Journal of Cell and Molecular Research (2016) 8(2), 90-97

Research Article

Purification and Characterization of an Extracellular Phosphatase Enzyme From *Bacillus* spp.

Maryam Parhamfar[1]*, Arastoo Badoei-Dalfard[1], Milad Parhamfar[2] , Shohreh Fahimi Rad[3]

[1] *Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran*
[2] *Department of Chemistry, Faculty of Science, Duissburg-Essen University, Essen, Germany*
[3] *Department of Biotechnology, Campus of Agriculture and Natural Resources, University of Tehran, Karaj, Iran*

Received 5 September 2016 Accepted 12 October 2016

Abstract

Phosphorus is one of the most important nutrients for plant growth and development. Chemical Pi fertilizer is used to provide the phosphorus for the plants, but it is mostly fixed in the soil into insoluble form and become unavailable to the plants. Phosphate-solubilizing bacteria have lots of application in agriculture as biological fertilizer. Consumption of biofertilizers instead of chemical fertilizers can lead to environmental pollution reduction and crop production enhancement using sustainable farming. In this study, a phosphatase-producing bacterium was isolated from agricultural soil in Kerman. Screening of phosphate solubilizing bacteria was performed on the PVK medium, based on clear area diameter. The best bacterium (AG41) was identified based on 16s rDNA gene. The optimum condition for production of phosphatase was also determined and it was purified and characterized. Sequence alignment and phylogenetic tree results show that AG41 is closely related to *Bacillus subtilis*, with 98% homology. Phosphatase activity was determined by end point method. The best carbon, nitrogen and phosphate sources for enzyme production were 1.0% glucose, 0.5% ammonium sulfate and (0.25%) sodium phytate +(0.25%) tricalcium phosphate, respectively. Bacterial phosphatase was partially purified using ammonium sulfate fractionation followed by dialysis. Results showed that the optimum temperature for the purified enzyme activity was 40ºC and it was stable at temperatures below 60°C. This enzyme was stable between pH 3.0-7.0, and the optimal pH activity was found to 5.0. These results indicated that this strain can be a notable candidate for using as biofertilizers.

***Keywords*:** Screening, Biofertilizer, Phosphate-solubilizing bacteria, Phosphatase

Introduction

Phosphorus (P) is one of the most important essential elements for crop production. Although phosphorus is quite abundant in many soils, it is one of the major nutrients limiting plant growth (Vassilev and Vassileva, 2003). With increasing demand of agricultural production and as the peak in global production will occur in the next decades, phosphorus is receiving more attention as a non renewable resource (Shen et al., 2011). Phosphorus is added to soil in the form of phosphate fertilizers but the overall P use efficiency is low because a large portion of the soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Rodríguez and Fraga, 1999). In addition, applications of chemical P fertilizers and animal manure to agricultural land cause environmental damage in the past decades (Shen et al., 2011).

On the other hand, several phosphate solubilizing bacteria (PSB) occur in soil, but usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P liberated by them is generally not sufficient for increase in situ plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement (Rodríguez and Fraga, 1999). Biofertilizers are the microorganisms that can convert useless nutrient to usable compounds. Using biofertilizers containing Pi solubilising bacteria hydrolyses P from various phosphate compounds resulting in decreasing application of the chemical Pi fertilizers. The principal mechanism of phosphate solubilization

