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Purification and characterization of Alkaline Phosphatase from *Pseudomonas aeruginosa* isolated from apple rhizosphere

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Abstract
An extracellular alkaline phosphatase was purified from potential phosphate solubilizing strain of *Pseudomonas aeruginosa* An-H isolated from rhizospheric soil of apple. Alkaline phosphatase was purified using ammonium sulphate precipitation and Sephadex G-100 gel filtration chromatography with specific activity of 1.93 U/mg of protein in crude extract and specific activity reached to 5.63 U/mg of protein in ammonium sulphate precipitation. On Sephadex G-100 column chromatography, specific activity reached to 30.29U/mg of protein with purification fold of 15.69. The molecular weight of purified *Pseudomonas aeruginosa* An-H was recorded as 66 kDa on 10% SDS gel electrophoresis. The pH optima of this enzyme is 9.0 in 0.1 M Tris-HCl buffer with optimum temperature of 47°C. Production and purification of this enzyme in NBRIP medium shows that the *Pseudomonas aeruginosa* An-H solubilize the phosphate associated with calcium ions and this available phosphorus is easily used by apple plants for their growth and development. Therefore, the aim of the study was to check the production and purification of alkaline phosphatase.

Keywords: apple rhizosphere, *Pseudomonas aeruginosa*, *alkaline phosphatase*

Introduction
Extracellular alkaline phosphatases (ALPs) (E.C. 3.1.3.1 orthophosphoric monoester hydrolysis) are widely present in various organisms which help in metabolism of different phosphorus containing organic compounds [1, 2]. Phosphatase has been known to be essential in most of microorganisms to release phosphate from organic compounds when inorganic phosphate is limited [3].

Solubilization and mineralization of phosphorus compounds by bacteria in soil seems to be one of the possible pathways by which plants takes inorganic phosphorus for their growth and development. 98% of Indian soils are deficient in phosphorus, because the concentration of free phosphorus, the form available to plants even in fertile soils is generally not higher than 10 µM even at pH 6.5 where it is most soluble [4]. Indian soils on an average contain 0.05% phosphorus that constitutes 0.2% of plant dry weight. Even in phosphorus rich soils, most of this element is in insoluble form and only a small proportion (0.01%) is available to plants [5]. Being an important constituent of nucleic acids, phytins, phospholipids, nucleotides, co-enzymes and enzymes, phosphorous is of great importance in the transformation of energy, transfer of heredity characters, fat and albumin formation and cell organization in plants [6].

Alkaline phosphatase is ubiquitous in nature, having already been identified in numerous organisms and tissues. In general, the alkaline phosphatases occur in very small quantities, are unstable in dilute solution, and are subject to surface denaturation in the pure state. These properties, and their tendency of structure and regulation [7] of alkaline phosphatase to occur in multiple forms, often make the isolation of this enzyme difficult [8]. Earlier, ALPs with molecular mass of 66 kDa were isolated from *Pseudomonas* sp. [9]. *Pseudomonas aeruginosa* An-H isolated from rhizospheric soil of apple is an important phosphate solubilzing strain alongwith the other plant growth promoting activities. It helps in solubilization of insoluble phosphorus to available form which is easily accessible to plants. Therefore in present study, attempts were made to purify and characterize alkaline phopshatase from *Pseudomonas aeruginosa* isolated from apple rhizosphere of Himachal Pradesh.

Materials and Methods

Organism and Cultivation conditions *Pseudomonas aeruginosa* strain An-H (GenBank accession no. KJ500026) isolated from temperate zones of H.P. used as a source of alkaline phosphatase activity. The alkaline phosphatase production by candidate strain was carried out by using optimized medium (glucose 1%, tri-calcium phosphate 0.5%, magnesium chloride 0.5%, magnesium sulphate 0.025%, potassium chloride 0.020% and ammonium sulphate 0.010%) in 250 ml flasks at 150 rpm at 28±2ºC for 3 days.

