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Effect of purified alkaline phosphatase from *Bacillus licheniformis* on growth of *Zea mays* L.

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Abstract

Some soil microbes have the capability to solubilize mineral phosphate into organic phosphorous and used as biofertilizer to improve crop productivity in agricultural field. In this study, phosphate solubilization assay was carried out onto media plates containing calcium phsophate precipitated nutrient agar media for bacterial strains like *Bacillus megaterium* MTCC 453, *Bacillus subtilis* MTCC 1134, *Bacillus licheniformis* MTCC 2312, *Pseudomonas aeruginosa* MTCC 424, *Escherichia coli* MTCC 570. Among these bacterial strains, *B. licheniformis* MTCC 2312 showed largest clear zone of phosphate solubilzation and maximum activity of alkaline phosphatase. The enzyme alkaline phosphatase was purified from *B. licheniformis* MTCC 2312 with purification fold 3.52 and specific activity 295.89 U mg^{-1} protein using DEAE-sepharose chromatography. This enzyme showed molecular weight as 60 KD, thermostability upto 50 ˚C, pH stability up to 8.5 and Michaelis constant (K_m) and maximum activity (V_{max}) as 2.30 mM and 2223 U ml^{-1} respectively. The lyophilized powder of this enzyme was further supplemented with media components for the growth of *Zea mays* for carrying tissue culture experiment. The sterilized soil supplemented with alkaline phosphatase improved the total height, dry weight, % phosphate content in the stem and root of *Zea mays* by 3.07, 3.15, 2.35 and 1.76 fold respectively compared to control set. This enzyme could be used at large extent as effective biofertilizer for the agricultural industry.

Keywords: Alkaline phosphatase; *Bacillus licheniformis*; Biofertilizer; *Zea mays*

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Introduction

Phosphate anions are extremely reactive and present in insoluble complex as phosphorylated derivatives of Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+} in soil samples and unavailable for plant (1, 2). Some soil microbes have the capability to solubilize these immobilized insoluble phosphate either by secreting organic acids or phosphohydrolase enzyme (3, 4). Several microbes have been reported to exhibit phosphate solubilization activity for hydrolyzing insoluble complex of phosphate like dicalcium phosphate, hydroxyapatite, tricalcium phosphate into inorganic phosphate (5, 6). Bacterial strains like *Bacillus, Pseudomonas, Aereobacter, Burkholderia, Erwinia, Rhizobium, Agrobacterium, Micrococcus, Achromobacter, Flavobacterium, Paenibacillus* exhibited phosphate solubilization activity (3, 7-15). Soil inoculated with these phosphate-solubilizing

bacteria (PSB) improved the yield and productivity of some crops (2). In conjugation with phosphate-solubilizing bacteria, these biofertilizers should provide a cheap source of chemical phosphate fertilizer for crop production (16). Hence, phosphate-solubilizing bacteria have the potential to improve crop production in this area. The performance of these microbes for hydrolysis of insoluble complex of phosphate is affected severely under climatic stress of high salt, pH and temperature. In the alkaline soils of the tropical field, the optimum concentration of salts, pH value and temperature range varies from 1-2%, 7.5-10.5, 35-45 °C respectively. These climate changes result variance in survivability of phosphate-solubilizing bacteria (17-19). *Bacillus* species like *B. brevis*, *B. licheniformis, B. megaterium, B. polymixa, B. thruringenisis* have unique characteristics of producing stress resistant spores which can withstand a wide range of pH and temperature of soil (3, 17). *Bacillus species* are also known to produce large amount of alkaline phosphatase enzyme extracellularly which easily solubilize mineralized phosphate of soil and thereby enhance the phosphorous uptake by the plant leading to improve crop productivity. There is not any scientific report available till date for use of purified alkaline phosphatase secreted from *Bacillus* spp. for improving productivity of crop plant. This study will highlight the biochemical characterization of alkaline phosphatase from *B. licheniformis* and its application as biofertilizer for growth of *Zea mays* plant.

