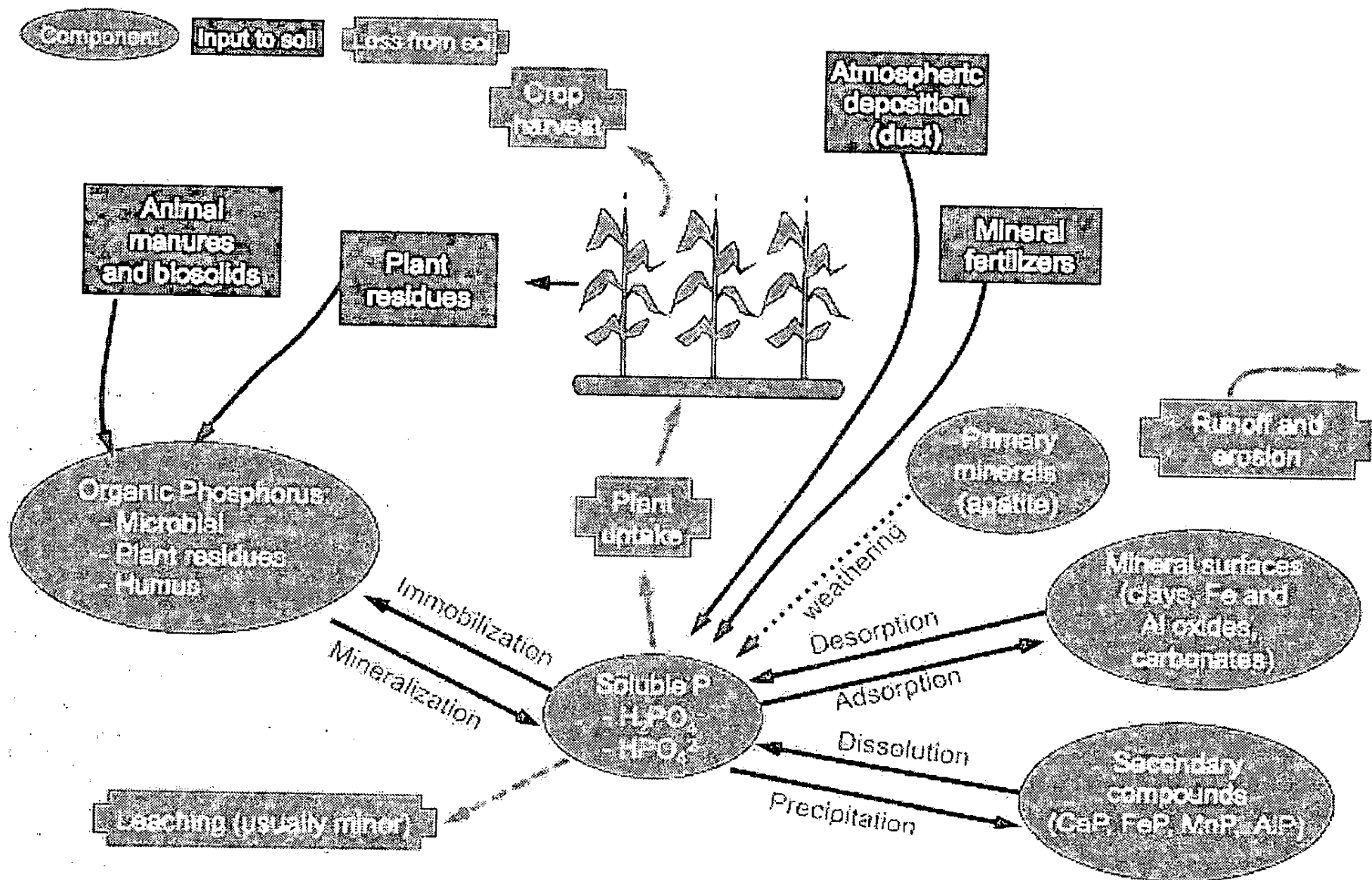


Fig.1.1 The Phosphorus cycle

Phosphorus (P) occurs in soils as both organic and inorganic forms. Large number of compounds makes up the organic P in soils, with the majority being of microbial origin. Organic P is held very tightly and is generally not available for plant uptake until the organic materials are decomposed and the phosphorus is released via the mineralization process. Mineralization is

carried out by microbes, and the rate of P release is affected by factors such as soil moisture, composition of the organic material, oxygen concentration and pH.

The reverse process, immobilization, refers to the tie-up of plant-available P by soil minerals and microbes that use phosphorus for their own nutritional needs. Microbes may compete with plants for P, if the decomposing organic materials are high in carbon and low in nitrogen and phosphorus. Mineralization and immobilization occur simultaneously in soil. If the P content of the organic material is high enough to fulfill the requirements of the microbial population, then mineralization will be the dominant process (Figure. 1.1).

1.4 Problems Associated with P-Fertilizers

The use of chemical fertilizers is reaching the theoretical maximum, beyond which there will be no further increase in crop yields. The repeated and injudicious applications of fertilizers, however, lead to the loss of soil fertility, disturbance to microbial diversity and their associated metabolic activities, and reduced yield of agronomic crops. Moreover, after application, a considerable amount of P is rapidly transformed into less available forms by complexing with Al or Fe in acid soils or with Ca in calcareous soils (Goldstein 1986; Toro 2007) (Figure. 1.2). The excessive application of fertilizer also leads to phosphate pollution of lakes, streams and groundwater. The consequence is an exuberant growth of algae and other aquatic plants in P-polluted water systems, a process known as eutrophication that kills fish and imbalances aquatic ecosystems (Brady and Well, 2002).

These concerns on P fertilizer requirements for crops, P economy and efficiency and its bad impact on environment have renewed interest in exploring alternatives for dissolution of immobilized P or cheapest supplementary P sources for use in sustainable agriculture.

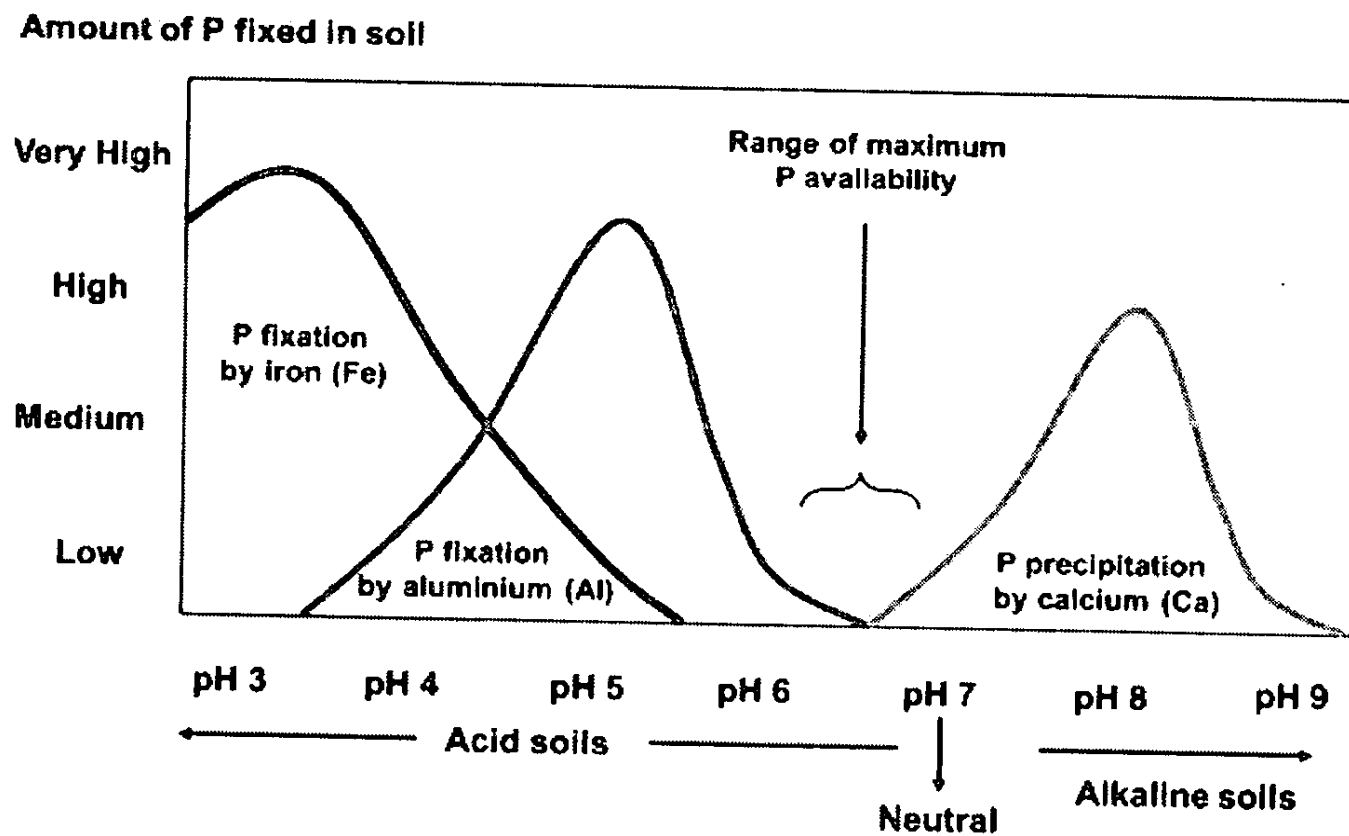


Fig. 1.2 The influence of soil pH on the immobilization of phosphorus

1.5 Rock Phosphate as Phosphate Fertilizer Alternative

Considerable interest in the past has gone into the use of natural rock phosphate as cheapest alternative P fertilizer. It is also the only economical source of phosphorus for production of chemical phosphate fertilizers. Nearly all the rock phosphate fertilizer production is based on some form of the mineral apatite. There are two main types of phosphate rock deposits - sedimentary and igneous.

Igneous phosphate deposits are often associated with carbonatites and/or alkalic (silica deficient) intrusions. Igneous phosphate rock concentrates are produced from deposits that are mainly exploited in Russia, the Republic of South Africa, Brazil, Finland and Zimbabwe. Igneous phosphate ores are often low in grade (less than 5 percent P_2O_5), but can be upgraded to high-grade products (from about 35 percent to over 40 percent P_2O_5). The apatite associated with igneous source rocks may be of a primary magmatic, hydrothermal or secondary origin. Primary apatite from igneous sources may be of fluorapatite, hydroxylapatite or chlorapatite varieties. Pure apatites from igneous deposits contain slightly over 42 percent P_2O_5 .

Sedimentary phosphate rocks occur throughout the geological time scale. Most sedimentary deposits were apparently formed in offshore marine conditions on continental shelves. They

exhibit a wide range of chemical compositions and great variations in physical form. The most desirable sedimentary phosphate rocks contain distinct phosphate particles that can be separated from the unwanted minerals. Most sedimentary deposits contain varieties of carbonate-fluorapatite called francolite. Francolite is defined as an apatite that contains significant CO₂ with >1 percent fluorine (McConnell, 1938). McClellan and Lehr (1969), McClellan (1980), McClellan and Van Kauwenbergh (1990) and Van Kauwenbergh (1995) studied francolite in commercial phosphate rock concentrates and documented that the contents of Ca, Na, Mg, P₂O₅, CO₂ and F can adequately describe most francolites. Sedimentary phosphate rocks containing the most highly carbonate-substituted apatites can only be beneficiated to maximum grades of 33-34 weight percent P₂O₅. Depending on the amount of carbonate substitution, the grade can increase to a maximum of about 42 weight percent P₂O₅ for sedimentary apatites with essentially no substitution.

1.5.1 World Rock phosphate Deposits

Phosphate rock is produced in some 40 countries and, out of which 12 countries have capitalized 92 percent of the world's production (Table 1.1). The United States is the world's leading producer with about 30% of the total production followed by China, Morocco and Russia. The other prominent phosphate production countries are Brazil, Israel, Kazakhstan, Senegal, South Africa, Togo and Tanzania. Eppawela deposit represents less than 1% of world Phosphate reserves.

In many countries rock phosphate are applied directly in large quantities as a P fertilizer each year to soils due to low availability of phosphorus in this native material. Due to the excessive use, reserves of inexpensive rock phosphate could be depleted in as little as 60-80 years (Runge-Metzger, 1995). For that reason, there should be a global effort to more effectively mine and process reserves of rock phosphate and utilize phosphate fertilizer as efficiently as possible.

Table 1.1 : World phosphate rock production, 1999

Producers of Rock phosphate	Product (1 000 tonnes)	World total %
United States of America	40 867	28.1
China	30 754	21.1
Morocco and Western Sahara	21 986	15.1
Russian Federation	11 219	7.7
Tunisia	8 006	5.5
Jordan	6 014	4.1
Brazil	4 301	2.9
Israel	4 128	2.8
South Africa	2 941	2
Syrian Arab Republic	2 084	1.4
Senegal	1 879	1.3
Togo	1 715	1.2
India	1 623	1.1
Algeria	1 093	0.8
Egypt	1 018	0.7
Mexico	955	0.7
Kazakhstan	900	0.6
Finland	734	0.5
Viet Nam	710	0.5
Christmas Island	683	0.5
Nauru	604	0.4
Iraq	415	0.3
Venezuela	366	0.3
Canada	350	0.3
Australia	145	0.1
Uzbekistan	139	< 0.1
Zimbabwe	124	< 0.1
Democratic People's Republic of Korea	70	< 0.1
Sri Lanka	30	< 0.1
Peru	15	< 0.1
Colombia	4	< 0.1
World total	145 472	100

1.6 Rock phosphate deposits, Sri Lanka

Sri Lanka is gifted with large and important natural phosphate deposit at Eppawala in the North central province (Fig. 1.3) with capacity of 40×10^6 metric tons of the ore (Dahanayake et al, 1995). Eppawala rock phosphate deposit was first discovered by Geological Survey Department of Sri Lanka in 1971. Large quantities of P in inorganic form of carbonate apatite, chloro apatite or hydroxyl apatite. Field observation recognized Eppawala rock phosphate as heterogeneous phosphate rock with several types of phosphate minerals on the basis of color, texture and structure. Laboratory studies have shown that Eppawala rock phosphate is an indiscriminated mixture of more soluble primary crystals (HERP) and less soluble secondary phosphate (ERP) matrix rich in aluminous and ferruginous compounds with an average P_2O_5 content of 30% (Dahanayake et al., 1995; Dahanayake and Subasinghe, 1991). The primary crystal fraction was reported to contain even higher amount of P_2O_5 , (35-42%).

Another rock phosphate recently discovered at Kawisigamuwa, near Ridigama (Fig. 1.3). Which is restricted to a smaller area, in comparison with the surficial distribution of the Eppawala deposit. The total P_2O_5 content of primary apatite is around 40% (w/w) whereas it differ from 25 to 35% (w/w) in the matrix.

1.7 Use of Eppawala rock as Phosphate Fertilizer in Sri Lanka

The use of ERP as fertilizer for direct application has gained attention in Sri Lanka since early 1990s. Finely ground phosphate rocks have been used as direct application fertilizer for long term crops such as tea, rubber and coconut (Dahanayake et al, 1995). However, due to the limited solubility of this fertilizer is not effective for short term crops like rice and vegetables (Goenadi et al, 2000). Therefore the rock phosphate needs to be converted into more soluble form which is suitable for the short term crops. Therefore, economic methods to increase the agronomic efficiency of ERP by solubilizing rock phosphate into plant available phosphate forms would be an attractive low cost alternative to save much needed foreign exchange for chemical fertilizers and some poor farmers to profitably apply ERP to their P deficient soils.

Rock phosphate solubilization can be achieved by chemical and biological means. By chemical mean rock phosphate can be converted into triple superphosphate (TSP) and single superphosphate (SSP) by treating with phosphoric acid or sulfuric acid under different suitable

conditions. Biological methods have more advantages than chemical methods, since it is an environment friendly, efficient and cost-effective solution. In biological method phosphate solubilizing microorganisms (PSM) are employed to solubilize the rock phosphate (Rodriguez and Fraga, 1999).

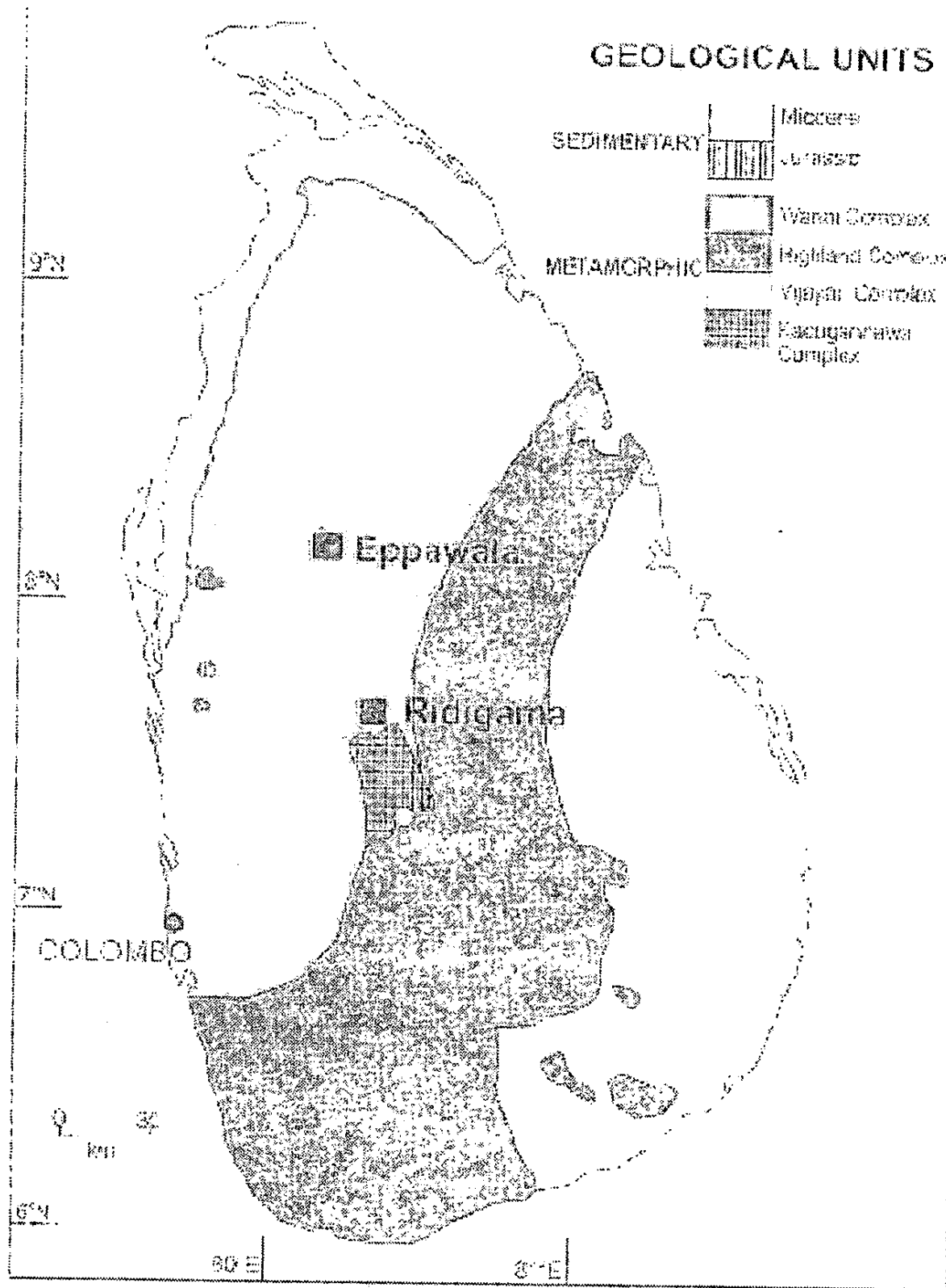


Fig. 1.3 Map showing Eppawala and Ridigama phosphate deposits, Sri Lanka

1.8 Phosphate Solubilizing Microorganisms

A large number of autotrophic and heterotrophic soil microorganisms have capacity to solubilize mineral rock phosphates. PSM are present in almost all the soils, although their number varies depending upon the soil and climatic conditions (Kucey et al. 1989).

Researchers have found that, Phosphate solubilizing microorganisms include various bacteria, fungal and actinomycetes forms which help to convert insoluble phosphate into simple and soluble forms. Bacteria are equally effective in phosphorus solubilizing as (Alam et al., 2002). Among the whole microbial population in soil, phosphate solubilizing bacteria (PSB) constitute 1 to 50 %, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5 % in P solubilization potential (Chen et al., 2006). Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers (Igual et al., 2001). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers (Whitelaw, 2000). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important species (Subbarao, 1988; Kucey et al., 1989). A nematofungus *Arthrobotrys oligospora* also has the ability to solubilize the phosphate rocks (Duponnois et al., 2006).

1.8.1 Phosphate Solubilizing Bacteria

An extensive range of soil bacteria including actinomycetes both aerobic and anaerobic are able to solubilize various forms of insoluble inorganic phosphate compounds, such as TCP, dicalcium phosphate, hydroxyapatite and rock phosphate (RP) (Goldstein 1986; Rodriguez et al. 2000; Gulati et al. 2008; Sulbarán et al. 2009). The prominent genera involved in mineral phosphate solubilization are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Erwinia*, *Alcaligenes*, *Escherichia*, *Serratia* and *Xanthomonas* sp. (Li 1981; Datta et al. 1982; Venkateswarlu et al. 1984; Fernandez et al. 1984; Thomas and Shantaram 1986; Surange and Kumar 1993; Farhat et al. 2009).

The *Pseudomonas* sp. includes *P. striata*, *P. aeruginosa*, *P. putida*, *P. vermiculosam* and *P. fluorescens* (Sen and Paul 1957; Gaiind and Gaur 1990a, b; Kumar et al. 2002; Buch et al. 2008).

Population of PSB mainly depends on different soil properties such as physical, chemical properties, organic matter and P content (Kim et al. 1998).

Major *Bacillus sp.* known for their mineral phosphate-solubilizing abilities are *B. polymyxa*, *B. subtilis*, *B. brevis*, *B. circulans*, *B. megaterium*, *B. mesentericus*, *B. mycoides*, *B. pulvifaciens*, etc. (Sen and Paul 1957; Sundara Rao and Sinha 1963; Paul and Sundara Rao 1971; Rodriguez and Fraga 1999; Swain and Ray 2009).

Most PSB were isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from bulk soil (Baya et al. 1981; Katznelson and Bose 1959; Vazquez et al. 2000).

Actinomycetes as PSM are of special interest since these gram-positive filamentous sporulating bacteria can flourish in extreme environments (Jiang et al. 2005; Pathom-Aree et al. 2006) and also produce various plant growth-promoting substances (Fabre et al. 1988; Manulis et al. 1994; Ikeda 2003; Jain and Jain 2007)

Many workers have used a composite inoculum of bacteria and fungi to enhance the P solubilization but the efficiency of such mixed inoculants depends on the compatibility among microorganisms. Kundu and Gaur (1981) found that inoculation of *P. striata* and *Aspergillus awamori* together solubilized more phosphates than dual inoculation of *B. polymyxa* and *A. awamori*.

1.9 Mechanisms of Phosphorus Solubilization

A wide range of microbial P solubilization mechanisms exist in nature (Banik and Dey, 1982; Toro, 2007) such as, acidification of the medium through secretion of simple organic acids (figure 1.4) (table 1.2), metal complexing, metal reduction and enzymatic phosphate dissolution. (Cunningham and Kuyack 1992; Illmer and Schinner 1995; Song et al. 2008).

Acidification is a result of proton release, for example, as the H⁺ antiport in the course of bacterial ammonium (NH₄⁺) assimilation, by means of which the cell maintains a neutral charge, or production of inorganic acids that do not form strong complexes with Ca, Al, or Fe³⁺ and easily release protons upon dissociation (Illmer and Schinner 1995; Illmer et al. 1995; Rodriguez and Fraga 1999; Whitelaw 1999). Ca-P solubilization occurred by simple acidification process

In metal complexing process, metal ion is coordinated by an anion. Specifically, metal chelation, in the case of a chelating ligand or anion that forms two or more bonds with the metal forming a ring structure, transforms an insoluble phosphate mineral into the metal complex and releases phosphate anions. Ca, Al and Fe phosphates can be solubilized by this mechanism

Phosphate solubilization by metal reduction can be happen either directly (for Fe^{3+} -reducing bacteria) or indirectly microbially mechanisms (such as via release of reductive secondary metabolites). This lead the formation of Fe^{2+} . It has long been known that ferrous (Fe^{2+}) salts are more soluble than the corresponding ferric (Fe^{3+}) salts.

Enzymatically driven dissolution of phosphate may be an essential process in organic phosphorus-rich soils, involving phosphatase-driven dissolution and mineralization of organic P-compounds to release inorganic phosphate in the rhizosphere (Rodriguez et al. 2006).

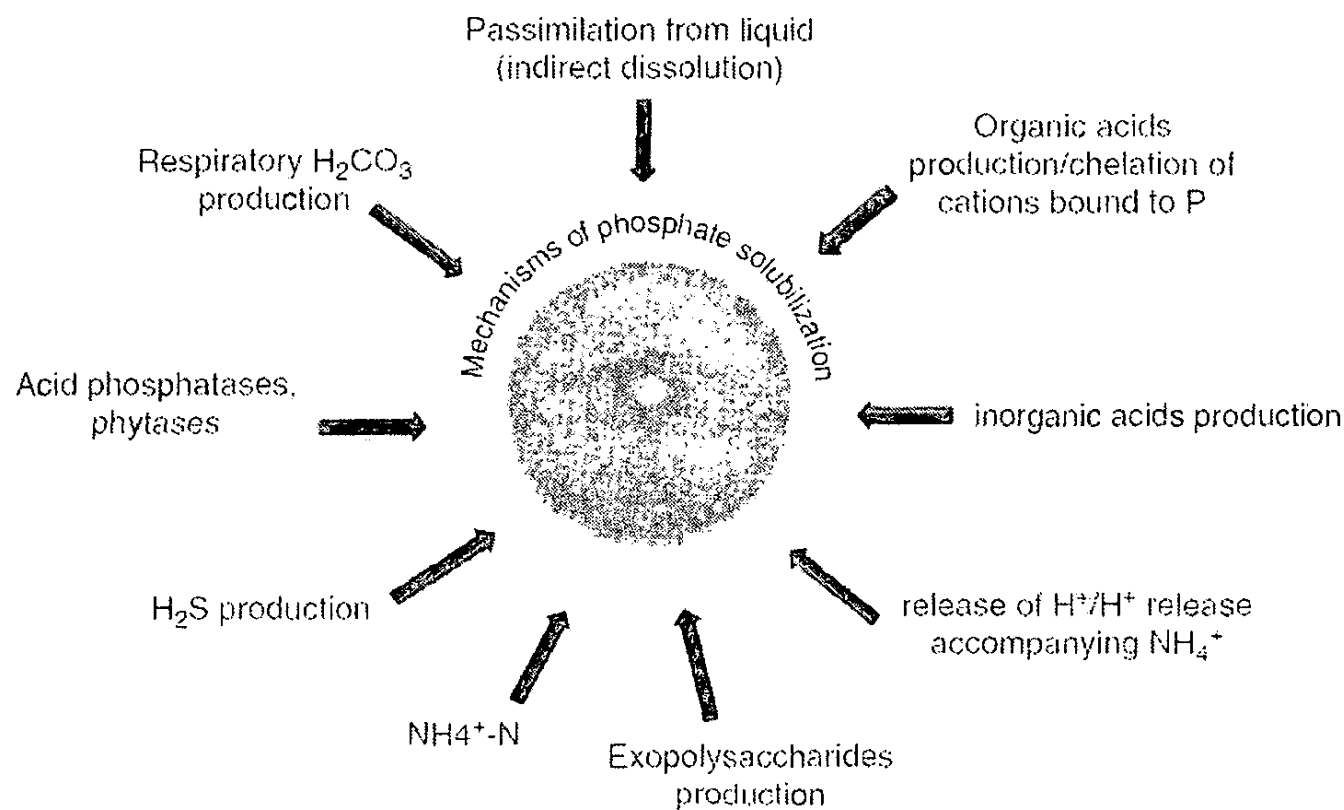


Fig.1.4 Mechanisms of P-solubilization by phosphate solubilizing bacteria

Table 1.2 : Organic acids involved in P-solubilization

Phosphate-solubilizing bacteria	Predominant acids	References
<i>Enterobacter intermedium</i>	2-ketogluconic acid	Vazquez et al., 2000
<i>Bacillus amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, xanthobacter agilis, Enterobacter aerogenes, E. taylorae, E. asburiae, Kluyvera, cryocrescens, Pseudomonas aerogenes, Chryseomonas luteola Pseudomonas cepacia</i>	Lactic, itaconic, isovaleric, isobutyric, acetic acid	
<i>Bacillus polymyxa, B. licheniformis, Bacillus spp. Pseudomonas striata</i>	Gluconic , 2-ketgluconic acid Oxalic, citric acid	Bar-Yosef et al., 1999 Gupta et al., 1994 Gaur, 1990
<i>Arthrobacter sp.</i>	Malic, glyoxalic, succinic, fumaric, tartaric, α -ketobutyric Oxalic, malonic acid	Banik and Dey, 1982
<i>Bacillus firmus</i>	2-ketogluconic, succinic acid	Banik and Dey, 1982
<i>Micrococcus spp.</i>	Oxalic acid	Banik and Dey, 1982
<i>Bacillus subtilis, Bacillus spp.</i>	Oxalic, succinic, citric, 2-ketgluconic acid	Banik and Dey, 1982

1.10 Isolation of Phosphate Solubilizing Bacteria (PSB)

Phosphate Solubilizing Bacteria are generally isolated from rhizosphere and nonrhizosphere soils, rhizoplane, phyllosphere, of plant and even from stressed soils using serial plate dilution method or by enrichment culture technique. The viable microbial preparations possessing P-solubilizing activity are generally termed microphos. The microphos production involves several stages, starting from screening and selection and in vitro evaluation of P-solubilizing potentials of the PSB. Since 1948, when Gerretsen suggested that microbes could dissolve not easily available forms of soil P and play an important role in providing P to plants, numerous media, such as Pikovskaya medium (Pikovskaya 1948), Pikovskaya medium incorporated with bromophenol blue dye (Gupta et al. 1994), and National Botanical Research Institute P (NBRIP)

medium (Nautiyal 1999), have been proposed. Basically selection of microorganism in these media are done by supplementing the media with insoluble P, as the sole P source and monitoring their ability to solubilize insoluble P source and grow. Solubilization of tricalcium phosphate (TCP) in agar medium has been used as the sole P source for isolation and enumeration of PSB. However, the extent of solubilization varies with the source of inorganic P and the microorganisms involved (Banik and Dey 1982). In addition to TCP, other P compounds are used for isolating and testing PSB, such as aluminum phosphate, ferric phosphate, rock phosphate, dicalcium phosphate and phytate (Reyes et al. 2006, Chabot et al. 1996; Antoun et al. 1998; Peix et al. 2003, 2004 and Unno et al. 2005). As TCP gets metabolized faster, it is normally used for initial screening studies compared to RPs (Singh and Kapoor 1994). Also, TCP solubilization is higher as compared to aluminium phosphate and ferric phosphate (Narsian et al. 1993). Both bacterial and fungal strains exhibiting PS activity are detected by the formation of clear halo (a sign of solubilization) around their colonies. The zone of clearance is due to solubilization of inorganic phosphate mainly due to the production of organic acids in the surrounding medium (Johnston 1952; Das 1963; Sethi and Subba Rao 1968; Pareek and Gaur 1973; Kundu and Gaur 1981; Kapoor et al. 1989; Halder et al. 1990; Singal et al. 1991; Yadav and Dadarwal 1997).

Gupta et al. (1994) suggested a modified PVK medium formulated using different concentrations of bromophenol blue (BPB) dye for screening PSM. Results revealed that as the concentration of dye increased, the clarity and visibility of the yellow coloured halo/zone improved, and most appropriate dye concentration was found to be 2.4 mg/ml. There is no correlation between halo zone formation and quantity of inorganic phosphate solubilized (Ostwal and Bhide 1972; Arora and Gaur 1979). Indicator medium containing bromothymol blue (BTB) has also been developed to enhance chances of picking up efficient PSB (Krishnaraj 1996). Advantage of using modified medium is that the incubation period required prior to selection of PSB is significantly reduced, i.e. minimum incubation period for PSB was 24 h than in original PVK medium that exceeds 7 days.

National Botanical Research Institute's phosphate growth medium (NBRIP), which is more efficient than PVK medium was developed for screening of PSM (Nautiyal 1999). NBRIP medium was comparable to PVK agar medium; however, in broth assay, NBRIP medium consistently resulted in a 3-fold increase in P solubilization than PVK liquid medium.

1.11 Characterization of PSB

1.11.1 Biochemical characterization

Selected bacteria capable of persistent and high phosphate solubilization can be identified by phenotypic characterization or genotypic characterization. Phenotypic characterization relies mainly on biochemical tests and was the traditional way to identify bacteria until the application of the application of new molecular techniques for classification and description of microorganisms.

Characterization of phosphate solubilizers based on the formation of visible halo/zone on agar plates is not a reliable technique, as many isolates which did not show any clear zone on agar plates solubilized insoluble inorganic phosphates in liquid medium. (Gupta et al., 1994). Once the PSB have been isolated, the P solubilization efficiency of that particular isolate need to be quantitatively assayed in liquid medium. Different PSB have the potential to solubilize different forms of insoluble phosphates (Calcium phosphate, ferric phosphate, and aluminum phosphate and rock phosphate) based on their P solubilization efficiency bacteria can be categorized as suitable biofertilizer candidate for acidic soils or basic soils. Example, the least efficient Fe-P and Al-P solubilizers are largely impractical for biofertilizer application in acidic soils.

Phosphate-solubilizing bacteria further be characterized according to kinetics and rate of P accumulation. Babenko et al., 1984, have isolated and grouped phosphate-solubilizing bacteria into four different types, these groups range from a linear increase of P concentration along with the growth of the culture, to oscillating behavior with variations in the soluble P levels giving rise to several peaks and troughs of P concentration. This last type of kinetic behavior has also been observed (Babenko et al., 1984, Rodríguez et al., 1996). These changes in P concentration could be a consequence of P precipitation of organic metabolites (Babenko et al., 1984) and/or the formation of organo-P compounds with secreted organic acids, which are subsequently used as an energy or nutrient source, this event being repeated several times in the culture [Illmer et al., 1992]. An alternative explanation could be the difference in the rate of P release and uptake. When the rate of uptake is higher than that of solubilization, a decrease of P concentration in the medium could be observed. When the uptake rate decreases (for instance as a consequence of decreasing growth or entry into stationary phase), the P level in the medium increases again. More probably, a combination of two or more phenomena could be involved in this behavior.

Thus, the P concentration in the culture broth as an indication of phosphate solubilization capacity should be viewed with caution, and a kinetic study.

1.11.2 Molecular characterization of microorganisms

Currently, the 16S rRNA gene has been widely used as phylogenetic marker for molecular characterization of PSB (Ludwig et al., 1998, Normand et al., 1996). As molecular chronometers, rRNA sequences have preserved their evolutionary history (Woese, 1987). Highly conserved events and changes that are most recent occur within less conserved positions or stretches. The degree of divergence of present day rRNA sequences gives as estimate of their phylogenetic distances.

PCR amplification of bacterial 16S rRNA sequence is carried out using universal primer such as fD1 and rD1. Partial characterization of isolated PSB at strain level can be achieved by 16S rRNA PCR-RFLP typing (Weisberg et al., 1991). The sequencing of obtained 16S rRNA amplicons and comparative analysis using comprehensive databases of bacterial 16S rRNA sequences (www.ncbi.nlm.nih.gov/BLAST/) with appropriate software allows rapid identification of unknown bacteria based on their rRNA sequence data (Ludwig et al., 1998, Maidak et al., 2001, Woese, 1987).

1.12 Effect of PSB on Plant growth

Under diverse soil and agro-climatic conditions, the organisms with phosphate-solubilizing abilities have proved to be an economically sound alternative to the more expensive superphosphates and possess a greater agronomic utility.

Phosphate rock minerals are often too insoluble to provide sufficient P for crop uptake. Use of PSMs can increase crop yields up to 70 percent (Verma, 1993). Combined inoculation of arbuscular mycorrhiza and PSB give better uptake of both native P from the soil and P coming from the phosphatic rock (Goenadi et al., 2000; Cabello et al., 2005). Higher crop yields result from solubilization of fixed soil P and applied phosphates by PSB (Zaidi, 1999). Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation (Kucey et al., 1989; Ponmurugan and

Gopi, 2006). *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (Son et al., 2006). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma et al., 2007), while co-inoculation of PSM and PGPR reduced P application by 50 % without affecting corn yield (Yazdani et al., 2009). Inoculation with PSB increased sugarcane yield by 12.6 percent (Sundara et al., 2002). Sole application of bacteria increased the biological yield, while the application of the same bacteria along with mycorrhizae achieved the maximum grain weight. (Mehrvarz et al., 2008). Single and dual inoculation along with P fertilizer was 30-40 % better than P fertilizer alone for improving grain yield of wheat, and dual inoculation without P fertilizer improved grain yield up to 20 % against sole P fertilization (Afzal and Bano, 2008). Mycorrhiza along with *Pseudomonas putida* increased leaf chlorophyll content in barley (Mehrvarz et al., 2008). Rhizospheric microorganisms can interact positively in promoting plant growth, as well as N and P uptake. Seed yield of green gram was enhanced by 24 % following triple inoculation of *Bradyrhizobium* + *Glomus fasciculatum* + *Bacillus subtilis* (Zaidi and Khan, 2006). Growth and phosphorus content in two alpine *Carex* species increased by inoculation with *Pseudomonas fortinii* (Bartholdy et al., 2001). Integration of half dose of NP fertilizer with biofertilizer gives crop yield as with full rate of fertilizer; and through reduced use of fertilizers the production cost is minimized and the net return maximized (Jilani et al., 2007).

ii. Scientific scope of the project

1.13 Aims and objectives of the study

Therefore isolation of efficient rock phosphate solubilizing PSB and uncovering their unique P solubilization that promotes the plant P intake is an important research trends in sustainable agriculture. Although the isolation and characterization and application of PSB have been done elsewhere, presently however, very little researches have been conducted in Sri Lanka. This is the first study which describes the isolation, characterization and application of novel PSB from Eppawala rock phosphate deposit, Sri Lanka. Therefore, this work seek to uncover the novel PSB which are well adapted to our edaphoclimatic conditions and brings new knowledge, methodologies and processes that may not be currently available for improving soil P of infertile cultivation lands of Sri Lanka.

