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INVESTIGATION OF ALKALINE PHOSPHATASE ENZYME OF A NOVEL *BACILLUS* SPECIES ISOLATED FROM RHIZOSPHERIC SOIL OF POTATO FIELD

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ABSTRACT: The present study reports isolation and biochemical/physiological characterization of a novel *Bacillus* strain (designated as PB-1) from the rhizospheric soil of potato field that exhibited phosphate solubilization activity. The organism grows optimally at pH 6.0-9.0; at temperature 28-37^{0}C and in presence of 1% NaCl. These data indicate that the bacterium is mostly a neutrophile, mesophile and non-halotolerant in nature that matches with the features of a typical soil-borne bacterium. It was also seen that the organism was able to ferment various types of carbohydrates including both mono-and disaccharides. In the second phase of the study, partial purification and characterization of the extracellular alkaline phosphatase enzyme from the *Bacillus* PB-1 strain was carried out. It was found that the said enzyme was optimally active at 40^{0}C temperature and at pH range 8.0-9.0. The enzyme activity increased consistently with increase in the concentration of inorganic phosphate in the growth medium up to 4%. Concentration of phosphate above that did not affect the enzyme activity any more suggesting that the substrate binding site of the enzyme may become saturated at 4% concentration of phosphate which is an important aspect to understand the kinetics of the enzyme. The effect of various carbon sources on the enzyme activity (production) was also studied and was observed that fructose was the best inducer of the enzyme rather than glucose which is conventionally used in the Pikovskaya's medium for detecting phosphatase activity. The production of phosphatase enzyme by this rhizospheric strain of *Bacillus* explores its pivotal role as a PGPR that increases soil fertility by de-mineralization of

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bound phosphates and help in promoting plant growth. Further experiments on the function of this bacterium on inducing crop growth and its molecular identification by 16srRNA sequencing remain to be done in future as an extension of the current work.

KEYWORDS: Rhizosphere, PGPR, *Bacillus*, Alkaline Phosphatase, Characterization, Purification

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1.INTRODUCTION

The genus *Bacillus* is widely distributed in nature, even in extreme environmental conditions. This genus is comprised of Gram-positive, rod-shaped, endospore-forming non-capsulated and aerobic bacteria that belong to the family *Firmicutes* which are phylogenetically and phenotypically heterogenous (Claus and Berkeley, 1986). Rhizosphere soil harbors many different species of bacteria including the *Bacillus* that might exert a positive effect on the growth of the plant and are, therefore, classified as Plant Growth Promoting Rhizobacteria (PGPR). Among the diverse types of PGPR, *Bacillus* being a spore-former has advantage over the other non-spore forming bacteria due to their resistant nature to high temperatures and strong chemicals used in the form of fertilizers [1]. Various species of *Bacillus* are also potent sources of many industrially important enzymes one of which is alkaline phosphatase (ALP or ALPase). The ALP enzyme is found to be present in most organisms starting form bacteria to higher animals the main function of which is to release phosphate from organic molecules when inorganic phosphate is limited in the growth environment[2].Alkaline phosphatase (E.C.3.1.3.1) is basically classified as phosphomonoesterases that exert their monoesterase activity through the formation of a phosphoseryl intermediate[2].The enzymes in this group, however, vary in their sizes, substrate specificities and metal ion requirements as co-factors[2]. The enzyme is widely used commercially in gene cloning methods and in other fields of biotechnology, as therapeutic agents and also has other uses in clinical medicine. As for example- one of its uses in clinical medicine is to label monoclonal antibodies during their production [3]. Additionally, in agriculture, phosphorus is considered to be a major nutrient that limits plant (crop) growth [4]. Phosphorus is presently receiving more attention as a non-renewable source (Shen et al., 2011) because of its role in agricultural production. The phosphorus in soil becomes immobilized and thus unavailable to plants even after its application in the form of phosphate fertilizer (Rodriguez and Fraga, 1999). In contrary, there are several Phosphate Solubilizing Bacteria (PSB) present in the rhizospheric soil as a part of the PGPR flora that can release the immobilized phosphate by the