Materials and Methods

Selection of potent phosphate solubilzing bacteria

Bacterial strains (procured from IMTECH Chandigarh) like *B. megaterium* MTCC 453, *B. subtilis* MTCC 1134, *B. licheniformis* MTCC 2312, *P. aeruginosa* MTCC 424, *E.coli* MTCC 570 were maintained in nutrient agar media (pH 7.5) and subcultured once in two weeks. They were grown in growth media (pH 7.5) containing 1% glucose, 0.1% yeast extract, 1% peptone, 0.002% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.5% NaCl and incubated at 35 ˚C, 120 rpm for 72 h. The phosphate solubilization assay was carried out by streaking calcium phsophate precipitated nutrient agar media plates containing 10% K_2HPO_4, 10% $CaCl_2$ and incubating at 27 ˚C for 72 h with the suspension of these bacterial strains (20). Clear zone of phosphate solubilization was measured around bacterial colony after 14 days and the bacterial strain showing largest zone was selected for further study.

The fermentative broth culture was centrifuged at 10,000 g at 30 ˚C for 15 min and cell free supernatant was used for estimation of activity of alkaline phosphatase. The activity was measured spectrophotometrically at 415 nm by monitoring the release of p-nitrophenol from p-nitrophenyl phosphate disodium salt (pNPP) (21, 22). One unit of alkaline phosphatase is defined as the amount of the enzyme required for liberation of 1 µmole of p-nitro phenol per ml of reaction mixture per minute under standard condition.

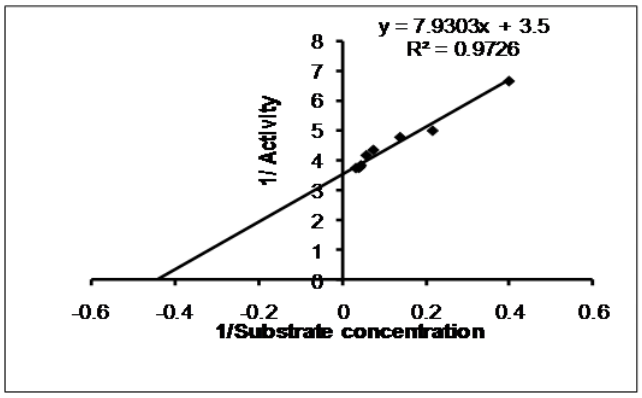

Fig. 1. Line weaver Burk plot for purified alkaline phosphatase.

Purification of alkaline phosphatase

The fermentative broth culture was centrifuged at 10000 × g for 15 min at 4 ˚C and collected supernatant was used as crude extract. The crude extract was partially purified by precipitating with 30–80% ammonium sulfate saturation and the pellet was dissolved in 50 mM Tris–HCl (pH 8.0). Each pellet suspension was dialysed against Tris HCl buffer and dialysed fraction was subjected to ion exchange chromatography using DEAE-Sephadex G-200. Activity of alkaline phosphatase was estimated in each fraction and total protein was simultaneously determined by Bradford method. SDS-PAGE electrophoresis was used for estimation of molecular weight of purified extract of alkaline phosphatase.

Characterization of alkaline Phosphatase

Kinetic constant values K_m and V_{max} for purified alkaline phosphatase were determined by plotting Lineweaver Burk plot for different substrate concentration (2.0-30 mM) (Fig. 1). The value of optimum pH was estimated by incubating the reaction mixture in different range of pH values (8.5 to 12.5) at 50 ˚C for 20 min and temperature was optimized by incubating the mixture with optimum pH at different temperature (40 to 100 ˚C) for 20 min. The thermostability was determined by incubating purified enzyme extract at temperature 50 ˚C for intervals of 2, 4, 6, 8, 10, 20, 40, 50 and 100 h. Substrate specificity test for alkaline phosphatase was done by analyzing inorganic phosphate obtained from hydrolysis of monosubstituted phosphate linkages compounds by alkaline phosphatase. Lowry-Lopez method (23) was used to determine the concentration of released inorganic phosphate. The reaction mixture containing alkaline phosphatase enzyme and phosphorylated compounds (5.4 mM-Tris-HCl, pH 9.5) was incubated at 50 ˚C for 20 min.

