Polish Journal of Microbiology
2016, Vol. 65, No 4, 407–412

ORIGINAL PAPER

Study of Acid Phosphatase in Solubilization of Inorganic Phosphates by *Piriformospora indica*

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Submitted 24 November 2013, revised and accepted 23 May 2016

Abstract

Phosphorus is an essential plant macronutrient present in the soil. Only a small portion of phosphorus in soil is taken up by plants and the rest of it becomes unavailable to plants as it is immobilized. Phosphate solubilizing microorganisms play a vital role in converting the insoluble form of phosphates to the soluble form. The present paper reports the solubilization of tricalcium phosphate, rock phosphate, single super phosphate, zinc phosphate and aluminum phosphate by *Piriformospora indica* with the production of organic acids as well as acid phosphatase. The amount of phosphate released (4.73 mg ml^{-1}) and titratable acidity (0.12%) was found to be the highest in the case of single super phosphate as compared to other phosphate sources. High performance liquid chromatography (HPLC) revealed the presence of oxalic acid, lactic acid, citric acid and succinic acid in the media. Highest phosphatase activity was observed with the cell membrane extract of the organism in the presence of zinc phosphate.

Key words: *Piriformospora indica*, acid phosphatase, organic acids, phosphate solubilization

Introduction

Phosphorus is an essential plant macronutrient which plays a significant role in the development of root, flowers and seed formation, helps in crop maturity and provides resistance to plant diseases (Khan *et al*., 2009). The functioning of certain key enzymes responsible for the regulation of metabolic pathways is also dependent on phosphorus availability (Tallapragada and Seshachala, 2012).

Most Indian soils do not contain a sufficient amount of available phosphorus which is necessary to maximize plant growth. Though large amount of inorganic phosphates are added to the soil in the form of chemical fertilizers, only a small fraction is utilized by plants and rest is converted to insoluble forms (due to soil pH) which becomes unavailable to the plants (Tallapragada and Seshachala, 2012). Phosphorus combines with iron and aluminum salts present in soil and forms complexes, as a result most of it is fixed in the soil. Acidic soils usually contain inorganic phosphates in the form of iron and aluminum salts where as neutral soil contains calcium phosphate (Gyaneshwar *et al*., 2002). Excessive utilization of chemical fertilizers in order to minimize phosphorus deficiency affects soil fertility and consequently affects crop yield (Nath *et al*., 2012).

A group of soil microorganisms known as phosphate solubilizing microorganisms (PSMs) plays a key role in converting the insoluble form of phosphates to soluble form thus making it available for the plants (Illmer and Schinner, 1995; Whitelaw, 2000). Microorganisms assimilate soluble phosphates and prevent it from adsorption or precipitation (Khan *et al*., 2009). PSMs are divided into two groups (i) Phosphate solubilizing bacteria (PSB) and (ii) Phosphate solubilizing fungi (PSF) (Tallapragada and Seshachala, 2012). Microbial communities involved in phosphorus acquisition include *Pseudomonas striata*, *Pseudomonas* fluorescens, *Bacillus megaterium*, *Bacillus polymyxa*, *Azotobacter* spp., *Burkholderia* spp. , *Rhizobium* spp., *Penicillium* spp., *Aspergillus* spp., *Trichoderma* spp. and various mycorrhizal fungi (Rodriguez and Fraga, 1999; Vyas and Gulati, 2009).

The mechanism of phosphate solubilization takes place via various microbial processes including (i) organic acids production, (ii) proton extrusion and (iii) phosphatase enzyme (Khan *et al*., 2009). Some microbes have potential for mineralization and solubilization of both inorganic and organic phosphorus. PSBs dissolve the soil phosphorus by producing weak organic acids such as gluconic acids, ketogluataric acids, succinic acid *etc*.

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Piriformospora indica, an arbuscular mycorrhiza like fungus plays an important role in the phosphate solubilization. It is known to solubilize different sources of organic phosphates as well as polyphosphates with the help of an enzyme acid phosphatase (66 kDa), present in its hyphal tips. *P. indica* exerts growth promotional effects on various plants by colonizing their roots. It has tremendous applications in the field of plant biotechnology as it acts as a biofertilizer, bioregulator, stimulator and a biocontrol agent (Malla *et al*., 2004).