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production of extracellular phosphatase enzymes (alkaline or acidic)[4] making phosphates utilizable by the crops and thus inducing better growth of the crops. Therefore, the enzyme has significant functions in various aspects of modern science as reflected by its biggest market volume share of $20 million[3]. This makes it an interesting and expanding arena of research to explore new types of ALPs with novel properties suitable for large scale industrial production and applications. The present study initially aims to isolate a species of *Bacillus* from the rhizospheric soil collected from potato field following its morphological and physiological characterization. In the second phase of the current work, an attempt had been made to screen the phosphatase activity of the isolated strain by growing the same on Pikovskaya's Agar medium followed by partial purification and characterization of the extracellular alkaline phosphatase enzyme secreted by this particular strain of *Bacillus* with reference to the enzyme's optimum temperature, pH as well as effect of phosphate and carbon sources on its activity in order to find out if the enzyme exhibits any novel properties that could be exploited for economical purposes. Similar work performed by Patel F.R.(2016) has led to the extraction and purification of the ALPase enzyme from a halotolerant facultative alkaliphilic strain of *Bacillus flexus* that has an opportunity to replace ALPases which are now economically obtained from calf intestine, *E.coli* or shrimps. In addition to this, the work done by Parhamfar M.et al. (2016) has focused on the extraction and characterization of an extracellular acidic phosphatase from *Bacillus* species. These works were preceded by Kannaiyram S.et al. (2015) who extracted and purified a thermostable ALP enzyme exhibiting optimum activity at 60^0C from a novel species of *Bacillus* isolated form rocky soil. Priya D.et al.(2014) has optimized the production of a mesophilic extracellular alkaline phosphatase from *Bacillus megaterium*. In another such research, Mahesh M.et al.(2010) also purified and characterized a thermostable ALPase enzyme from a novel strain of *Bacillus* that equally has a promising scope in industries where phosphatase activity at high temperature is required. More recently, Bhattacharjee M.et al. (2017) has reported the extraction and purification of an ALP enzyme from a strain of *E.coli* isolated from water sample that showed partial thermostable nature. Thus, the investigation of ALP enzyme isolated from a potato rhizosphere soil- borne species of *Bacillus* seems to be an interesting and worthy research problem that has been done in this study underscoring its importance as a PSB in rhizospheric soil that could play a role as PGPR.

2. MATERIAL AND METHODS

Collection of the sample: Rhizospheric soil samples were collected from two different potato fields within 5km.distance located near Arambagh, Hoogly district, West Bengal, India (latitude 22° 52' 31.6668" N and longitude 87° 47' 33.5328" E). Two samples of soil were collected from each plot within air-tight plastic containers. The samples were processed in the lab facility within 12 hrs.of collection.

Isolation of bacterial flora from the soil samples: For the purpose of culture, 1g of each of the

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four soil samples were suspended in10 ml.of sterilized distilled water in separate test tubes. The samples were then serially diluted up to 10^{-6} dilution factor using sterile distilled water as the diluents. 1ml aliquot was taken from each of the 10^{-6} dilution tubes (one for each of the four soil samples) and was then plated on pre-sterilized nutrient agar (NA) plates (containing 2% agar, pH 7.0). The medium was purchased from HiMedia®, India. The plates were then incubated at 30^0C for 36-48 hrs.(Atlas, 1993). All the plates were marked and labeled accordingly to distinguish among the soil samples. After the incubation period, four different isolates from the four plates (one plate for each of the four soil samples) was randomly chosen and their colony morphologies were studied. The selected colonies were then subjected to Gram staining and endospore staining (by Schaeffer-Fulton Method) to detect the possible candidates of the genus *Bacillus* followed by microscopic observation under 100x magnification. The promising colonies that showed positive results for these tests were sub-cultured on NA slants (containing 2% agar, pH 7.0) and the slants were then preserved at 4^0C temperature under refrigeration as pure cultures of the potato rhizospheric soil isolates.

Screening for extracellular phosphatase activity: The pure cultures of the selected soil isolates were then inoculated on Pikovskaya's Agar medium under aseptic conditions using continuous streaking method. The agar plates were then incubated at 30^0C temperature for a period of 48-72 hrs. The plates were then observed for the appearance of clear zone around the growth line that indicates the solubilization of phosphate and hence, confirms the production of the extracellular alkaline phosphatase enzyme [5]. The isolate that exhibited best positive result was identified (designated as PB-1) and was subjected to further biochemical and physiological characterization to confirm the presence of the genus *Bacillus*.