Growth of plant in treated and untreated soil

Seeds of *Zea mays* were washed with autoclaved water and sterilized with sodium hypochlorite (0.5%). These sterilized seeds were germinated in pot filled with sterilized soil supplemented with calcium phosphate [$Ca_5(PO_4)_3OH$] at different doses (0, 200 and 375 mg kg^{-1} soil). Three pots were filled with soil having different doses of calcium phosphate as control sets and three pots were filled with calcium phosphate supplemented sterilized soil along with lyophiillized powder of alkaline phosphatase as experimental sets. Sterilized seeds of *Zea mays* were inserted into all these pots and allowed to germinate for 60days in greenhouse under controlled conditions at temperature varying 35-50 ˚C. Height of the plant and percent of phosphate content in stem and roots was recorded. Plant samples of each control and experimental set after harvesting were dried in oven at 65 ˚C to obtain total plant biomass (Dry weight).

Determination of percent phosphate content

Vanado-molybdophosphoric acid reagent was prepared by mixing ammonium molybdate (7.5 g l^{-1}) and concentrated ammonium metavandate (0.6875 g l^{-1}). Standard phosphate solution (50 mg l^{-1}) was prepared by adding 0.2195 g KH_2PO_4 to 100 ml distilled water and acidifying with 25 ml of 7N H_2SO_4. Phosphate content in plant sample was estimated by mixing 10 ml acid digest of plant sample with 10 ml of the vanadate-molybdate reagent, diluting solutions to 50 ml and measuring absorbance at 420 nm after 10 min. The standard curve obtained for estimation of phosphate content has been shown in Fig. 2.

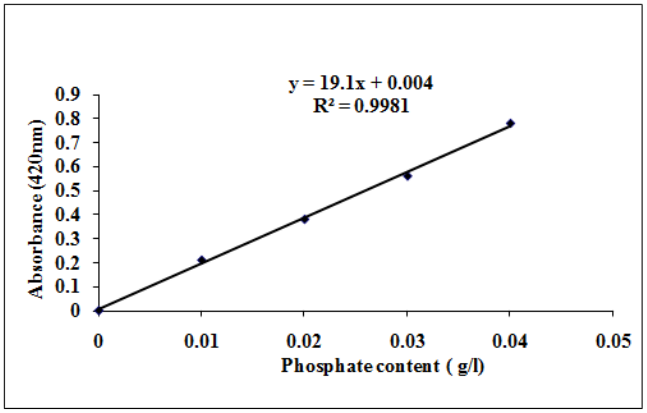

Fig. 2. Standard curve for estimation of phosphate content.

Results and Discussion

Bacterial strains like *B. megaterium* MTCC 453, *B. subtilis* MTCC 1134, *B. licheniformis* MTCC 2312, *P. aeruginosa* MTCC 424, *E. coli* MTCC 570 were streaked on nutrient agar media plates supplemented with calcium phosphate and screened on the basis of zone of clearance due to phosphate solubilizing assay (Fig. 3) and estimation of activity of alkaline phosphatase (Fig. 4). *B. licheniformis* MTCC 2312 showed largest clear zone of phosphate solubilization in compare to other strains. This visual analysis method has been considered as general reliable method for morphological characterization of phosphate-solubilizing-microbes (3, 7, 8, 24-26). Alkaline phosphatase activity was estimated for all the bacterial strains and maximum activity was found as 1550 U ml^{-1} for *B. licheniformis* MTCC 2312 as shown in Fig. 4. *B. licheniformis* MTCC 2312 was selected as potent bacterial strain for purification of alkaline phosphatase. Alkaline phosphatase was purified by fractional precipitation with 30-80% ammonium sulfate and DEAE column with purification fold 3.52 fold and 1.614% of recovery (Table 1).