1.13.1 Overall objective of the study

To screen, isolate, characterize and evaluate the promiscuous PSB occurring naturally in Sri Lanka, in Eppawala rock phosphate deposit for uncovering the existence of efficient rock phosphate solubilizers to make use that resource information for developing biofertilizer in use.

1.13.2 Specific Objectives

This experiment is designed to,

- Isolation of Phosphate Solubilizing Bacteria (PSB) near Eppawala rock phosphate deposits.
- Evaluate PSB for their ability to solubilize Al-PO₄, Fe-PO₄, Ca-PO₄ and Eppawala rock phosphate on modified PVK solid medium.
- Identification of efficient Eppawala rock phosphate solubilizing bacteria in modified PVK liquid medium.
- The effect of Eppawala rock phosphate concentration and optimum particle size on phosphate solubilization.
- Evaluate efficient Eppawala rock phosphate solubilizing bacteria strains for their ability to solubilize Al-PO₄, Fe-PO₄ and Ca-PO₄ in liquid medium.
- Type and partially characterize, efficient Eppawala rock phosphate phosphate solubilizing bacteria by PCR-RFLP.

- To evaluate, efficient Eppawala rock phosphate bacteria strains for their ability to solubilize Eppawala rock phosphate in soil and their effect on the plant growth.

iii . Materials and Methods

2.1 Materials

Higher grade primary Eppawala Rock Phosphate (HERP) with 38% P_2O_5 content was used as rock phosphate (Rock-P) source in this study.

2.2 Soil sampling

Rizospheric soil samples of well grown plant species, near Eppawala rock phosphate mine, Eppawala, Sri Lanka (Figure 2.1) were collected to a depth of 0-15 cm. Two sites (sites 1 and site 2) were selected for collecting soil samples. In total, thirty soil samples were collected from both sites, under sterile conditions and transported immediately to the laboratory at the university of Kelaniya. Soil samples from each site were pooled separately to give two sub samples and subjected each to soil chemical analysis (section 2.3). The sub soil samples from site 1 and site 2 were then pooled to give one composite sample and stored in a sterile container at $4^{\circ}C$ for isolation of phosphate solubilizing bacteria.

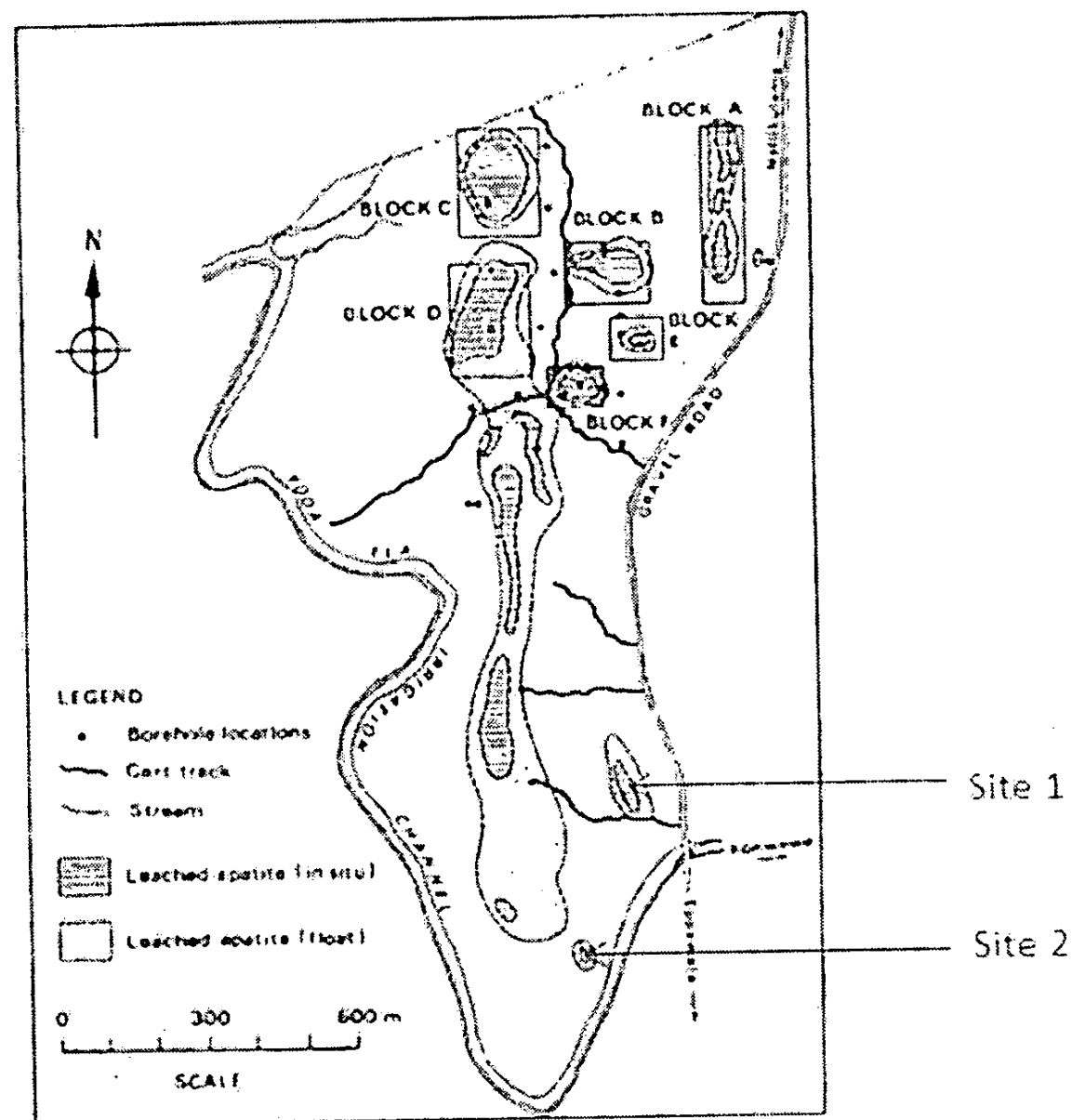


Fig. 2.1 Map of Eppawala rock phosphate deposit showing sampling site 1 and 2

2.3 Soil chemical analysis

The physicochemical properties, such as pH and soluble phosphate of soils from site 1 and 2 were determined as follows. A soil sample, from the University of Kelaniya premises was also analysed as a reference, exactly according to above procedures, for available P and pH for comparison purpose.

2.3.1 Determination of soil pH

A portion of soil sample was air dried, ground and passed through a 2 mm sieve. 20 g dry soil was transferred into a sterile 250 ml reagent bottle, 100 ml sterile distilled water was added (1:5, soil: distilled water) and stirred well. The solution was allowed to settle for 1 hr. The supernatant was separated by decanting. pH of the supernatant was measured using calibrated pH meter.

2.3.2 Determination of soluble phosphorous (P) in soil

Measurement of available P

20.00 g of finely ground (< 2 mm) soil sample was placed in a reagent bottle, 100 ml of distilled water was added and shaken gently for 30 min. It was allowed to stand for 15 min and filtered. 1.2 ml filtrate and 2.8 ml test solution were mixed and incubated for 45 min at 30⁰C. Test solution was prepared by mixing 0.42% Ammonium Molybdate in 1N Sulphuric acid and 10% ascorbic acid in 6:1 ratio. Absorbance readings were measured at 620 nm using a spectrophotometer. Distilled water was used as the blank. Available soil P concentration was determined using the calibration curve (section 2.3.2).

Construction of the calibration curve

Nine phosphorus standards, ranging 0.5, 1, 2.5, 5, 7.5, 10, 25, 50, 75 μ M KH_2PO_4 were prepared. 2.8 ml test solution and 1.2 ml of each standard solution were mixed as above (section 2.3.2) and incubated for 45 min at 30⁰C. Absorbance readings were taken at 620 nm using a spectrophotometer and used to construct a calibration curve.

2.4 Isolation of phosphate solubilizing bacteria

10 g of composite soil sample from rock phosphate deposit was suspended in 90 ml sterile distilled water and shaken for ~1 hr. The soil extract was serially diluted to give 10^{-2} – 10^{-7} dilutions. Aliquots of each dilution were separately spreaded on modified Pikovskaya (PVK) medium (Table 2.1) (Pikovskaya, 1948) supplemented with agar (2%, w/v), bromophenol blue (0.025 mg/ml) and CaHPO_4 (5.56 g/l) and incubated for two days at 30°C . The plates were examined for the presence of bacterial colonies surrounded by characteristic yellow halo zones (Pikovskaya, 1948). The colonies that formed halo zones were picked and repeatedly sub cultured on the same medium for several rounds until pure colonies were obtained. Agar slants and glycerol stocks of the isolates were maintained at 4°C and -20°C respectively.

Table 2.1 Composition of modified PVK medium (Pikovskaya, 1948)

Components	Concentration (g l^{-1})
Glucose	10.00
Ca_3HPO_4	5.56
$(\text{NH}_4)_2\text{SO}_4$	0.50
NaCl	0.20
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.10
KCl	0.20
Yeast extract	0.50
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.0020
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.0020
Adjusted the pH to 7.0	

2.5 Qualitative estimation of phosphate solubilisation on various inorganic phosphates

Modified PVK agar media (section 2.4) containing each inorganic P source, CaHPO_4 (5.56 g/l), AlPO_4 (4.51 g/l), FePO_4 (7.19 g/l) and Rock-P (5.98 g/l) were prepared. They were inoculated with each isolate, and incubated for two days at 30°C . Bacterial colonies forming halo zones were identified and their phosphate solubilization indices were calculated using equation (1) given in figure 2.2.

$$\text{Phosphate Solubilization Index} = \frac{b}{a} \text{----- (1)}$$

a: Diameter of the bacterial colony

b: Total diameter of the halo zone (colony + halo zone)

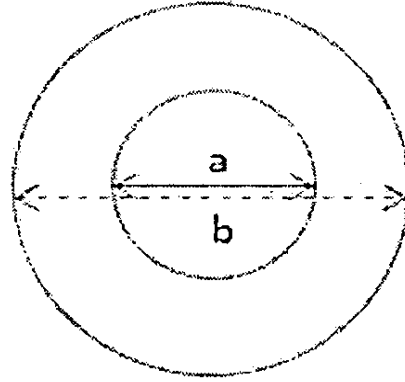


Fig. 2.2 Calculation of phosphate solubilization index based on the size of the halo zone

2.6 Quantitative estimation of Rock-P solubilization in liquid media

Each bacterial isolate, that formed characteristic halo zones on agar media (section 2.5) was analyzed for their ability to solubilise Rock-P in modified PVK liquid medium (Table 2.1). 25 ml of PVK liquid medium containing 0.12 g of finely grounded (< 0.3 mm) Rock-P was inoculated with approximately 300 µl of cell suspension (2×10^8 cfu/ml), in 100 ml Erlenmeyer flasks. Experiment was performed in triplicate. Inoculated flasks and un-inoculated controls were incubated at 160 rpm for three days, at 30°C. After incubation period, cultures were centrifuged at 10,000 rpm for 10 min. Cell free supernatants were decanted and used to determine pH, titratable acidity and soluble P concentrations. pH was measured using a calibrated pH meter. Titratable acidity was determined by an acid base titration. Briefly, few drops of phenolphthalein indicator was added to 5.00 ml of culture supernatant and titrated with 0.01 N NaOH. The titratable acidity was expressed as ml of 0.01N NaOH consumed per 5.00 ml of culture filtrate. Soluble P concentrations of cell free supernatants were determined as described previously in section 2.3.2.

2.7 Effect of Rock-P particle size on phosphate solubilization

Five different Rock-P particle sizes ranging >0.3, 0.3-0.15, 0.15-0.075, 0.075-0.037 and <0.037 mm were tested for their ability to release soluble P in modified PVK liquid medium. The experiment was performed as described in section 2.6 except different Rock-P particles sizes

were used instead of one size. Culture pH, titratable acidity and soluble P concentrations for each particle size were also determined as described above in section 2.6.

2.8 Effect of Rock-P concentration on phosphate solubilization

Three concentrations of Rock-P, 1 %, 3 %, and 5 % (w/v) were tested for their ability to release soluble P in modified PVK liquid medium. The experiment was performed exactly according to section 2.6 except, different amounts of Rock-P were used instead of one. Culture pH, titratable acidity and soluble P concentrations for each particle size were also determined as described in section 2.6.

2.9 Quantitative estimation of Inorganic phosphate solubilization in liquid medium

The ability of isolated bacteria to release soluble P from various inorganic phosphates were tested using CaHPO_4 , AlPO_4 , FePO_4 and also Rock-P. Modified PVK media containing each P source, 14 g CaHPO_4 , 0.11g AlPO_4 , and 0.18 g FePO_4 , were inoculated with each isolate and the experiment was performed as described in section 2.6 except different inorganic phosphates were used. Culture pH, titratable acidity, and soluble P were also determined as described in section 2.6.

2.10 Molecular characterization of efficient Rock-P solubilizing strains by PCR-RFLP

The most efficient Rock-P solubilizing bacterial strains were characterized by PCR-RFLP .

2.10.1 Extraction of genomic DNA

Bacteria isolates were inoculated in LB broth and incubated overnight at 30⁰C. One ml of overnight grown culture was pelleted by centrifugation at 3000 g for 5 min. Cell pellet was resuspended in 567 μl TE buffer by wide bore in-out pipetting. Cells were lysed by incubating in 30 μl 10% SDS and 3 μl proteinase K solution (20 mg/ml) for 1hr at 37⁰C. Salt concentration was adjusted by adding 100 μl 5M NaCl followed by 80 μl CTAB/NaCl (10% CTAB/0.7 M NaCl) solution. The solution was mixed thoroughly and incubated for 10 min at 65⁰C. After extraction with 0.7 volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) followed by equal

volume of Chloroform: Isoamyl alcohol (24:1), DNA was precipitated by adding x 0.6 volume of ice cold isopropanol. DNA was pelleted by centrifugation and washed with ice cold ethanol (70%). Finally pellet was air dried and resuspended in 50 µl of ultra-pure water. The DNA samples were stored at 4⁰C for until further analysis. The amount of genomic DNA was estimated by agarose gel electrophoresis against λDNA *EcoRI/HindIII* as the standard.

2.10.2 16S rRNA amplification

The 16S rRNA gene was amplified from genomic DNA extracts by PCR, using universal set of primers fD1(5'-AGAGTTTGATCCTGGGCTCAG-3') and rD1(5'AAGGAGGTGATCCAGCC-3') (Weisberg et al, 1991). These primers have been designed to amplify nearly full length of 16S rRNA from most eubacteria (Weisberg et al, 1991). The amplification was preceded with 35 cycles of denaturation at 94⁰C for 1 min, annealing at 55⁰C for 1 min and extension at 72⁰C for 2 min followed by final extension at 72⁰C for 5 min. The reaction mixture contained 20 ng of genomic DNA, 0.5x PCR buffer (Flexy colored buffer, Promega Corporation,USA), 1.5 mM of MgCl₂, 15 pmol of each primer, 0.2 mM of each dNTP (Promega Corporation,USA), and 1U of taq DNA polymerase. Amplified DNA was quantified as described in section 2.10.1.

2.10.3 Restriction enzyme analysis

Each amplified 16s rRNA products was digested with 1-1.5 U of each restriction enzyme *Alu* I, *Hae* III and *Hinf* in a final volume of 25 µl containing 1 x reaction buffer, 2 µg of BSA. The digestion was performed at 37⁰C for 3 hr, followed by incubation at 70⁰C for 10 min. Each restriction enzyme digest was analyzed as described in section 2.10.4.

2.10.4 Analysis of DNA

All DNA products, genomic DNA, 16S rRNA PCR products and restriction digests were analysed by agarose gel electrophoresis (SCIE-PLAS,Harvard Bioscience Inc,USA). The gel was prepared according to Sambrook et al, 1989 (table 2.2). The amount of agarose varied with size of the DNA fragments analyzed. Concentrations of agarose used to analyze different sized DNA fragment during this study are shown in Table 2.2.

Table 2.2 The concentrations of agarose gels used in this study

Agarose concentration (% w/v)	Types of DNA analysed
0.8	Genomic DNA
1	16SrRNA PCR products
1.2	Restriction enzyme digests

DNA sample to be analyzed was mixed with 6 X loading buffer (2 μ l) (Sambrook et al, 1989). The mixture was loaded onto a agarose gel and electrophoresis was performed at constant voltage of 60V for 1hr and 30 min in 0.5 x Tris-Borate-EDTA (TBE) buffer containing ethidium bromide (5 mg/ml) (Sambrook et al, 1989). The DNA were visualized at 254 nm on an UV transilluminator (Hoefer Inc.,San Francisco,USA) and photographed. The sizes of DNA fragments were estimated against λ DNA/ *EcoRI/HindIII* standard.

2.11 Effect of Rock-P solubilizing bacteria on early growth of rice and dissolution of Rock-P in rice rhizosphere

Two rice varieties, a modern and a traditional variety, BG352 and Suduheenati, were used in this study. The seeds of these varieties were kindly provided by the plant genetic resources centre (PGRC), Gannoruwa, Kandy.

2.11.1 Green house experiment and estimation of growth parameters

The rice seeds were surface sterilized by treating with 70% ethanol for one min followed by 10% sodium hypochlorite solution for four min. The seeds were then carefully washed four times with sterile distilled water. Surface sterilized seeds were allowed to germinate on sterilized wet paper towel in Petri dishes for 5 days. Pots for the cultivation of rice plants were prepared by filling 500 g of autoclaved soil into each pot. Two Germinated seeds were planted per pot and allowed to grow. Treatments were set up for rice varieties (BG352, and Suduheenati) in complete randomized design (CRD) with three replicates as follow.

Treatment 01: No fertilizer application and no inoculation

Treatment 02: 60 kg N/ha, 50 kg P₂O₅/ha and 30 kg K₂O/ha without inoculation

Treatment 03: 60 kg N/ha, 50 kg HERP/ha and 30 kg K₂O/ha without inoculation

Treatment 04: 60 kg N/ha, 50 kg HERP/ha and 30 kg K₂O/ha with 0.5×10^7 bacteria cells

Treatment 05: 60 kg N/ha, 50 kg HERP/ha and 30 kg K₂O/ha with 1×10^7 bacteria cells

Treatment 06: 60 kg N/ha, 50 kg HERP/ha and 30 kg K₂O/ha with 2×10^7 bacteria cells

The fertilizers were applied on 8th day after planting. Inoculum contained most efficient Rock-P solubilizing strain(s) Inoculant was applied on the 10th day in the form of a liquid cell suspension (approximately 10^7 cells resuspended in LB medium) near to the root of rice seedling as needed for each treatment levels 04-06. Treatments, 01-03 were treated with equal volume of sterile LB as the blank instead. The plants were irrigated with autoclaved tap water once every two days or as required. Pots were maintained under submerged conditions. On 40th day, a random samples were taken from each pot representing all treatment levels to determine plant growth parameters, such as, plant dry weight (g), shoot length (cm) and root length (cm).

2.11.2 Quantitative estimation of solubilized Rock-P in the rice rhizosphere

In a parallel experiment on 40th day, rice seedlings were uprooted and the loosely adhering bulk soil was removed by gentle tapping. 10 g of roots and soil adhered to it, were suspended in a conical flask containing, 25 ml sterile distilled water and shaken at 160 rpm for 2 hrs. Concentration of soluble phosphate in the soil solution was determined as described in section 2.6.

2.11.3 Quantitative estimation of plant acquired P

Harvested rice plants were dried to a constant weight, in an oven at 80⁰C. Finley grounded plant material was transferred into a crucible, 5 ml 0.5 N MgNO₃ and 10 ml distilled water was added. The solutions were completely evaporated by placing the crucibles on a steam bath. Then crucibles were placed in muffle furnace and ignited at 600⁰C for 30 min. Crucibles were removed from muffle furnace and allowed to cool until liquid can be added without spattering. Then 15 ml H₂SO₄ (2 N) was transferred in to crucibles and revolved until that the acid come into contact with all of the ash. 15 ml Distilled water was added in to the crucibles before placed them on steam bath. Liquid was allowed to evaporate until the final volume decreased to 5ml. Finally 10 ml distilled water was added into the remaining solution and transferred into 15 ml

falcon tubes, and stored in freezer at 4⁰C. Soluble P in plant extract was determined exactly according to section 2.6.

iv. Results

3.1 Soil analysis

Chemical analysis of soil samples from site 1 and site 2 of Eppawala rock phosphate deposit, showed that available P content of soil, near the deposit was much higher and ranged from 157.3 -165.0 ppm. Also, the soil pH at site 1 and 2 were 5.5 and 5.8 respectively (table 3.1). In comparison to the above, the available soluble P concentration of the soil sample at the University premises remained 14.27 ppm, which is substantially lower than that at the deposit. Similarly as expected, somewhat higher pH value of 6.54 was observed for the reference sample (table 3.1).

Table 3.1: pH and soluble phosphate contents of soil samples at site 1 & 2

Soil sample	pH	Soluble P concentration (ppm)
Site1	5.5	157.3
Site 2	5.8	165.0
University	6.54	14.27±0.07

3.2 Isolation of phosphate solubilizing bacteria

For the isolation of PSB, soil extract were serially diluted and spreaded on modified PVK media containing Ca-P, and bromocresol blue indicator dye. Large number of microbial colonies of varying morphologies were observed on culture plates. PSB colonies were identified by their characteristic yellow halo zone formed around their colonies. Halo formation was assumed as an indicator of the phosphate solubilizing activity. Colonies were further purified by replating them on the same medium (figure 3.1).

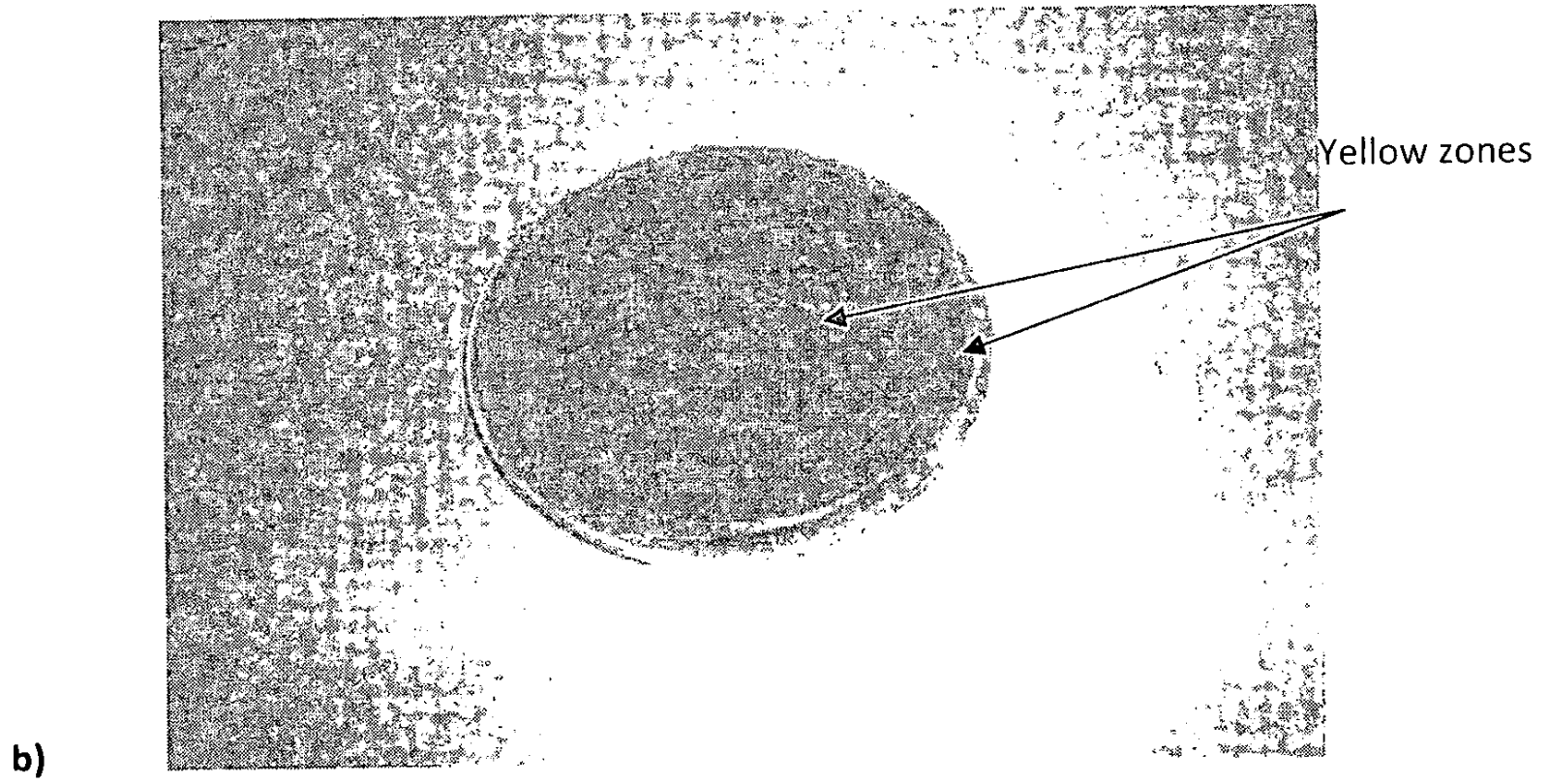
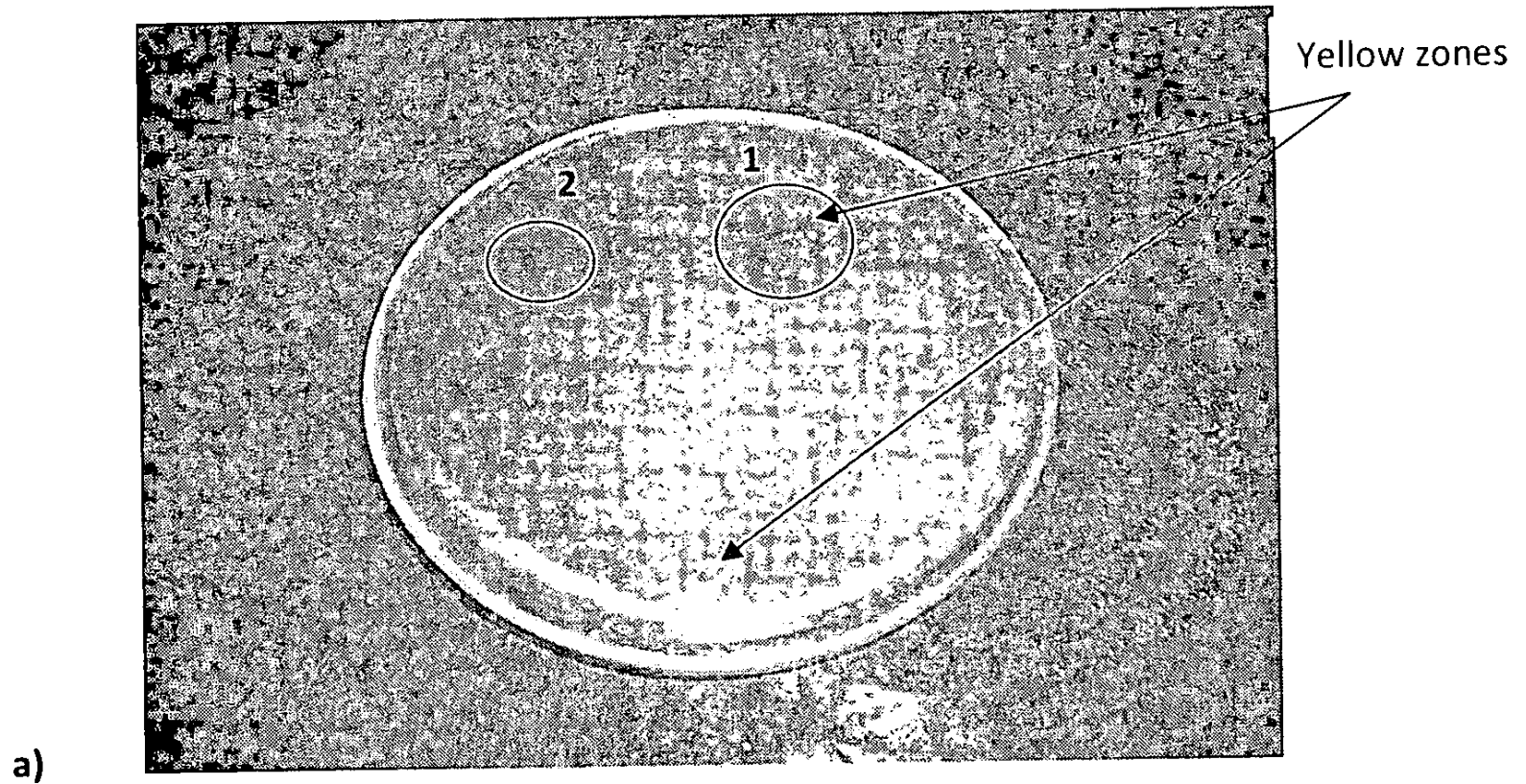


Fig. 3.1 a,b : Isolated phosphate solubilizing bacterial colonies showing yellow zones on modified Pikovskaya's agar medium



Fig. 3.2 : EP 02 strain streaked on modified PVK's agar containing Ca-P as the sole P source

In total, 20 strains EP 02,EP 03,EP 05,EP 11,EP 12,EP 13,EP 14,EP 15,EP 16,EP 17,EP 20,EP 21,EP 22,EP 23,EP 24,EP 25,EP 27,EP 28, EP 29 and EP31 were isolated in the first screening step. This preliminary observation suggested the existence of PSB exhibiting different degrees of phosphate solubilizing activity at the Eppawala rock phosphate deposit.

To determine their phosphate solubilizing efficiency on various inorganic phosphate sources, such as Ca-P, Fe-P, Al-P and Rock-P, bacterial strains were inoculated on modified PVK agar media containing each inorganic phosphate, as the sole P source. Results showed that all 20 bacteria were able to solubilize Ca-P in modified PVK agar medium. The different sizes of halo zones suggested that bacterial isolates have different degree of Ca-P solubility. Solubilization indices of these isolates on Ca-P ranged from 0.97 to 4.9.cm (table 3.2) (figure.3). Notably, strain EP-13 gave the highest index value and came out as the most efficient Ca-P solubilizer followed by strain EP-11.

Likewise, 19 isolates EP 02,EP 03,EP 05,EP 11,EP 12,EP 13,EP 14,EP 15,EP 16,EP 17,EP 20,EP 21,EP 22,EP 23,EP 24,EP 25,EP 27,EP 29 and EP31) were able to solubilize both Fe-P and Al-P. The halo sizes of these isolates ranged from 0.00 to 3.62 cm for Fe-P and 0.00 to 3.53 cm for Al-P (table 3.2)(figure.3). Above all, EP 11 showed the highest solubilization indices for these two substrates and identified as the most efficient Fe-P and Al-P solublizer.

Table 3. 2 : Solubilization indices of bacterial strains in modified PVK agar medium containing each Ca-P,Fe-P,Al-P and Rock-P as the sole P source.

Bacterial strain	Ca-P	Bacterial strain	Fe-P	Bacterial strain	Al-P	Bacterial strain	Rock-P
EP13	4.9 A	EP11	3.62A	EP11	3.53A	EP11	3.13A
EP11	4.58B	EP13	2.97B	EP23	2.37B	EP5	2.00B
EP31	4.53B	EP2	2.47C	EP2	2.32B	EP2	1.78C
EP3	3.80C	EP23	2.00D	EP13	1.952C	EP23	1.71CD
EP2	3.58C	EP21	2.00D	EP21	1.732CD	EP13	1.68CD
EP14	2.72D	EP5	2.00D	EP5	1.72CD	EP31	1.63CD
EP5	2.62DE	EP15	1.98D	EP24	1.582DE	EP3	1.60D
EP15	2.43EF	EP3	1.83DE	EP17	1.452EF	EP15	1.29E
EP27	2.40EFG	EP17	1.72E	EP14	1.432EF	EP21	1.22EF
EP21	2.40EFG	EP31	1.70E	EP3	1.37EF	EP20	1.08FG
EP17	2.33FG	EP24	1.70E	EP15	1.32FG	EP17	1.07G
EP23	2.25FG	EP14	1.68E	EP31	1.30FG	EP22	1.00G
EP16	2.22FGH	EP16	1.64E	EP27	1.28FG	EP16	1.00G
EP20	2.17GHI	EP20	1.61E	EP20	1.28FG	EP14	1.00G
EP24	2.00HIJ	EP25	1.28F	EP29	1.20FGH	EP27	0.97G
EP22	1.93IJ	EP29	1.13FG	EP16	1.10GH	EP25	0.78H
EP25	1.90J	EP22	1.13FG	EP22	1.00H	EP29	0.00I
EP29	1.40K	EP27	1.00G	EP12	1.00H	EP28	0.00I
EP12	1.05L	EP12	0.97G	EP25	0.97H	EP24	0.00I
EP28	0.97L	EP28	0.00H	EP28	0.00I	EP12	0.00I

Means followed by the same letter did not differ significantly at 5% Tukey test

When Rock-P was used as the sole P source, 16 isolates, EP 02, EP 03, EP 05, EP 11, EP 13, EP 14, EP 15, EP 16, EP 17, EP 20, EP 21, EP 22, EP 23, EP 25, EP 27 and EP31 showed halos on PVK agar medium. Different sized halos around colonies, indicated their varying degrees of Rock-P solubilization ability. Sizes of the halo zones ranged from 0.00-3.13cm. EP11 strain showed the highest solubilization index and, identified to be the best Rock-P solubilizer on PVK agar. However, some strains such as EP 12, EP24, EP28 and EP 29, did not produce any detectable halos on Rock-P (table 3.2) (figure.3.3).

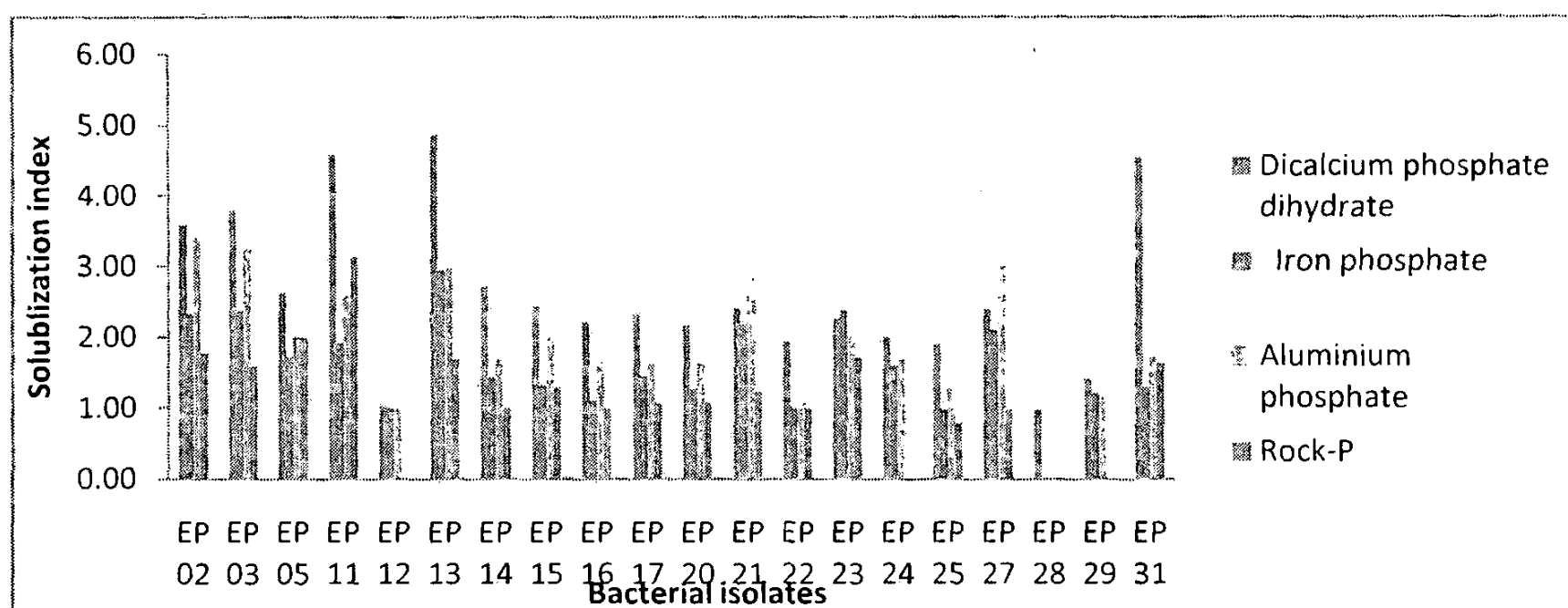


Fig. 3.3: Solubilization indices of each PSB strain on various inorganic P sources Ca-P, Fe-P, Al-P and Rock-P); Each value is a mean of three replicates;
Solubilization index = (Colony + halozone diameter/colony diameter).