Corresponding authors E-mail:
* *mary_parhamfar@yahoo.com*

bacteria for mineral phosphate solubilization is the production of organic acids and acid phosphatases (Rodríguez and Fraga, 1999). Actually, the major source of phosphatase activity in soil is considered to be of microbial origin (Sharma et al., 2013). Phosphatases or phosphoric monoester hydrolases (EC 3.1.3) are clustered in acid or alkaline. The acid phosphohydrolases, show optimal catalytic activity at acidic to neutral pH values. Also, they can be further classified as specific or nonspecific acid phosphatases, in relation to their substrate specificity (Rodríguez and Fraga, 1999). Secretion of phosphatase enzymes occurs in response to both phosphate starvation and environmental pH signaling, instigating the microbes to utilize phosphorus containing substrates (Ferreira-Nozawa et al., 2003). On the other hand, activity of phosphatase enzyme is affected by some factors, such as the amount and type of substrate, pH, temperature, concentration of enzyme and product (Fitriatin et al., 2011). It should be noted, proper and economical production of biofertilizers depends on the mass production of biofertilizer forming microorganisms which are obtained by suitable culture medium and fermentation process (Nautiyal et al., 2000).

In this study, phosphate solubilizing bacteria was isolated and characterized from an agricultural soil in tropical regions. Bacteria with the highest phosphatase producing ability purified on the specific media and identified. The optimum condition for growth and production of phosphatase was also determined. Phosphatase from bacteria was partially purified and stability and activity of phosphatase was assayed in different temperatures, pHs.

Materials and Methods

Screening of Phosphate-Solubilizing Bacteria (PSB)

Samples picked up from agricultural soil in Kerman, located the south-eastern of Iran. 1 g of soil samples was suspended in 10 ml of 0.9% saline solution and 1 ml of this suspension was inoculated in 50 ml of Pikovskaya (PVK) broth [1% glucose, 0.5% $Ca_3(PO_4)_2$, 0.05% Yeast extract, 0.05% $(NH_4)_2SO_4$, 0.02% KCl, 0.01% $MgSO_4.7H_2O$, 0.02% NaCl, 0.0002% $FeSO_4$, 0.0002% $MnSO_4$, pH 7] and were incubated for 2 days at 37ºC in an orbital shaker (180 rpm) (Shekhar Nautiyal, 1999). Then 0.1 ml of the cultured samples were streaked on PVK agar (at 37ºC for 48 h). A clearing zone around the bacterial colony on PVK plates represents extracellular phosphatase activity (Hu et al., 2010; Fitriatin et al., 2011). A promising colony showing the highest clear zone was purified on the specific media and designated as AG41. Isolate AG41 was selected for further study and identified up to the genus level based on the morphological and biochemical properties.

PCR Amplification and 16S rDNA Sequencing

Genomic DNA of isolate AG41 was extracted according to Sambrook and Russell protocols (Sambrook and Russell, 2001) and its purity was checked by the A260/A280. Then universal 16S rRNA PCR forward primer (5-AGTTTGATCCTGGCTCAG-3) and reverse primer (5-GGC/TACCTTGTTACGACTT-3) were used for the amplification of 16S rRNA gene. The reaction conditions were as follows: (1) 94°C for 5 min as initial temperature, (2) a run of 30 cycles with each cycle consisting of 45 s at 94°C, 45 s at 54°C and 90 s at 72°C, and (3) 8 min at 72°C to permit for the extension of any incomplete products. Products were purified by DNA extraction kit (Cinaclone) and DNA sequencing was performed on both strands directly by SEQ-LAB.

The phylogenetic tree was made based on the comparison of 16S rRNA sequences of *Bacillus* sp. AG41 strain with other strains of *Bacillus* species that were obtained from the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov). All sequences were aligned with Clustal Omega that was obtained from: http://www.seqtool.sdsc.edu/ CGI/Omega.cgi (Sievers et al., 2011) and phylogenetic tree was made in the MEGA program version 4 (Tamura et al., 2007).

Optimization of Culture Media for Phosphatase Production

Effect of Carbon Source on Phosphatase Production

Effect of different carbon sources on phosphatase production was determined by addition of 0.1% of respective sugar (1% galactose and 0.5% glucose + 0.5% galactose) instead of glucose in the PVK medium. Strain AG41 incubated in this medium in shaker at 37°C for 3 days. Samples were picked up at each 24 h interval and phosphatase activity was determined. Phosphatase activity and phosphate releasing was determined by end point method.