Solubilization of tricalcium phosphate by diffe rent microorganisms is available in the literature (Agnihotri *et al*., 1970; Nath *et al*., 2012; Tallapragada and Seshachala, 2012). Few reports are also available on solubilization of other phosphate sources such as rock phosphate, zinc phosphate and aluminum phosphate by different microorganisms (Sagervanshi *et al*., 2012; Xiao *et al*., 2013). However, there are no reports available related to solubilization of single super phosphate by any organism.

Many insoluble forms of calcium, iron and aluminum phosphate occur in soil; however, few studies are reported related to the solubilization of aluminum and iron phosphate. Presently, rock phosphate and single super phosphates are being chiefly employed to sustain soil phosphorus level in available form for plants. Also zinc is one of the limiting factors in crop production. Hence the present study was undertaken to study the solubilization of different phosphate sources by replacing tricalcium phosphate(TCP) (present in Pikovskaya's media) with rock phosphate (RP), single super phosphate (SSP), zinc phosphate (ZnP) and aluminum phosphate (AlP) by *P. indica*. Both organic acids and phosphatase enzyme produced by the organism have contributed to the solubilization of phosphates.

Experimental

Materials and Methods

Materials. Tricalcium phosphates, zinc phosphate and aluminum phosphate used in the present study were obtained from Qualigens and S. D. Fine chemicals, Bangalore India. Single super phosphate and rock phosphate were obtained from the University of Agricultural Sciences, Bangalore, India. Molecular weight marker was obtained from Genei Pvt Ltd, Bangalore India.

Microorganism and culture maintenance. The culture of *P. indica* with an accession number of AF014929 USA; was obtained from Prof. Ajit Varma (Amity Institute of Herbal and Microbial Studies, Noida, India). The stock culture was maintained on potato dextrose agar at optimum conditions and stored at 4°C for further studies.

Phosphate solubilization in liquid medium. Phosphate solubilization was performed by inoculating 1×10^6 ml^{-1} in Pikovskaya's broth with five different phosphate sources TCP, RP, SSP, ZnP and AlP at initial pH adjusted to 7 and incubated at 30°C for 15 days. Parameters such as phosphate estimation, drop in pH, titratable acidity, production of organic acid and phosphatase activity were studied.

Phosphate estimation. Amount of phosphate released was estimated according to Fiske-Subbarow method (Fiske *et al*., 1925). The supernatant (0.5 ml) was mixed with 1 ml of 2.5 M sulphuric acid and 2.5% ammonium molybdate. To the above mixture 1 ml of the reducing agent (0.2 g of 1 amino-2-naphthol-4 sulfonic acid and 1.2 g of sodium sulfite in 100 ml distilled water) was added, incubated at room temperature for 10 minutes and the absorbance was read at 650 nm using potassium dihydrogen phosphate as standard.

Titratable acidity. Titratable acidity was estimated by titrating the known amount of culture filtrate with 0.1 M NaOH in the presence of phenolphthalein as an indicator (Nenwani *et al*., 2010). Drop in pH of the media was also recorded using digital pH meter (Elico make).

Organic acid production. Presence of organic acids in the media was detected by inoculating *P. indica* in Reyes basal medium containing five phosphate sources in presence of an indicator bromocresol green. The change in the colour of the media indicates the production of organic acids which was further identified and confirmed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Reyes *et al*., 1999; Gadagi and Sa, 2002).

Thin layer chromatography. Presence of organic acids produced in the media was detected by TLC using silica gel. The culture filtrate was reduced to $1/10^{th}$ of its volume prior to spotting. Five different solvent systems: (i) n-propanol: water: ammonia (60:20:20), (ii) benzene: methanol: acetic acid (90:16:8), (iii) methanol: 5 mol/l ammonia (80:20), (iv) ethanol: water: 25% ammonia (100:12:16), (v) n-propanol: 2N ammonia (7:3); were used to develop chromatograms which were later dipped in 0.4% (w/v) of bromocresol green in ethanol to detect the organic acid (Kraiker and Burch, 1973).

High performance liquid chromatography (HPLC). In order to identify and quantify the organic acid produced in the culture medium containing five different phosphate sources, 20 µl of the culture supernatant was subjected to HPLC analysis (Waters make) using C 18 column with acetonitrile and water in the ratio 7: 3 and pH of 3.5. The flow rate was maintained at 1 ml min^{-1} and detected by UV detector at 210 nm (Mardad *et al*., 2013).

Protein estimation. The concentration of proteins was measured by Lowry's method using bovine serum albumin as a standard (Lowry *et al*., 1951).