Table 3.3 : Ranking of bacterial strains based on the solubilization indices on various inorganic P sources

Bacterial strain	Ca-P	Rank	Bacterial strain	Al-P	Rank	Bacterial strain	Fe-P	Rank	Bacterial strain	Rock-P	Rank
13	4.87	1	11	3.62	1	11	3.53	1	11	3.13	1
11	4.58	2	13	2.97	2	23	2.37	2	5	2.00	2
31	4.53	3	2	2.47	3	2	2.32	3	2	1.78	3
3	3.80	4	5	2.00	4	13	1.95	4	23	1.71	4
2	3.58	5	21	2.00	5	21	1.73	5	13	1.68	5
14	2.72	6	23	2.00	6	5	1.72	6	31	1.64	6
5	2.62	7	15	1.98	7	24	1.58	7	3	1.60	7
15	2.43	8	3	1.83	8	17	1.45	8	15	1.29	8
21	2.40	9	17	1.72	9	14	1.43	9	21	1.22	9
27	2.40	10	24	1.70	10	3	1.37	10	20	1.08	10
17	2.33	11	31	1.70	11	15	1.32	11	17	1.07	11
23	2.25	12	14	1.68	12	31	1.30	12	14	1.00	12
16	2.22	13	16	1.64	13	27	1.28	13	16	1.00	13
20	2.17	14	20	1.61	14	20	1.28	14	22	1.00	14
24	2.00	15	25	1.28	15	29	1.20	15	27	0.97	15
22	1.93	16	22	1.13	16	16	1.10	16	25	0.78	16
25	1.90	17	29	1.13	17	12	1.00	17	12	0.00	17
29	1.40	18	27	1.00	18	22	1.00	18	24	0.00	18
12	1.05	19	12	0.97	19	25	0.97	19	28	0.00	19
28	0.97	20	28	0.00	20	28	0.00	20	29	0.00	20

Means followed by the same letter did not differ significantly at 5% Tukey test

Table 3. 4: Concentration of soluble P released after three days by each isolated PSB strain in modified PVK liquid broth containing 4.8 g/l Rock-P.

PSB strain	Soluble P concentration (ppm)
EP11	57.17A
EP27	24.49B
EP21	15.63C
EP2	14.97D
EP3	13.59E
EP13	10.19F
EP17	7.49 G
EP29	6.10 H
EP16	4.42 I
EP20	4.07 J
EP14	3.04 K
EP31	3.03 K
EP25	3.03 K
EP24	3.03 K
EP28	2.37 L
EP22	0.32 M
EP12	0.32 M
EP23	0.32 M
EP5	0.32 M
EP15	0.32 M

Means followed by the same letter did not differ significantly at 5% Tukey test

The soluble P concentration released by these strains ranged between 0.32-57.17 ppm with variation among different isolates. EP 11 released the maximum concentration of soluble P in the presence of Rock-P, which was 57.17 ppm, followed by EP27 which released 24.49 ppm soluble P (figure 3.4, table 3.4).

The solubilization of Rock-P in liquid medium by different isolates was accompanied by significant drop in pH from an initial pH of 7.0 to 3.55 -5.04. Maximum drop in pH from 7.0 to 3.55 was observed with the most efficient solubilizing strain EP11 (figure 3.4).

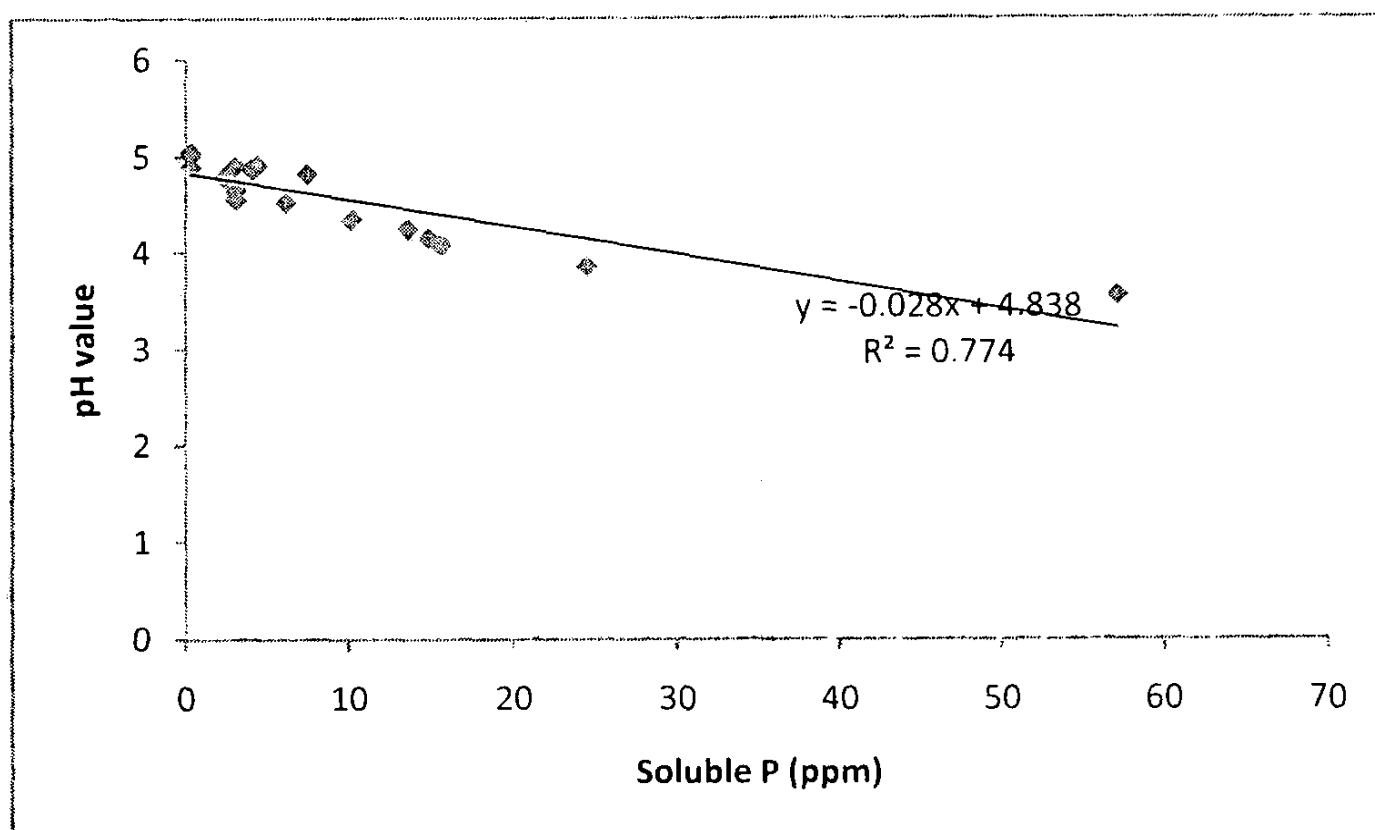


Fig. 3.5: Relationship between soluble P concentration released by all PSB strains and their corresponding final culture pH.

Strains, EP5, EP12, EP20, EP22 and EP23 released the lowest amount of soluble P. Compared to more efficient strains, cultures of these strains showed only a slight drop of pH from its initial value, of 7.0 to 4.89, 5.04, 5.03, 4.89 and 5.01, respectively.

Therefore, it can be observed that the lower the pH of the medium the higher the concentration of soluble released. To elaborate this relationship further we constructed a graph of soluble P against final culture pH from all 20 strains (figure 3.5). Analysis of the graph indicate that there is a strong correlation ($R = 0.774$) between the soluble P concentration and the final pH of the culture for each strain. However graph drawn between soluble P concentration against the final titrable acidity of the culture does not show such a strong correlation, especially at high soluble P concentrations (figure 3.6).

P solubilization efficiency of six strains, EP11, EP 27, EP 21, EP2, EP3, EP 13, was quite prominent. These strains released the highest concentration of soluble P from Rock-P in liquid cultures. They all lowered the pH approximately by three pH units to a value below pH 4 in liquid cultures (figure 3.4, figure 3.6). Their titrable acidity values were also remarkably higher than the other strains (figure 3.7, figure 3.8). Therefore considering their overall performance in liquid medium in the presence of Rock-P and also in solid media with various other inorganic phosphates, these six strains were selected as the most efficient PSBs for subsequent work.

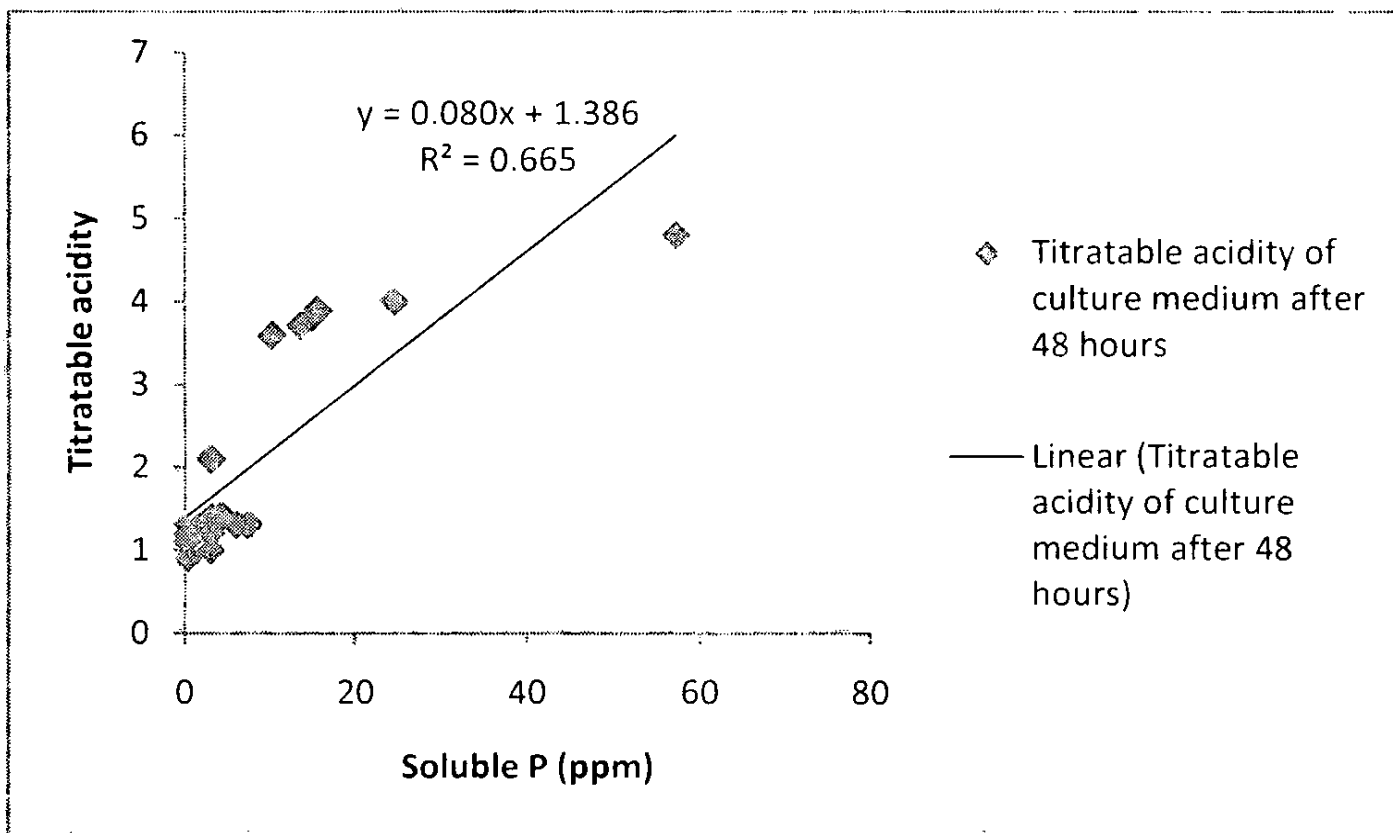


Fig. 3.6: Relationship between solubilized P concentration and final titratable acidity of the liquid culture

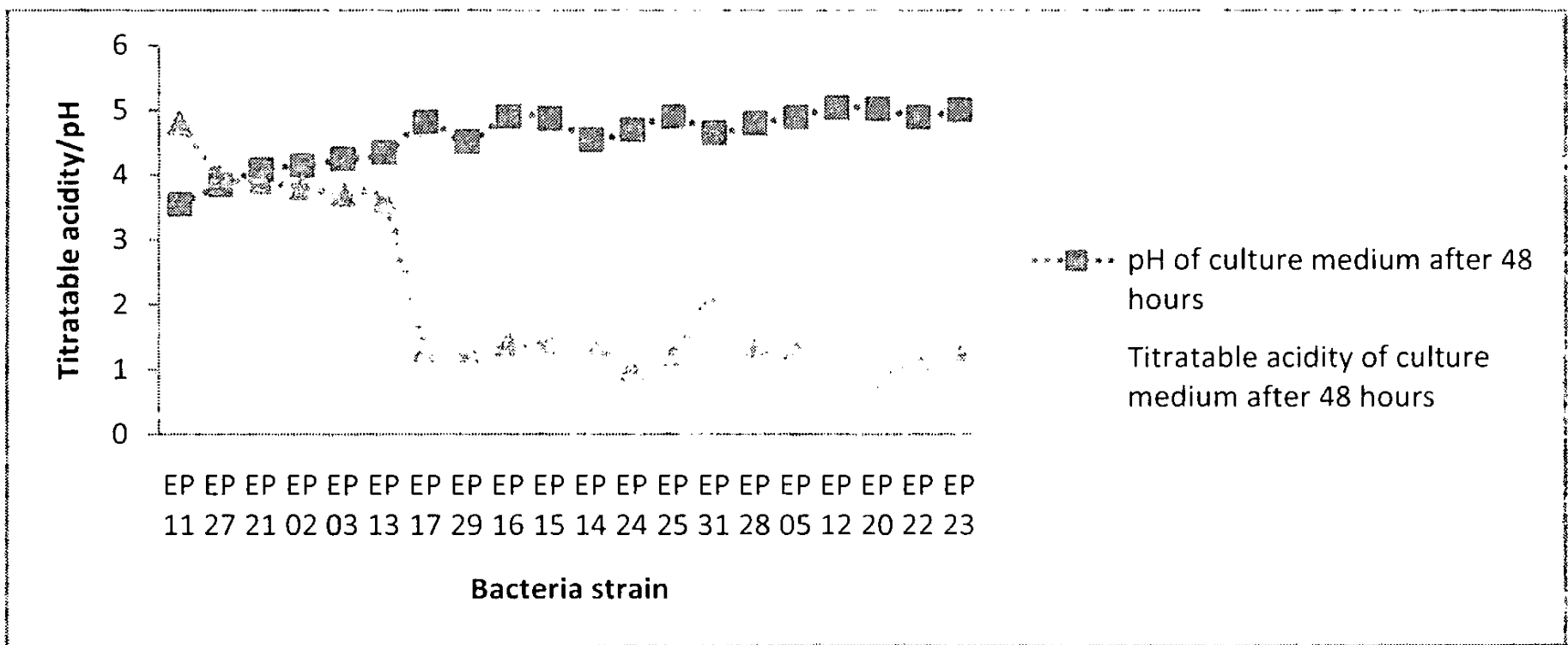


Fig. 3.7: Changes in pH and titratable acidity by the PSB in modified Pikovskaya's liquid medium containing 4.8 g/l Eppawala rock phosphate; Each value in the panel represents mean of three replicates.

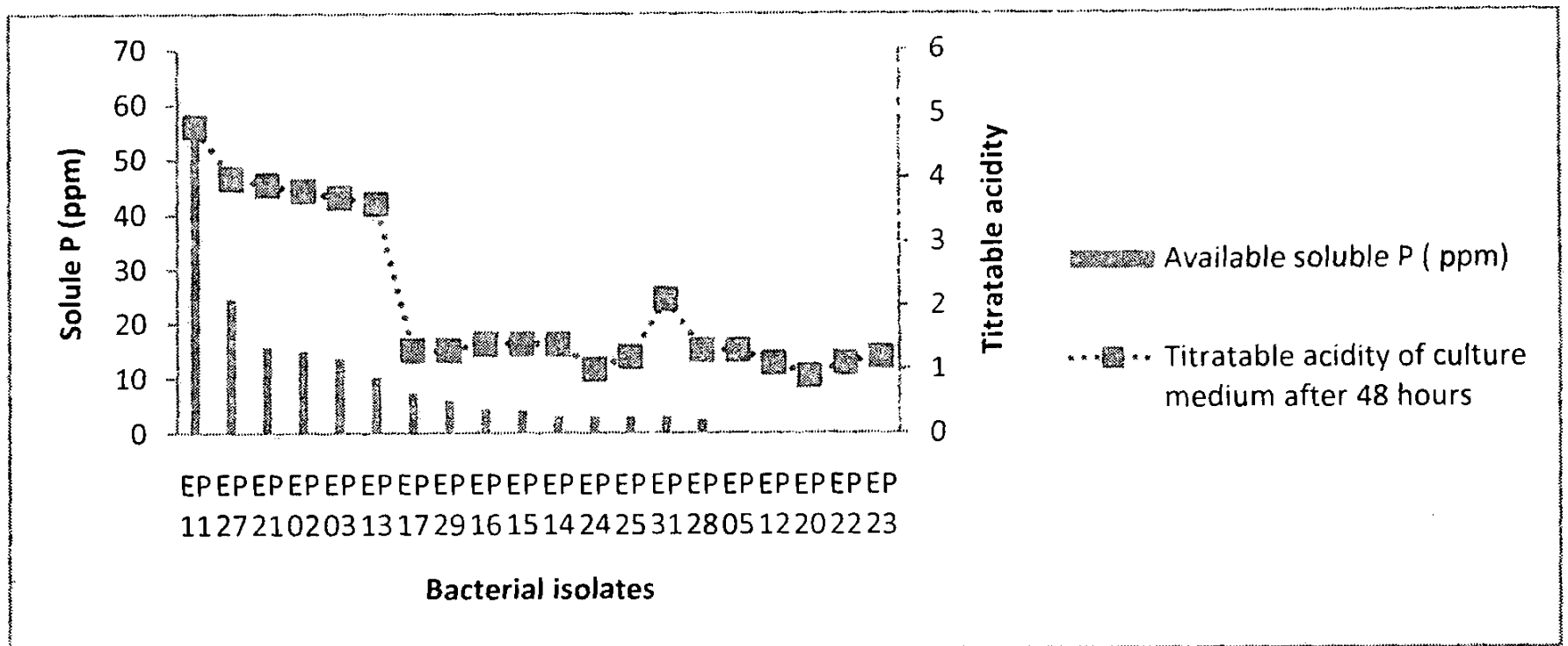
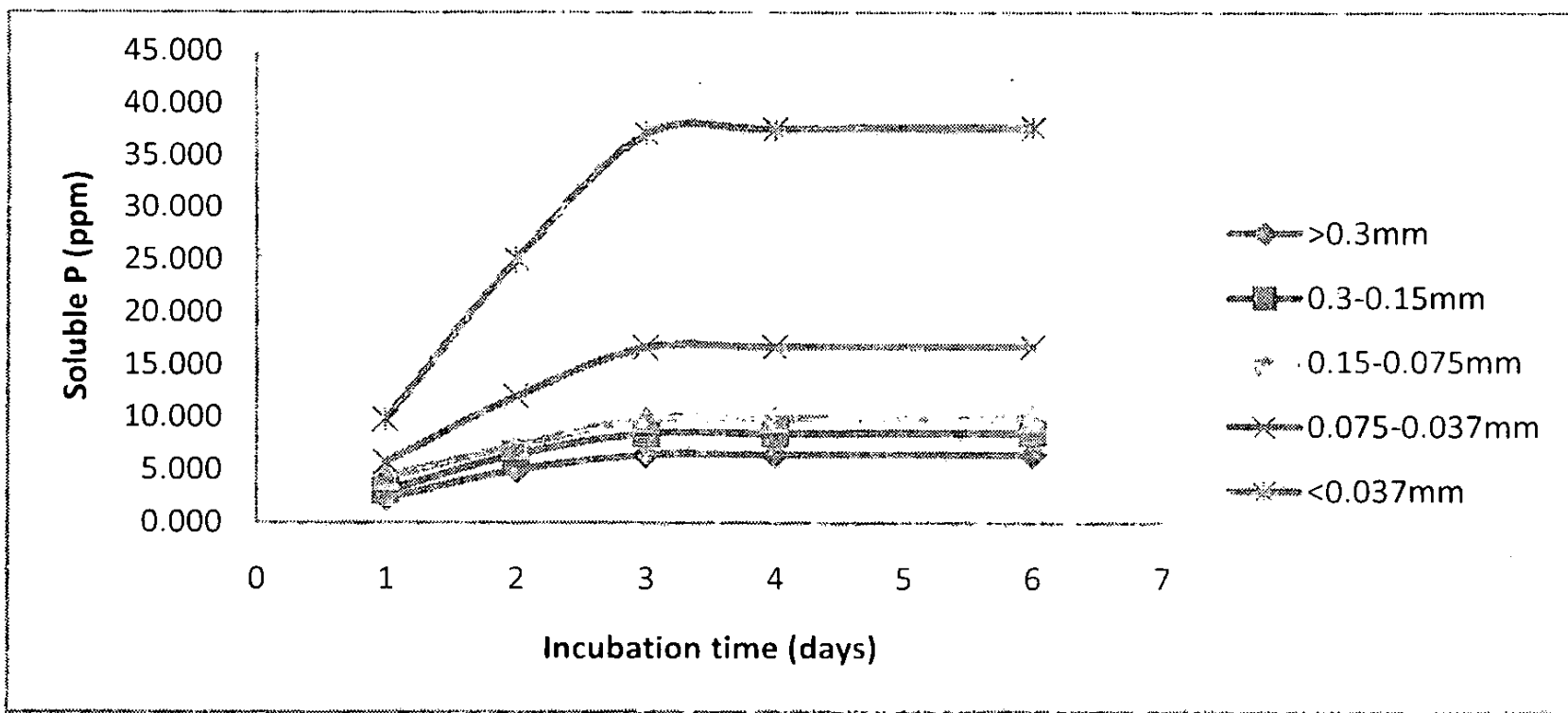


Fig. 3.8: Change in titrable acidity and soluble P by the PSB in modified Pikovskaya's liquid medium containing 4.8 g/l Eppawala rock phosphate, each value in the panel represents mean of three replicates.

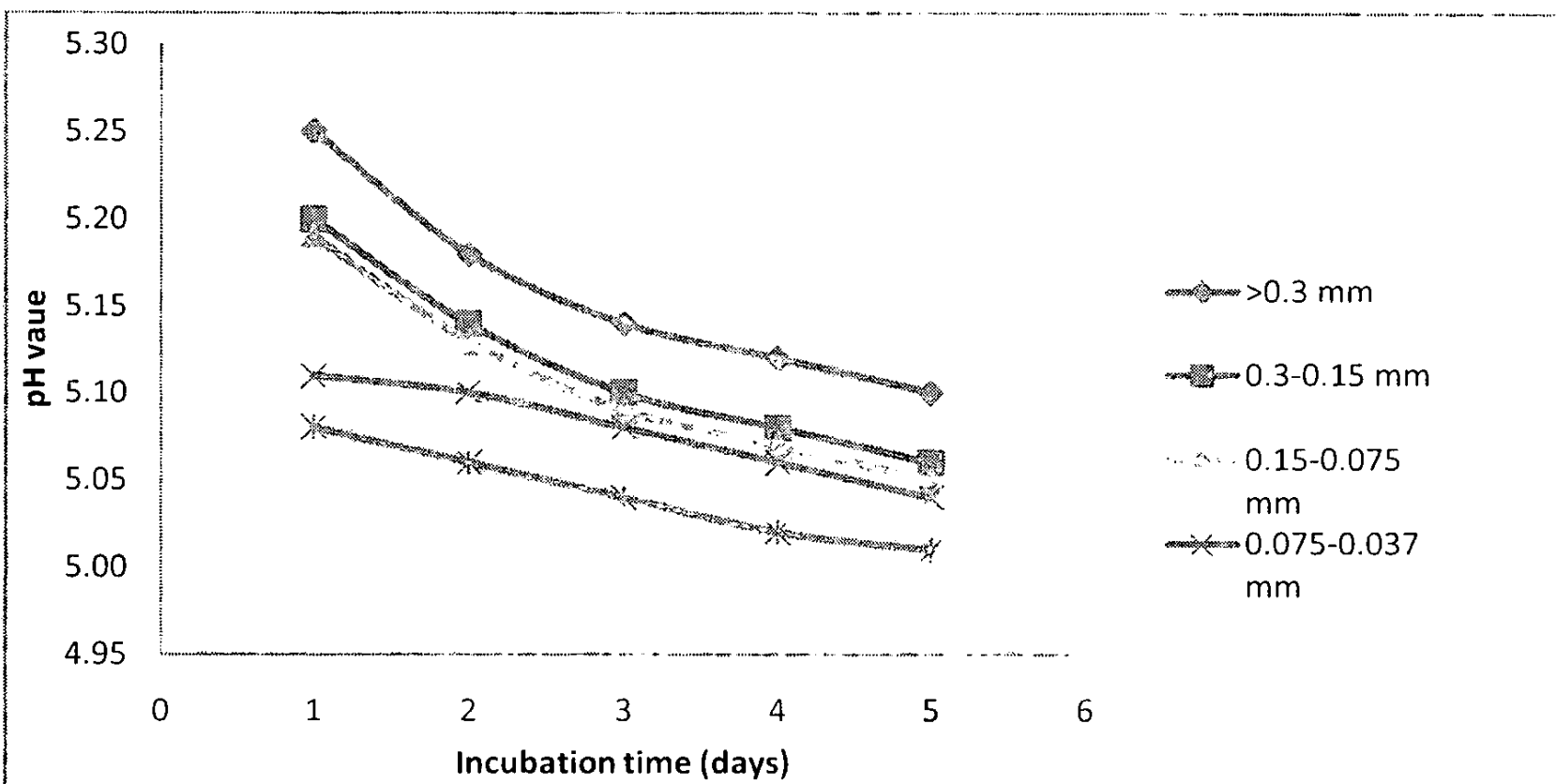
3.3 The effect of particle size on Rock-P solubilization

To determine the effect of particle size on Rock-P solubilization, five different particle sizes, >0.3, 0.3-0.15, 0.15-0.075, 0.075-0.037 and <0.037mm in modified PVK's liquid medium were inoculated with each strain. The observed solubilization patterns and associated culture pH changes during three day incubation period, are shown in figure 3.9 -3.15.

P concentrations from dissolving Rock-P showed non linear increase with time (figure 3.9 -3.14). Soluble P increased in the first few days and then became constant with the remainder of the experiment. The increase is quite rapid especially with the smallest particles, <0.037 mm where they released the highest concentration of soluble P (figure 3.9-3.14) with all strains, indicating a higher dissolution rates of smallest particles <0.037 mm over the larger particle sizes > 0.037 mm. The soluble P concentration in the media ranged from 14-69.78 ppm among six strains and decreased in the order, EP11>EP27>EP21>EP2>EP03>EP13. The highest soluble P concentration was recorded by EP 11, 69.18 ppm and the lowest soluble P concentration, 6.49 ppm by EP 13.



a

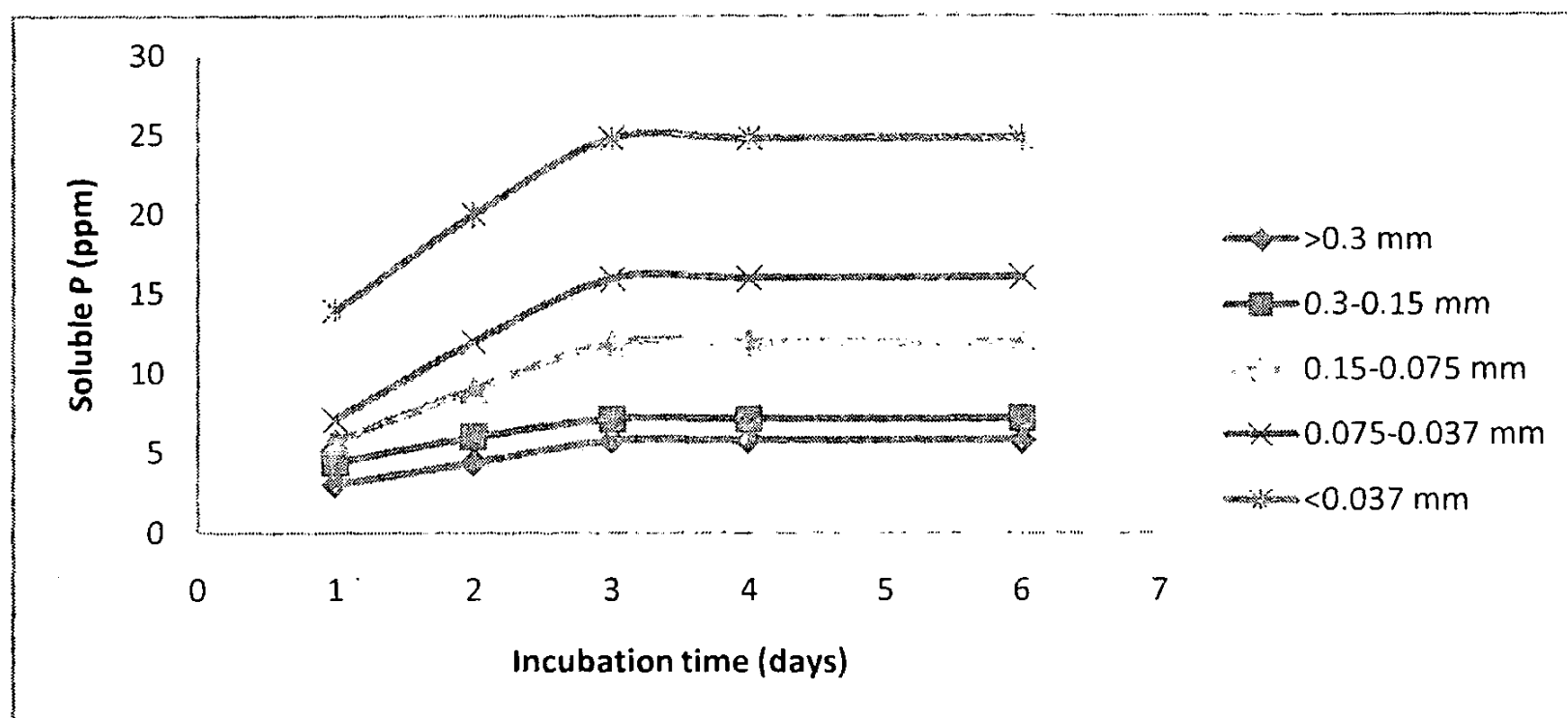


b

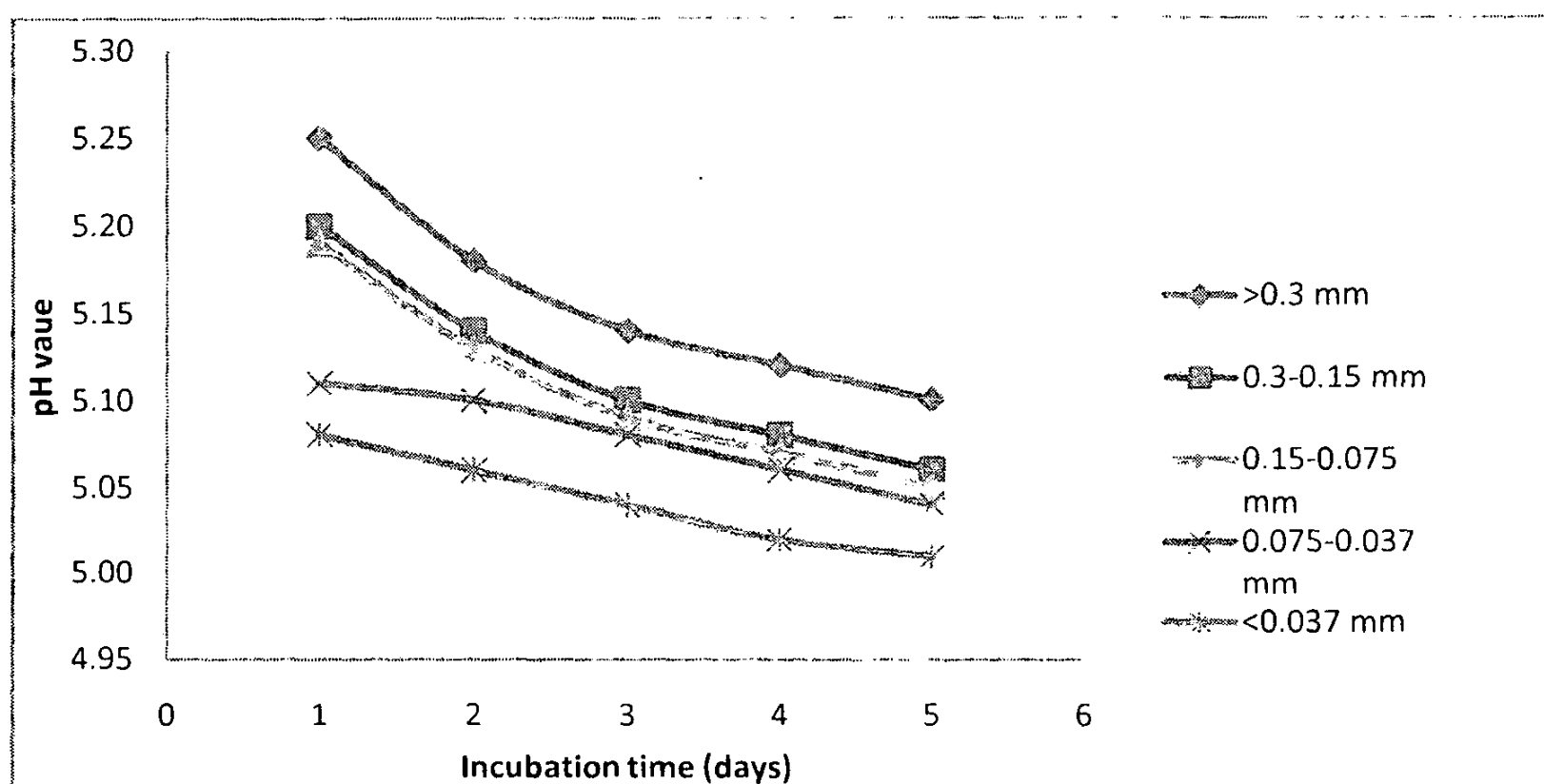
Fig. 3.9 a,b : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different sized Rock-P particles (9.6 g/L) by strain EP 2 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates.

The rate of solubilization and the average concentration of soluble P in the culture however, appears to decrease with increasing particle size (figure 3.9-3.14). The maximum soluble P concentration released from the largest particle sizes >0.037 mm by the most efficient solubilizer EP 11, was 48.72 ppm whereas the minimum soluble P concentration, of 6.49 ppm was reported with EP 13.

Other strains were moderately effective and showed intermediate soluble P concentrations with each particle size ranges between 0.15 - 0.037mm (figure 3.9 -3.14).