In this method, 1 ml of medium containing grown bacteria was centrifuged for 10 min at 4000 rpm. The reaction mixture consisted of 100 µl supernatant (enzyme) and 300 µl 0.1 M sodium acetate buffer of pH 6.0, containing 2 mM sodium phytate (substrate)

and 2 mM $CaCl_2$. The reaction mixture was incubated at 37°C for 30 minutes then stopped by adding 400 µl of 5% (w/v) trichloroacetic acid (TCA). Then 400 µl of ammonium molybdate reagent was added (containing 1:4 mixture of 2.7% FeSO4 and 1.5% ammonium molybdate in 4.4% H_2SO_4).

After 10 min incubation at room temperature, absorbance at 700 nm was read. Measurement of phosphatase activity is based on the colorimetrical quantification at 700 nm of free phosphorus released by the hydrolysis of phosphate, using ammonium molybdate as color reagent (molybdate-blue method) (Kerovuo and Tynkkynen, 2000). One enzyme unit (U) is the amount of enzyme librating 1 µM of inorganic phosphate in 1 minute under the assay.

Effect of Nitrogen Source on Phosphatase Production

To determine the effect of nitrogen source on phosphatase production, the activity of this enzyme in PVK medium with 0.5% glycine, 0.5% nitrate sodium and 0.5% ammonium sulfate was assayed. The best strain (AG41) was inoculated into 50 ml of PVK broth in a 250 ml erlenmeyer flask and incubated on shaker a 37°C for 3 days.

Effect of Phosphate Source on Phosphatase Production

To study the effect of phosphate on phosphatase production, the activity of this enzyme in nutrient broth, PVK medium with tricalcium phosphate (0.5%), sodium phytate (0.5%) and medium with sodium phytate (0.25%) + tricalcium phosphate (0.25%) was assayed. The medium was incubated under same condition as above. The enzyme activity was measured for 3 days. The chosen carbon, nitrogen and phosphate sources were used in the subsequent experiments.

Partial Purification of Enzyme and Phosphatase Activity Assay

Partial purification of phosphatase was by ammonium sulfate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulfate up to 65%. The content was incubated over night and centrifuged at 12,000 rpm for 10 min. Supernatant was collected and investigated for enzyme activity. Pellet was collected and transferred to dialysis bag and immersed in Tris/HCl buffer (pH 7.5) at 4°C for 24 h. Then phosphatase activity was determined by end point method.

Effect of Temperature on Enzyme Activity and Stability

Phosphatase activity was determined at various temperatures as following; 10-70°C. For the thermostability, the enzyme was incubated for 30 min at various temperatures as described previously. Then phosphatase activity and phosphate releasing was determined by molybdate-blue method.

Effect of pH on Enzyme Activity and Stability

Phosphatase activity was determined at various pHs as following; pH 3-10. For the pH stability, the enzyme was incubated at various pH for 30 min as described previously. The residual activity was determined.

Results

Screening and Identification of Phosphatase Producing Bacteria

A total of 20 strains that can solubilize tricalcium phosphate and form peripheral halo zone on phosphatase specific agar medium around colonies were isolated. Amongst these, isolate AG41 showing the highest clear zones on PVK screening medium was selected for the further studies (Figure 1). Based on their cultural, biochemical and morphological characteristics, AG41 isolate was identified that belong to *Bacillus* genus.