Biochemical and physiological characterization of the selected isolate:

Biochemical Characterization:

a) IMViC Tests: Indole, Methyl Red, Vogues-Proskauer and Citrate Utilization Tests were performed for the bacterium under question according to the standard protocol (Dubey and Maheshwari, 2011) [6] and the results were tabulated. All the regents/media required for the tests were purchased either form Merck®, India or SRL®, India and were of analytical grade.

b) Starch Hydrolysis Test: Starch could be hydrolyzed by amylase, an extracellular enzyme produced by many bacterial species. In order to detect the starch degrading activity of the selected isolate, it was grown in Starch Agar medium (containing 1% starch; pH 7.0) and after incubation at 30^0C temperature for a period of 48 hrs, the plates (in duplicates) were observed for the growth of the isolated bacterium. The hydrolysis of starch was detected by the appearance of a clear zone on the surface of the starch agar plates around the colonies after the addition of 2-4 ml.of 0.1 N iodine solution as an indicator (Aneja K.R., 2003) and the observation was tabulated.

c) Gelatinase Test: This test is done to determine the ability of the test organism to produce gelatinase enzyme by which it can hydrolyze gelatin and utilize it as the source of carbon and /or

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nitrogen for its growth. Pure cultures of the selected bacterial species grown on Gelatin Agar medium (purchased from HiMedia®, India; containing 30g/L gelatin and 15g/L agar-agar; pH 7.0) and the tubes (in duplicates) were incubated at 30^0C for 72 hrs. Following the incubation period, the culture tubes were subjected to low temperature treatment at 4^0C for 30 minutes. The tubes which were positive for gelatin remain liquefied due to the production of gelatinase enzyme by the isolate while the other tubes become solidified at low temperature due to lack of gelatinase activity. The result was then tabulated accordingly.

d) Urease Test: With an aim to detect the production of urease enzyme capable of hydrolyzing urea to release ammonia by the test bacterial strain, the isolate was grown in test tubes (in duplicates) with Urease Test Broth (purchased from HiMedia®, containing 20g/L urea and Phenol Red as the pH indicator dye). The tubes were then incubated at 30^0C temperature for 24-48 hrs. and were observed for the color change of the broth media from yellow to red that indicates a positive reaction.

e) Carbohydrate Fermentation Profile: In order to assay the fermentation of various carbohydrates viz. glucose, galactose, lactose, sucrose, mannitol and fructose, the test bacterial isolate was inoculated into appropriate fermentation media containing 1% of each of the carbohydrates in sterile test tubes with Durham's tubes to detect gas production. Phenol Red was used as the pH indicator dye that turns red under acidic condition (due to formation of mixed acids) and gives a positive reaction for acid production. Formation of gas could be detected by the presence of bubbles inside the small Durham's tubes. The result was recorded after 48 hrs. of incubation for all the culture tubes at 30^0C temperature.

f) Catalase Test: Catalase is an important enzyme that neutralizes the toxic hydrogen peroxide formed during oxygen metabolism in all aerobic bacterial species. The enzyme converts H_2O_2 into water and molecular oxygen. The test is performed simply by taking a loopful of the pure culture of the test isolate onto a clean and dry slide followed by addition of 4-5 drops of 30% hydrogen peroxide (purchased from Merck®, India). Occurrence of bubbles due to production of O_2 by the activity of catalase (effervescence) is an indicator of positive reaction. The observation was recorded and tabulated.

g) Oxidase Test: This test detects the presence of Cytochrome c oxidase in the bacterial respiratory chain. When a loopful of pure bacterial culture was mixed with tetramethyl p-phylene diamine dihydrochloride (purchased form Sigma-Aldrich®, India) on a piece of dry filter paper, the color of the dye changes to blue due to reduction of the compound by the oxidase enzyme (Benson, 1994). This color change represents a positive reaction. The result for test bacterial strain for this test was recorded accordingly.

h) Cellulase Test: In order to find whether the bacterial isolate can produce cellulase enzyme, it was inoculated onto Carboxy-Methyl Cellulose (CMC) agar medium (purchased from HiMedia®, India) by spread plate method and the plates (in duplicates) were incubated at 30^0C temperature for