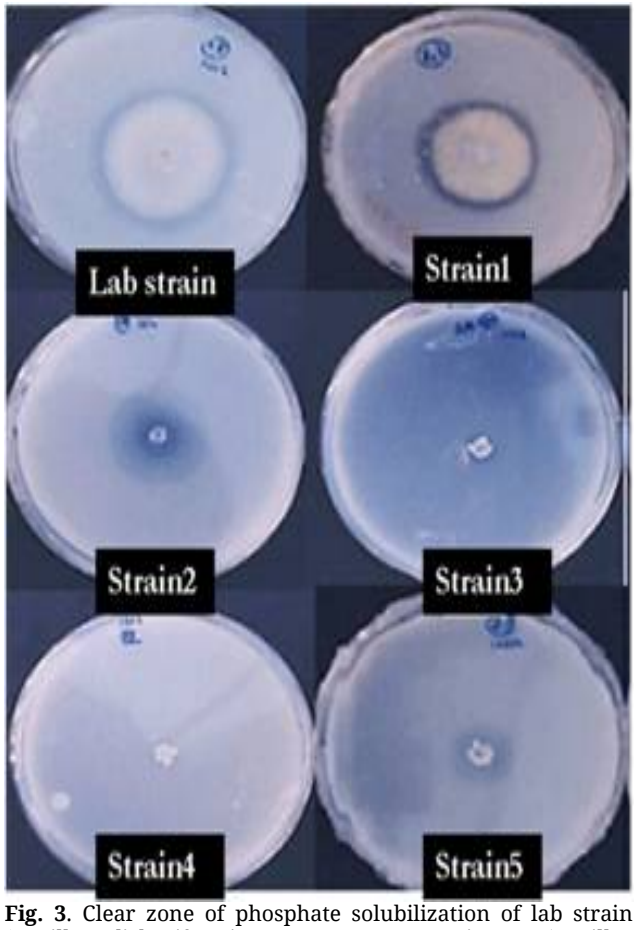

Fig. 3. Clear zone of phosphate solubilization of lab strain (*Bacillus licheniformis* MTCC 2312, strain 1 (*Bacillus megaterium* MTCC 453), strain2 (*Bacillus subtilis* MTCC 1134), strain 3,4 *Escherichia coli* MTCC 570 and Strain 5 (*Pseudomonas aeruginosa* MTCC 424).

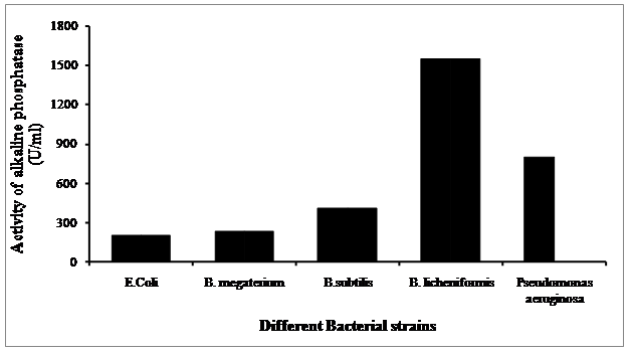

Fig. 4. Screening of bacterial strains on the basis of their ability to produce alkaline phoshatase.

The specific activity for this enzyme was obtained as 95.89 U mg^{-1} of protein which showed high purity of this enzyme. The purified fraction of DEAE-sepharose column showed a molecular weight of 60 kD after SDS-PAGE electrophoresis (Fig. 5). The low molecular weight of this enzyme is comparable with most alkaline phosphatases isolated from other bacterial strains like *Bacillus, Pseudomonas* (22, 27-31) which is lower than mammalian alkaline phosphatases (120-200 KD).

Table 1. Purification scheme of *B. licheniformis* MTCC1483 alkaline phosphatases by DEAE column chromatography.