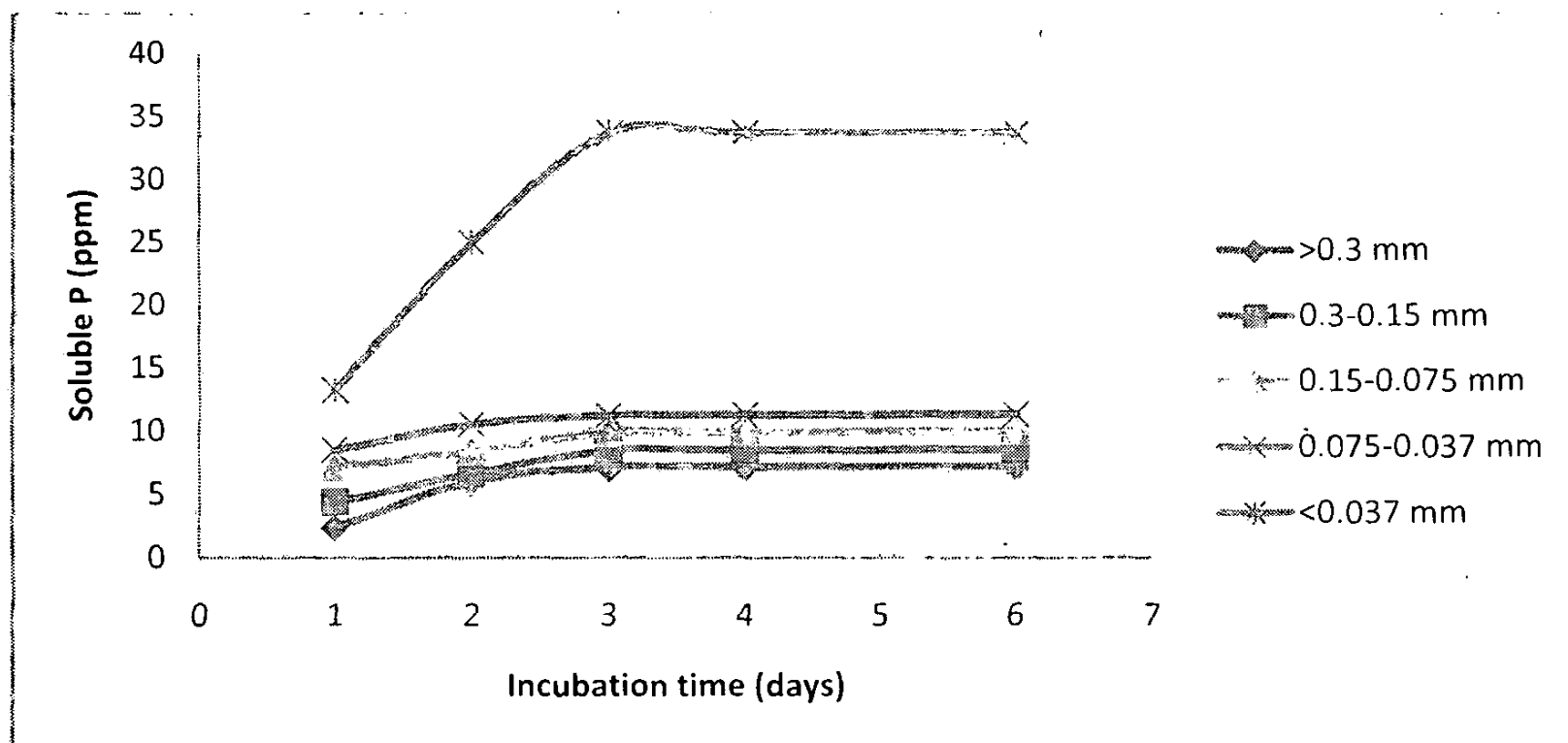
a



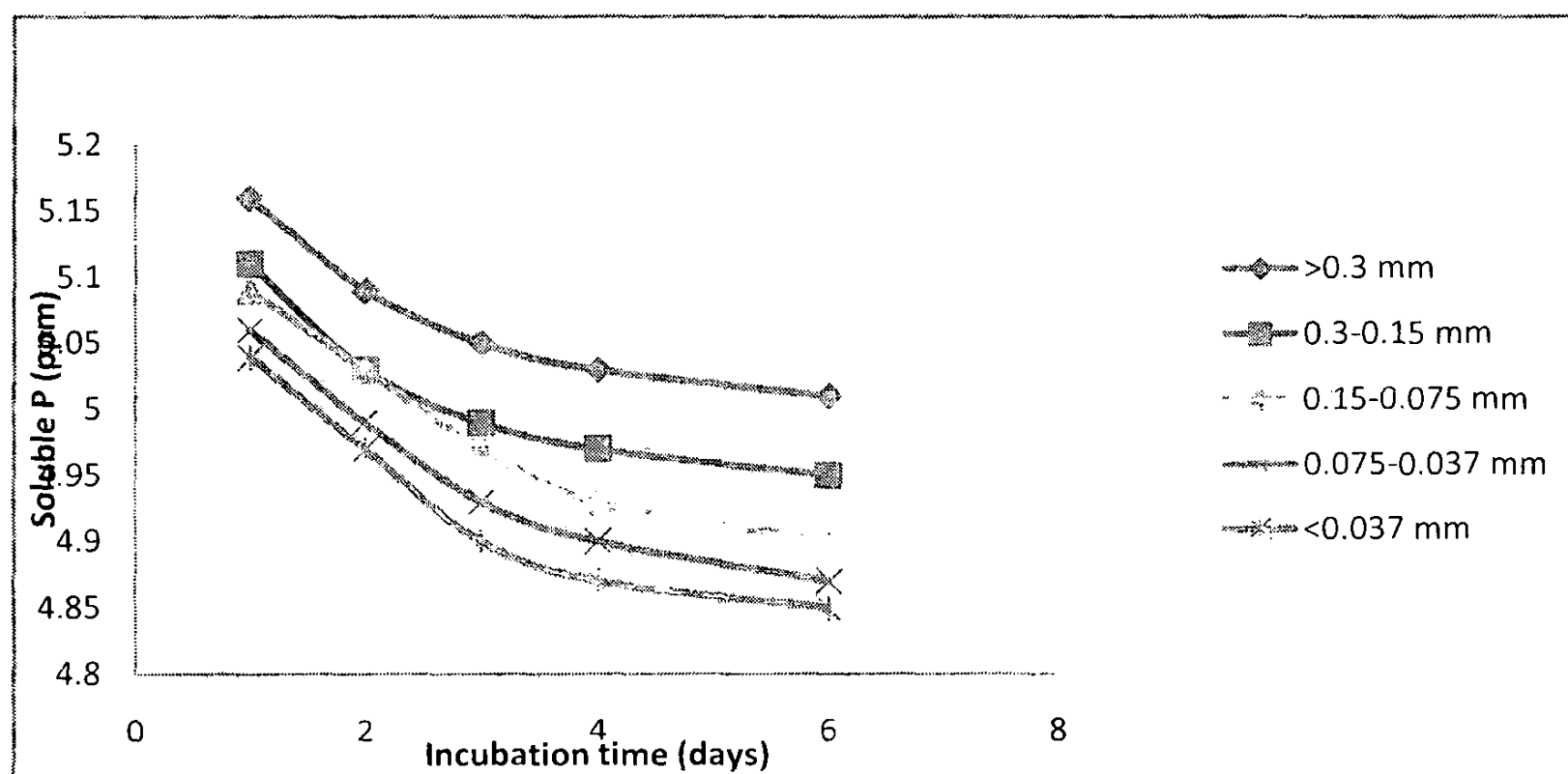
b

Fig. 3.10 a, b : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different sized Rock-P particles (9.6 g/L) by strain EP 27 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

P Solubilization is also accompanied with a corresponding decrease in pH in the medium. pH drop is somewhat rapid in the first few days and then increased slightly with EP11, EP21, and EP13 strains, whereas it continued to decrease with EP2, EP27, EP3.



a



b

Fig. 3.11: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different sized Rock-P particles (9.6 g/L) by strain EP 3 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

Maximum drop of culture pH by EP2, EP27 and EP3 strains was observed when culture was amended with the smallest particle sizes <0.037 mm. Conversely the maximum drop in culture pH with other three strains EP 11, Ep 21 and EP 13 were observed with the largest particle sizes.

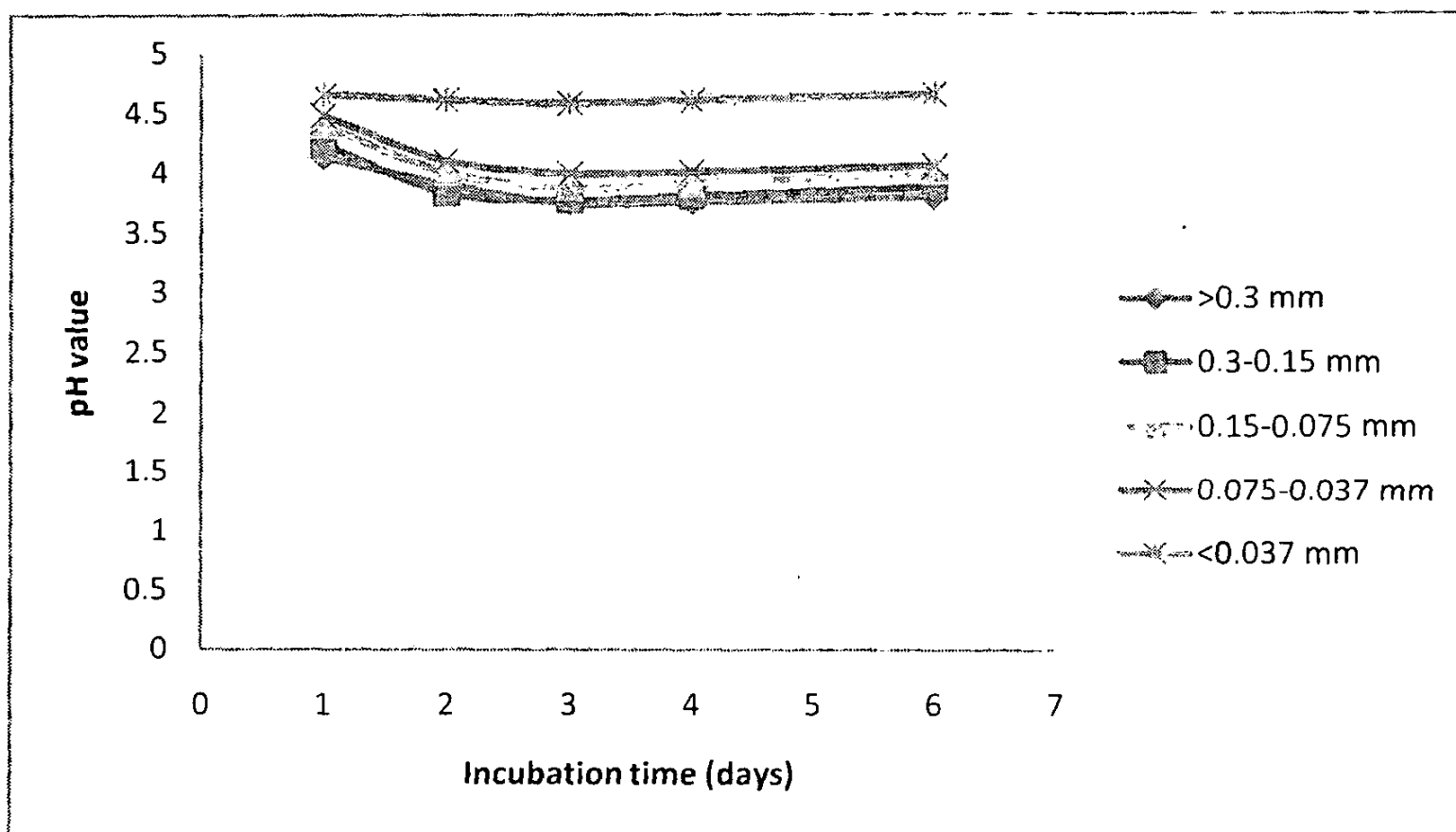
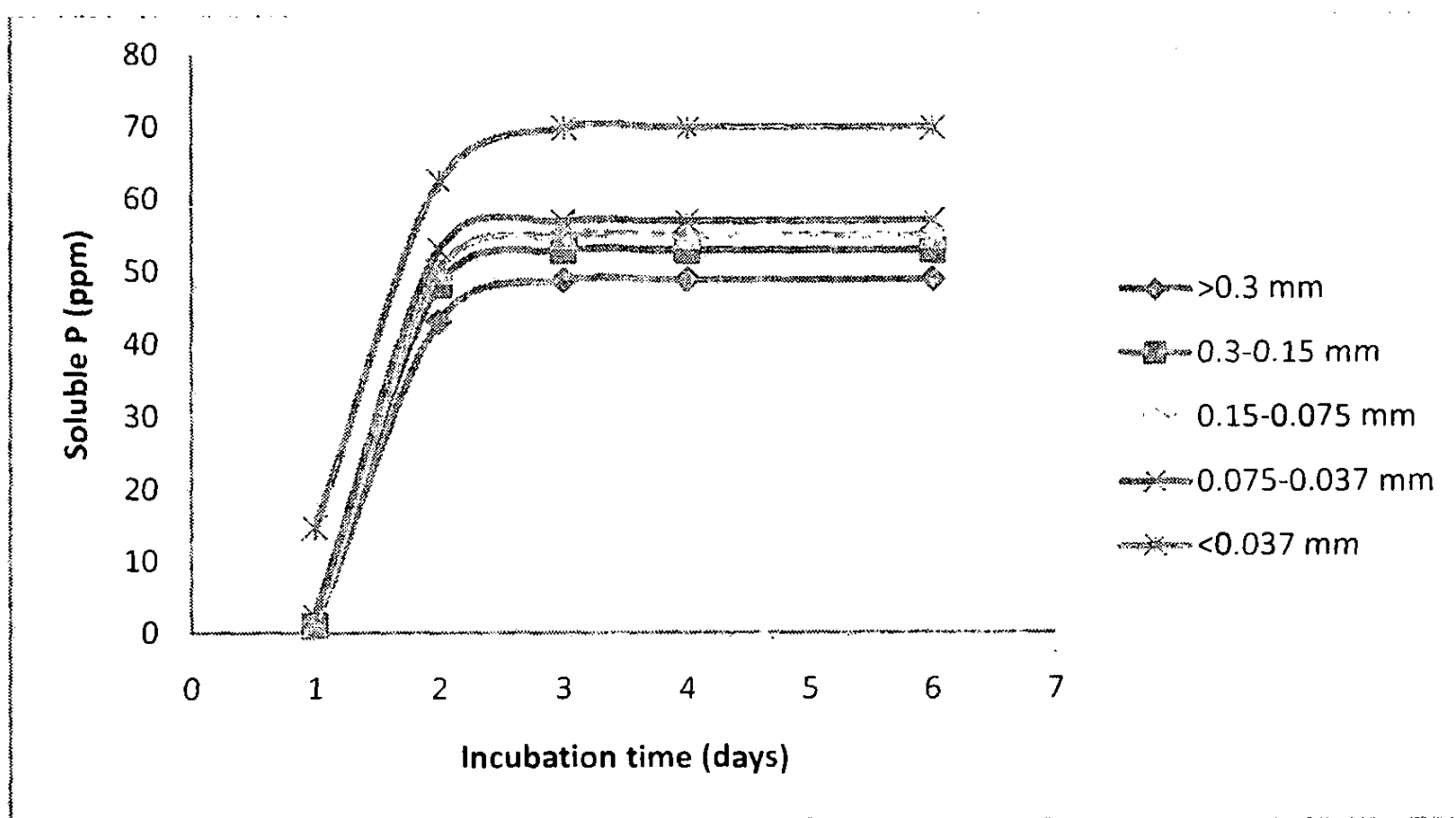


Fig. 3.12 : : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different sized Rock-P particles (9.6 g/L) by strain EP11 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

Analysis of Rock-P solubilization of each strain indicate they share some similarities as well as some differences among one another. Solubilization patterns of strain EP 2 (figure 3.9), is somewhat similar to that of EP27, (figure 3.10). However the rate of solubilization and average P concentrations released by EP 2 is much higher than that of EP27.

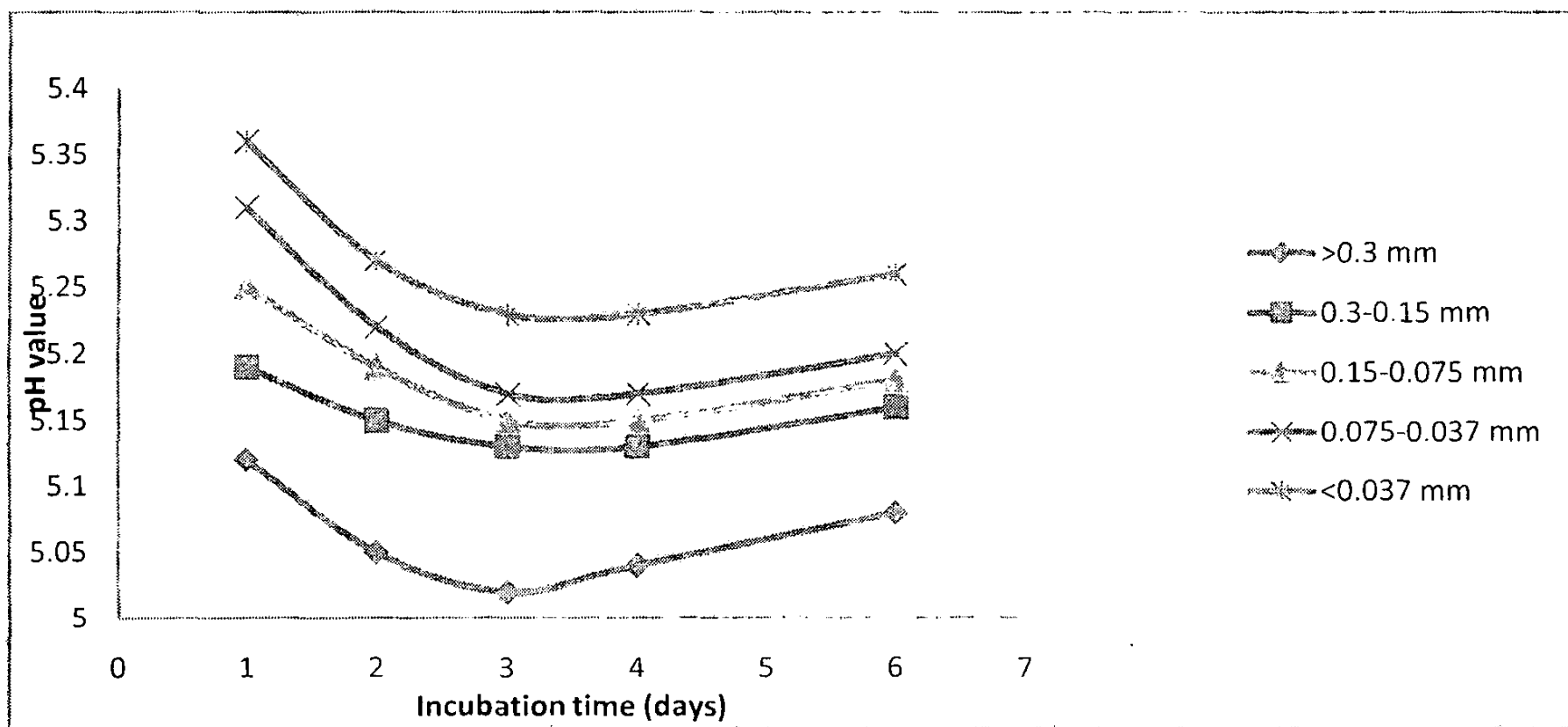
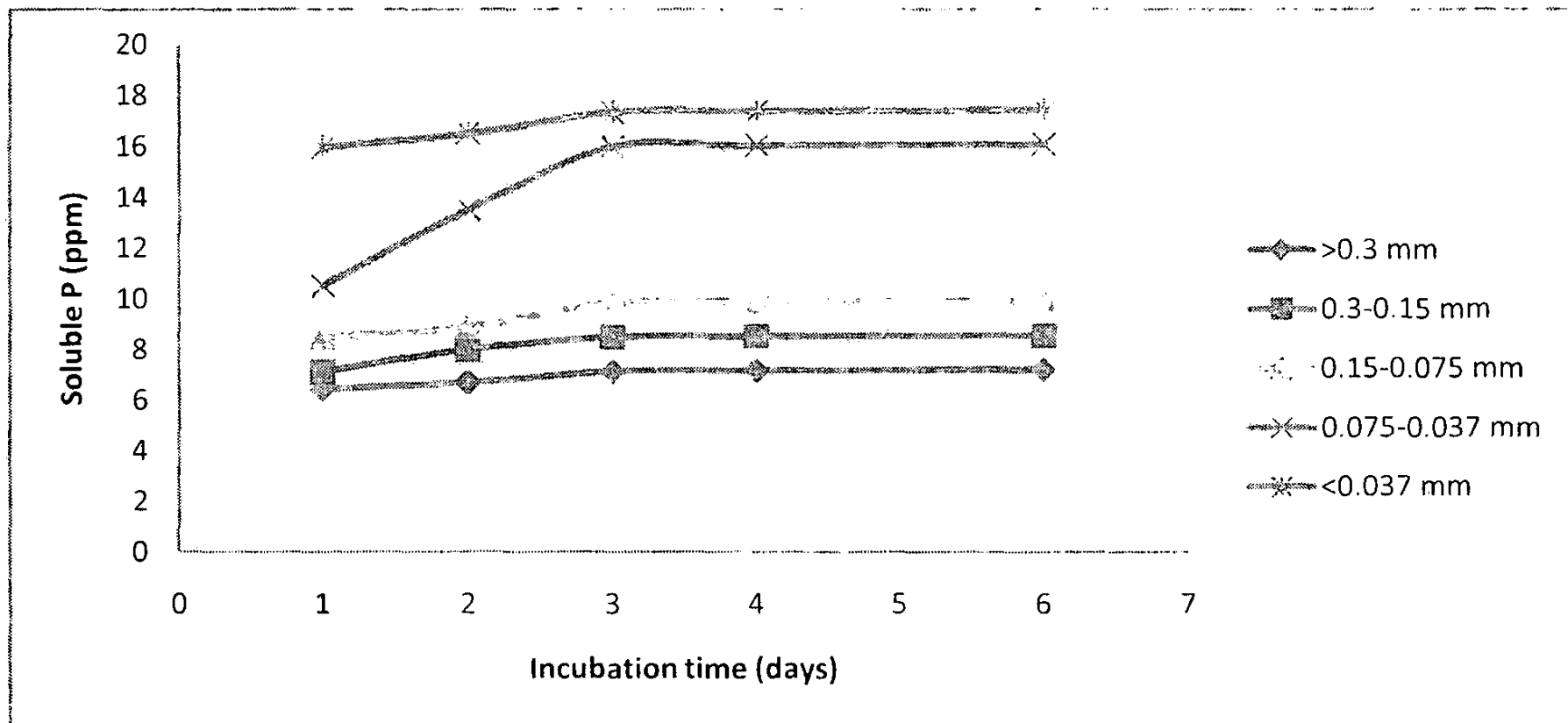


Fig. 3.13: : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different sized Rock-P (9.6 g/L) by strain EP 13 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

Strain EP3 (figure 3.11) also follows the similar solubilization pattern to that of EP2 and EP27. Rapid early increase of soluble P, with the smallest particle size is again notable with strain EP3. Average soluble P concentration released with smallest particles by EP3, also close to that of EP2. However, solubilization of larger particle sizes above 0.037 mm occurred at considerable lower rates with EP3, thereby releasing only low, 5-10 ppm levels of soluble P from larger particles. pH of EP3 culture medium follows the same trend as with EP2 and EP 27.

Effect of Rock-P particle size on P solubilization by EP 11 appears to share similarities as well as some important differences to that of other strains. Strain EP 11 shows the typical nonlinear increase of soluble P with time. It shows the highest solubilization rate with the smallest particles <0.037 mm as usual and released the highest soluble P concentration observed in this series of experiments. In addition, to smallest size particles, EP 11 seems to have solubilized larger Rock-P particles, above >0.037 mm also efficiently. For this reason soluble P concentrations released from larger particle also reached quite high, 48.72 ppm, which is close to that released from the smallest particle, 69.18 ppm. This is also evident from their solubilization rates, where all particle sizes appear to have been solubilized approximately at the same rate (figure 3.12) by EP11. Therefore strain EP11 observed to be more efficient solubilizer for releasing soluble P from various Rock-P particle sizes. It was also noticed that pH of the culture medium containing smallest particle size dropped only slightly and remained constant during the solubilization period. In contrast, pH of the cultures containing larger particle sizes above 0.037 mm dropped pH in substantial amounts in the first three days and then started to rise slightly in the remainder of the solubilization process. This pattern of changing pH observed with EP11 is different from that observed with EP2, EP27 and EP3 where pH decreased continuously during the solubilization process (figure 3.12).

Effect of Rock-P particle sizes on P solubilization by remaining EP13 and EP21 strains follow similar solubilization patterns to each other. Though, dissolution pattern was non linear, usual rapid early increase of soluble P, was not prominent or even absent with the smallest particle size. Average soluble P concentration released with smallest particles also considerably low. Changes in culture pH are similar to that of strain EP11 for both strains (figure 3.13 and 3.14).

In summary, figure 3.15 shows the overall effect of different particle sizes on the solubilization of Rock-P by six bacterial strains, tested. The release of soluble P gradually increase with the decrease of particle size with all strains.

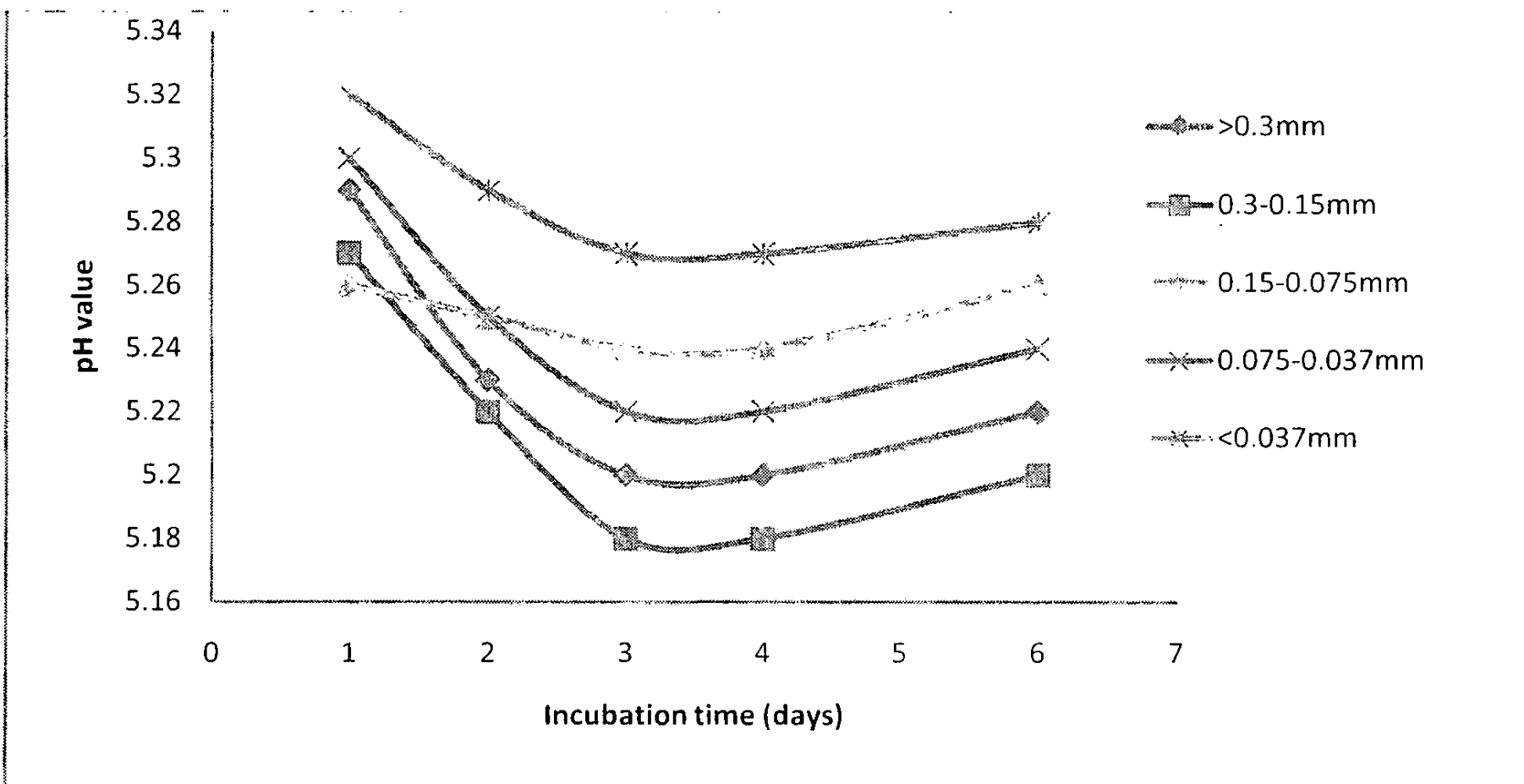
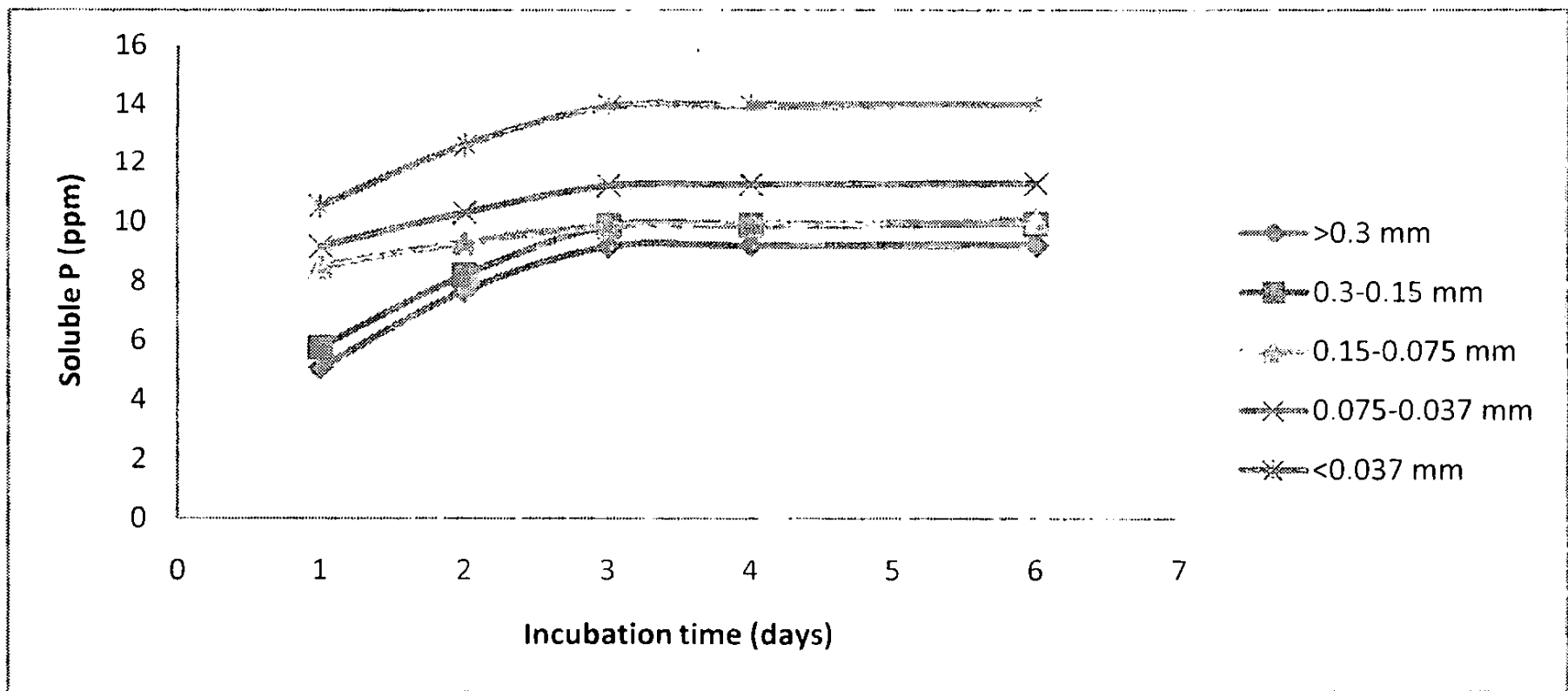


Fig. 3.14: : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different sized Rock-P particles (9.6 g/L) by strain EP21 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

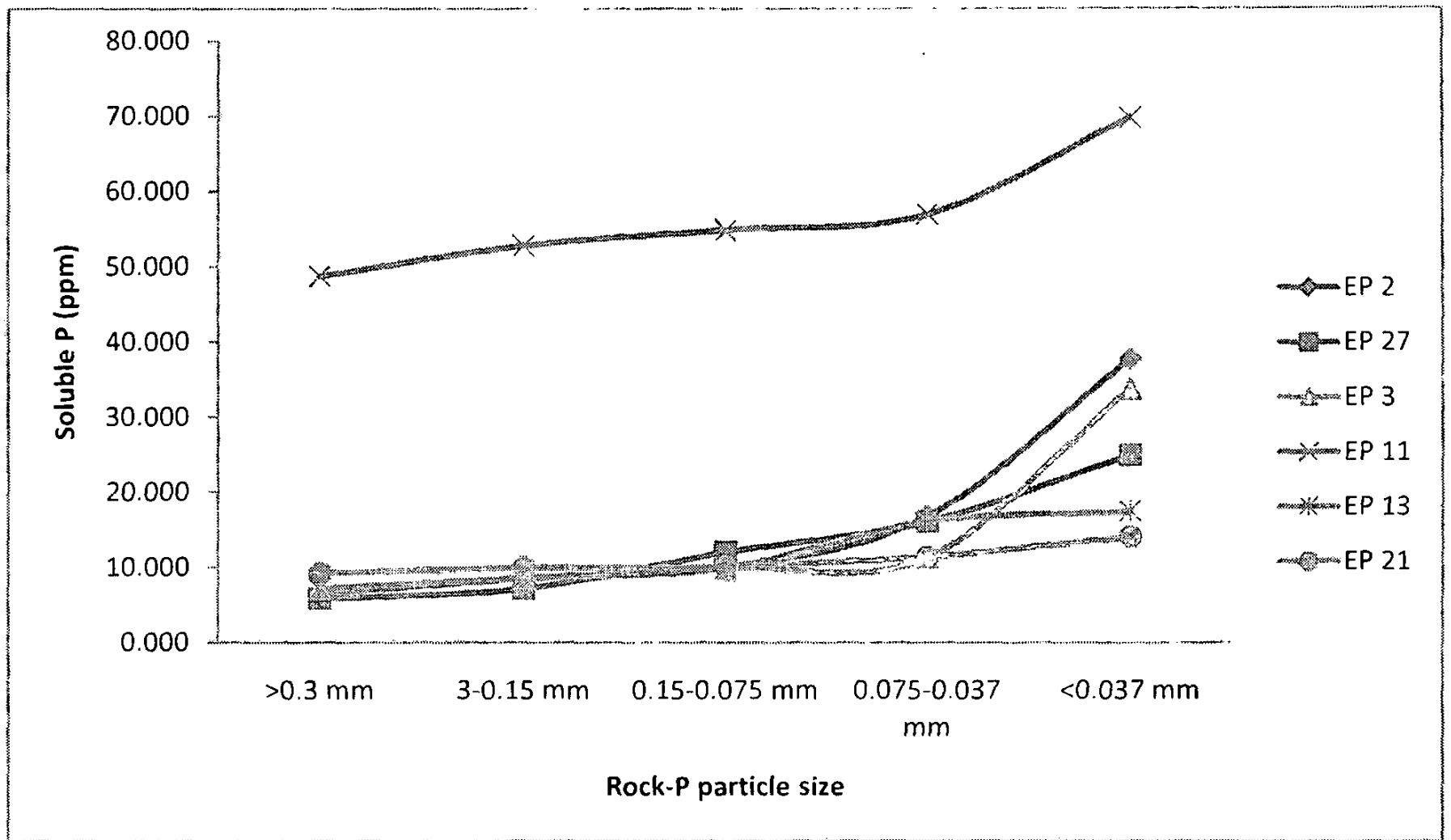


Fig. 3.15 Effect of Rock-P particle size on soluble P concentration after 6 days of incubation. Bacterial Strains: EP2, EP 27, EP3, EP11, EP13, and EP21, Each value in the panel represents mean of three replicates

The smaller the particle size, the higher the concentration of soluble P released. This is clearly observed with all strains. In addition EP11, solubilized larger particles efficiently and released soluble P concentration which is in level with that released from the smallest particle sizes. Among all the isolates the concentration of soluble P released by EP11 was the highest.

3.4 The effect of Rock-P concentration on Rock-P solubilization

To determine the effect of different Rock-P concentrations on their solubilization, modified PVK liquid media containing varying concentrations of Rock-P, 1%, 3%, and 5% (w/v) were inoculated with each bacterial strain. The observed solubilization patterns and associated culture pH changes during six day incubation period, are shown in figure 3.16 -3.22.