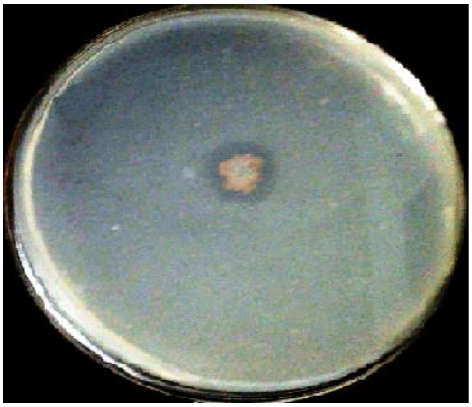

Figure 1. Zones of clearance by AG41 isolate on PVK screening medium

Bacillus isolate (AG41) was subjected to molecular identification using 16S rRNA. The PCR product of 16S rRNA gene was about 1400 bp. The phylogenetic tree was made by neighbor-joining method by MEGA4 software (Figure 2) (Sievers et al., 2011). Result shows that *Bacillus* sp. AG41is strongly related to *Bacillus subtilis* with 98% homology.

Research Article

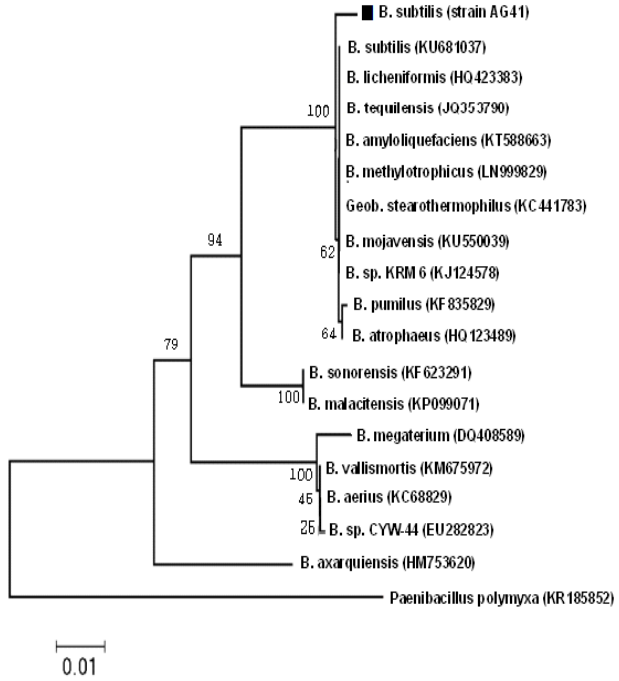

Figure 2. The phylogenetic tree constructed by the neighbor-joining method showing the position of isolate AG41

Optimization of Culture Condition

Among different carbon sources studied, glucose showed the maximum phosphatase activity (Figure 3).

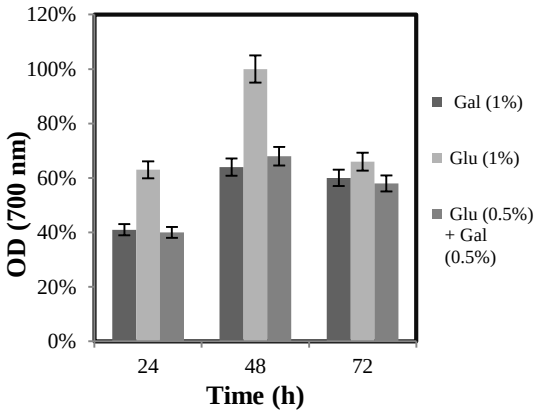

Figure 3. Effect of carbon source on phosphatase production

Results show that all three carbon sources improved phosphatase production at 48 h of incubation and slightly decrease at 72 h.

To investigate the effect of nitrogen sources on the growth and production of phosphatase, ammonium sulfate showed the maximum enzyme production and phosphate releasing (Figure 4).

Assay activity of this enzyme carried out in four media with different phosphorus sources.

Result showed phosphatase activity in medium with sodium phytate (0.25%) + tricalcium phosphate (0.25%) increased after 2 days.

Thus, this enzyme seems to enable degradationof sodium phytate through possibly phosphatase with phytase activity (Figure 5).