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a period of 96 hrs. After the incubation period, the plates were flooded with 0.3% Congo Red Dye for 30 minutes. The stain was drained off and the plates were washed with 0.1M NaCl and were observed for the appearance of clear zones around the colonies that indicate that the isolate is a cellulase producer.

i) Nitrate Reduction Test: To assay the production of the enzyme nitrate reductase by the bacterial isolate PB-1, 1 ml of pure culture in nutrient broth was inoculated in nitrate broth (Purchased from Sigma-Aldrich®, India; containing 5g/l peptone, 3g/l meat extract and 1g/l potassium nitrate; pH 7.2) and the Erlenmeyer flasks (in duplicates) containing the inoculated medium were incubated at 30^0C temperature for a period of 48 hrs. After the incubation is over, 6-8 drops of sulphanilic acid (8g/l of 5N acetic acid) and equal amount of 1- naphthylamine (6g/l of 5N acetic acid) were added to the broth medium. If the color turns to red or pink within a few minutes of addition of the regents without the addition of zinc powder, the sample is considered as positive for nitrate reductase activity. The observed result for the isolate PB-1 was tabulated.

j) Arginine Hydrolysis Test: This test was performed to test the arginine decarboxylase (dihyrolase) activity of the isolate by inoculating the pure culture of the same into Arginine dihydrolase broth (with Bromo Cresol Purple as the pH indicator dye) and incubating the inoculated flasks (in duplicates) at 30^0C temperature for 48 hrs. Color change from purple to yellow and then back to purple indicates a positive result. The observation was made after the due time and was recorded.

Physiological Characterization

a) Effect of pH on Growth: To determine the effects of pH on the growth of the selected bacterial isolate PB-1, nutrient broth media were prepared and the pH of the broth was adjusted accordingly (pH 4.0, 6.0, 7.0, 9.0 and 11.0) by using 1M NaOH and 5 N HCl. Separate Erlenmeyer flasks were used for each pH. All the flasks were incubated at 30^0C for 48 hrs.and the growth was measured spectrophotometrically (instrument purchased from Systronics, India; Model No.-AU-2603) at 540 nm wavelength. The resulting data was recorded to depict the growth variation of the *Bacillus* isolate at different pH ranges.

b) Effect of NaCl on Growth: To study the variation in the growth pattern of the bacterial strain at different concentrations of NaCl (1%, 2%, 3%, 4%, 5% and 6%), the organism was grown in different nutrient broth media with respective salt concentrations in separate Erlenmeyer flasks. Following incubation at 30^0C for 48 hrs, the absorbance was measured at 540 nm spectrophotometrically and the observed data was recorded.

c) Effect of temperature on Growth: The effect of temperature on the growth pattern of the selected bacterium was studied by exposing the pre-inoculated nutrient broth media at specified temperatures (10^0C, 28^0C, 37^0C, 45^0C, 60^0C and 80^0C) for 15 minutes followed by incubating the flasks at 30^0C for 48 hrs. The growth variation was then determined by measuring the absorbance spectrophotometrically at 540 nm wavelength.

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Production and Purification of the ALP enzyme from isolate PB-1: A fresh culture of the isolated strain PB-1 was prepared in 250ml of Pikovskaya's Broth medium after 48 hrs.of incubation at 30^0C temperature in a shaker incubator with 130 rpm motion. The purification of ALPase enzyme was carried out as described by Patel F.R. [7] with slight modifications. A cell-free supernatant was prepared after centrifugation of the broth medium at 10,000 rpm for 20 minutes at 4^0C temperature. This supernatant was treated as the crude enzyme extract that was further purified by precipitation by addition of a solution of 65% w/v ammonium sulphate to the cell-free extract. The precipitate was stored overnight at 4^0C temperature under refrigeration and was then recovered by centrifugation at 8,000 rpm for 10 minutes. The supernatant was then again re-precipitated by adding 20% w/v ammonium sulphate solution and stored again at 4^0C for 6-8 hrs. All the precipitates were then collected and re-suspended in 50mM Tris-HCl buffer, pH 8.0. This protein suspension was then dialyzed in a dialysis bag at 4^0C temperature for overnight in presence of the Tris-HCl reaction buffer, pH 8.0. After this, the dialysis bag was dipped in 25% sucrose solution for 3 hrs. in order to remove the impurities. This concentrated protein mixture was treated as the partially purified enzyme source and was next subjected to further course of study.