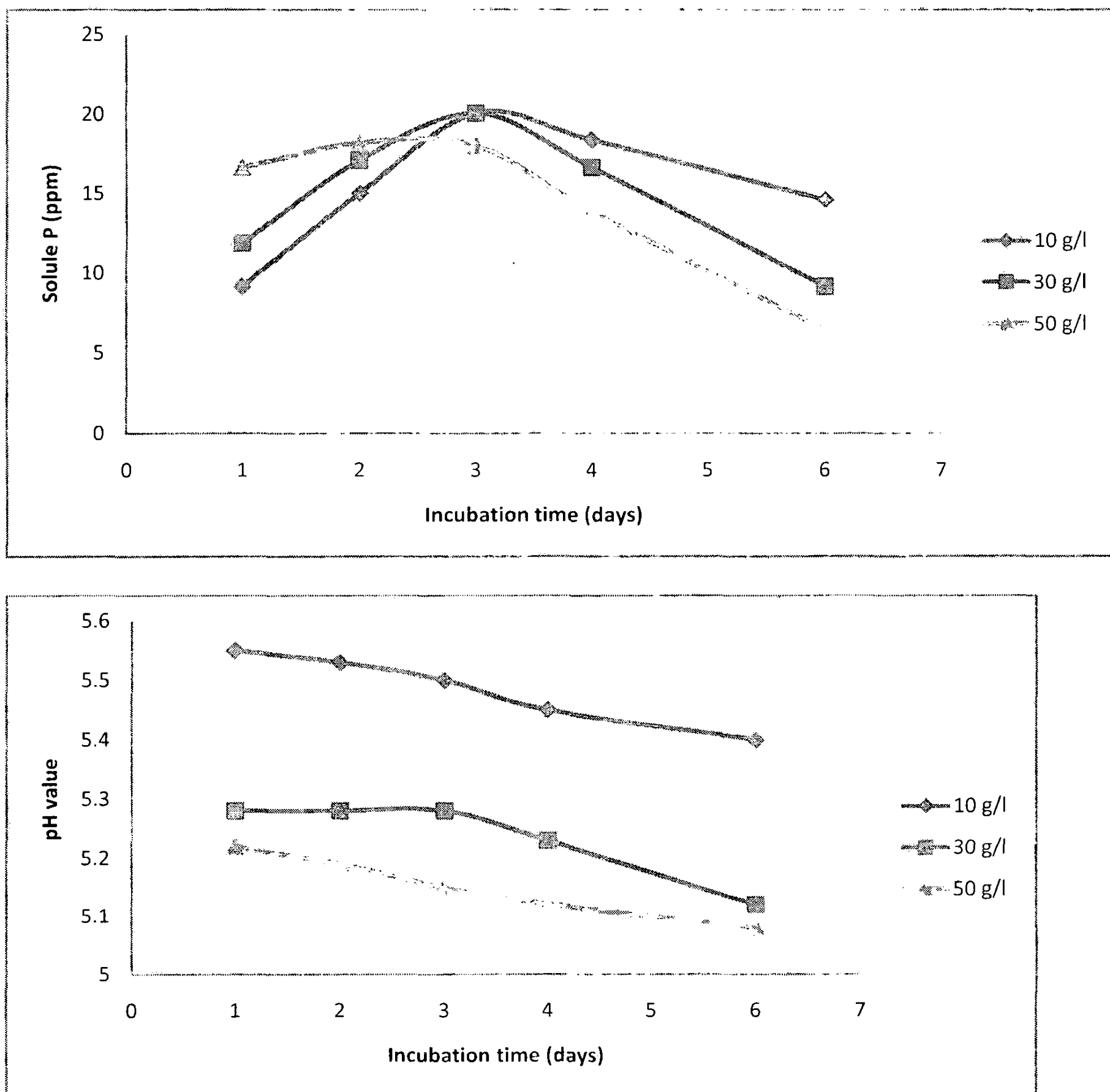


Fig. 3.16: : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different concentrations of Rock-P by strain EP2 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

At 1% Rock-P concentration, strain EP11, released 118 ppm, the highest concentration of soluble P, into the medium (figure 3.18), whereas EP 27 exhibited 13.25 ppm, the lowest concentration of soluble P (figure 3.21) (table 3.5).

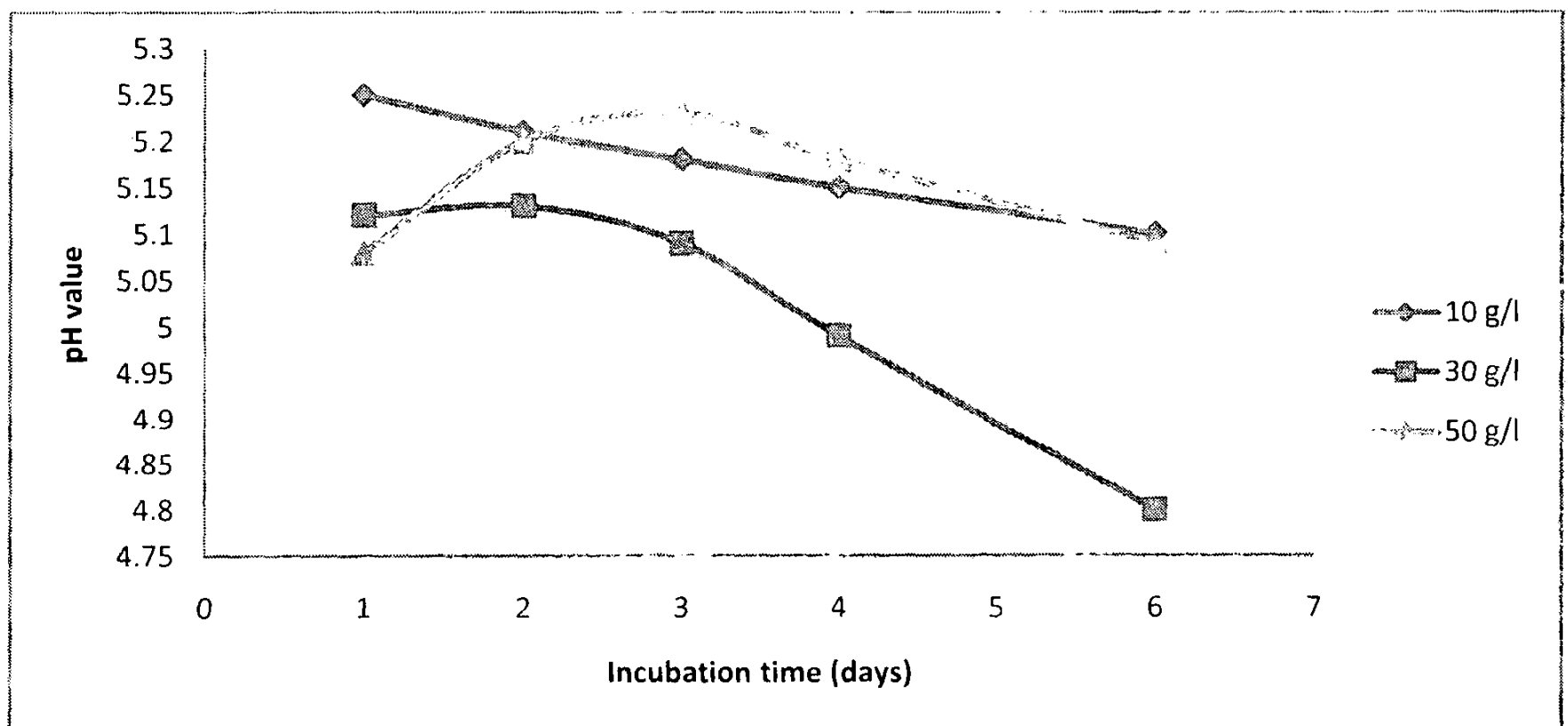
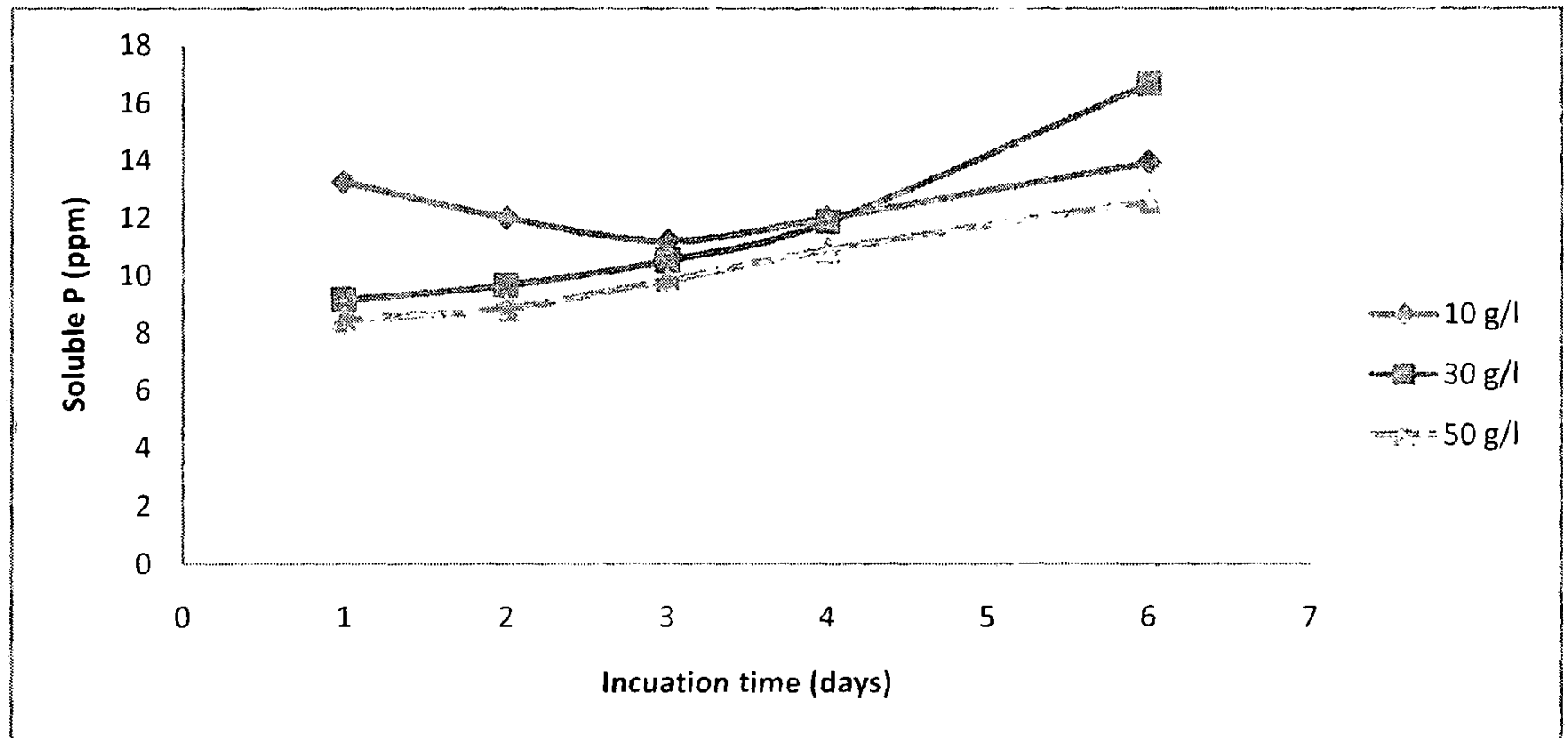


Fig. 3.17: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different concentrations of Rock-P by strain EP3 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

Concentration of soluble P released decreased in the order EP11>EP13>EP27,EP21>EP2>EP3 (figure 3.16 -3.21)(table 3.5). As noticed earlier, P solubilization was accompanied with a concomitant drop of pH in the culture medium.

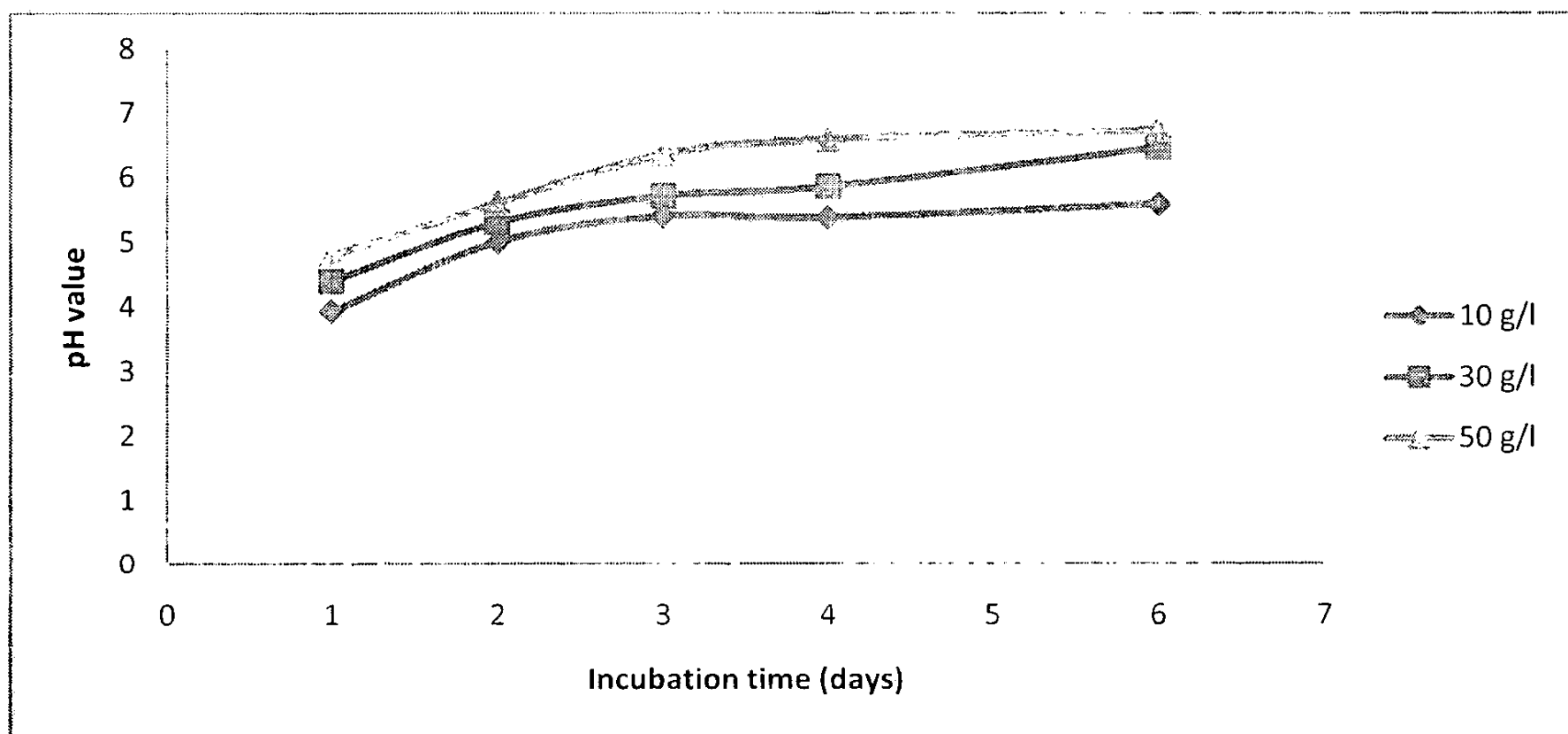
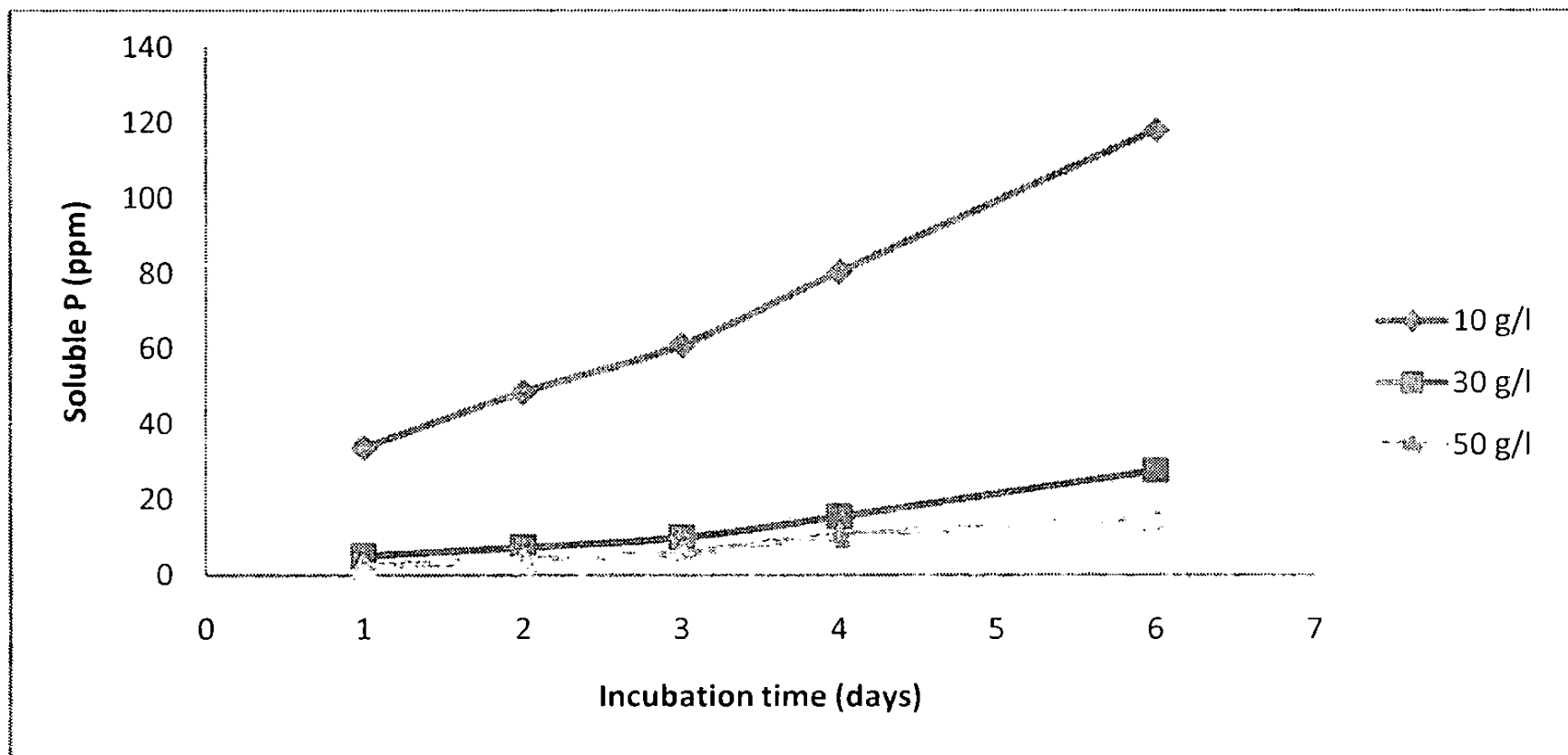


Fig. 3.18: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different concentrations of Rock-P by strain EP11 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

The strain EP11 which released the highest amount of soluble P drop the pH to 5.58, whereas the least efficient strain EP27, dropped the pH further to 4.84 (figure 3.18).

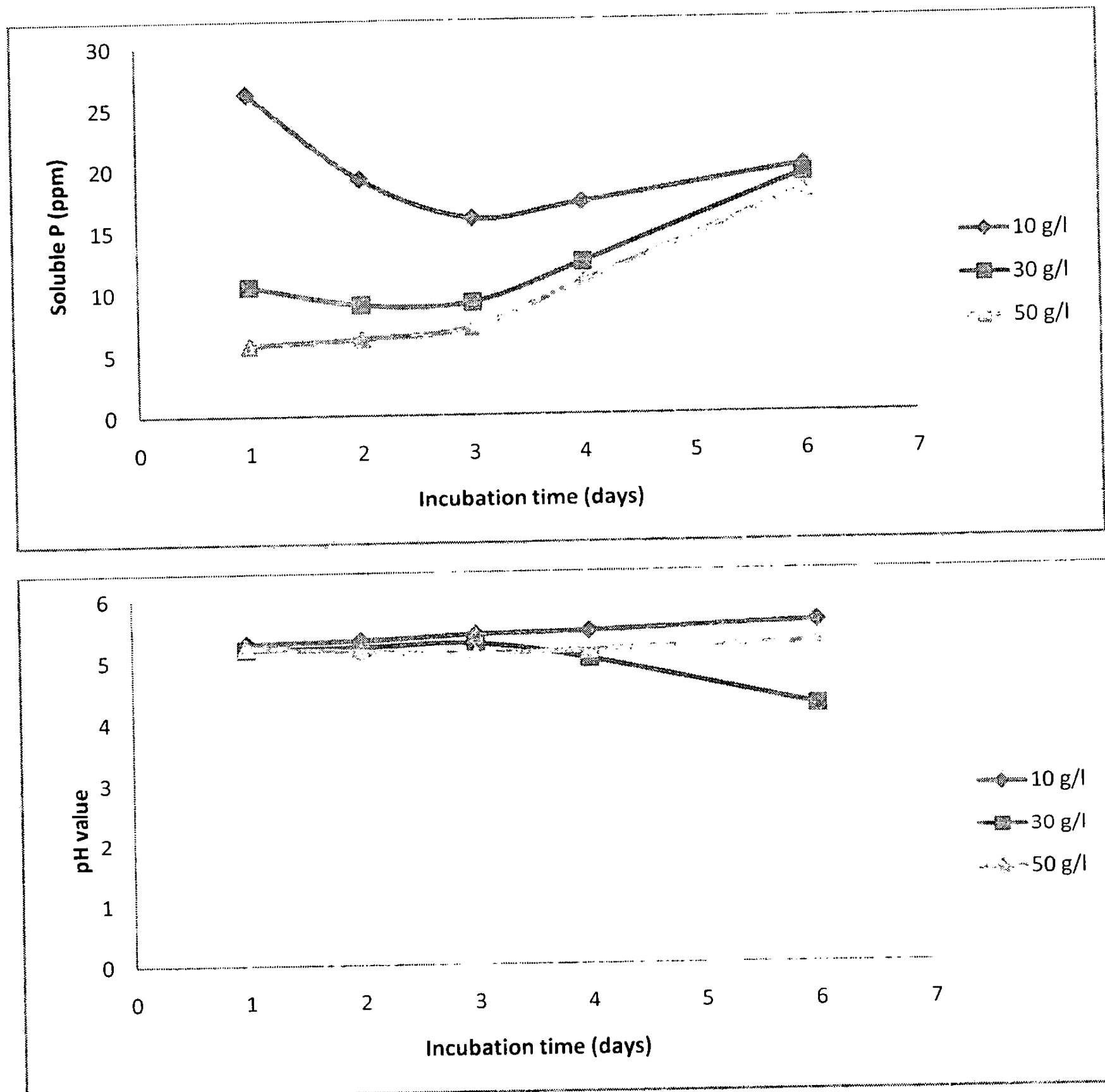


Fig. 3.19: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different concentrations of Rock-P by strain EP13 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

When the concentration of Rock-P was increased to 3% the release of soluble P by efficient solubilizer EP11 dropped drastically. At 3% Rock-P concentration (30g/l), EP11 released only 27.54 ppm, which is much lower compared to soluble P, released previously at 1% Rock-P, approximately by a factor of four (table 3.5).

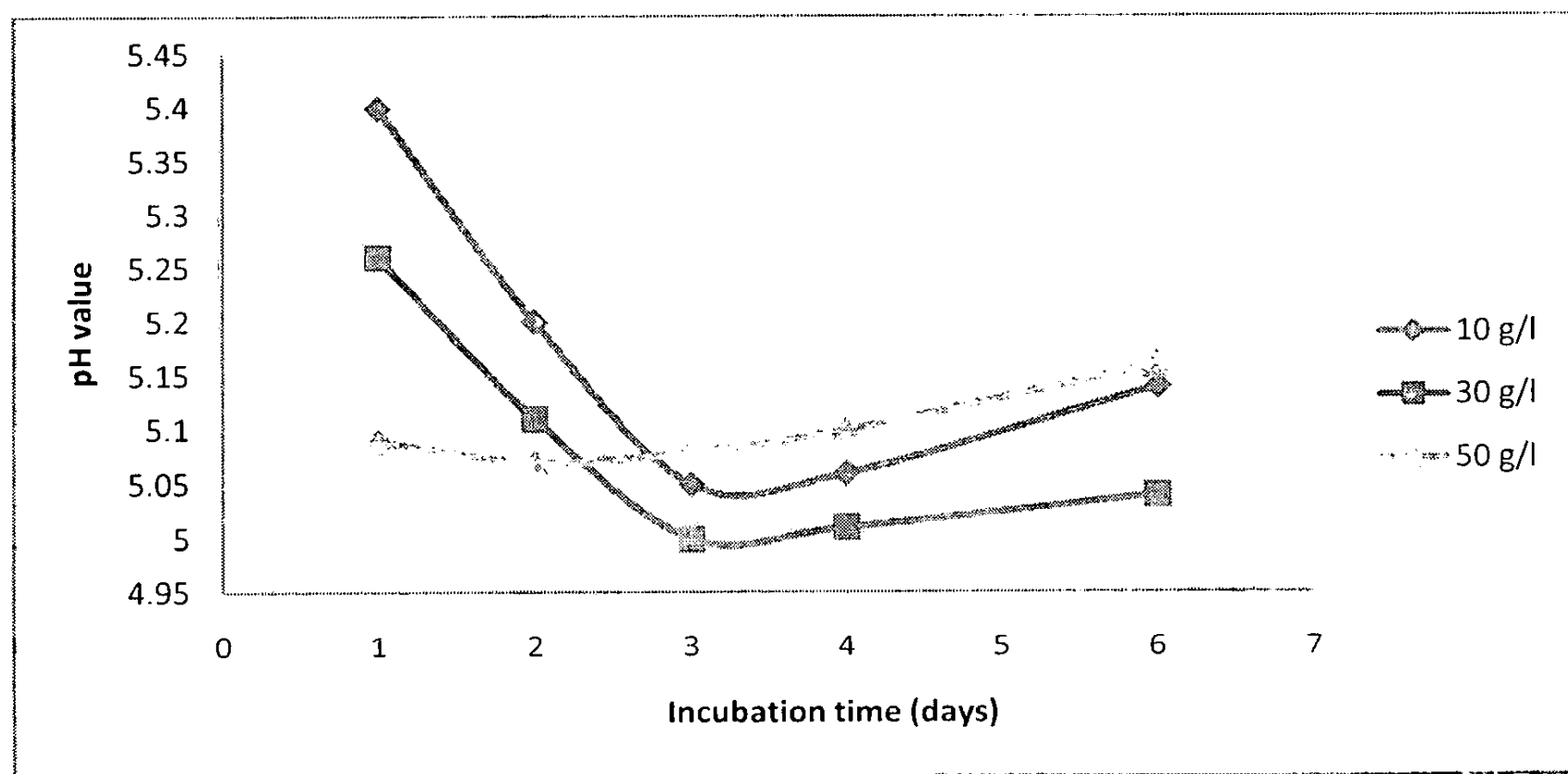
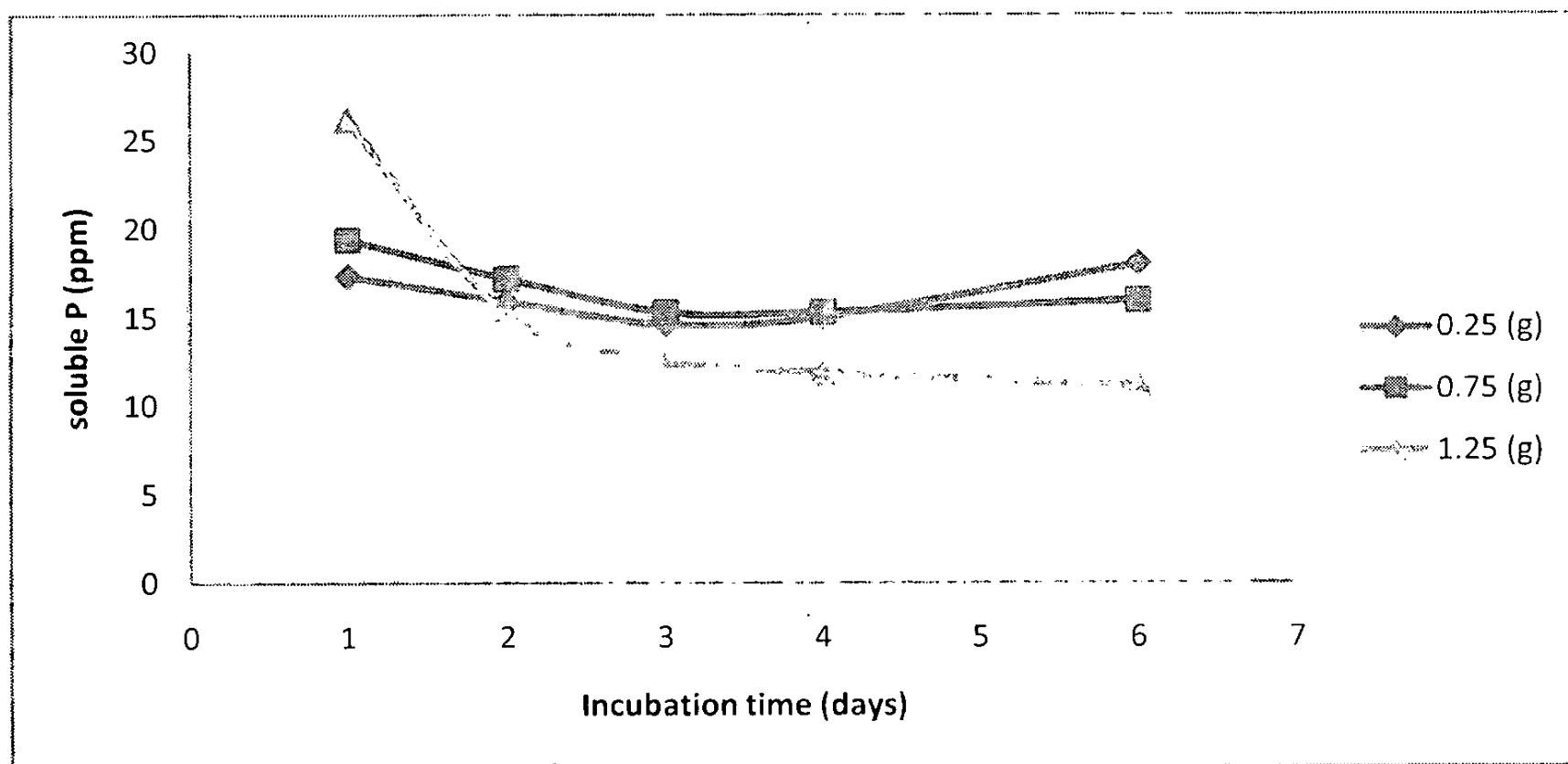


Fig. 3.20: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different concentrations of Rock-P by strain EP21 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

However this is still the highest concentration observed at 3% Rock-P. In addition to EP11, EP27 also released the same concentration of soluble P, which is however a slight increase from its soluble P released at 1% Rock-P. Strain EP2 exhibited the lowest concentration of soluble P, which was 9.16 ppm (figure 3.16).

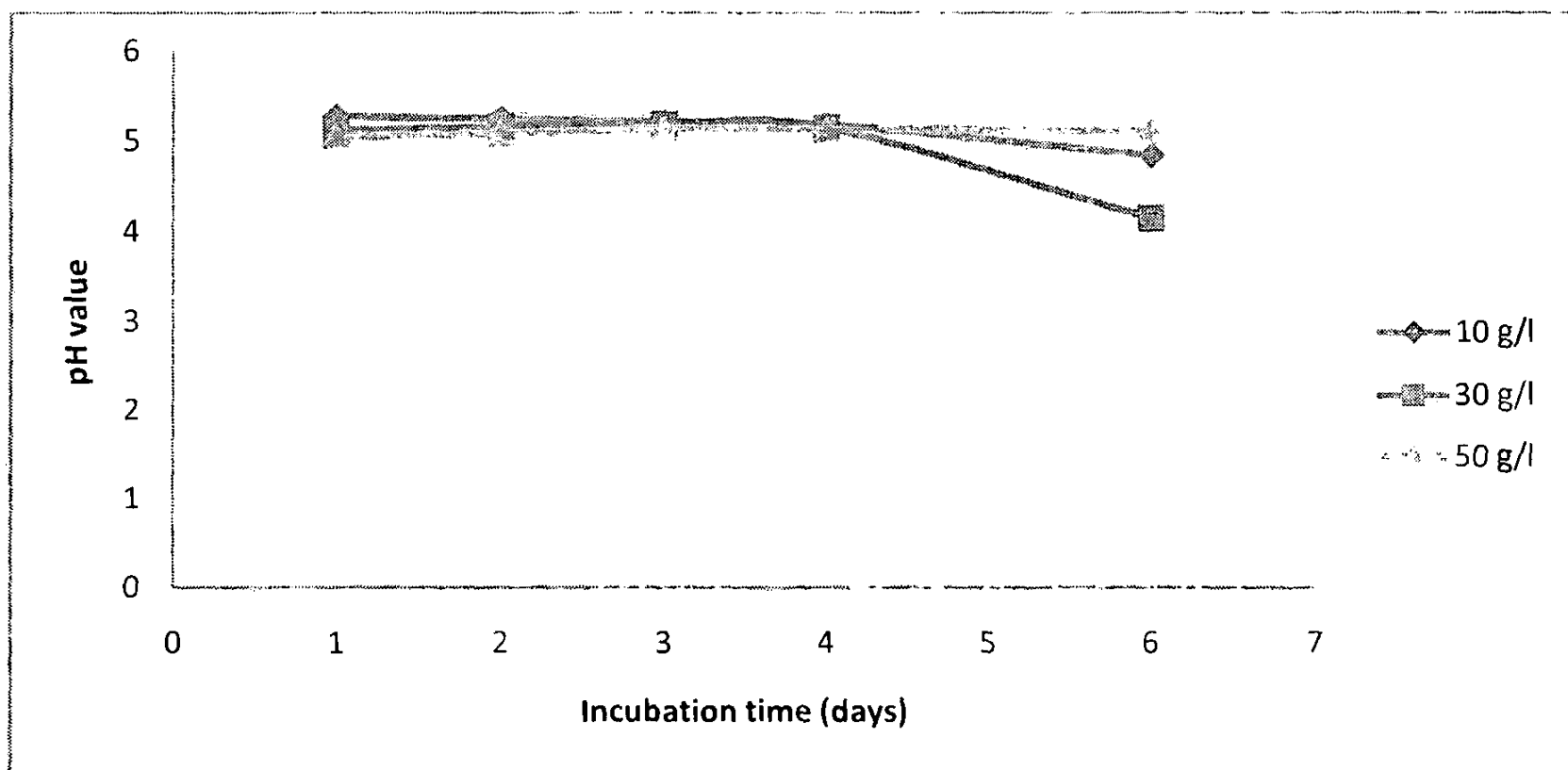
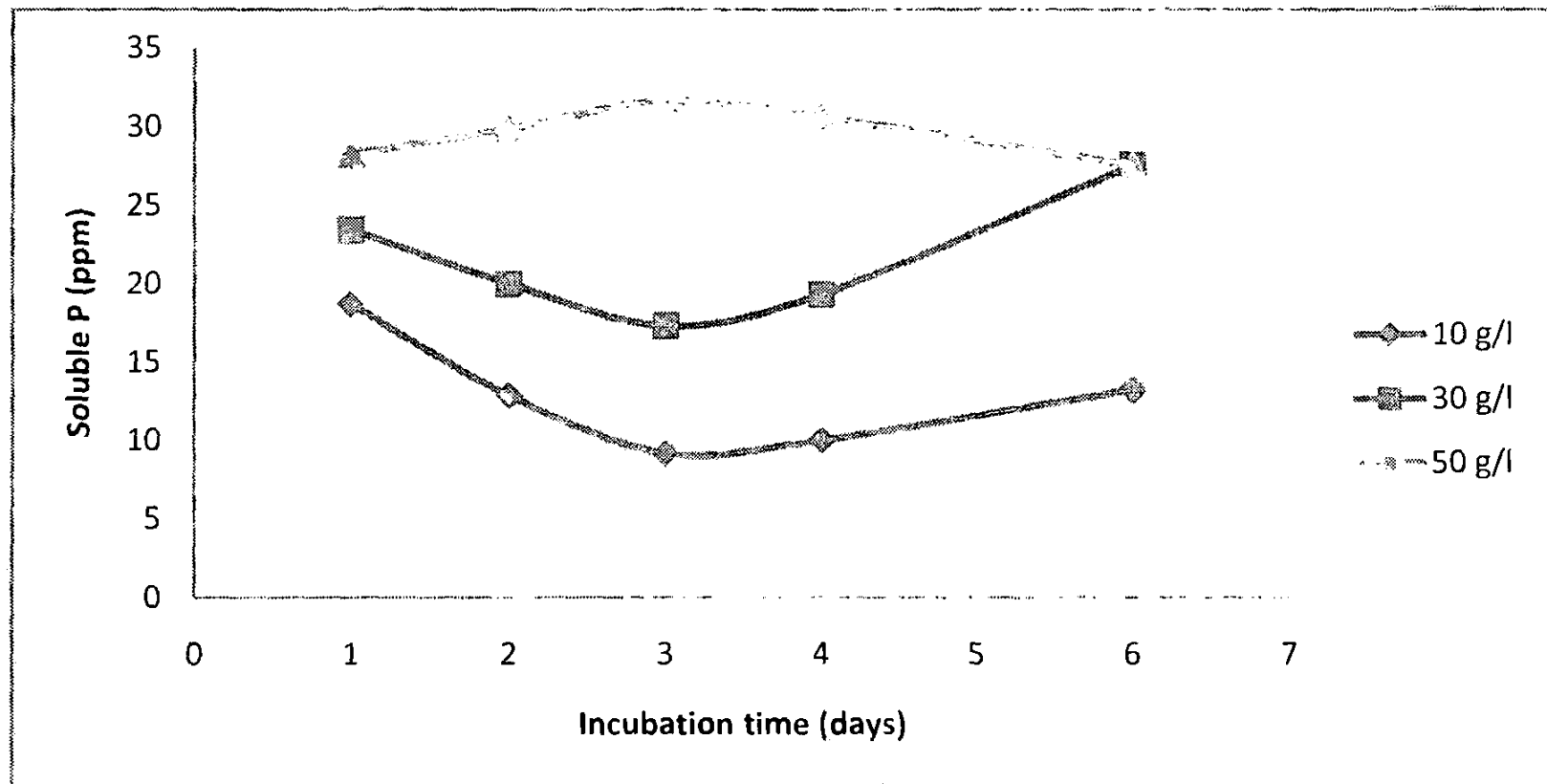


Fig. 3.21: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of different concentrations of Rock-P by strain EP27 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

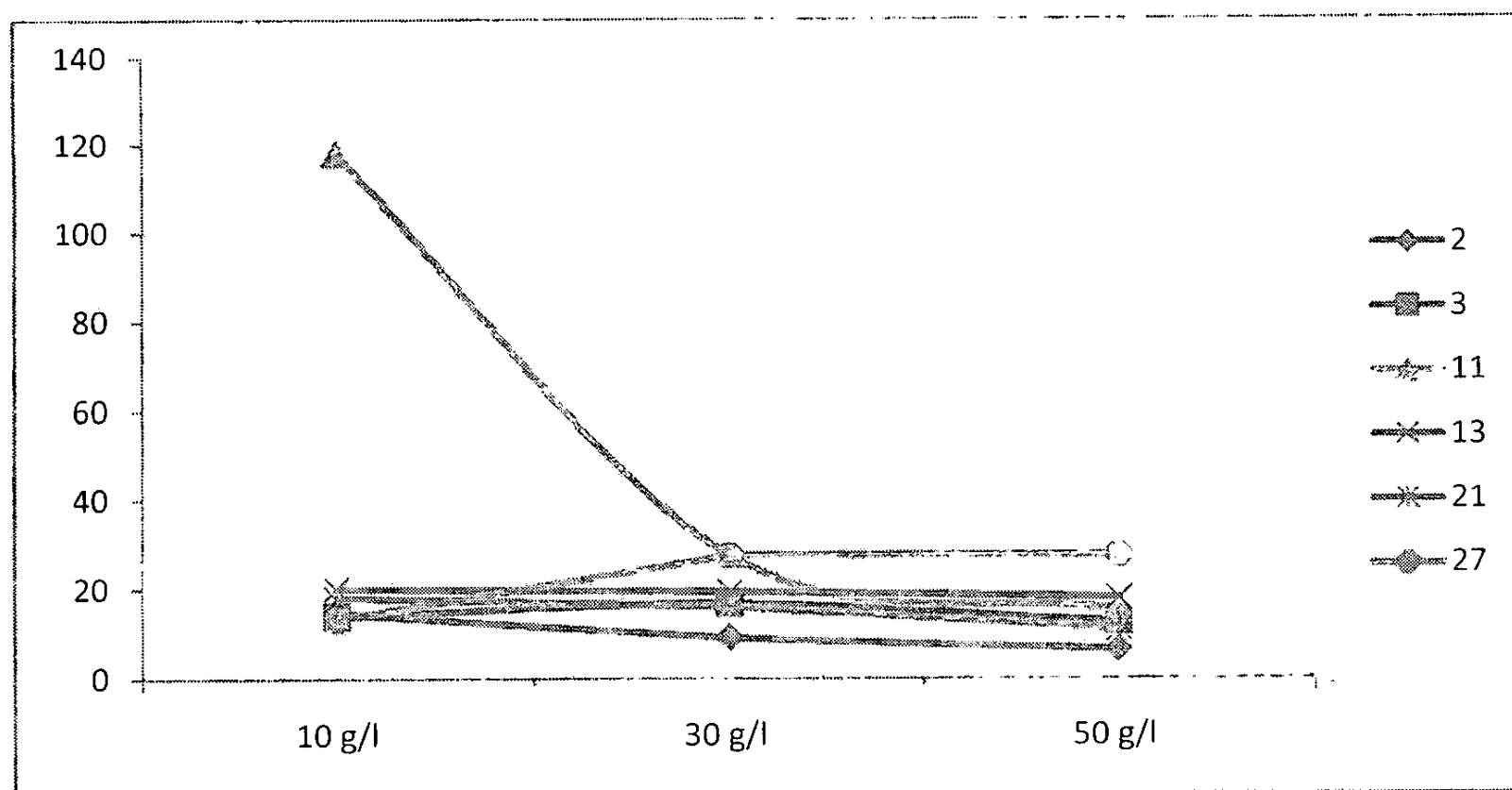


Figure 3.22: Effect of Rock-P concentration on soluble P after 6 days of incubation. Bacterial Strains: EP2, EP 27, EP3, EP11, EP13, and EP21, Each value in the panel represents mean of three replicates

The efficiency of releasing soluble P at 3% Rock-P by bacterial strains, decreased in the order EP11, EP27>EP13,EP21>EP3>EP2. pH drop in the culture was recorded as 4.14, 6.45, and 5.12 for EP27, EP 11 and EP 2 respectively (table 3.5).