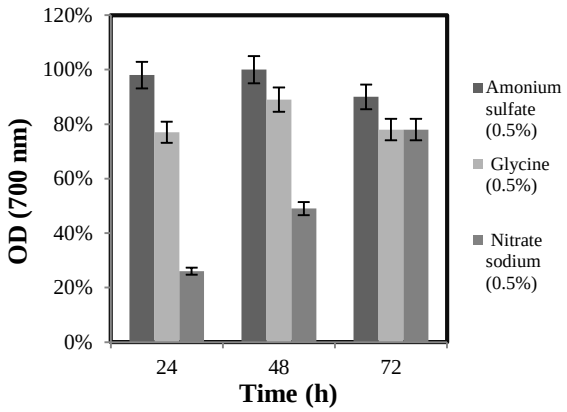

Figure 4. Effect of nitrogen source on phosphatase production

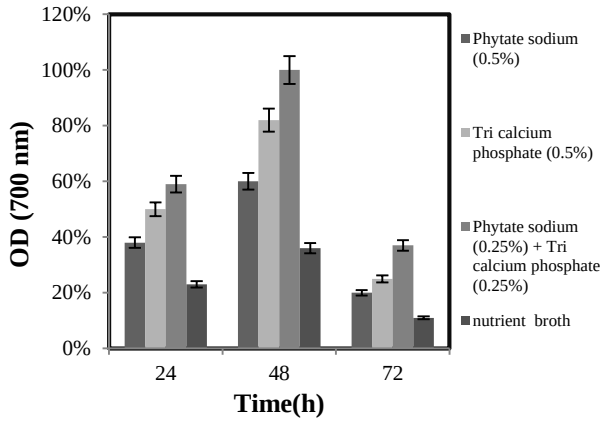

Figure 5. Effect of phosphate source on phosphatase production

Enzymatic Properties

Temperature Optimization and Thermal Stability

The temperature profile of the purified phosphatase was determined from 10°C to 70°C using the standard phosphatase assay. The optimum temperature was found to be 40°C (Figure 6a).

The apparent activation energy was estimated at pH 6.0.

In order to check thermal stability, the purified enzyme was incubated at different temperatures,

cooled to 4°C and assayed using the standard phosphatase assay.

The data showed excellent linearity from 10°C to 50°C. This phosphatase enzyme, when exposed for 30 min at 60°C, retained 80% and at 70°C, 50% of the initial activity, which indicate that the enzyme is thermostable (Figure 6b).

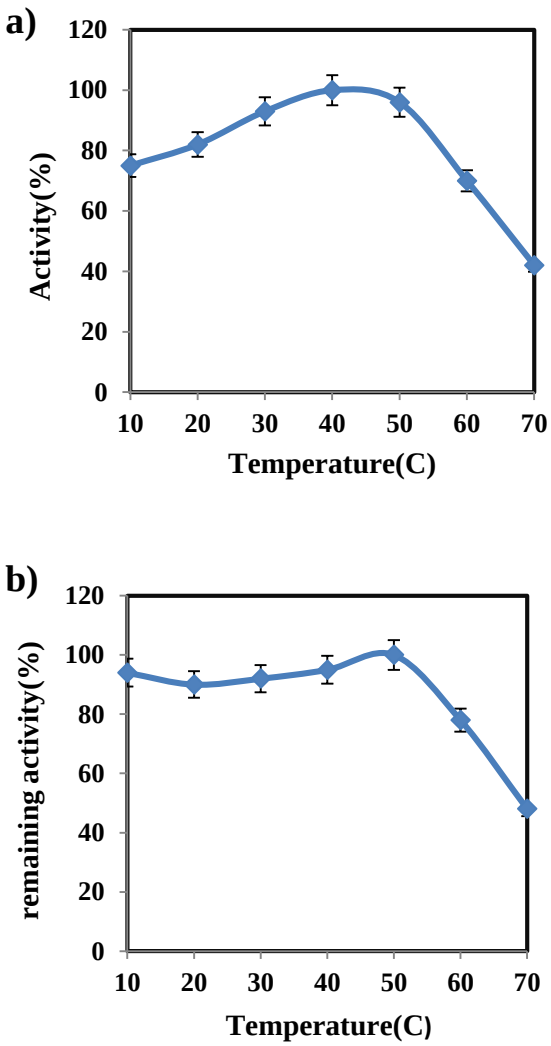

Figure 6. Effect of temperature on phosphatase activity (a) and stability (b) of *Bacillus* sp. AG41.

pH Optimization and pH Stability

The purified enzyme had a single optimum pH at pH 5.0. At pH 6.0, 82% and at pH 7.0, 76% of the activity at optimal pH was observed.