Purification steps	Total Activity (Unit)	Total protein (mg)	Specific Activity (U mg^{-1})	Purification fold
Crude Extract	33803	402.56	83.97	1
$(NH_4)_2SO_4$ precipitation	28250	218.00	129.59	1.54
DEAE-Sepharose	15650	52.89	295.89	3.52

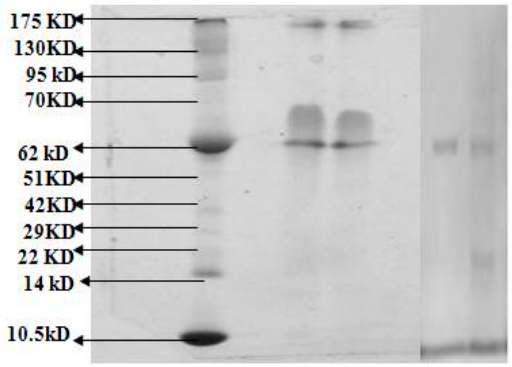

Fig. 5. Purified band for alkaline phosphatase for ladder (lane1), crude extract (Lane2), ammonium sulphate precipitation (lane3), fraction 1 of DEAE-Sepharose (Lane 4), fraction2 (lane 5).

The activity of alkaline phosphatase increased with increase of pH value from 6 and optimum activity was estimated at pH 8.5 as shown in Fig. 6. The activity of this enzyme was found to be increased with increase of temperature and maximum activity was estimated at 50 ˚C (Fig. 7).

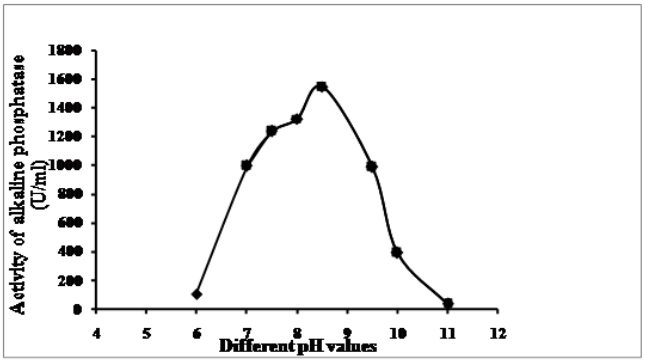

Fig. 6. Effect of pH on activity of alkaline phosphatase from *B. licheniformis.*

Alkaline phosphatases are non-specific to hydrolyse many phosphorylated substrates like phosphomonoesters, diesters and triesters (27-29, 32). In this study, purified alkaline phosphatase showed substrate specificity for a wide variety of phosphorylated compounds like para nitro-phenyl

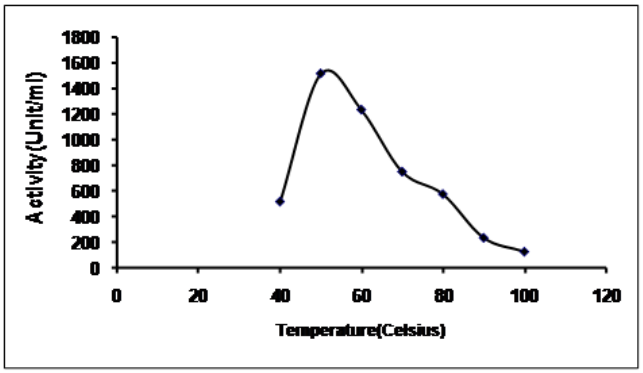

Fig. 7. Effect of temperature on activity of alkaline phosphatase from *B. licheniformis.*

phosphate, Guanosine mono-phosphate (GMP), Adenosine monophosphate (AMP), Adenosine Di-Phosphate (ADP), Adenosine Tri-phosphate (ATP), Glucose-6-Phosphate and phosphoenol pyruvate (PEP) as shown in Table 2. Here monoester-phosphorous compounds like pNPP, GMP, AMP, PEP showed more specificity than diester or trimester compounds suggesting its phosphomonoesterase nature. The kinetic constant values of K_m and V_{max} was obtained as 2.30 mM and 2223 U ml^{-1} respectively for this enzyme with para nitrophenyl phosphate sustrate. It is evident from Table 3 that alkaline phosphatse retained its 50% activity upto 8 h which can be reported as its half life time. Alkaline phosphatase was found to be thermostable up to 50 h at temperature 50 ˚C. The stability of alkaline phosphatase produced from *B. licheniformis* MTCC 2312 at high pH and high temperature is comparable to stability of alkaline phosphatase secreted from thermophilic bacteria (33-35).