Table 3.5: Concentration of soluble P released by each PSB strain in modified PVK liquid broth containing different concentrations of Rock-P.

Bacterial strain	Rock-P concentration		
	1%, (10g/l)	3%, (30g/l)	5%, (50g/l)
EP11	118.08A	27.54A	15.29C
EP13	20.06B	19.38B	18.01B
EP27	18.02C	27.54A	27.57A
EP21	18.02C	15.98D	11.13E
EP2	14.62D	9.18E	6.43F
EP3	13.94E	16.67C	12.57D

Means followed by the same letter did not differ significantly at 5% Tukey test

Increasing concentration of Rock-P further to 5% did not increase the release of soluble P. Instead, concentration of soluble P decreased with all strains. Concentration of soluble P released by the efficient solubilizer EP 11 decreased further to 15.29 ppm, whereas the concentration of

soluble P released by EP 27 remained the same as at 3% Rock-P. The lowest concentration of soluble P, 6.44 ppm was exhibited by isolate EP2. The efficiency of releasing soluble P at 5% Rock-P by bacterial strains, appears to decrease in the order EP27, EP13>EP11>EP3>EP21>EP2 (table 3.5). pH drop in the culture occurred as 5.11, and 5.08 for EP27 and EP2 respectively.

Therefore, increasing the Rock-P concentration from 1% to 3% or to 5% Rock-P, did not result any significant release of soluble P concentration. Instead there was a visible decrease in the concentration of soluble P. The highest concentration of soluble P was observed when Rock-P concentration in the medium was 1%, and inoculated with EP11 strain. Therefore results showed that 1% or lower Rock-P concentrations appears to increase the Rock-P solubilization capability in the liquid media.

3.5 Quantitative measurement of inorganic phosphate solubilization in liquid medium

To characterize P solubilization capabilities of these strains further, modified PVK liquid medium containing in various other inorganic P sources such as Ca-P, Fe-P, Al-P, was inoculated with each strain. All inorganic phosphate sources used as substrates were carefully washed to remove contaminating soluble P, which otherwise could interfere with the analysis.

When Ca-P was used as the P source, average soluble P concentrations released by strains were quite high and ranged from 81.68 ppm to 136.13 ppm with variations among different strains (table 3.6). Highest soluble P concentration was observed at the beginning of the incubation period and then it decreased slowly with time. The highest soluble P concentration observed was 136.13 ppm, by EP3 followed by 122.52 ppm soluble P by two strains EP11 and EP2. However, after six days, some amount of soluble P was also observed in the uninoculated control. Therefore the net amount of P solubilized by each strain was determined by subtracting the soluble P concentration of the uninoculated control.

Ca-P solubilization was accompanied by a substantial drop in pH from initial pH of 7.0 to 5.18, 5.38 and 5.21 by strains EP3, EP2, and EP 11 respectively. pH drop during Ca-P solubilization seem to be weakly correlated with the concentration of soluble P released. For example EP2 released 122.52 ppm soluble P into the medium, and dropped the pH to 5.38. EP13, on the other hand released lesser concentration (88.49 ppm) of soluble P, but dropped the pH to 5.04. The abilities of Ca-P solubilization in modified PVK liquid culture and modified PVK solid agar were also appears to be inconsistent. EP 13 was identified as most efficient Ca-P solubilizer on

modified PVK agar but it was only moderately effective in modified PVK liquid culture. On the other hand moderately effective EP 3 on modified PVK agar was identified as the best Ca-P solubilizer in liquid medium.

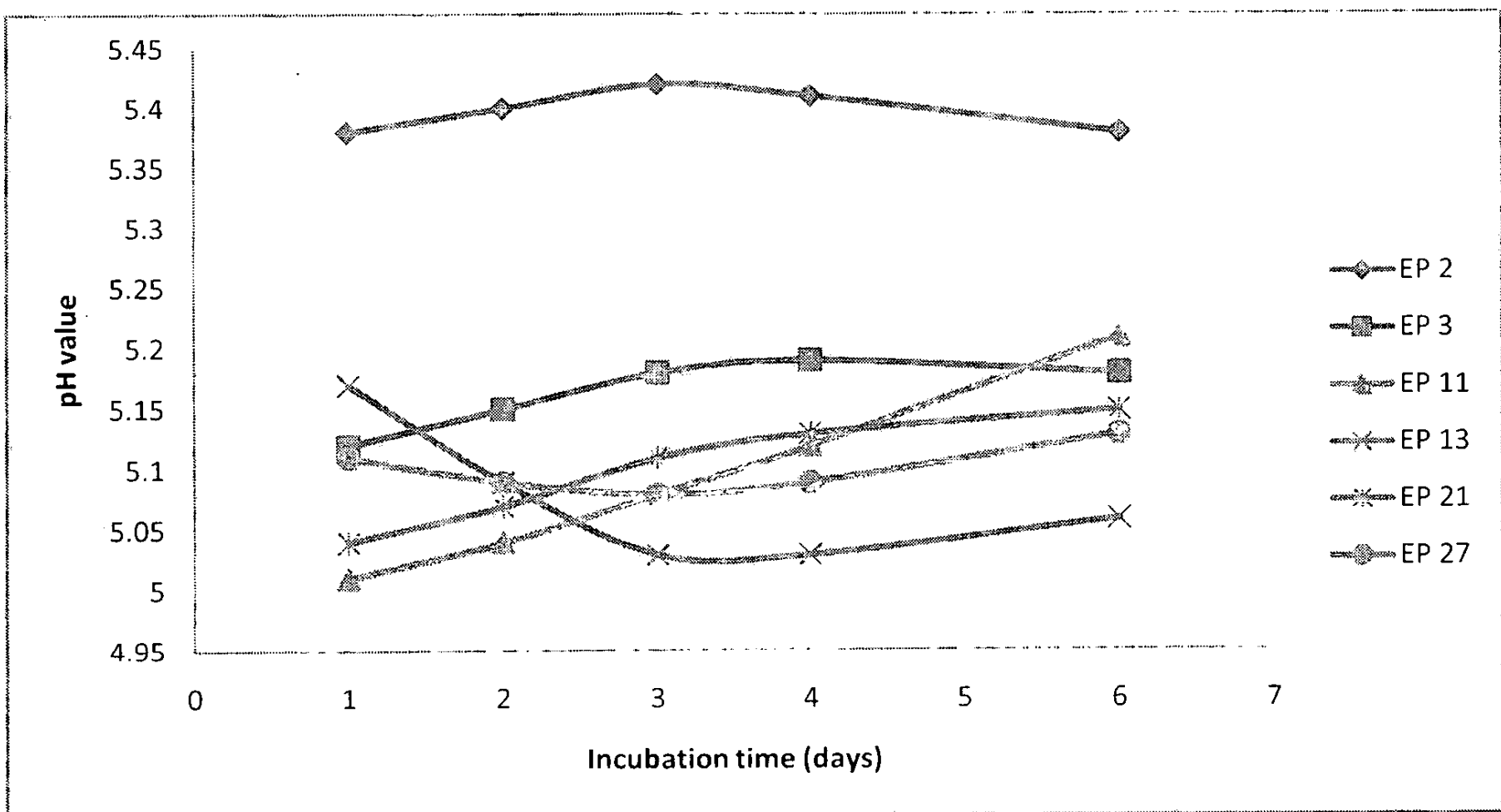
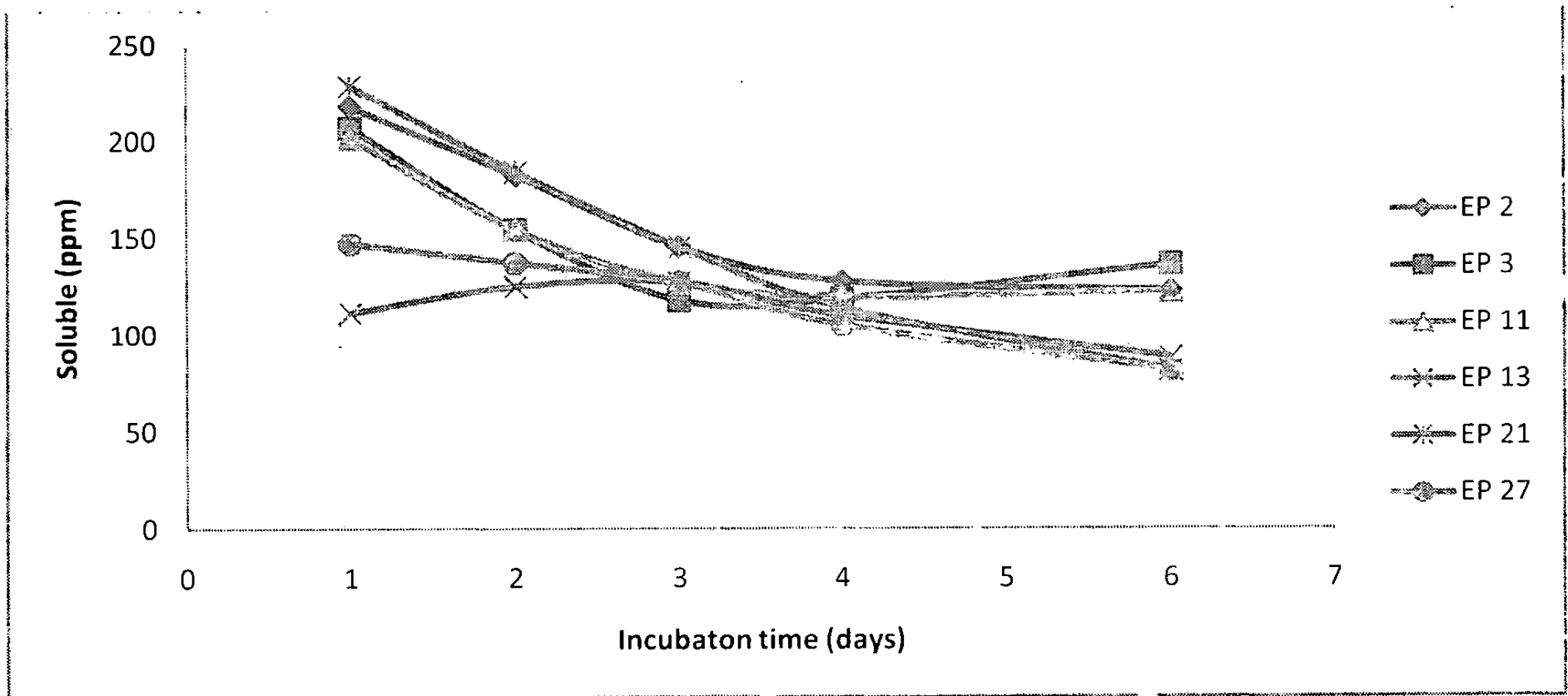


Fig. 3.23: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of Ca-P (5.56 g/L) by strain EP2, EP3, EP11, EP13, EP 21, and EP27 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

Table 3.6 : Concentration of soluble P released by each PSB strain in modified PVK liquid broth containing various inorganic phosphates

Bacterial isolates	Ca-P(ppm)	Fe-P(ppm)	Al-P(ppm)	Rock-P(ppm)
EP3	136.12A	0.00B	0.90B	2.92E
EP11	122.52B	E	F	83.94A
EP2	122.52B	4.09A	C	7.02D
EP13	88.49C	D	1.37A	7.95C
EP21	81.69D	C	0.00D	12.04B
EP27	81.69D	0.00B	E	7.02D

Means followed by the same letter did not differ significantly at 5% Tukey test

When Fe-P was used as sole P source, all the tested strains released only trace amounts of soluble P into the medium. Some soluble P was also observed in uninoculated controls. Therefore the net amount of P solubilized by each strain was calculated by subtracting the soluble P concentration accumulated in the uninoculated control. Similarly, some strains recorded negative values for soluble P concentrations. This could be due to their higher soluble P consumption rates compared to P release rates. Therefore the values presented in table 3.6 correspond to net soluble P concentrations in the medium. Strain EP2 released 4.09 ppm net soluble P after six days. Its soluble P concentration appears to be continuously increased upon further incubation. Other five strains though, followed the same pattern, showed negative soluble P values, due to the consumption of available soluble P from the medium. The highest consumption was observed in EP11 strain, where the available soluble P level dropped to the lowest value observed. In addition, to the above, EP11 also dropped the culture pH to the lowest value observed 3.1, whereas that in all other strains remained above pH 5.

Similar observations were also made, when Al-P was used as the substrate. The concentration of soluble P in these cultures were even lower than that with Fe-P. Two strains, EP13 and EP 3 released 1.37 ppm and 0.90 ppm net soluble P. Although the patterns of Al-P solubilization follows that of Fe-P, further incubation does not seem to increase soluble P levels substantially. Again the highest consumption of soluble P was observed in EP 11 and dropped the soluble P

level to the lowest value observed. Similarly, EP11 dropped the culture pH to the same extent as observed with Ee-P.

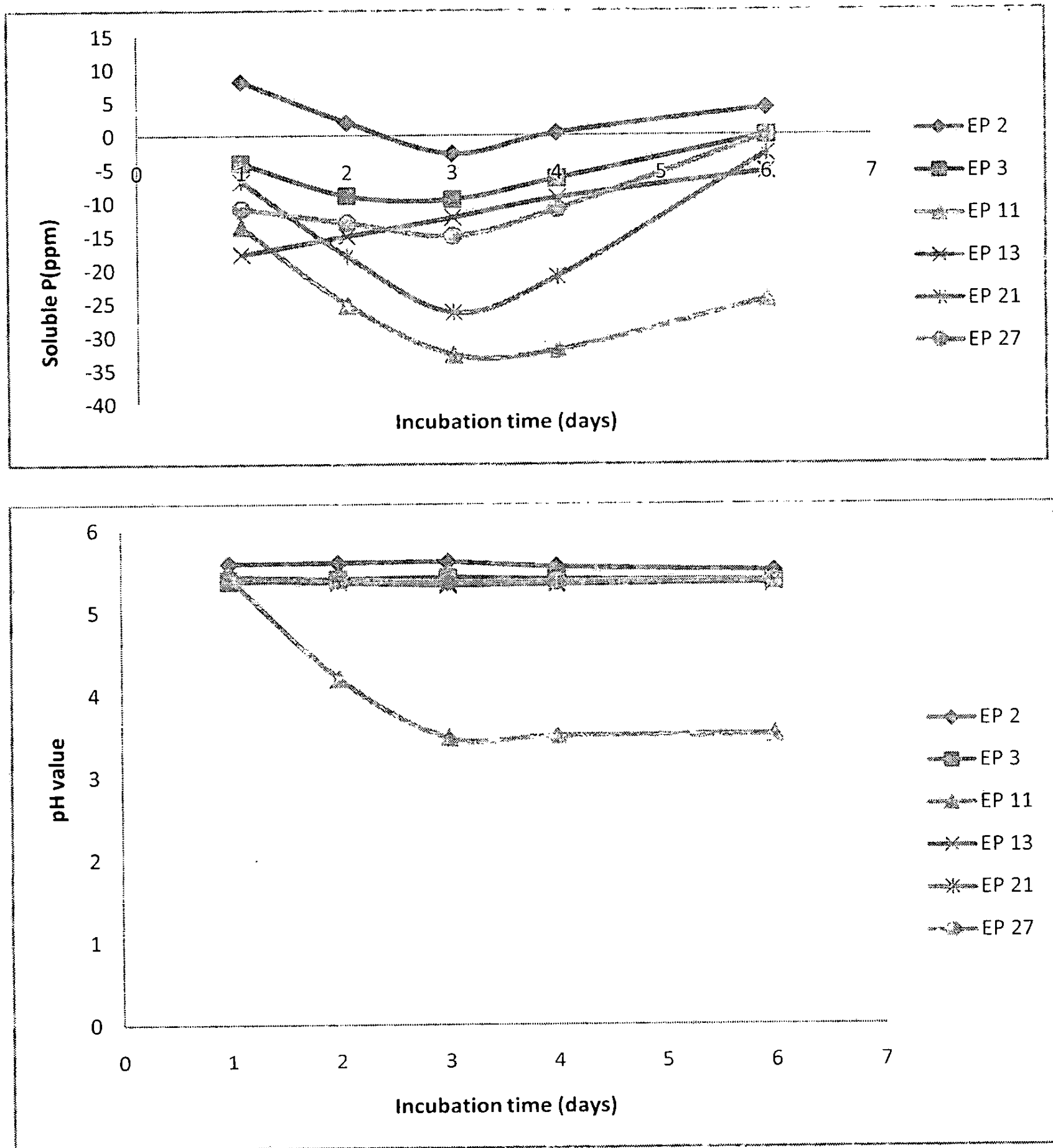


Fig. 3.24: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of Fe-P (7.19 g/L) by strain EP2, EP3, EP11, EP13, EP 21, and EP27 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

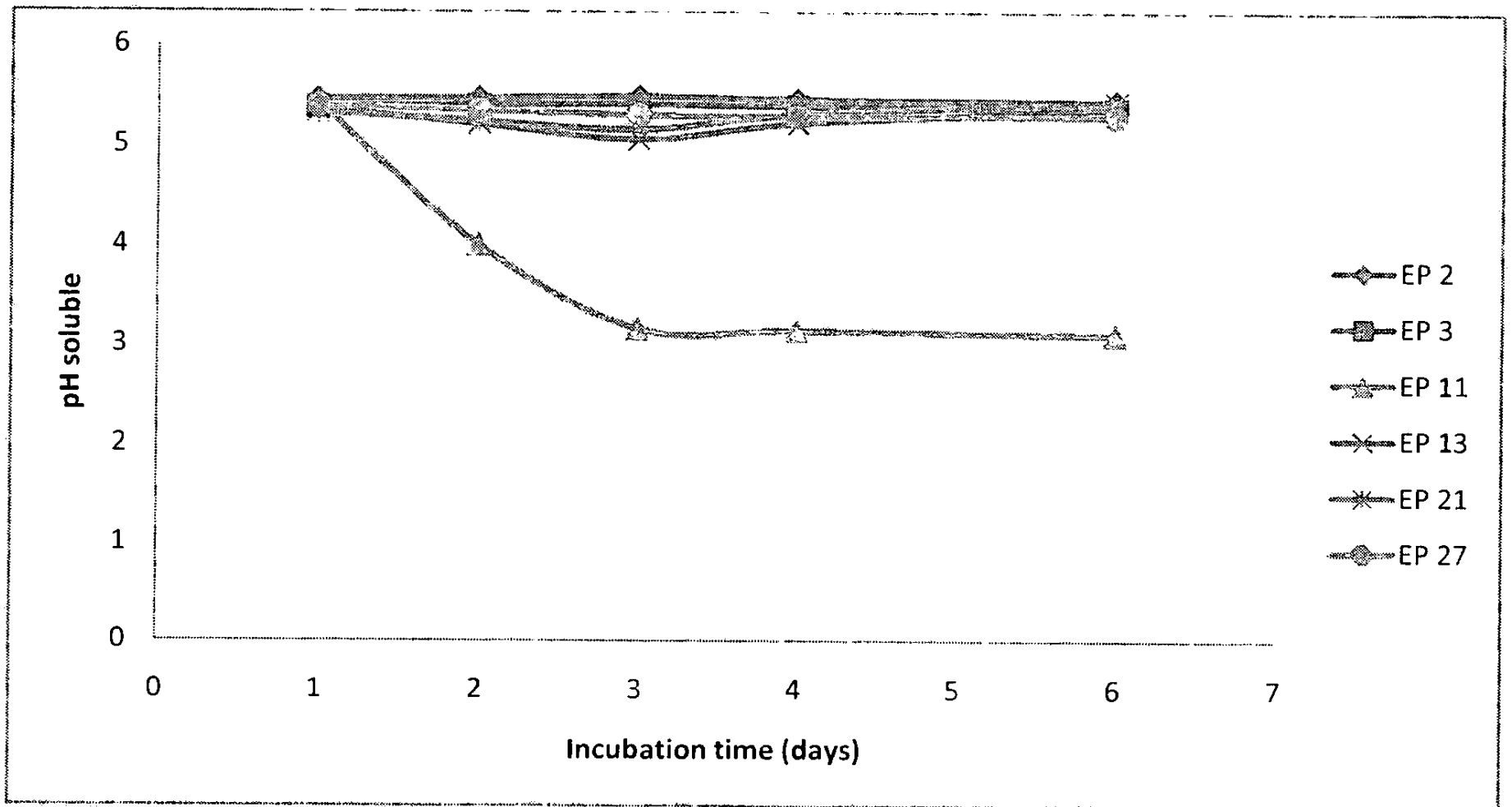
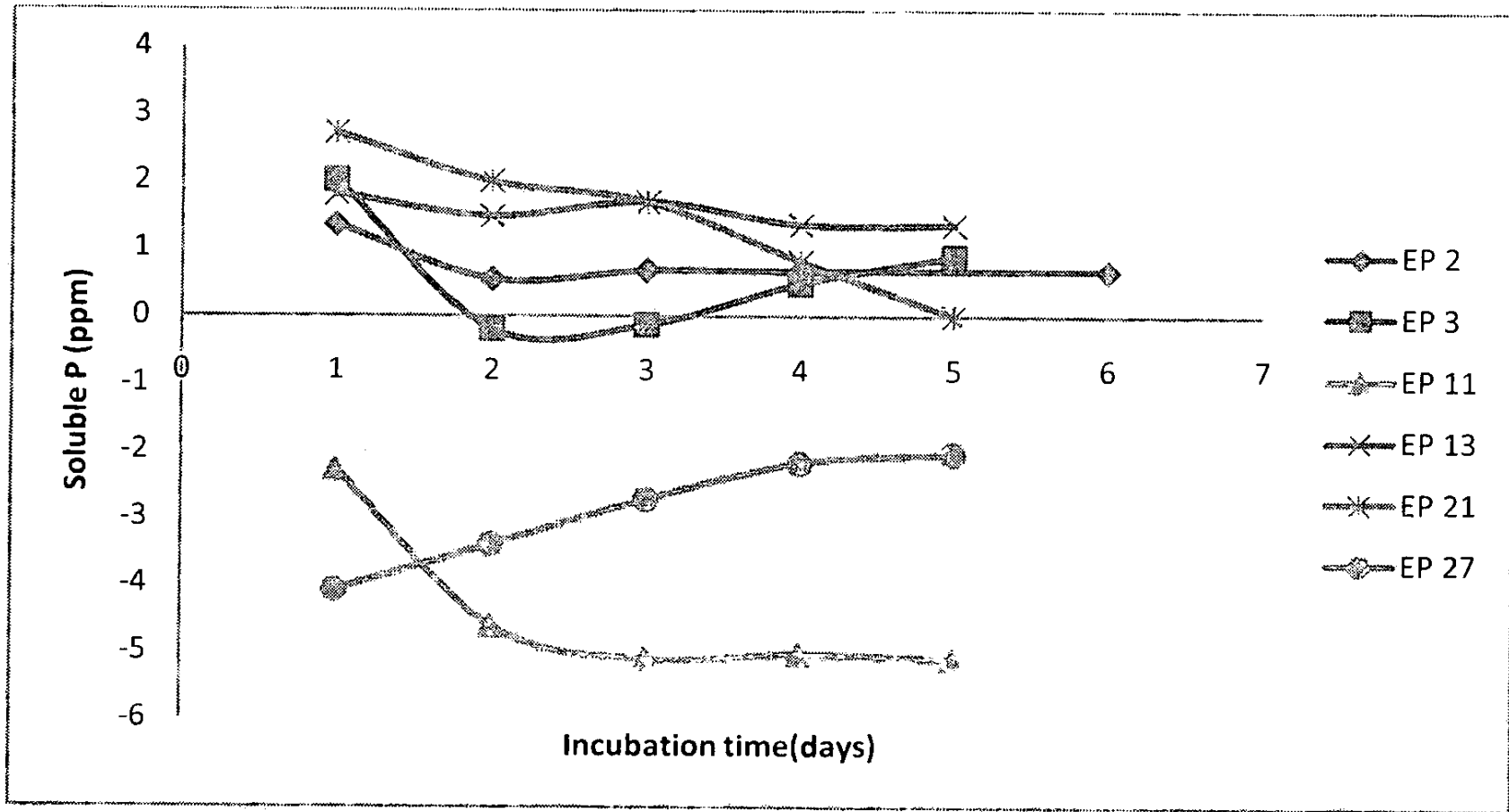


Fig. 3.25: Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of Ca-P (4.51 g/L) by strain EP2, EP3, EP11, EP13, EP 21, and EP27 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

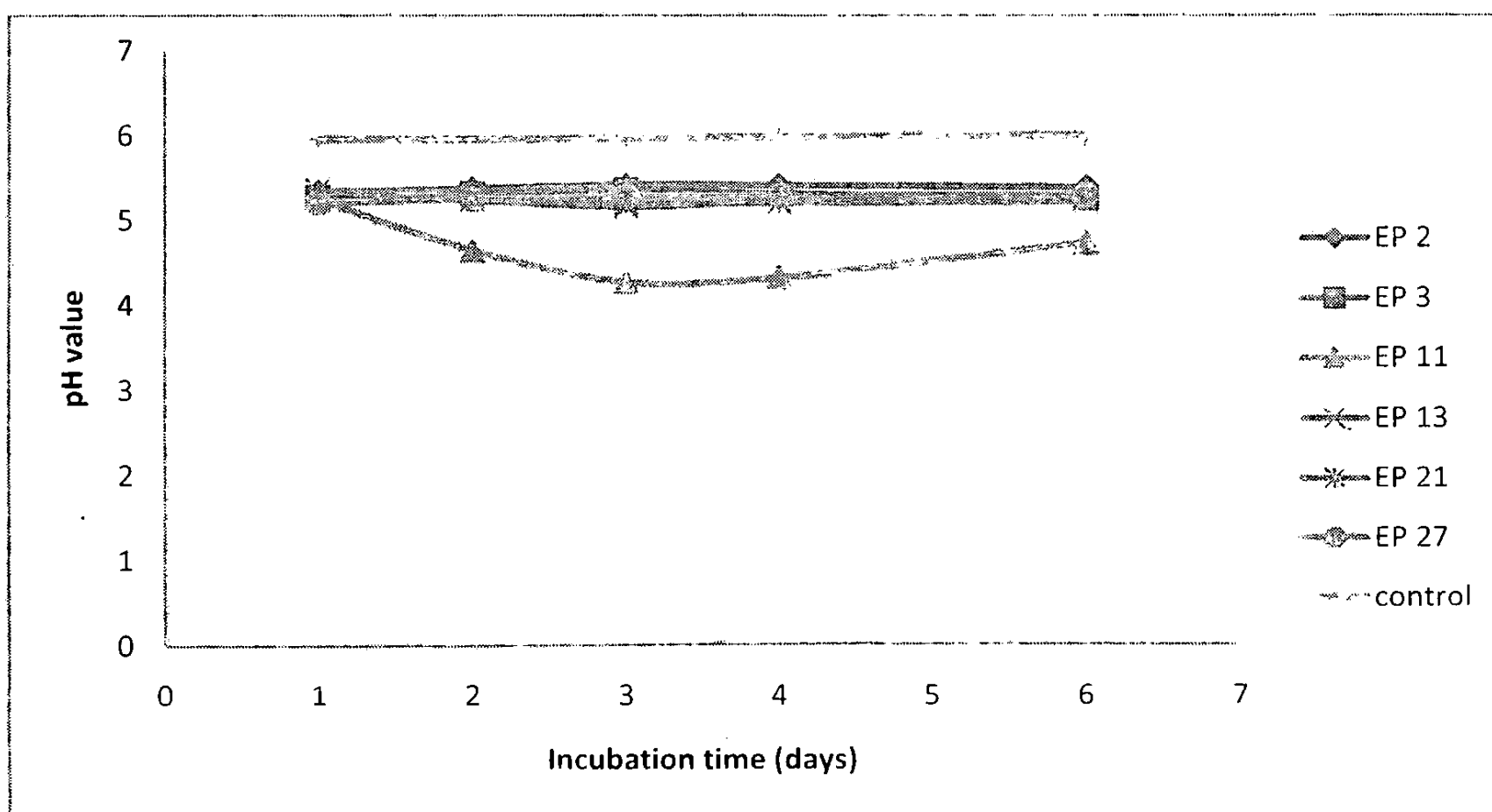
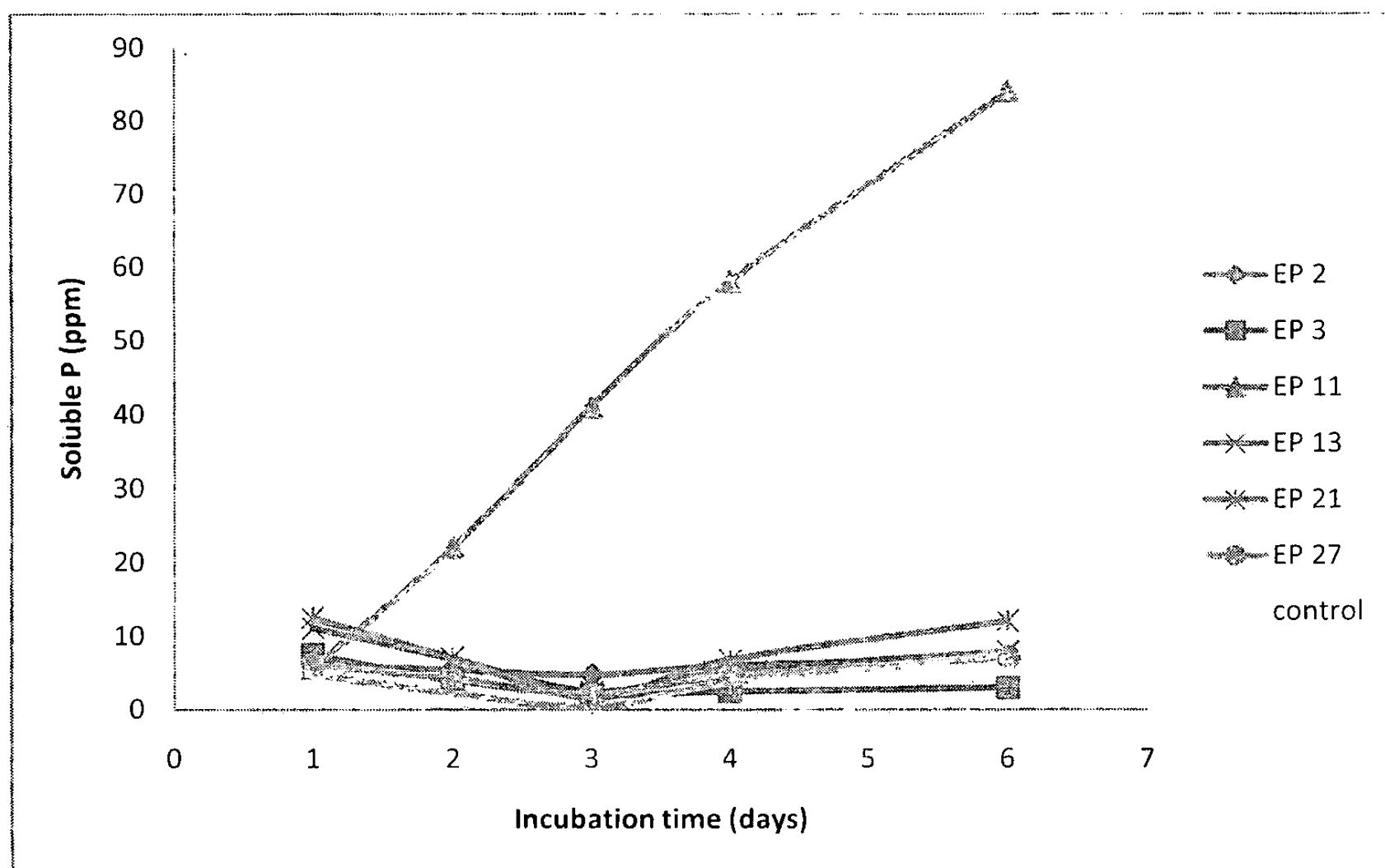


Figure 3.26 : Changes in the soluble P concentration and corresponding pH of the culture during the solubilization of Rock-P (<0.037mm, 5.98 g/L) by strain EP2, EP3, EP11, EP13, EP

21, and EP27 in modified Pikovskaya's liquid medium; Each value in the panel represents mean of three replicates

The experiments were further extended to analyze the release of soluble P also from Rock-P. In the presence of Rock-P (<0.037mm,) the highest P release was recorded by EP11, which released 83.95ppm soluble P, followed 12.0 ppm soluble P by EP21. Among the isolates the lowest concentration of soluble P was observed in the cultures of EP3. The performance of EP11 again seem to be extraordinary. From the first day, the soluble P concentration was increased rapidly and continued to increase further. Whereas release of soluble P by all other strains remained comparatively low, below 15 ppm, even after six days. Phosphate solubilization was accompanied by a significant drop in pH. The most efficient strain dropped the pH of the culture to 4.74, whereas E21 and the least efficient strain EP3 dropped from initial pH of 6.9 to 5.39 and 5.26. The soluble P concentration of uninoculated controls remained zero throughout the experiment.