The effect on enzyme stability was studied in the pH range 3.0–10.0 at 4°C. Within 30 minutes the phosphatase did not show activity in the pH 10.0, but at pH 6.0, 98% and at pH 8.0, 75% of the initial activity was retained (Table 1).

Table 1. Effect of pH on phosphatase activity and stability

pH	Activity (%)	Remaining activity (%)
3	50	90
4	90	95
5	100	100
6	82	98
7	76	90
8	56	75
9	45	40
10	30	17

Discussion

Phosphatase is one of the important enzymes produced by several soil microorganisms. Also, the pH of most soils ranges from acidic to neutral values. Thus, acid phosphatases should play the major role in phosphate solubilization. (Rodríguez and Fraga, 1999).

On the other hand, there are fewer reports of phosphatase activity shown by microbes in tropical soils (Nopparat et al., 2007; Nenwani et al., 2010). Currently, most of PSB studied and applied to date have been mesophiles that could only be used under mesophilic conditions (Jatoth et al., 2015). Therefore, these types of microbes are not appropriate as biofertilizer for tropical regions. Also, they are not suitable for biofertilizer preparation at the high temperatures (over 50ºC) that occur during the first stage of composting (Chen et al., 2007). Thus, it is very important and necessary to isolate and screen some phosphate-solubilizing bacteria that can adapt to the environment for enhancing the utilization of phosphorus in the agriculture. Thermal stability of phosphatase is considered to be an important and useful criterion for application in agriculture as biofertilizers in acidic and tropical soils.

In this study soil sample of agriculture (in tropical regions) was chosen to isolate the phosphate solubilisers. The bacterial strain isolated from soil will have phosphate solubilizing capacity by producing extracellular phosphatase. 20 strains

showed phosphatase activity on PVK agar. The highest phosphatase producing strain, AG41, was selected based on a clear zone around the strain. In present study, strain AG41 is strongly related to *Bacillus subtilis* using 16S rDNA sequencing analysis.

The parameters like carbon, nitrogen and phosphate sources were optimized for better phosphate solubilization and production of phosphatase enzyme. The nature of the carbon compound and the concentration may stimulate or down modulate the production of enzymes in the microorganisms. Among the different carbon sources, 1.0% glucose shown the maximum production of enzyme. Nitrogen is considered as another energy source for growth of the microorganism and phosphatase production. The better nitrogen source for enzyme production and phosphate releasing was ammonium sulfate. Also, the enzyme production was maximum when the medium was modified with sodium phytate (0.25%) + tricalcium phosphate (0.25%) as phosphate sources.

The best carbon and phosphate sources for maximum phosphatase production by *Bacillus licheniformis* isolated from hot spring were glucose and tricalcium phosphate, respectively (Parhamfar et al., 2014). Phosphate solubilization activity of *Aspergillus* sp. was investigated in the presence of five carbon and seven nitrogen sources. This strain demonstrated diverse levels of phosphate solubilization activity in the presence of various carbon and nitrogen sources, but glucose and $(NH_4)_2SO_4$ were found as the best carbon and nitrogen sources (Pradhan and Sukla, 2012). It was previously reported that the best carbon and nitrogen sources for production of alkaline phosphatase by *Bacillus subtilis* were starch and Egg albumin, respectively (Jatoth et al., 2015).