Enzyme assay. Acid phosphatase activity was determined by spectrophotometer using para-nitrophenol

(pNPP) as substrate. Extraction of phosphatase enzyme from the cell membrane of *P. indica* was performed according to Malla *et al.* (2004). The protein concentration of the extract was estimated by the Lowry's method. Five hundred microlitres of the crude enzyme extract was mixed with 1 ml of pNPP solution along with 1 ml of sodium acetate buffer pH 5.3. The reaction mixture was incubated at 40°C for 30 minutes. The reaction was stopped by the addition of 2 ml of 0.05 M NaOH and the absorbance was measured at 410 nm. The enzyme activity is defined as the amount of enzyme required to release p-nitrophenol per ml, per minute under standard conditions.

Electrophoresis. SDS-PAGE was performed according to Laemalli (1970) to determine the molecular weight of the protein. Cell membrane extract was loaded into 10% gel along with a molecular weight marker.

Statistical analysis. All experiments were conducted in triplicates. The data obtained from three independent experiments were analyzed using Origin Pro 8.1 version and Microsoft Excel. Each value represents the mean of three independent experiments performed in triplicate. Student 't' test was performed for all experiments.

Results and Discussions

Phosphate solubilization was performed in Pikovskaya's broth with five phosphate sources TCP, RP, SSP, ZnP and AlP. Increase in soluble phosphate was in correlation to decrease in pH, which suggests the role of $[H^+]$ in solubilization mechanism as reported by Kang *et al.* (2002) which was in accordance with our results. Malla *et al.* (2004) has also reported the utilization of complex insoluble phosphates like TCP and RP by *P. indica*. Similar studies on solubilization of TCP, RP and AlP by different microorganisms were reported earlier (Kang *et al.*, 2002; Frankem *et al.*, 2008; Nath *et al.*, 2012). Panhwar *et al.* (2011) reported the solubilization of triple super phosphate by two *Bacillus* spp. PSB 9 and PSB 16.

Phosphate estimation. Figure 1A represents the phosphate estimation plots for all the five substrates. Amount of phosphate liberated was highest with SSP (4.73 mg ml^{-1}) followed by TCP (2.89 $mgml^{-1}$). RP was found to be better compared to ZnP and AlP which released same amount of phosphate.

Titratable acidity and pH. Titratable acidity and pH also followed the similar trend. Percentage of titratable acidity was found to be highest (0.12%) for SSP with a comparative lower pH of 4.12 as depicted in Figure 1B. Titratable acidity of 0.01% for AlP was found to be least as compared to RP and ZnP. These results clearly explain the fact that the phosphate solubilization occurs by synthesized carboxylic acids released by microorganisms, as a result pH of the media decreases. Decrease in pH as well as increase in titratable acidity was observed with all the phosphate sources which are in accordance with Nenwani *et al.* (2010).

Organic acid production. Change in colour from blue to yellow in Reyes basal medium indicated the solubilization of phosphate by organic acid production. The sucrose content in the basal medium might have contributed to the increased production of organic acid as a result change in colour was observed (Reyes *et al.*, 1999). Solubilization of aluminum phosphate in Reyes basal media containing bromocresol green/ bromothymol blue dye by *Pseudomonas striata* and *Penicillium oxalicum* has been reported by Gadagi and Sa (2002).

Thin layer chromatography. Out of five different solvent systems, n-propanol and 2 N ammonia in the ratio 7 : 3 and benzene, methanol and acetic acid in the ratio 90 : 16 : 8 was found to be best solvent system.

High performance liquid chromatography. HPLC analysis of the culture filtrate was performed to identify and quantify the organic acids produced during