Alkaline Phosphatase Assay

The standard assay method was used [10]. ALP activity was carried out at 37ºC for 10 min. using 0.5 ml of 15.2 mM p-nitrophenylphosphate (pNPP) (Sigma Chemical) as a substrate in 0.5 ml of 100 mM glycine buffer pH 9.0 containing 0.1 mM $MgCl_2$. The volume of reaction mixture was 1.0 ml. 100µl of enzyme preparation was used for routine enzymatic assay. The enzyme reaction was terminated by adding 4 ml of 20 mM sodium hydroxide (NaOH) solution and the absorbance was measured spectrophotometrically at 410 nm as the difference between the assay and control samples by using appropriate blank. One unit of ALP activity was defined as the amount of enzyme required to release 1.0 µm of p-nitrophenol from pNPP in 1 min. the specific activity is given as units/mg of protein. The protein content in the sample was estimated by using Bradford reagent (Sigma, USA) [11].

Purification of Alkaline phosphatase

The cell free extract (500 ml) collected was subjected to ammonium sulphate (30-70% saturation at 4ºC) precipitation. After overnight incubation, the mixture was centrifuged and the pellet was collected and diluted with minimal amount of 100 mM Tris- HCl buffer (pH 8.0) and dialyzed against same buffer. Then, the dialyzed protein was loaded on Sephadex G-100 column (32-1.5cm) (Sigma Aldrich, USA) equilibrated with 100 mM Tris HCl (pH 8.0). Elution with same buffer was carried out at a constant flow rate of 24 ml/hrs and fractions of 3 ml were collected in refrigerated conditions. After each fraction was analyzed for absorbance at 280 nm and ALP activity, the highest active fractions were pooled by using. The active fractions were pooled and concentrated.

Characterization of purified Alkaline phosphatase

Electrophoretic analysis: Molecular weight of ALP was determined through Sodium Dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis with 10% running gel and 3% stacking gel on Mini protein cell apparatus (BioRad Laboratories) according to the method of Laemmli [12]. The protein bands were stained with Coomassie brilliant blue R-250. For destaining solution, 100 ml of methanol was mixed with 100 ml of glacial acetic acid. After mixing the contents, make final volume 1000 ml with distilled water.

Zymogram analysis

Zymogram analysis was performed through electrophoresis under non-denaturing conditions. The unstained gel was put on the Pikovskaya's agar medium prepared in 0.05M Tris buffer, pH 7.0.

Protein molecular weight marker

The protein molecular weight markers PAGE mark™ Protein Marker (broad range 20.1 kDa-205 kDa) from G-Biosciences already mixed with protein dye were used for SDS-PAGE.

Effect of pH on Alkaline Phosphatase activity

The optimum pH of the purified alkaline phosphatase was determined by incubating the suitably diluted column purified enzyme for 10 min. at 47^0C in the following buffer (0.1M): citrate phosphate buffer (pH 4.0-5.0); (0.1M): potassium phosphate buffer (pH 6.0-7.0) and(0.1M): Tris-HCl buffer (pH8.0) at different pH range and enzyme activity was assayed. The results were recorded in the form of enzyme units.

Effect of temperature on alkaline phosphatase activity

The optimum temperature of the purified alkaline phosphatase were determined in glycine buffer with 0.1 mM magnesium chloride (100mM; pH 8.8) by varying reaction temperature from (17ºC to 67ºC) and enzyme activity was assayed. The results were recorded in the form of enzyme units.

Effect of substrate concentration on Alkaline Phosphatase activity

Alkaline phosphatase activity was determined by using the different concentration of p-nitro phenylphosphate (1.0 mM-16.0 mM) and enzyme activity was assayed. The results were observed in the form of enzyme units.

Statistical Design

The data obtained has been analyzed as per analysis of variance technique, one way classification of data using statistical software SPSS 17.0.

Results and Discussion

The strain *P. aeruginosa* An-H isolated from the rhizosphere soil of apple produced extracellular ALP with specific activity of 1.93 U/mg of protein in crude extract. In ammonium sulphate precipitation specific activity reached to 5.63 U/mg of protein (Table 1). On gel filtration chromatography, the enzyme was eluted as one major peak and two minor peaks. The major peak reveals a series of fractions from 10-19 have most alkaline phosphatase activity (Fig. 1). In this step enzyme showed a specific activity of 30.29U/mg of protein with purification fold of 15.69 (Table 1).

Electrophoretic analysis

However the concentration of protein might be low in culture supernatant, purification is the essential step in getting concentrated supernatant [13]. Ion exchange and Gel filtration chromatography are widely used for purification techniques.