Partial purification of enzyme phosphatase was by ammonium sulfate precipitation followed by dialysis. The optimum temperature for enzyme purified activity was 40ºC and the enzyme was stable at temperatures below 60°C when preincubated at various temperatures for 30 min. Cheng and Yang (2009) reported Thermo-tolerant phosphate-solubilizing microbes with six types of enzyme activities and three types of inorganic phosphate-solubilizing activities at 25 and 50ºC were isolated from the composts and biofertilizers. Acid phosphatase of *Penicillium citrinum* (cold-tolerant fungus) had an optimum temperature of 60ºC. The dried enzyme extract is stable at a temperature of up to 50ºC for at least 1 h (Gawas-Sakhalkar et al., 2012). In addition, pH had a statistical effect on the activity of acid phosphatase where, in more acid media, a higher activity of acid phosphatase was evaluated. As an example, Mclachlan (1980) reported the greatest phosphatase activity in the acidic range pH optima 5-6 for all species. It seems, increase in pH effect the charges on the amino acids with in the active site such that the enzyme is not to be able to form enzyme substrate complex. Therefore, there is decrease in enzyme activity (Mahesh et al., 2010).

The optimum pH of the AG41 phosphatase was 5.0 and it was relatively stable over a pH range of 3.0 to 7.0. So, it can be concluded that the purified enzyme phosphatase is a thermostable acid phosphatase with phytase activity. Acid phosphatase isolated from *Burkholderia gladioli* was stable after 6 h of incubation at 45°C in 100 mM acetate buffer at pH 6.0. The rate of hydrolysis enzyme reached a maximum at pH 6.0 (Rombola et al., 2014). Boyce and Walsh (2007) reported an acid phosphatase with activity on phytate produced by *Mucor hiemalis Wehmer* that exhibited a maximum activity at a temperature of 55ºC and pH 5.0-5.5. An alkaline phosphatase was produced from *Bacillus* spp, isolated from soil samples shows its optimum activity at pH 8.8 and temperature 65ºC, which indicate that the enzyme is thermostable (Mahesh et al., 2010).

This study reports biological production of phosphatase by *Bacillus* sp. AG41. Enzyme was able to tolerate high temperature and acidic pH. In addition, phosphatase enzyme production capacity, activity and stability in various conditions and phosphate solubilizing potential in different carbon and phosphate sources show that this strain has considerable importance as biofertilizers. Also, efforts have been made to encapsulate phosphate solubilizing bacteria for use in agriculture because these bacteria involved in both mineral and organic phosphate solubilization and increase soluble phosphorus in soil, stimulate root growth and promote sprouting on different plant species through the increase in P uptake.

References

1. Boyce A. and Walsh G. (2007) Purification and characterization of an acid phosphatase with phytase activity from *Mucor hiemalis Wehmer*. Journal of Biotechnology 132(1): 82-7.
2. Chang C. H. and Yang S. S. (2009) Thermo-tolerant phosphate-solubilizing microbes for multi-functional

biofertilizer preparation. Bioresource Technology 100: 1648–1658.