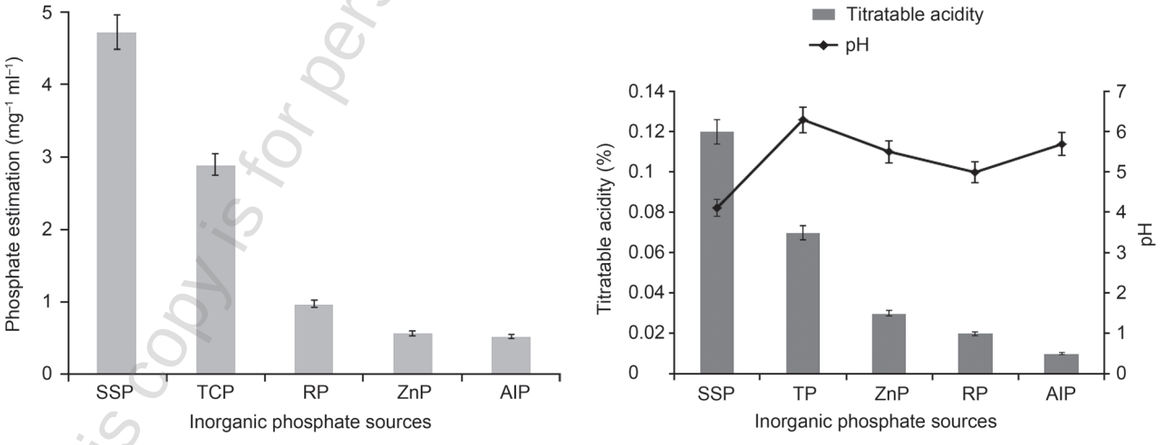

Fig. 1A and 1B. Plots depicting phosphate estimation (1A) and titrable acidity, pH (1B)

Table I
Quantification of the organic acid produced with respect to five different inorganic phosphate sources

Organic Acids	TCP	RP	SSP	ZnP	AlP
Oxalic Acid	0.3 mg ml^{-1}	2 mg ml^{-1}	0.48 mg ml^{-1}	–	0.74 mg ml^{-1}
Lactic Acid	3.8 mg ml^{-1}	0.49 mg ml^{-1}	3.75 mg ml^{-1}	4.02 mg ml^{-1}	–
Citric Acid	–	–	0.29 mg ml^{-1}	–	–
Succinic Acid	–	–	–	–	1 mg ml^{-1}

the solubilization of TCP, RP, SSP, ZnP and AlP. The quantitative difference in the production of organic acids during solubilization of different phosphate sources is as given in Table I. Both oxalic acid and lactic acid were produced during the solubilization of TCP and RP. Similarly with SSP, citric acid was produced along with oxalic acid and lactic acid. Lactic acid alone was produced during the solubilization of ZnP whereas with AlP, both lactic acid and succinic acids were produced. Two or three different unknown acids were also produced during the solubilization of all five phosphate sources.

The lactic acid and oxalic acid produced during the solubilization of phosphate sources varied in their quantity. Acid production during phosphate solubilization appears to be a common event of occurrence and the type of acid produced depends upon the type of phosphate source (Vyas and Gulati, 2009; Mardad *et al*., 2013). Among the carboxylic acids produced oxalic acid, tartaric acid, malic acid, fumaric acid, malonic acid and citric acids are more effective for phosphorus solubilization (Ryan *et al*., 2001). Our results are in accordance with Vazquez *et al*. (2000), who have also reported the production of lactic acid, succinic acid *etc*.

Phosphate solubilization is the combined effect of both drop in pH and organic acid production. Microorganisms secrete different organic acids which dissociates the bound form of phosphates. Phosphate solubilization of TCP involves the acidification of the microbial cells surrounded by proton substitution reaction that releases phosphate and calcium ions (Khan *et al*., 2009). The organic anions and associated protons are effective in solubilizing precipitated forms of soil phosphates such as Al, Fe *etc*. by chelating metal ions that may be associated with complex forms of phosphates or may facilitate the release of phosphates through ligand exchange reaction (Jones, 1998; Omar, 1998).

Enzyme activity. Phosphatase enzymes are believed to be very important in the uptake of phosphorus. Phosphatases have wide specificity which cleaves phosphate ester bonds and plays an important role in hydrolysis of insoluble polyphosphates and organic phosphates. They are also involved in phosphate transport within the cells. Phosphatases produced by plants and microorganisms are presumed to convert insoluble form of phosphates to soluble form there by helping plants to take up phosphate easily. The phosphatase activity of *P. indica* cell membrane extract with ZnP (0.088 Uml^{-1}) was found to be highest followed by RP (0.076 Uml^{-1}), SSP (0.065 Uml^{-1}) AlP (0.045 Uml^{-1}) and TCP (0.022 Uml^{-1}) which explains the importance of the type of phosphate source used (Fig. 2A). The molecular weight of acid phosphatase when analyzed on 10% polyacrylamide gel was 66 kDa (Fig. 2B) similar to the earlier report of Malla *et al*. (2004). According to Calleja *et al*. (1980) cell bound phosphatase is thought to be most important in cleavage and procure-