On 10% SDS-PAGE under denaturing conditions, a single band of 66 kDa was observed after gel filtration chromatography (Fig2). Yellow coloured band on phosphate solubilizing Pikovskaya's agar medium showed the presence of alkaline phosphatase enzyme on gel under non-denaturing conditions. It was observed from the present results that alkaline phosphatase purified from *Pseudomonas aeruginosa* helps in solubilization of insoluble phosphorus. Other molecular weight studies of alkaline phosphatase from a marine bacterium *C. marina* was determined by gel filtration to be 62 kDa [14], 32kDa in *Bacillus steriothermophilus* [15], 66 kDa by *Pseudomonas* sp. [9] and 54 kDa in *E.coli* [16].

Table 1: Purification data of extracellular acid and alkaline phosphatases from culture supernatant of *Pseudomonas aeruginosa* An-H

Steps	Vol. of Fraction (ml)	Protein (mg/ml)	Total protein (mg)	Enzyme activity (U/ml)	Total units	Specific activity (Units/mg protein)	*Purification fold	**% recovery yield
1	500	2.2	1100	4.24	2120	1.93	1	100
2	3	1.4	4.2	7.88	23.64	5.63	2.92	1.12
3	2.7	0.24	0.648	7.27	19.63	30.29	15.69	0.925

72h old culture supernatant
Ammonium sulphate precipitation (70% saturation)
Molecular sieving through Sephadex G-100

*Purification fold= specific activity of purified /Specific activity of crude
**% Recovery = Total units in purified/Total units in crude×100

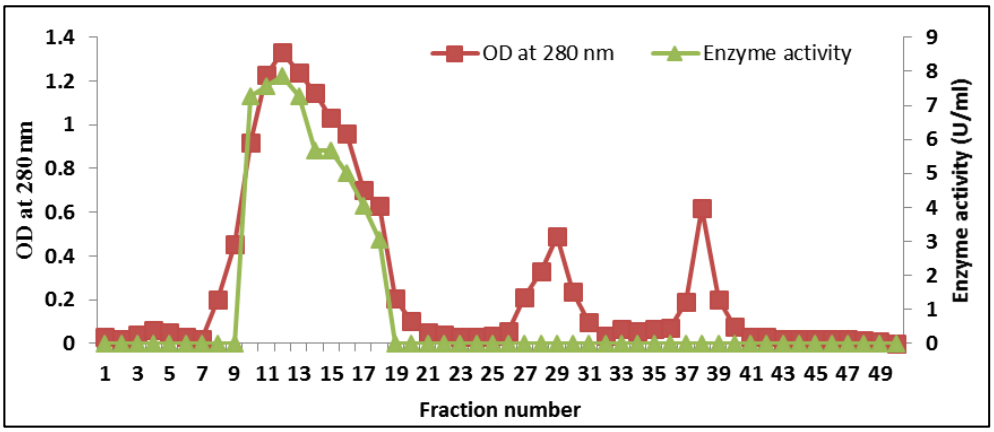
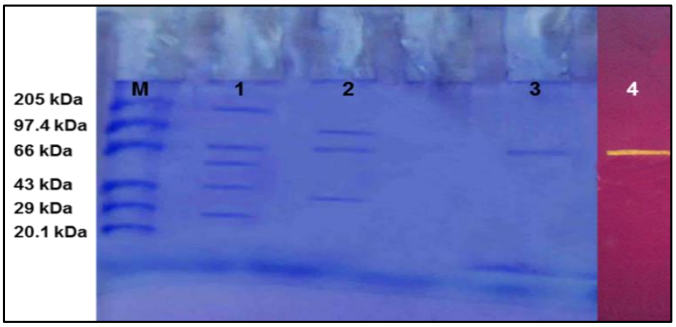


Fig 1: Sephadex G-100 gel filtration of ammonium sulphate precipitate fractions of alkaline phosphate phosphatase from *Pseudomonas aeruginosa* An-H



M. Molecular weight marker
1 Crude enzyme
2 Ammonium sulphate precipitate fraction
3 Column purified fractions
4 Zymogram

Plate 1: SDS-Polyacrylamide gel electrophoresis of crude enzyme and purified preparation of alkaline phosphatase

Optimum pH

The optimum pH of this enzyme is 9.0 in 0.1 M Tris-HCl buffer with pNPP substrate as shown in Fig.3 and above and below this pH activity decreases. It was reported that the maximum activity of alkaline phosphatase at pH 10.0 for calf intestine [17], pH 11.0 for *Bacillus* sp. [18] and pH 8.0 for *Bacillus subtilus* [19].