3. Chen K. S., Lin Y. S. and Yang S. S. (2007) Application of thermotolerant microorganisms for biofertilizer preparation. Journal of Microbiology, Immunology and Infection 40(6): 462-73.
4. Ferreira-Nozawa M. S., Nozawa S. R., Martinez-Rossi N. M. and Rossi A. (2003) The dermatophyte Trichophyton rubrum secretes an EDTA-sensitive alkaline phosphatase on high-phosphate medium. Brazilian Journal of Microbiology 34(2): 161-164.
5. Fitriatin B. N., Arief D. H., Simarmata T., Santosa D. A. and Joy B. (2011) Phosphatase-producing bacteria isolated from Sanggabuana forest and their capability to hydrolyze organic phosphate. Journal of Soil Science and Environmental Management 2(10): 299-303.
6. Gawas-Sakhalkar P., Singh S. M., Naik S. and Ravindra R. (2012) High-temperature optima phosphatases from the cold-tolerant Arctic fungus *Penicillium citrinum*. Polar Research.
7. Hu X. J., Li Z. J., Cao Y. C., Zhang J., Gong Y. X. and Yang Y. F. (2010) Isolation and identification of a phosphate-solubilizing bacterium *Pantoea stewartii* subsp. *stewartii* g6, and effects of temperature, salinity, and pH on its growth under indoor culture conditions. Aquaculture International 18(6): 1079–1091.
8. Jatoth K., Shantipriya A., Mangilal T. and Junapudi S. (2015) Optimization for the production of extracellular alkaline phosphatase from *Bacillus subtilis*. International Journal of Current Microbiology and Applied Sciences 4(10): 829-838.
9. Kerovuo J. and Tynkkynen S. (2000) Expression of *Bacillus subtilis* phytase in *Lactobacillus plantarum* 755. Letters in Applied Microbiology 30: 325-329.
10. Mahesh M., Neha G., Rajesh T. S., Somashekhar R. and Puttaiah E. T. (2010) Isolation and characterization of extracellular thermostable alkaline phosphatase enzyme from *Bacillus* spp. International Journal of Applied Biology and Pharmaceutical Technology 1(1): 21-33.
11. Mclachlan K. D. (1980) Acid phosphatase activity of intact roots and phosphorus nutrition in plants. I. Assay conditions and phosphatase activity. Australian Journal of Agricultural Research 31: 429-440.
12. Nautiyal C. S., Bhadauria S., Kumar P., Lal H., Mondal R. and Verma D. (2000) Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiology Letters 182(2): 291-6.
13. Nenwani V., Doshi P., Saha T. and Rajkumar S. (2010) Isolation and characterization of a fungal isolate for phosphate solubilization and plant growth promoting activity. Journal of Yeast and Fungal Research 1: 9-14.
14. Nopparat C., Jatupornpipat M. and Rittiboon A. (2007) Isolation of phosphate solubilizing fungi in soil from Kanchanaburi, Thailand. KMITL Science and Technology Journal 7: 137-146.
15. Parhamfar M., Badoei-Dalfard A., Khaleghi M. and Hassanshahian M. (2014) Isolation of phosphatase-producing phosphate solubilizing bacteria from Loriya hot spring: Investigation of phosphate solubilizing in the presence of different parameters. Biological Journal of Microorganism 3(9): 75-88.
16. Pradhan N. and Sukla L. B. (2005) Solubilization of inorganic phosphates by fungi isolated from agriculture soil. African Journal of Biotechnology 5(10): 850-854.
17. Rodríguez H. and Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17: 319–339.
18. Rombola T. H., Pedrinho E. A., de Macedo Lemos E. G., Gonçalves A. M., dos Santos L. F. and Pizauro J. M. (2014) Identification and enzymatic characterization of acid phosphatase from

Burkholderia gladioli. BMC Research Notes 7: 221.

19. Sambrook J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
20. Sharma S. B., Sayyed R. Z., Trivedi M. H. and Gobi T. A. (2013) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springer Plus 2:587.
21. Shekhar Nautiyal C. (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiology Letters 170(1): 265-270.
22. Shen J., Yuan L., Zhang J., Li H., Bai Z., Chen X., Zhang W. and Zhang F. (2011) Phosphorus dynamics: From soil to plant. Plant Physiology 156: 997–1005.
23. Sievers F., Wilm A., Dineen D. G., Gibson T. J., Karplus K., Lopez W., Li R., McWilliam H., Remmert M., Söding J., Thompson J. D. and Higgins D. G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular Systems Biology 7: 1-9.
24. Tamura K., Dudley J., Nei M. and Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.
25. Vassilev N. and Vassileva M. (2003) Biotechnological solubilization of rock phosphate on media containing agro-industrial wastes. Applied Microbiology and Biotechnology 61: 435–440