3.6 Effect of EP-11 on rice growth promotion

The ability of phosphate solubilizing bacteria to promote rice growth through enhanced phosphorus (P) uptake from Rock-P was studied in a pot experiment under glass house conditions. Rice seeds of two varieties, BG 352 and Suduheenati were selected to represent modern and traditional varieties. Rice seedlings in pots underwent 6 different treatments, No fertilizer, chemical fertilizer, Rock-P powder, Rock-P powder amended with 1×10^8 EP 11 cells, Rock-P powder amended with 2×10^8 EP cells and Rock-P powder amended with 0.5×10^8 EP 11 cells, The effect of each treatment was assessed after 40 days of growth period.

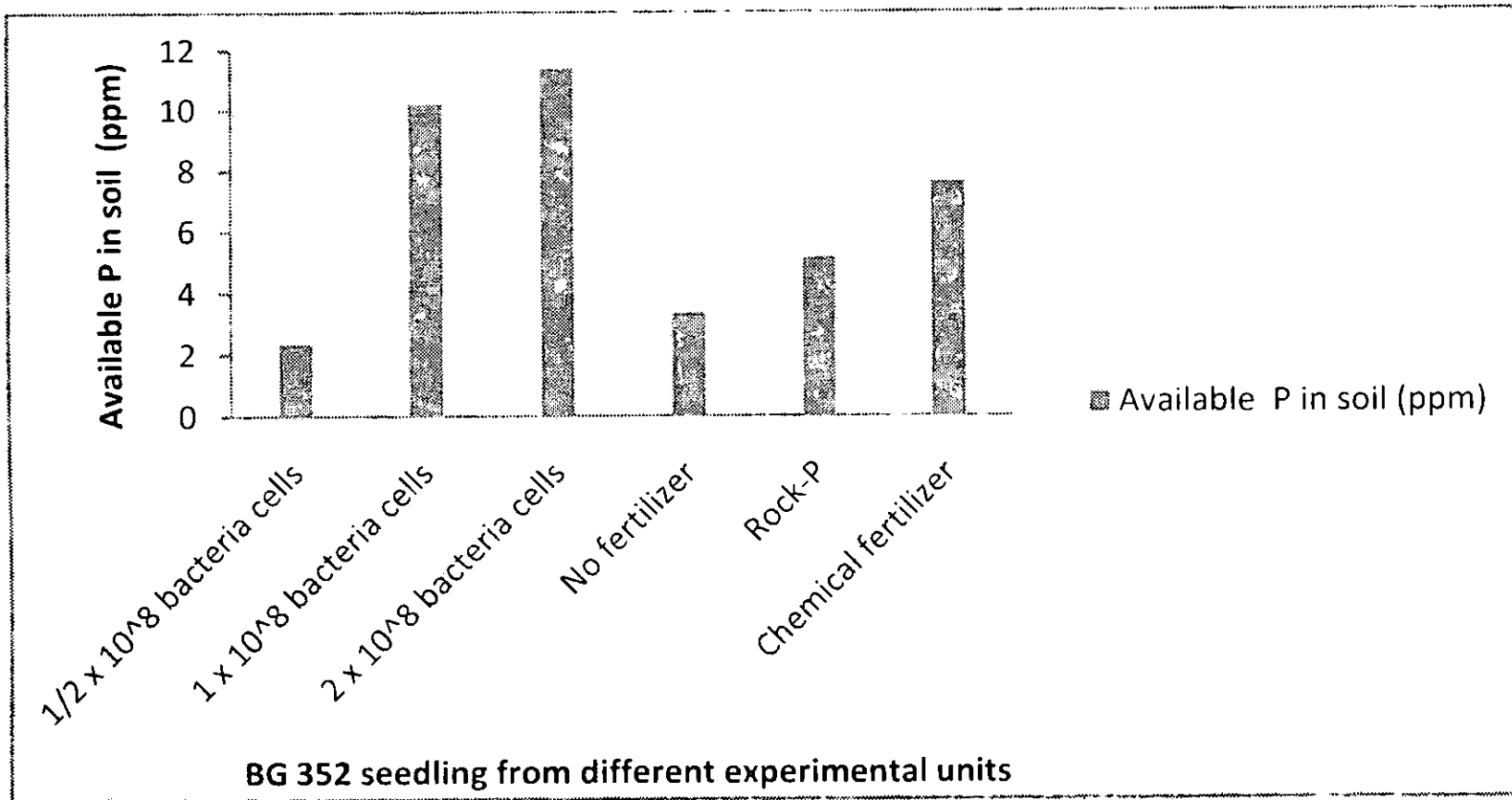


Fig. 3.27 The effect of EP11 inoculation, on available soluble P content of BG 352 rhizosphere soil amended with Rock-P

(Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; No fertilizer or Rock-P/No EP11 cells; Rock-P only; NPK Chemical Fertilizer only)

The effect of EP 11 on soluble P content of BG 352 cultivated soil

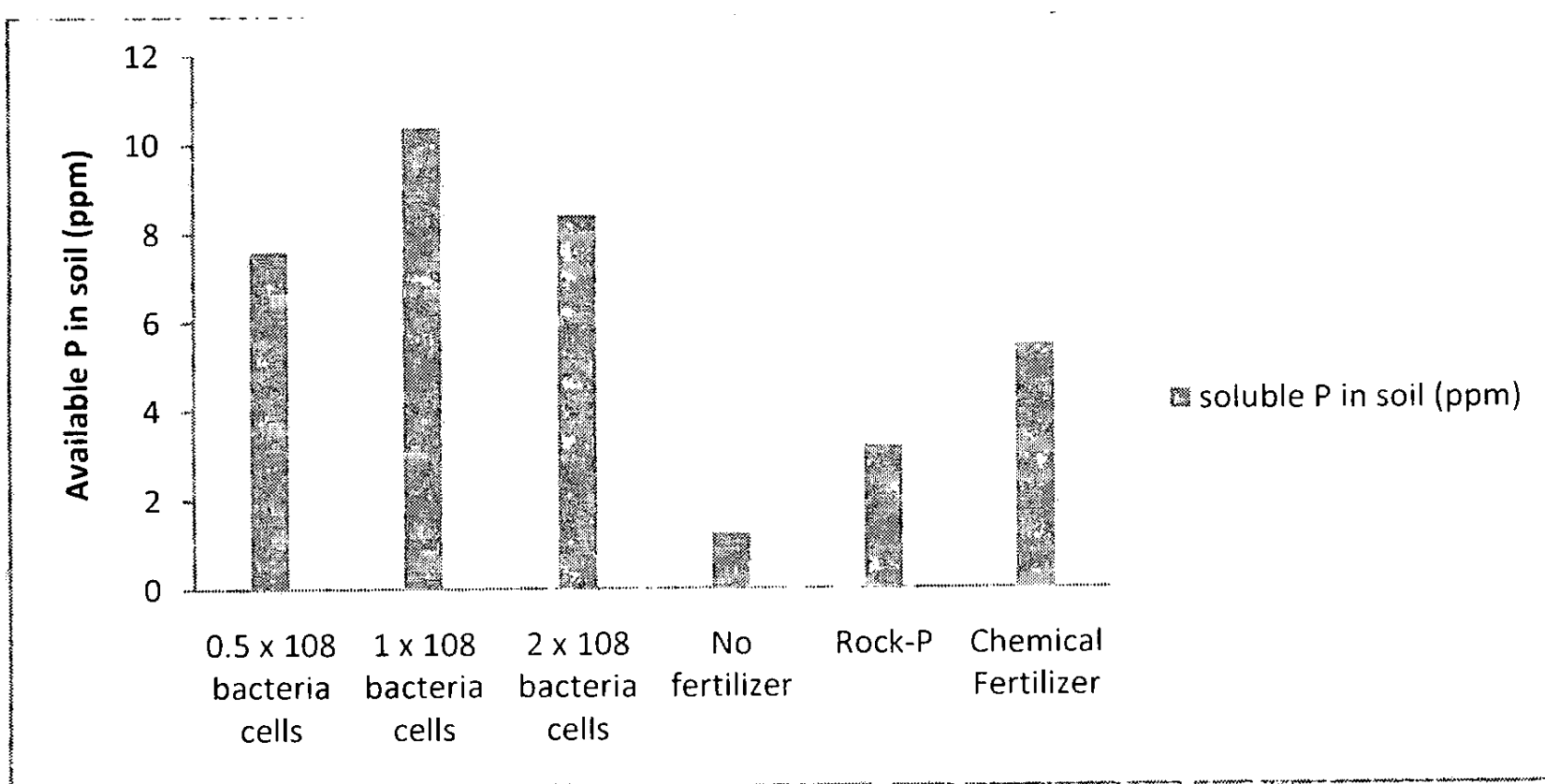


Fig. 3.28 The effect of EP11 inoculation, on available soluble P content of Suduheenati rhizosphere soil amended with Rock-P

(Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; No fertilizer or Rock-P/No EP11 cells; Rock-P only; NPK Chemical Fertilizer only)

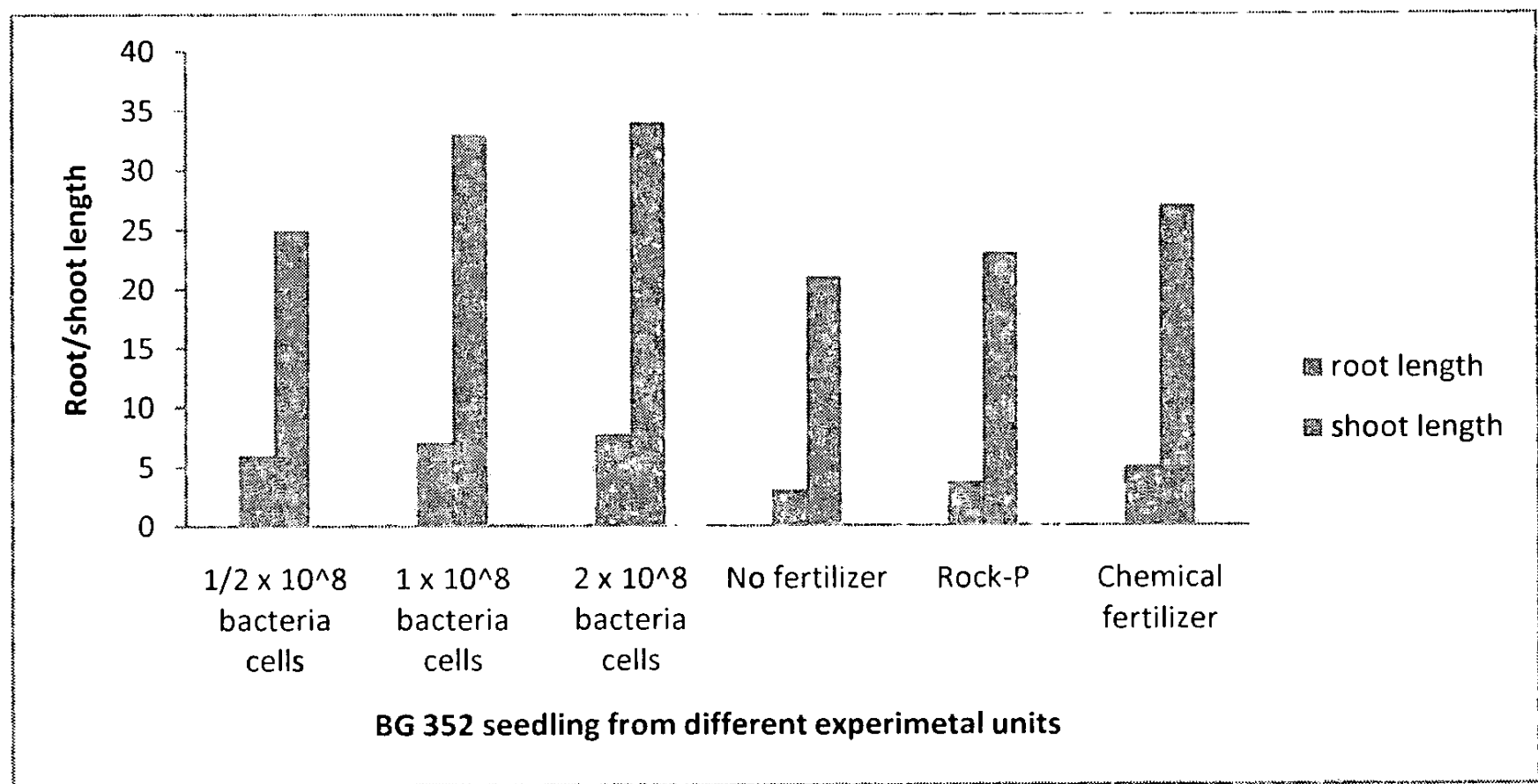


Fig. 3.29 The effect of EP11 inoculation, on growth parameters of BG 352 rice seedlings (Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; No fertilizer or Rock-P/No EP11 cells; Rock-P only; NPK Chemical Fertilizer only) The effect of EP 11 on soluble P content of BG 352 cultivated soil

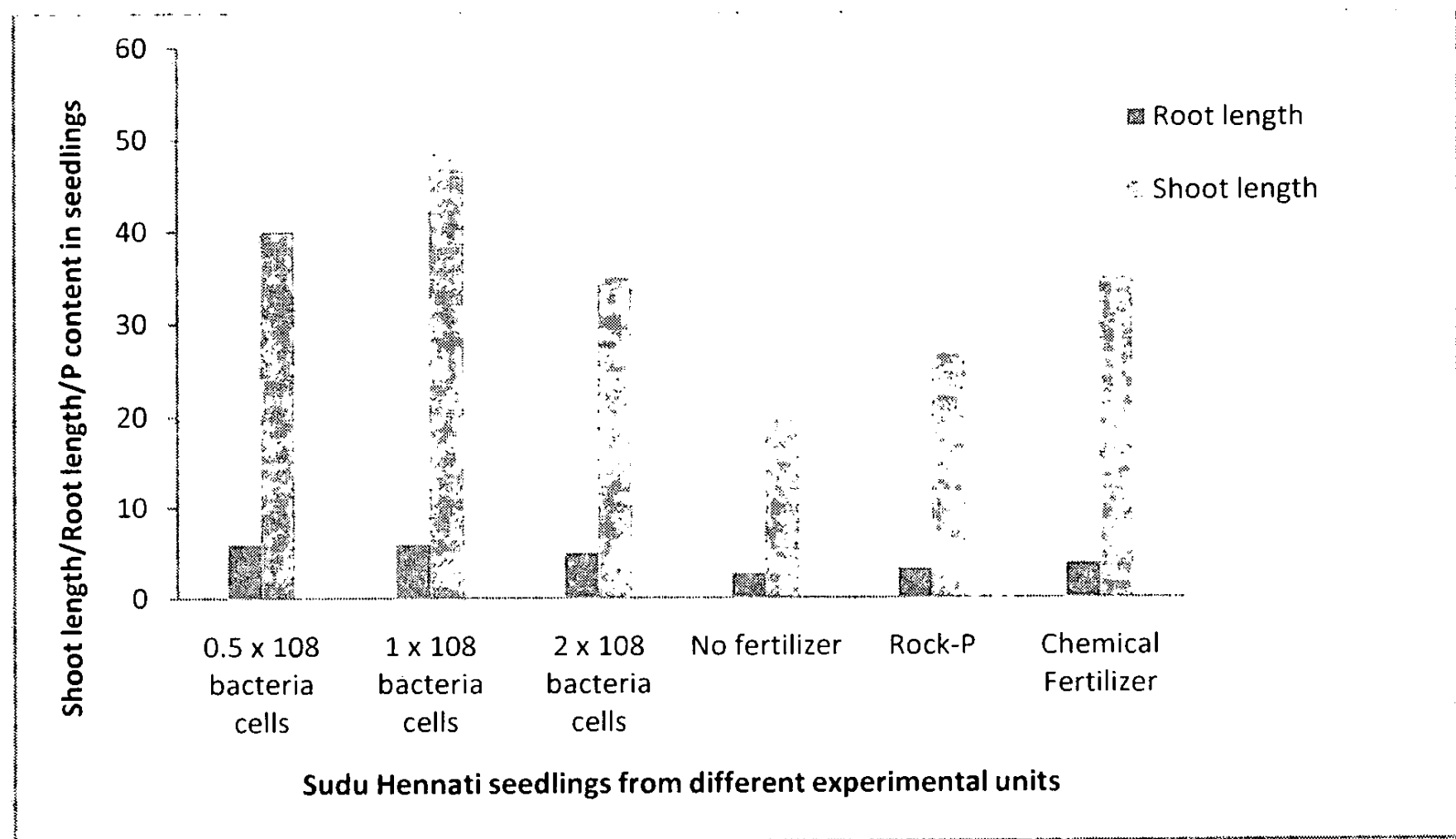


Fig. 3.30: The effect of EP11 inoculation, on growth parameters of Suduheenati (Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; No fertilizer or Rock-P/No EP11 cells; Rock-P only; NPK Chemical Fertilizer only)

Table 3.6 : Effect of inoculation with EP 11 strain on soil available P, plant growth parameters and P uptake of BG 352 rice seedlings

Treatments	Soluble P in soil (ppm)	Plant dry weight (g)	Root length (cm)	Shoot length (cm)	Plant P content (ppm)
Rock-P + 1 x 10 ⁸ EP 11 cells	10.21B	0.45A	7.03B	33.03B	19.08C
Rock-P + 2 x 10 ⁸ EP 11 cells	11.35A	0.36B	7.7A	34.03A	29.59B
Rock-P +0.5 x 10 ⁸ EP 11 cells	2.38F	0.45A	6.03C	25.03D	17.8787D
Rock-P only	5.14D	0.18D	3.7E	23.033E	17.41A
NPK Chemical Fertilizer only	7.59C	0.33C	5.03D	27.033C	48.79F
No fertilizer/No Rock-P/No EP11 cells	3.35E	0.14E	3.03F	21.00F	12.05E

Means followed by the same letter did not differ significantly at 5% Tukey test

Table 3.7 : Effect of inoculation with EP 11 strain on soil available P, plant growth parameters and P uptake of Suduheenati rice seedlings

Treatments	Soluble P in soil (ppm)	Plant dry weight(g)	Root length (cm)	Shoot length(cm)	Plant P content (ppm)
Rock-P + 1 x 10 ⁸ cells	10.39A	0.84A	6.03A	48.53A	66.77A
Rock-P + 2 x 10 ⁸ cells	8.41B	0.69B	5.03B	35.03C	22.25D
Rock-P +0.5 x 10 ⁸ cells	7.6C	0.63C	6.03A	40.03B	53.33B
Rock-P only	3.18E	0.21E	3.23D	27.03D	52.25C
NPK Chemical Fertilizer only	5.47D	0.42D	3.83C	35.03C	12.23F
No fertilizer/No Rock-P/No EP11 cells	1.22F	0.15F	2.73E	20.03E	19.08E

Means followed by the same letter did not differ significantly at 5% Tukey test

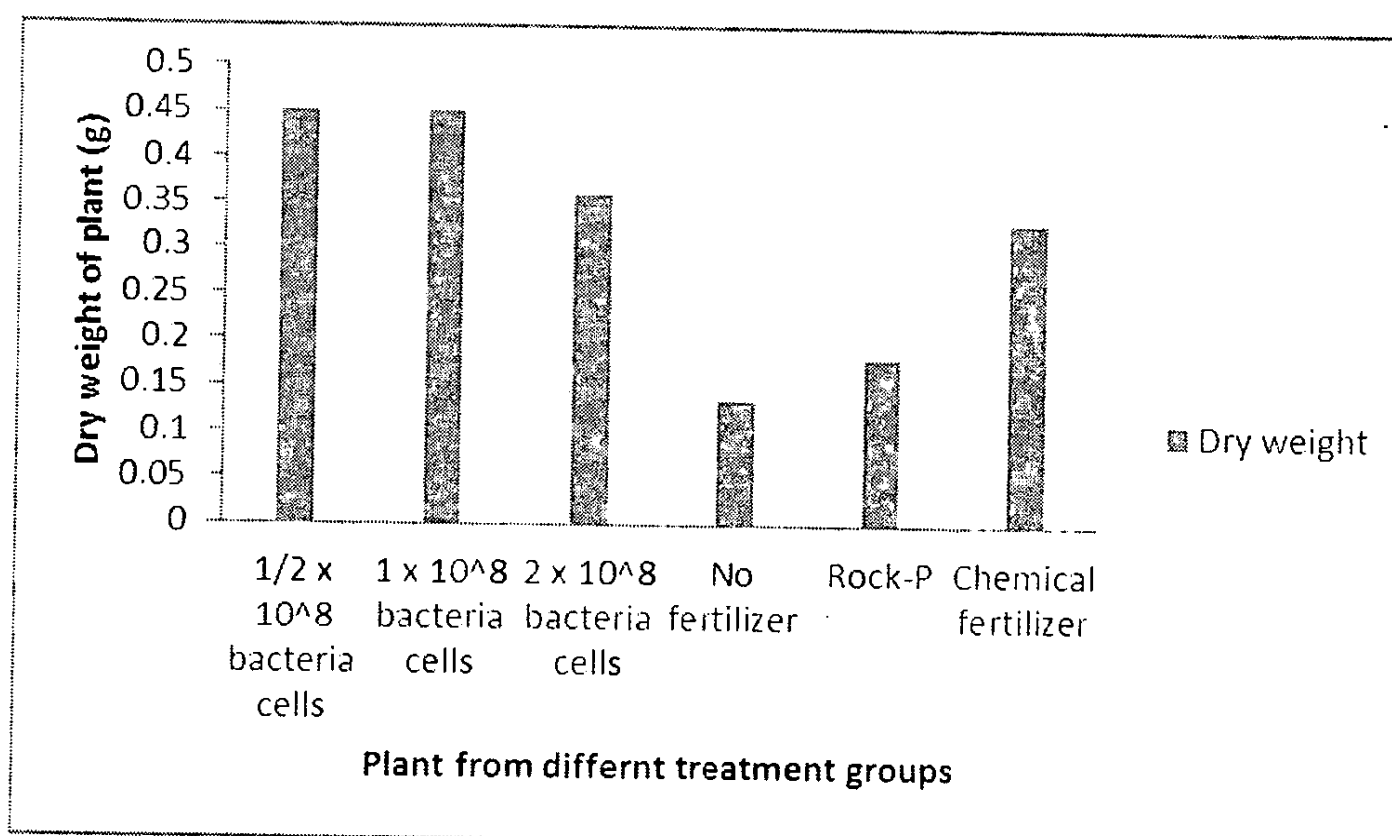


Fig. 3.31 The effect of EP11 inoculation, on dry weight of BG 352 rice seedlings (Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; No fertilizer or Rock-P/No EP11 cells; Rock-P only; NPK Chemical Fertilizer only) The effect of EP 11 on soluble P content of BG 352 cultivated soil

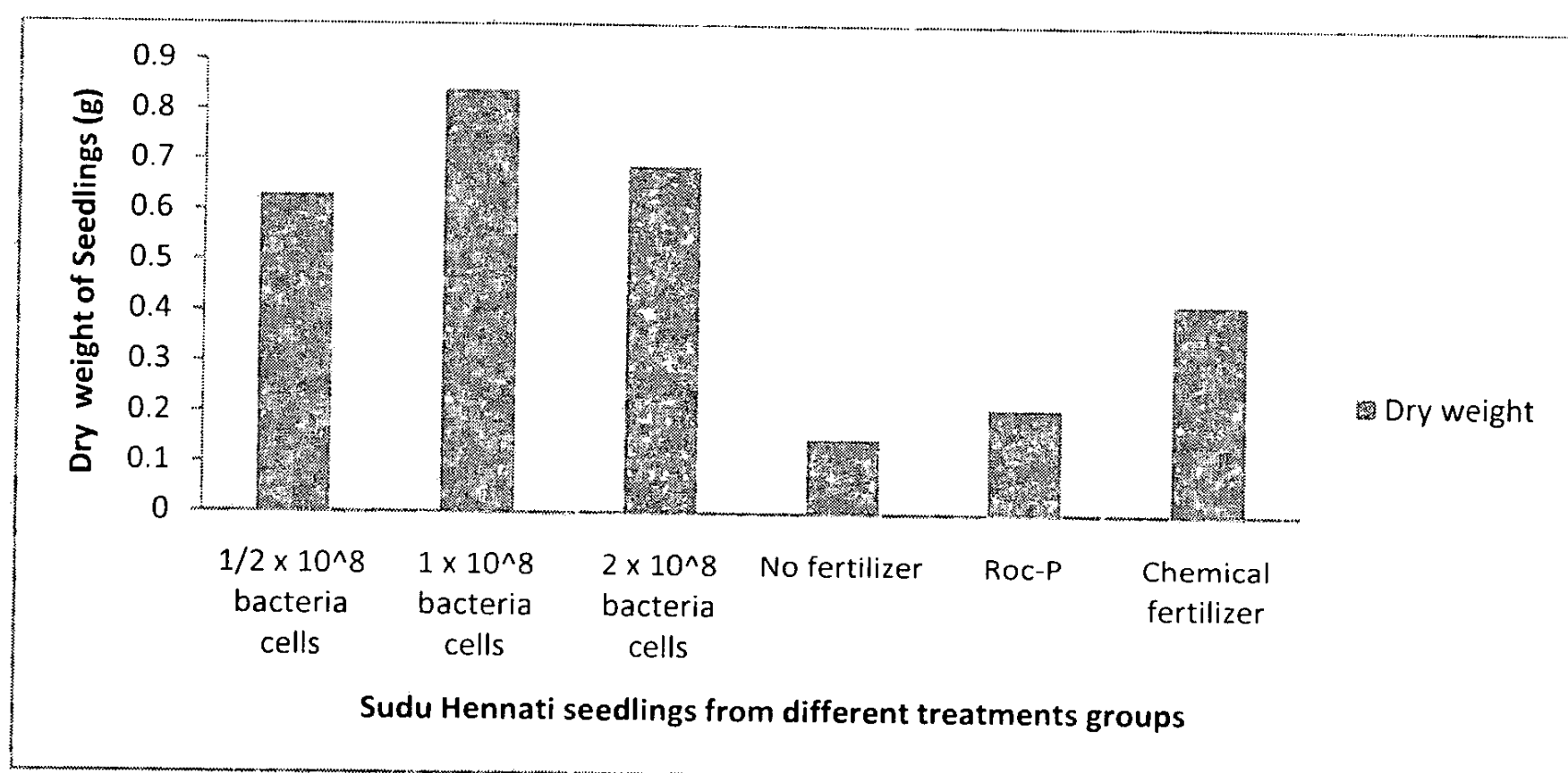


Fig. 3.32: The effect of EP11 inoculation, on dry weight of Suduheenati rice seedlings (Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; No fertilizer or Rock-P/No EP11 cells; Rock-P powder only; NPK Chemical Fertilizer only) The effect of EP 11 on soluble P content of BG 352 cultivated soil

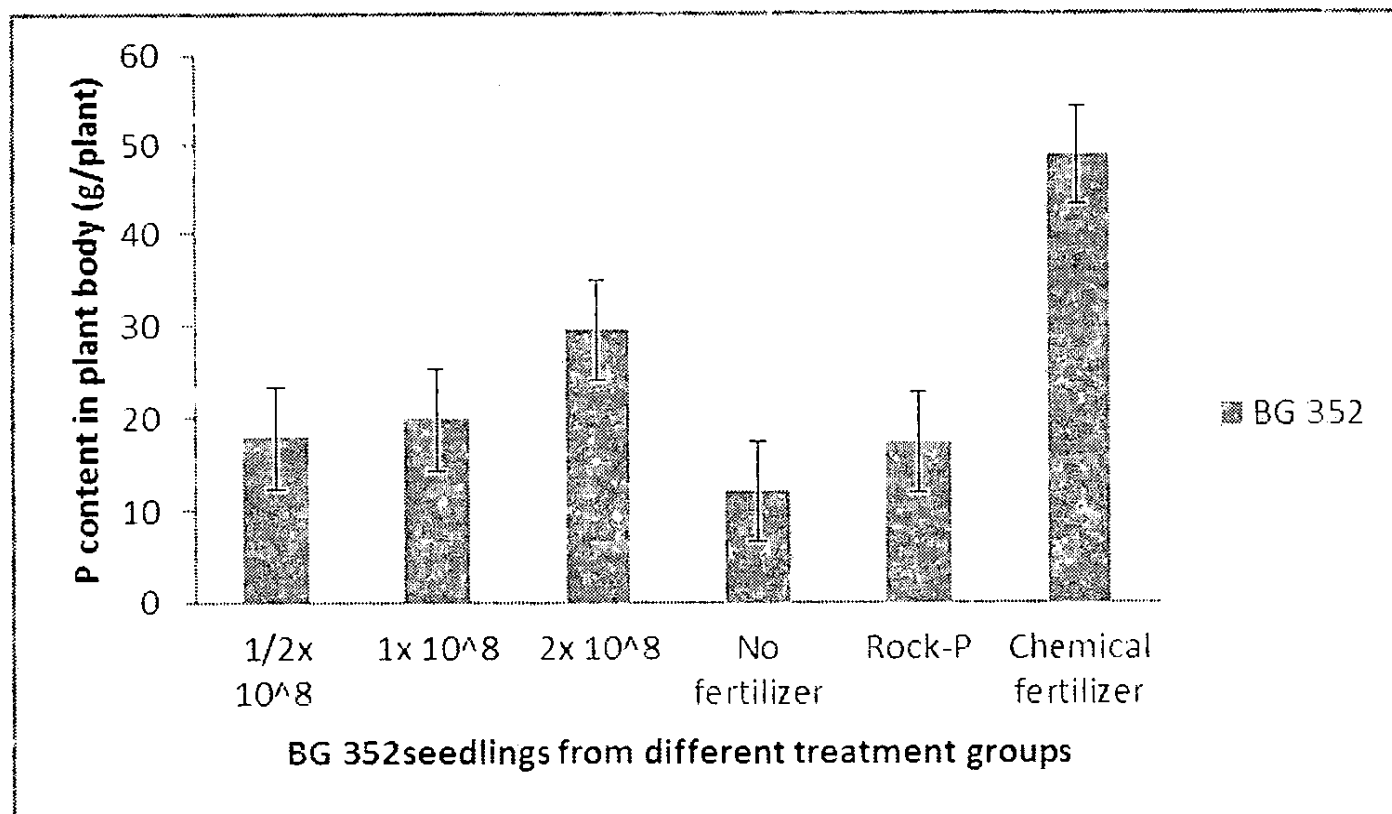


Fig. 3.33 The effect of EP11 inoculation, on BG 352 plant P content (Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells; Rock-P only; NPK Chemical Fertilizer only; No fertilizer or Rock-P/No EP11 cells)

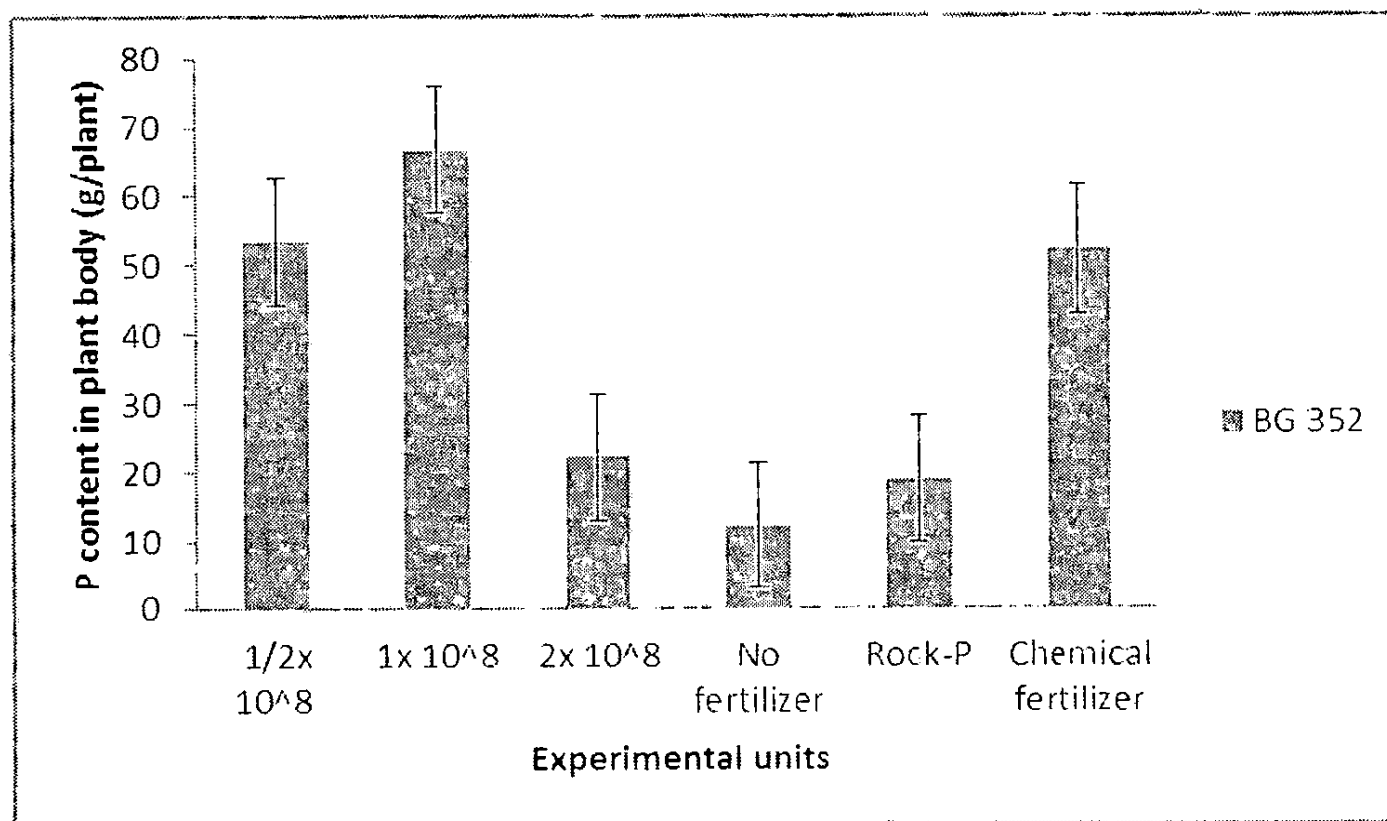


Figure 3.34: The effect of EP11 inoculation, on available soluble P content of Suduheenati rhizosphere soil amended with Rock-P (Treatments : Rock-P + 0.5×10^8 EP 11 cells; Rock-P + 1×10^8 EP 11 cells; Rock-P + 2×10^8 EP 11 cells;; Rock-P only; NPK Chemical Fertilizer only, No fertilizer or Rock-P/No EP11 cells)

Inoculation with EP 11 increased soil available P content in the rhizospheres of both BG 352 and Suduheenati rice varieties. The effect was more pronounced with Suduheenati rhizosphere at

0.5 x 10⁸ inoculum size (figure 3.27 and figure 3.28). Increasing inoculum size from 0.5 x 10⁸ to 1.0 x 10⁸, increased the amount of soil available P by 36% and 93% (figure 3,27 and figure 3,28) in BG352 and Suduheenati respectively. However increasing the inoculum size further to 2 x 10⁸ had only slight effect on the available soil P levels in both varieties. Highest amount of soil P, 11.35 ppm was observed in BG352 rhizosphere whereas that in Sidu heenati 10.39 ppm, which correspond to 121 % and 226 % increase in rhizosphere soluble P levels compared to uninoculated control. It is interesting to note that EP11 inoculation of rice rhizosphere amended with Rock phosphate led to increase soil available P even compared with the application of 50 kg P₂O₅/ha without inoculation (figure 3.27 and figure 3.28).

Effect of EP11 inoculation on rice growth parameters such as root length and shoot length in both rice varieties are shown in Figure 3.29 and Figure 3.30. In terms of the promotion of root length, the effect of inoculation was not conspicuous. In contrast, the effect on shoot length was more pronounced at 1x 10⁸ inoculum size, in both varieties. The highest shoot lengths observed were 34 cm and 48 cm in BG352 and Suduheenati, which correspond to 48% and 80% increase in shoot length compared to uninoculated control.

Both BG 352 and Suduheenati seedlings responded to inoculation by increasing the plant dry weight. The increase is more pronounced with Suduheenati, which correspond to 300 % increase in plant dry weight (figure 3.31 and figure 3.32) compared to uninoculated control. In contrast, the dry weight increase in BG 352 was lower than that of Suduheenati, where the increase was 150% compared to uninoculated control. Despite these variations in growth parameters among rice varieties, the inoculation increased the solubilization of Rock-P in plant rhizosphere and uptake of solubilized Rock- P to promote early rice growth. This is evident from the analysis of total plant P contents. Compared to BG 352, effect of inoculation on plant total P contents was more pronounced in Suduheenati variety.

3.7 16SrRNA-RFLP Genotyping

Nearly full length 16S rRNAs of efficient PSB were amplified via PCR with universal primers fD1 and rD1 (section). PCR yield amplification at approximately 1600bp on agarose gel (figure) for all the isolates. This size corresponds to the expected size of the 16S rRNA gene among bacteria (Weisberg et al., 1991).