Table 2. Effect of different substrate on hydrolysis of extracellular alkaline phosphatase from *B. licheniformis*.

Different types of phosphorylated Substrates	Relative activity (%)
p-Nitrophenyl phosphate	100
Glucose-6- Phosphate	18.12
Adenosine monophosphate	47.23
Adenosine diphosphate	13.23
Adenosine triphosphate	23.24
Guanosine monophosphate	63.48
Phosphoenol Pyruvic acid	12.41

Table 3. Thermostability of alkaline phosphatase for different time interval at temperature 50 ˚C.

Time (h)	Alkaline Phosphatase Activity (Unit ml^{-1})	% Thermostability
0	1550.00	100
2	1304.24	86.95
4	1173.71	78.25
6	1063.18	70.88
8	752.65	50.18
10	531.59	35.44
20	421.06	28.07
40	378.95	25.26
50	210.53	14.03
100	0	0

Some *Bacillus* spp. have phosphate solubilizing property and used as biofertilzer for improving crop productivity in alkaline soil due to having unique characteristic of producing stress resistant spores against high pH and high temperature range (3, 7, 10). The solubilization of insoluble complex of phosphate into free inorganic phosphate has been reported in phosphate solubilizing microbes by secretion of various types of organic acids like malonic, gluconic, oxalic, glycolic, and succinic acid (8, 9, 16, 36, 37). The hydrolysis of organic phosphorous compounds by these microbes has been reported due to secretion of phosphohydrolase enzymes. These dephosphorylation reactions are mainly caused by the hydrolysis of phosphoester or phosphoanhydride bonds in the presence of phosphohydrolases (8, 9, 38-40).

The purified extract of alkaline phosphatase was lyophollized and its powder was supplemented with sterilized soil with calcium phosphate to observe its effect on growth and yield of *Zea mays* crop. The height of plant and total dry weight of *Z. mays* per pot was found to be increased by 3.07 and 3.15 fold in experimental sets compared to control (Table 4). The percentage of phosphate content in stem and root of *Z. mays* was also found to be increased by 2.35 and 1.76 fold respectively (Table 4). Phosphohydrolase enzymes secreted from some phosphate solubilizing microbes has the capability of hydrolysis of inorganic or organic phosphate to improve plant growth performance (8, 41). Many plants like potato, rice, sugar beet, tomato, lettuce, wheat, maize, sorghum, etc showed improved growth after supplementation of immobilized beads of phosphate solubilizing bacteria as biofertilizer (8, 9, 42-47). There are no scientific reports available for use of the lyophilized powder of alkaline phopshatase to improve crop productivity in alkaline soil of arid region till date.

Table 4. Estimation of plant growth, *p*hosphatase activity and % phosphate content.

Treatments	Height plant (cm)	Total Dry Weight (g)	% phosphate	
			Stem	Root
Control C_1	29.7	5.1	0.015	0.019
C_2	33.9	4.9	0.026	0.023
C_3	21.1	6.1	0.023	0.016
Experimental E1	78.4	15.5	0.058	0.035
E2	94.7	20.3	0.043	0.039
E3	87.2	14.9	0.049	0.028

Conclusion

Alkaline phopshatase from *B. licheniformis* could be used as effective biofertilizer for agricultural industry to improve crop productivity. This microbial product has high range of pH stability along with thermostability and therefore could be used as biofertilizer to improve the crop productivity in arid region under severe climate condition.

Conflict of interest

The authors declare that they have no conflict of interest. This research article does not contain any studies with human participants or animals performed by any of the authors. This research work has not been funded by any funding agency.

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