Optimum temperature

The ALP was stable when incubated at temperature from 17-67ºC with maximum activity at 47ºC but at 67ºC only 30% activity was lost. Therefore the optimum temperature for this enzyme is 40-50ºC (Fig.4). The present results are at par with the results of *Pseudomonas* sp. [9], *A. caesplitosus* [20] and *Rhizopus microspores* [21].

Optimum Substrate concentration

Optimum substrate concentration was 10.0mM when enzyme was test over a range from 1.0 mM to 16.0 mM concentration. Above and below this concentration activity decreases.

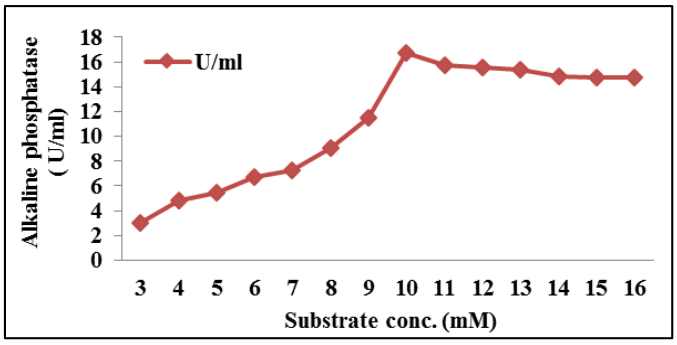


Fig 2: Effect of substrate concentrate on the activity of alkaline phosphatase from pseudomonas aeruginosa An-H

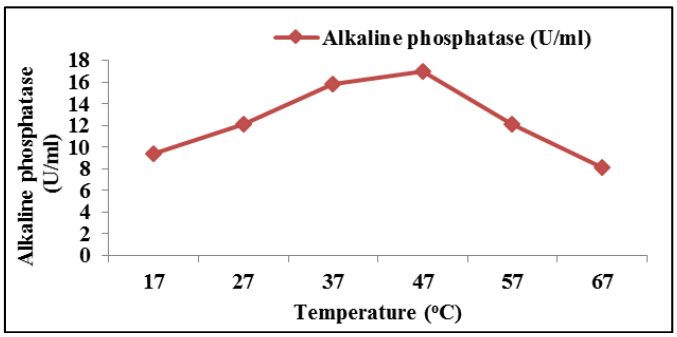


Fig 3: Effect of temperature of incubation on the activity of alkaline phosphatase from pseudomonas aeruginosa An-H

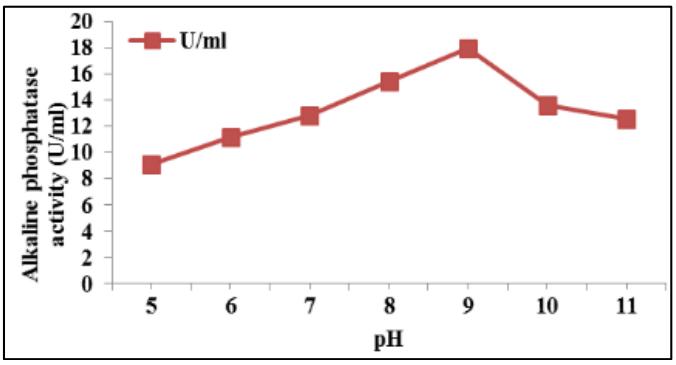


Fig 4: Effect of pH on the activity of alkaline phosphatase from pseudomonas aeruginosa An-H

Conclusions

Pseudomonas aeruginosa An-H isolated from rhizospheric soil of apple has been found to be able to secrete extracellular alkaline phosphatase with molecular weight 66 kDa on 10% SDS-PAGE. Alkaline phosphatase showed its optimum activity at pH 9, temperature 47ºC and substrate concentrate 10 mM which indicate that the enzyme is thermostable and alkaline in nature. Production of this enzyme in NBRIP medium shows that the *Pseudomonas aeruginosa* An-H solubilize the phosphate associated with calcium ions and this available phosphorus is easily used by apple plants for their growth and development. Therefore, the aim of the study was to check the production and purification of alkaline phosphatase.

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