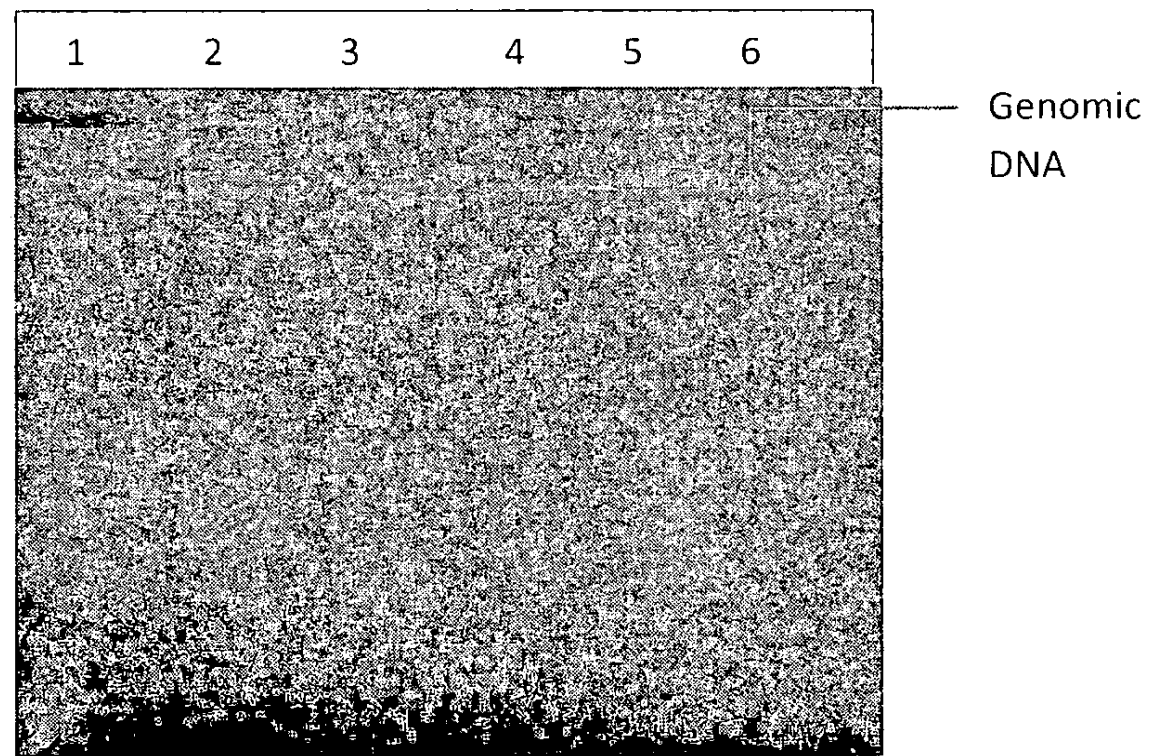


Figure 3.35: High molecular weight genomic DNA extracted by CTAB procedure, visualized in 0.8% agarose gel, lane 1-6, strains EP-2,EP- 3,EP-11,EP-13,EP-21,EP-27

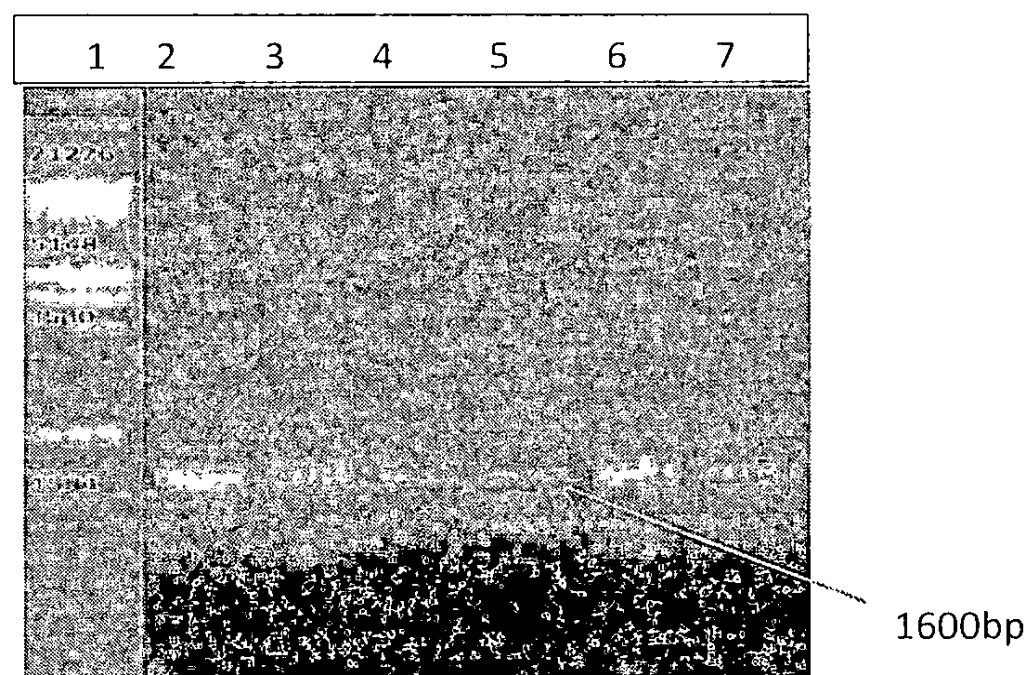


Figure 3.36: Amplified 16SrRNA PCR products from high molecular weight genomic DNA of phosphate solubilizing bacteria strains on 1% agarose gel. Lane 1, λ EcoRI/HindIII marker; Lane 2-7, strains EP-2,EP-3,EP-11,EP-13,EP-21,EP-27.

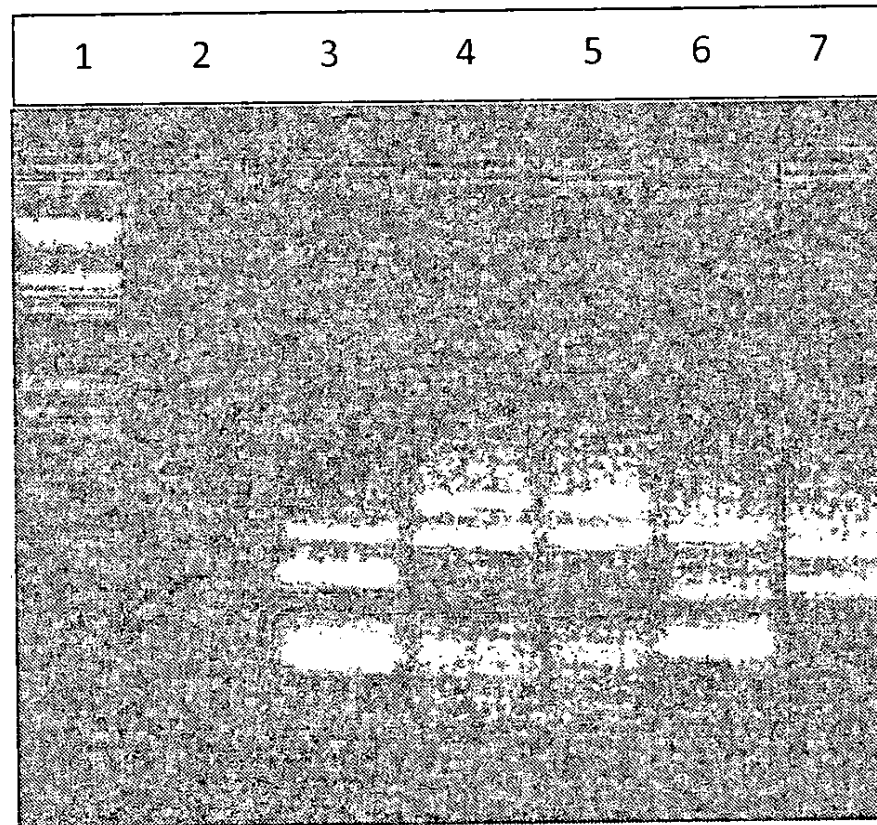


Figure 3.37: Banding patterns of *Alu I* digested 16S rRNA PCR product of the isolated phosphate solubilizing bacteria on 1.2% agarose gel. Lane 1, DNA ladder; Lane 2, EP – 2; Lane 3, EP-3; Lane 4, EP – 13; Lane 5, EP-21; Lane 6, EP – 27; Lane 7, EP-11.

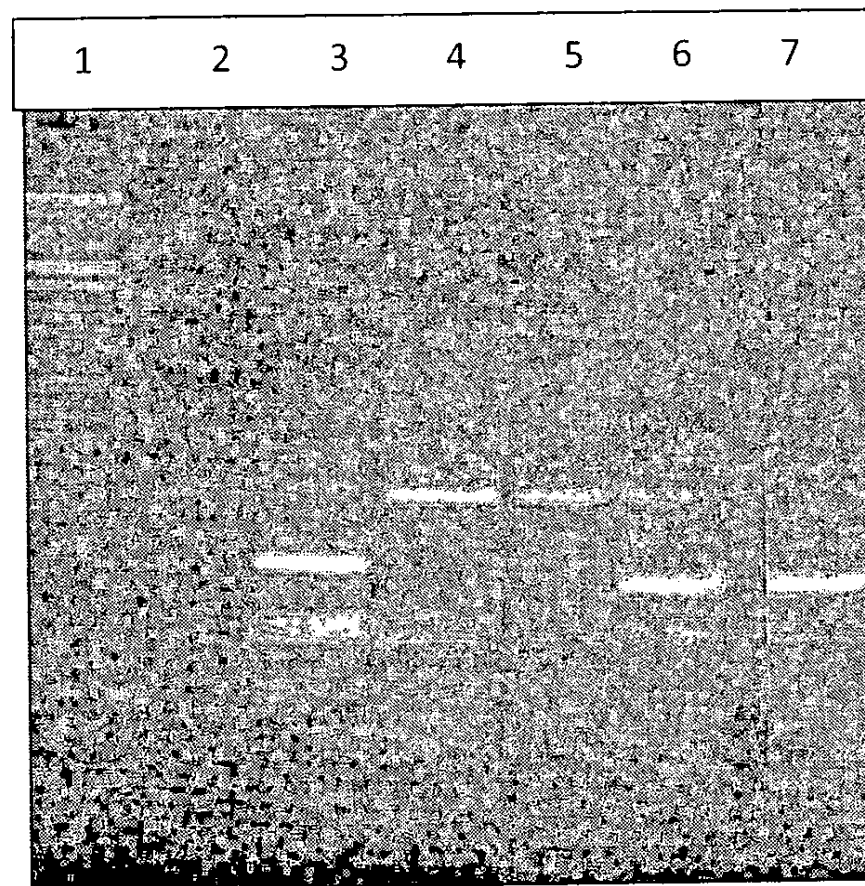


Figure 3.38: Banding patterns of *Hinf I* digested 16S rRNA PCR product of the isolated phosphate solubilizing bacteria on 1.2% agarose gel. Lane 1, DNA ladder; Lane 2, EP – 2; Lane 3, EP-3; Lane 4, EP – 13; Lane 5, EP-21; Lane 6, EP – 27; Lane 7, EP-11.

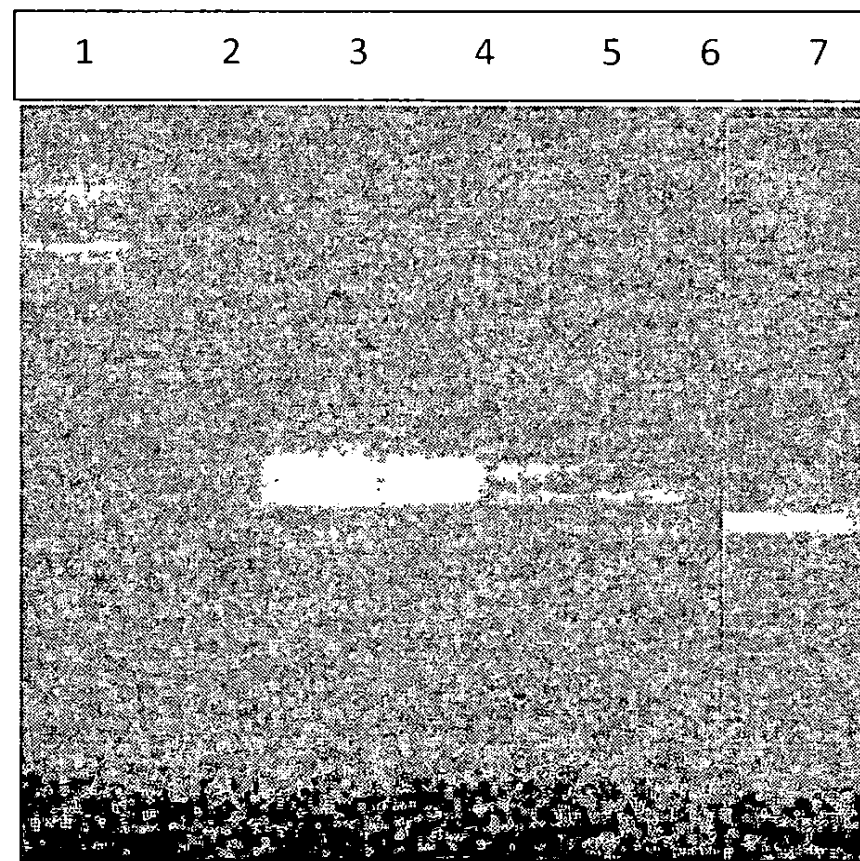


Figure 3.39: Banding patterns of *Hae III* digested 16S rRNA PCR product of the isolated phosphate solubilizing bacteria on 1.2% agarose gel. Lane 1, DNA ladder; Lane 2, EP – 2; Lane 3, EP-3; Lane 4, EP – 13; Lane 5, EP-21; Lane 6, EP – 27; Lane 7, EP-11.

All amplified 16S rRNA amplicons were subjected to restriction digestion with *AluI*, *HinfI* and *HaeIII* and products were analyzed on 1.2% agarose gel. Analysis showed that RFLP fragment sizes ranged from ~100bp to ~1000bp. Individual RFLP pattern of efficient bacterial strains are shown in figure and figure

Analysis showed that some PSB strains appear to share similar or identical pattern (figure) analysis of individual RFLP patterns, obtained with all 3 enzymes, from all strains, revealed, 03 unique RFLP patterns.

v. Discussion

4.1 Soil sampling

The main objective of this study was to isolate efficient Eppawala rock phosphate solubilizing bacteria for use as biofertilizers along with Eppawala rock phosphate.

Eppawala Rock Phosphate deposit was selected as the site for sampling. These deposits are rich in weathered simple P compounds, minerals, organic substances which provide microenvironments for growth of P utilizing microorganisms. Other rock dwelling organisms and plant rhizospheres also create a rich conducive living environment for microbial establishment. Taken together it can be hypothesized that phosphate rocks exposed to abiotic and biotic processes create an ecological niche likely to harbor microorganisms especially equipped to solubilize Rock phosphates. In terms of the above, the Eppawala rock could be considered as a potent biologically active deposit, where such indigenous microflora may have been evolved for long periods to solubilize this specific rock phosphate structures. Therefore the site itself would be a promising source for isolation of Rock-P solubilizers for dissolution of rock phosphates.

4.2 Soil chemical analysis

More clues to the potent biological activity of the rock come from soil analytical studies. Observed soil pH values in these sites were quite low. They were 5.5-5.8 compared to outside the deposit which was 6.5 (table 3.1). Lower pH could be associated with the acidification of the soil, by potent microbial activities (Rodrigo and Fraga, 1999). It can also be due to accumulation fluorides released from the decaying rock by both abiotic and biotic processes. Similarly, it is important to notice that soluble P concentrations at the rock sites are tenfold higher than that of outside the deposit. These findings further suggest that a potent, acidification process followed by a dissolution process resulted from abiotic weathering processes or microbial dissolution processes or both processes may be associated with this rock.

The chemical composition of the rock deposit, shows that it consist of two rock-P components (Dahanayake et al, 1995) (table 4.1).

Table 4.1 Chemical composition of primary appetite and fine matrix from Eppawala phosphate rock deposits.

	Eppawala Primary Apatite %	Eppawala Fine Matrix %
P ₂ O ₅	40.18-38.72	33.22-28.26
CaO	53.80-51.38	43.19-34.90
F	02.39-02.09	02.72-02.06
SiO ₂	00.19-00.11	00.79-00.14
Al ₂ O ₃	00.05-00.02	02.02-01.38
Fe ₂ O ₃	00.38-00.32	12.21-10.78
Na ₂ O	00.16-00.11	00.19-00.12
K ₂ O	00.05-00.02	00.04-00.02
MgO	00.18-00.11	00.19-00.13
Cl	01.98-01.84	01.41-01.16

The primary component consists of coarse, yellowish green, primary apatite crystals whereas secondary component has a finer brown colored matrix. Both components contain mainly two types of apatite, Chloro-fluro-hydroxy appatite as the major and fluroapatite as the minor. Primary apatite is rich in P, and contains 38-40% P₂O₅, 51-54 % CaO, 2-2.4 % F as major compounds, on the other hand fine matrix has lesser amount of P, and consist of 28-33 P₂O₅, 35-43% CaO as the major components.

4.3 Isolation of Phosphate solubilizing bacteria

To isolate PSB a defined selective medium containing insoluble P source, but lacking any soluble P, is needed as the isolation strategy. Such a selective medium was proposed by Pikovskaya (1948). Theoretically, any isolates growing in this medium must have phosphate solubilizing ability. In this study, modified PVK media containing CaHPO₄ as the sole P source, and bromocresol blue dye was used for isolation of PSB. Similar culture condition was employed by many researches during the isolation of soil PSB (Kumar and Narula 1999; Rodrigues and Fraga 1999; Katiyar and Goel 2003; Chen et al. 2006; Rajkumar et al. 2006; Son et al. 2006; Ahmad et al. 2008; Jorquera et al.2008; Oliveira et al. 2009; Park et al. 2010; Liu et al. 2011).

In order to isolate the PSB, soil sample extract was serially diluted and spread on modified PVK agar media containing Ca-P as the sole P source, and bromocresol blue dye. Large number of

PSB colonies among many other microbes were observed on culture plates. PSB colonies exhibiting characteristic yellow halo zones were carefully selected and further purified by replating on the same medium. This preliminary observation suggested the existence of bacterial isolates exhibiting different degrees of phosphate solubilization in the sampling site. Similar results were reported in previous studies on phosphate solubilizing bacteria from different phosphate mines. For instance, Hamdali et al. (2008) showed that the abundance of actinobacteria solubilizing Moroccan rock phosphate. Similarly, Ben Farhat et al. (2009) demonstrated that phosphate solubilizing bacteria (mainly *Serratia marcescens*) from the Tunisian phosphate mine of Gafsa. Reyes et al. (2006) have found that phosphate solubilizing bacteria (mainly *Azotobacter* sp.) from the Monte-Fresco RP mine in Venezuela.

The isolated bacteria were further screened in the presences Ca-P, Fe-P, Al-P and ERP to monitor their phosphate solubilizing ability on various other inorganic phosphate sources. Most of the strains could be able to decrease the pH in the zone surrounding the colony during the solubilization of Ca-P, Al-P, Fe-P and Rock-P. Even though the color change was directly linked to pH decrease of the medium, P solubilization efficiency of isolates was evaluated by determining the size of halo zone followed by calculating the solubilization indices (section 3.2).

All the PSB formed large halo zone on PVK agar medium supplement with Ca-P. The results showed that inorganic phosphate solubilization by PSB was decreasing in the order of Ca-P>Al-P>Fe-P>ERP. This is similar to a previous study by Puente et al. (2004), which shows that PSB from the rhizoplane of desert plant were effective in solubilizing Ca-P on solid medium but did not produce halos on agar plates amended with Al-P or Fe-P. Bacterial growth in the absence of any visible sign of phosphate solubilization in plates containing Fe-P or Al-P or Rock-P was also observed. This could be due to the slow diffusion rate of organic acids utilized during the solubilization process. However, since it has been demonstrated that many PSB not producing halo zone on agar plates are indeed able to solubilize different types of insoluble inorganic phosphates in liquid medium (Gupta et al., 1994), we decided to monitor the P solubilization of the bacterial isolates in modified PVK's liquid broth supplemented with Rock-P as the sole source of P.

4.4 Quantitative evaluation of Rock-P solubilization in liquid medium

The liquid culture study shows that PSB exhibit different degree of Rock-P solubility. Among the 20 isolates, EP-2, EP-3, EP-11, EP-13, EP-21 and EP-27 were identified as the potent Rock-P solubilizers in PVK broth. In fact the strain EP-11, identified as the most efficient Rock-P solubilizer. Most of the Rock-P solubilizers in broth culture showed similar P solubilizing trend to that seen on solid agar medium, except bacteria EP 24, EP 28 and EP 29. Some bacteria were unable to form halo zone on PVK's agar medium in the presence of Rock-P but they have solubilized Rock-P to a certain extent in liquid culture. These results indicate that the halo zone as criteria is not enough for selection and isolation of phosphate solubilizing bacteria. However it is somewhat reliable methods for isolation and preliminary characterization of PSB.

The solubilization of Rock-P by efficient strains was concomitant with medium acidification. Acidification by means of producing organic acids may be the key mechanism attributed to increased phosphate solubilization as revealed by the strong negative correlation ($r = -0.78$) (figure 3.5). Previous studies have shown that the importance of organic acids in the process of P solubilization. Illmer and Schinner, 1992 reported that, a major well characterized mechanism of mineral solubilization in Gram negative PSB is gluconic acid secretion. Similar finding was reported by Buch et al. 2008; Hameeda et al. 2008 and Kim et al. 1997. This low molecular weight organic acid results from the extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase. This enzyme requires pyrroloquinoline quinone (PQQ) as cofactor, whose biosynthesis involves *pqq* genes (Kim et al. 1998; Meulenberg et al. 1992).

The high tirtatable acidity values along with low pH values were observed in the cultures of EP11, EP2, EP3, EP13, EP21 and EP27. This might indicate that the organic acids produced by these bacteria may be highly dissociated or produced at high concentrations. Which facilitated the decrease in culture pH during the P solubilization. The high concentration of soluble P observed in these cultures clearly indicate the role of organic acid in Rock-P solubilization.

Conversely, The moderately high pH and low values of titratable acidity were observed in the culture of EP 05, EP12, EP 14, EP 15, EP 16, EP 17, EP 20, EP 22, EP 23, EP24, EP 25, EP28, EP29, and EP 31. This may indicates that the organic acids secreted by these bacteria was not completely dissociated or their concentration in the medium was quite low. Therefore these

bacteria could not be able to release soluble P into the culture medium. Out of 20 bacterial isolates, six isolates (EP11, EP2, EP3, EP13, EP21 and EP27), which showed highest solubilized Rock-P, ranging from 10.18-57.15 ppm, were selected for the further study.

4.5 The effect of particle size on Rock-P solubilization

Particle size of the rock phosphate is the vital factor in P solubilization. To investigate the effect of ERP particle size on the phosphate solubilization, various particle sizes (>0.3 mm, 3-0.15 mm, 0.15-0.075 mm, 0.075-0.037 mm and <0.037 mm) were added to the PVK liquid medium and bacteria samples were inoculated. In the present study maximum solubilization occurred with smallest particle size (below 0.037mm), similar observation was reported by Ivanova et al. (2006), which was that PSB were efficient in solubilizing smallest size Tunisian rock phosphate (below 0.2mm particle size) compared to particle size ranged from 0.4-0.63 mm. Small particle size Rock-P was more readily degraded by PSB than large particles. Decreasing the particle size, increases the surface area of the Rock-P particles so that dissolution rate of the rock phosphate can be increased. Based on these results, particle sizes, below 0.037mm was selected as optimal particle size for efficient P solubilization.

4.6 The effect of Rock-P concentration on P solubilization

Another important factor in P solubilization is the concentration of the rock phosphate. To investigate the effect of Rock-P concentrations on the phosphate solubilization, various concentrations of Rock-P ranging from 1% to 5% (w/v) were added to the PVK liquid medium. The isolates EP-11, EP-2, EP- 13 were efficient in P solubilization at a concentration of 1% (w/v) Rock-P, but P solubilization decreased when concentration was increased above 1% (w/v). However EP 3 and EP 27 were effective in solubilizing 3% (w/v) Rock-P. Since the ultimate goal of this research was to release the maximum possible amounts of soluble P, strains EP -2, EP 11, EP 13 and EP 21 can be more suitable candidates compared to EP 27 and EP-3. Among those bacteria, EP 11 was identified as most efficient Rock-P solubilizer, which exhibited the maximum soluble P concentration (118.09 ppm) at 1 % (w/v) Rock-P concentration. The data

indicate that lower the quantity of Rock-P, the greater the P solubilization. Similar observation was reported by Ivanova et al.2006. When the rock phosphate concentration was increased from 0.5 to 1 and 2% (w/v) phosphate solubilization became insignificant. Venkaterswarlu et al.1984 reported that when the rock phosphate concentration was increased over 0.25% P solubilization was insignificant with *Aspergillus niger*. Taken together, the smallest particle sizes, which are below 0.037 mm Rock-P and 1 % (w/v) Rock-P concentration may provide optimum conditions for the efficient P solubilization by the selected PSB.

4.7 Quantitative evaluation of inorganic phosphate solubilization

Efficacy of P solubilizers to solubilize various other P sources, were further evaluated in modified PVK liquid medium in the presence of Ca-P, Fe-P, Al-P, and ERP as sole P source. CaHPO_4 was solubilized more effectively than other three sources. This is in agreement with previous studies where PSB were highly effective in solubilizing P from Ca-P, but exhibit a much lower P solubilization in the presence of insoluble Fe-P or Al-P (Banik and Dey, 1983; Jones et al., 1991; Illmer and Schinner, 1995; Whitelaw et al., 1999; Reyes et al., 1999; Barroso and Nahas, 2005; Son et al.,2006). Furthermore, Reyes et al., 1999 reported that even mutant PSB with increased phosphate solubilization activity, were less efficient in solubilizing FePO_4 .

All the inorganic phosphate sources were washed several times before used as P source in liquid medium. However even after washing soluble P was observed in uninoculated controls of Ca-P, Fe-P and Al-P. This shows that, P was released from inorganic P sources into the culture medium. This could be due to the use of partially degraded inorganic phosphates, or induction of dissolution by abiotic processes such as by dissolved carbon dioxide in uninoculated controls. Gerretsen 1948, Pierre 1948, Waksman, 1952 and Norman, 1953 reported that carbon dioxide produced by microorganisms in the rhizosphere seems to be an effective mechanism in increasing the availability of phosphate. Similar principle may be applied to explain the presence of soluble P in uninoculated controls.

However solubilization of FePO_4 and AlPO_4 in inoculated culture occurred only to a lesser extent compared to controls. Due to presence of soluble P in uninoculated controls, net soluble P in culture medium was determined by subtracting the total soluble P values from that in controls. For this reason, in some cultures, negative values were observed for P concentration, for example

EP11 (figure 3.24 – 3.26). This is perhaps not surprising, because a significant proportion of soluble P was incorporated into biomass during the growth while phosphate solubilization was occurred at low rate. To avoid an underestimation of P solubilization rate, culture medium could have been supplemented with laboratory-synthesized fresh insoluble P sources, where no traces of soluble P was present. The use of such approach will allow the discriminating between different bacterial isolates, showing different phosphate solubilizing mechanisms.

Acidification seemed to be main strategy used by PSB isolates used in this present study. Indeed, a clear relationship could be established between culture medium acidification and P solubilization. It has been well established that, as a common strategy to mobilize P from insoluble mineral sources, microorganism produce organic acids and /or chelating agents (Rodriguez and Fraga,1999). The most common acid secreted by PSB, is gluconic acid, resulting from extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase (Goldstein 1994). However, in some cases gluconic acid production was not detected during the P solubilization, though pH of the culture medium was decreased. Researchers believed that, in these cases, the acidification might be due to the production and excretion of other organic acids, which may also act as agents for mobilizing insoluble P. Park et al., 1990 reported that, oxalic acid produced by *Penicillium* like fungus has been shown to particular P solubilization process for Fe-P. Moreover, citrate released by *Pseudomonas fluorescens* has been shown to effectively mobilize P from goethite (Hoberg et al., 2005). The carboxylate anions of these organic acids may compete with phosphate group for binding sites and chelate Fe and Al, finally P released in solution (Cline et al., 1982; Fox et al., 1990; Gerke, 1992). Furthermore more mechanisms have also been proposed to explain bacterial mediated P solubilization. Apart from the production and excretion of gluconic acid, they include the production of chelating substances, the release of protons originated by ammonium ion assimilation and the production of inorganic acids (Illmer and Schinner,1995; Rodriguez and Fraga, 1999).

However Solubilization of rock phosphate by PSB is not only depends on culture medium pH but it is an overall effect of the extracellular compounds such as low molecular weight organic acids participating as chelators and in exchange reactions, although other mechanisms could be involved during the ERP solubilization (Vazques et al., 2000 and Bhattacharyya et al., 2000). Citric acid has shown to be superior to other acids in its rock phosphate solubilizing ability (Kpombrekou-A and Tabatabai 1994, Xu et al.2004). *Aspergillus niger*, used in the industrial

production of citric acid, has been recognized as one of the most effective organisms for rock phosphate solubilization (Abd-Alla and Omar 2001, Agnihotri 1970, Omar 1998, Sperber 1958 b). Therefore we suspected that EP11 may have the ability synthesis citric acid during the P solubilization, which aid the release of high P from HERP.

During the liquid culture study changes in P concentration was observed at different time points. This P concentration changes with time can be in linked with bacterial growth kinetic to get a more reliable picture of bacterial cellular behavior toward P solubilization. The concentration difference could be due to the rate of P release and uptake by bacteria. When bacteria at exponential phase rate of uptake are higher than that of solubilization therefore decrease of P concentration in the medium could be observed. When bacteria move to stationary phase, the P uptake rate decreases as a result P level in the medium increases again. There could be a combination of several phenomena involved in this behavior.

Together , our results clearly shows that modified PVK medium containing $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, FePO_4 and AlPO_4 as an P solubilization indicator was biased toward the identification of HERP solubilizers. Therefore these steps can be omitted and rock phosphate solubilizers can be directly evaluated in PVK medium supplemented with ERP as sole P source.

4.8 Effect of EP-11 on rice growth promotion

Pot experiment was employed to investigate the ability of the efficient PSB (EP11) to solubilize ERP in soil. Ten days old rice seedlings were (BG 352 and Suduheenati Varity) fertilized with ERP (particle size of less than 0.037mm) and bacteria was inoculated at the rizospheric area of plants. The results showed two varieties of (BG 352 and Suduheenati) of rice were displayed significant improvement in bio mass, P content, shoot and root development after being inoculated with EP 11. This shows that after the inoculation of phosphate slubilizing bacteria EP 11 the growth of rice varieties was efficiently promoted in soil that is deficient in available P. During this study, bacteria inoculated soil had higher amount of soluble P in the soil solution compared to controls (figure) and it could be the reason for the efficient P uptake in plants and resulted in higher plant biomass. This finding was supported by Hinsinger, (2001), reported that the dry weight of pea was increased after the inoculation with a phosphate solubilizing fungus *pencillium bilaii*. German et al., (2000) observed that associative PSB play a positive role in

promoting the increase of root length and plant size; similar observation was obtained from our study too.

During the pot experiment less pH decline in soil was observed than phosphate solubilization in culture medium. This could be due to the buffering nature of soil. The inorganic acids and H⁺ ions of PSB and H⁺ ions released from plant roots during the ammonium assimilation are also reported to influence the soil pH (Illmer P and Schinner, 1995; Gaur, 1990)

Growth promoting capability of EP 11 on different rice varieties was also various. The plant genotype is vital factor that should be considered during the selection of PSB since it may act as selective filter between PSB and the associated plants. The results show that more efficient genotype/bacterial interaction could have been established between EP 11 and Suduheenati rice variety compared to the modern variety (BG352). Kan and Chen, 2002 reported that a strain that is effective in some variety might be poor to another variety.

The most interesting feature of EP-11 was its constant in solubilizing HERP on agar plate, liquid culture and in soil. Therefore EP 11 can be used as effective and fertilizer-saving way to supply P to the rice seedlings for sustains plant growth and yield, especially in P deficient Sri Lankan paddy fields. Sharma et al. 2010 also reported similar findings with Mussoori rock phosphate in the presence of PSB and suggested the potential of PSB as a useful substitute to inorganic P fertilizer in the rice, rapeseed and mungbean cultivation.

4.9 16SrRNA-RFLP Genotyping

The 16S rRNAs sequences contain sufficient sequence variability so that relationships between closely related bacterial strains can be determined. The 16S rRNA genes are more accessible for sequencing and identification of bacteria. The conserved nature of 16S rRNA enabled the design of primers that amplify nearly full length 16S rRNA sequences. The rRNA genes are ubiquitous, and the primers used in rRNA gene amplifications are often designed for broad phylogenetic bacterial groups. In this work the variation of conserved 16S rRNA among the efficient strains were estimated and used to classify bacteria into 16S rRNA genotypes. A simple way to see the variation in 16S rRNA gene is to amplifying the particular gene by PCR technique. The PCR results followed by agarose gel electrophoresis shows that all the bacterial isolates giving approximately 1500 bp sized fragment and the size of the band was determined by comparing against ladder.

vi. CONCLUSION

This study was undertaken to screen, isolate, categorize and evaluate the promiscuous Phosphate solubilizing bacteria, occurring naturally, in Eppawala rock phosphate deposits of Sri Lanka, for uncovering the existence of such PSB with the ability to solubilize Eppawala rock phosphate and make use that resource information for developing biofertilizer for crops.

1. This study confirmed the existence of Phosphate solubilizing bacteria at Eppawala rock phosphate deposits in Sri Lanka;
2. Strain EP-11 was identified as the most efficient ERP solubilizer and released 118 ppm of soluble P from rock phosphate in liquid medium.
3. The study also established that ERP smallest particle size (less than 0.037mm) and minimal ERP concentration (10g/l) have significantly influenced the solubilization of Eppawala rock phosphate.
4. The most efficient strain, EP 11, has promoted the early growth of traditional rice variety (Suduheenati) and modern variety under submerged greenhouse. Dry weight and P content of Suduheenati variety was increased by 560% and 546%, respectively compare to non-fertilized plants. Whereas 245% of P content and 333% of dry weight increase were observed in BG352.
5. The strain EP11 can be recommended as a biofertilizer for rice cultivation under wet land conditions in Sri Lanka.

vi. REFERENCES

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vii. Problems if any, encountered during the implementation of the project

- The major problem encountered during this project was to recruit a qualified student for the project. Several B.Sc students were recruited, however due to lack of proper skills in molecular biology, microbiology, and in ability to perform field visits and bio assay the students resigned in the middle of the ongoing project. This also made delaying the project work considerably and to request to extend the project duration further by another year.
- The above incidents also interrupted smooth running of the project, and generation of results on time. Therefore, I myself also engaged in research activities, especially in the last two phases of the project to bring it into success.
- Lack of a working UV spectrophotometer facility in the department of chemistry has also made the conduction of the project delayed and inconvenient.

Section 4

Impact of research results

1) Relevance of results achieved to scientific advancement

This study brought new scientific knowledge on the occurrence of efficient phosphate solubilizing bacteria, associated with in Eppawala rock soil deposits. The methods of their isolation, measuring phosphate solubilizing activity, characterizing bacteria at molecular level etc. have been uncovered. Previously published methods have been optimized specifically for analysis of local bacterial flora. The methods, techniques developed in this study therefore be highly useful in related research in future.

2) Relevance of results achieved to national / socio-economic development

The proposed preliminary research project, sought to solve an economically, important, timely problem, where it attempted to develop a low cost biofertilizer for increasing the agronomic efficiency of Rock Phosphate (EPR) minerals. This was achieved biotically by converting EPR into more soluble plant available phosphate forms by the action of phosphate solubilizing bacteria. Isolation, of efficient, phosphate solubilizing bacteria, which is the key objective of this project, has opened new avenues for utilizing EPR as a low cost, phosphate soil fertile improvement, for crop plants. Therefore, the results of this project would bring high impact on small scale farmer, agricultural sector, society and finally the economy.

3) Dissemination / application of research output

Output of this preliminary research will be highly useful to design reinoculation experiments to observe their efficacy in colonization in plant roots and solubilization of phosphates in the crop plant rhizospheres. Further steps in field level can lead to development of possible biofertilizer formulation for use with EPR for commercially important crops. Which in turn would help to cut down the cost at least to an certain extent for P fertilizer consumed by the farmer. On the other hand the methodologies developed can be used in related research to investigate the presence of more beneficial bacteria from various plant rhizospheres for uncovering better biofertilizer candidates. The results of the proposed project is planned to be included at least in two complete publications in following journals, Plant and Soil, or/and Soil Biology and Biochemistry

Section 5

Miscellaneous

- 1) List of major equipment acquired during the project period and their functionality

<u>Item/Description</u>	<u>Status</u>
1. Shaking Incubator (Gemmy; Model IN 666, 75L)	Working
2. pH meter (OHAUS, Model ST 300)	Working

- 2) List of publications / communications arising from the project and / or presentations made at seminars, workshops etc.

A Manuscript is in preparation

Section 6

Summary statement of expenditure*#

Personnel :	Rs.673,066.66
Equipment:	Rs.210,100.00
Consumables:	Rs.730,136.98
Travel and Subsistence:	Rs. 40,625.00
Miscellaneous:	Rs. 14,000.00

Subjected to confirmation by the Accounts branch, University of Kelaniya

*The original Final, Financial statement and the unspent balance of Rs.13,813.02 will be sent to the NSF by the University of Kelaniya

Section 7

i) Grantees' Signature

.....

ii) Comments of the Head of the Department/Signature

.....

iii) Head of the Institution

.....

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