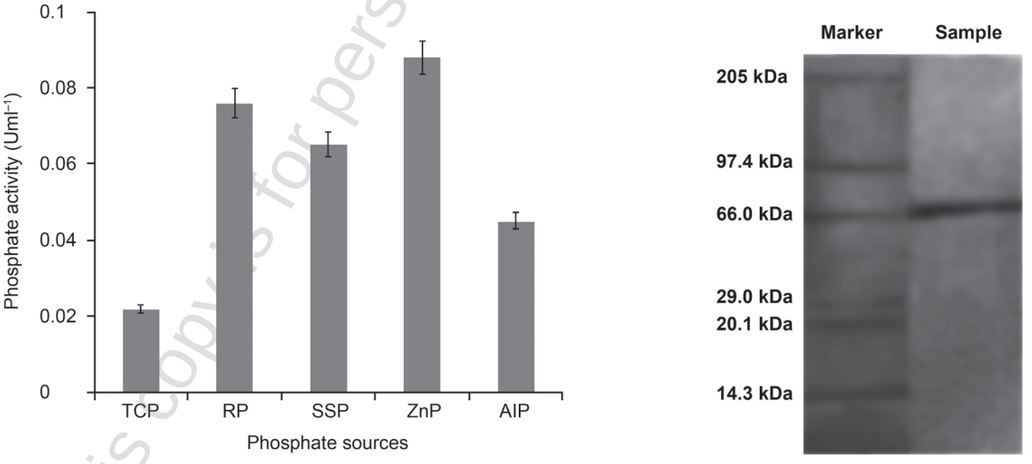

Fig. 2A and 2B. Plots depicting phosphatase activity (2A) and molecular weight of the protein obtained on polyacrylamide gel (2B)

ment of inorganic phosphates. Singh *et al.* (2002) have reported that *P. indica* secretes acid phosphatases to mobilize complex forms of phosphate present in rhizosphere there by facilitating the host plant to have better accessibility to soil phosphorus. Not many reports are available on the phosphate solubilization by phosphatase enzyme.

Phosphate solubilizing microorganisms and plants form a synergistic relationship in nature where in the PSM's provides soluble phosphates to plants and in return plants supply nutrients to microbes in the form of root exudates that promotes microbial growth. Organisms' having potential to solubilize the phosphates increases the availability of soluble phosphate and thereby enhances the plant growth and crop productivity. The mechanism of mineral phosphate solubilization has been a subject of analysis since long time and is still a matter of curiosity (Bagyaraj *et al*., 2000). Organic acids (mono, di and tricarboxylic acids) produced by the phosphate solubilizing microorganisms have been mainly involved in chelating the insoluble complexes of phosphate (Bagyaraj *et al*., 2000). According to Fankem *et al*. (2006) phosphate solubilization is the result of combined effect of decrease in pH and organic acids production. Phosphatase enzymes are likely involved in hydrolysis of insoluble phosphate complexes (Singh *et al*., 2002; Malla *et al*., 2004). Solubilization of phosphate by *P. indica* occurs due to the combined effect of both phosphatase enzyme and organic acid production. However the exact mechanism and correlation between the two needs further study for scientific validation.

Conclusion

The present study reports the solubilization of five different inorganic phosphate sources namely TCP, RP, SSP, ZnP and AlP by *P. indica*. A maximum amount of 4.73 $mgml^{-1}$ soluble phosphate with respect to SSP was solubilized by *P. indica*. Titratable acidity was found to be 0.12% and a drop in pH from 7 to 4.12 was observed with SSP. Organic acids present in the medium were detected and identified as oxalic acid, lactic acid, citric acid and succinic acid by TLC and HPLC analysis. Phosphatase activity was reported in *P. indica* with a maximum activity of 0.088 Uml^{-1} with respect to cell membrane extract of ZnP sample. The molecular weight of the phosphatase was found to be 66 kDa. Organisms such as *P. indica* having the potential to solubilize different sources of inorganic phosphates and the ability to increase the availability of soluble phosphate without the over application of chemical fertilizer enhance plant growth and crop productivity. The above outcome can be explored further and used as an innovative technology in organic farming for better crop productivity.

Acknowledgements

The authors wish to acknowledge Dr. Krishna Venkatesh, Director, Centre for Emerging Technologies, Jain University, Bangalore for his help and support during the study. The authors are also thankful to Dr. Ajit Varma for providing the culture.

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