Preperation of Standard Curve of p-nitro phenol (PNP): Different aliquots (200µl, 400µl, 600µl, 800µl and 1000µl) of stock PNP solution (100µg PNP/ml of 0.5M Glycin buffer of pH 8.5) were pipette out into different test tubes. The volume in each tube was made up to 1ml with appropriate volumes of 0.5M Glycin buffer (P^H 8.5) solution. Then the tubes were incubated for 10 min.at room temperature and the absorbance of the resultant yellow solution was read at 405 nm wavelength against an appropriate blank using the UV-visible spectrophotometer. PNP is the end product of alkaline phosphatase reaction when p-nitro phenyl-phosphate i.e. PNPP is used as the substrate. The absorbance (O.D.) was plotted in the Y-axis and the respective concentrations of PNP were plotted on the X-axis to obtain a standard curve which was used as the reference curve for determining the ALP activity under different parameters in the experiment as follows.

Effect of Temperature

Effect of different temperatures (30^0C, 40^0C, 60^0C and 80^0C) on partially purified alkaline phosphatase enzyme of the isolate PB-1 was investigated. In each tube, an aliquot of 200µl of the crude enzyme source was mixed with 1500 µl of the 10mM PNPP solution and the total volume was made upto 2000µl by adding 0.5 M of Glycin buffer of pH 8.5. All the tubes containing the reaction mixture were incubated at the specified temperatures for a period of 10 minutes and the O.D.values for each tube were determined at 405 nm wavelength against appropriate blank(without the enzyme source) spectrophotometrically. The amount of PNP released due to the activity of the crude ALP enzyme at each of the defined temperatures was then calculated by plotting the absorbance of the released PNP onto the reference standard curve. The ALP activity was calculated using the following formula [8] --

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$$\text{ALP acitivity} = \frac{\text{Amount of PNP released (in µg)}}{\text{Mol.wt.of PNP(gm.) x vol.of enzyme source (ml.) x Reaction Time (in minutes)}} \text{ µmol/ml/min}$$

Effect of pH

Effect of different pH ranges (pH 4.0, 6.0, 8.0, 9.0, 10.0 and 11.0) on the partially purified ALP enzyme was investigated by following the method as described above. The total volume in each tube was made up to 2000 µl by adding buffers of respective pH values such as acetate buffer for pH 4.0, citrate buffer for pH 6.0, sodium phosphate buffer for pH 8.0, Glycin buffer for pH 9.0 and 10.0 and sodium carbonate buffer for pH 11.0. The strength of all the buffer solutions was adjusted to 0.5M. All tubes were incubated at room temperature for a period of 10 min. The absorbance of the released PNP was read at 405nm against appropriate blanks in each case and the ALP activity was calculated by using the same method described as above.

Effect of Phosphate Concentration

Effect of different concentrations of inorganic phosphates (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%,4.0%, 5.0% and 6.0% of sodium di-hydrogen phosphate, purchased from SRL®, India) on production of partially purified periplasmic alkaline phosphatase enzyme by PB-1 isolate was determined. The appropriate amounts of phosphates were added in Pikovskaya's broth medium in separate Erlenmeyer flasks. The pH of the broth was adjusted to 7.0 in each flask. All the broth media with different concentrations of phosphates were inoculated with 1.0ml of pure broth culture of the PB-1 isolate and the flasks were incubated at 30^0C for overnight with continuous shaking at 120 rpm. The crude extracellular ALP extracts were prepared from the broth cultures as described already. The activity of ALP produced under different phosphate concentrations was measured spectrophotometrically at room temperature (28-30^0C) and at pH 8.5(Glycin buffer) by the method as already described.

Effect of Carbon Sources

Effect of different carbon sources (glucose, lactose, sucrose, fructose and maltose; all purchased form Merck®, India) on partially purified extracellular alkaline phosphatase enzymes produced by PB-1 isolate was studied. 1% of each of the carbon source mentioned was added in Pikovskaya's Broth basal media in separate flasks and the pH was adjusted to 7.0 in each case. All the flasks were then inoculated with 1.0ml of pure broth culture of the isolated strain and the flasks were then incubated at 30^0C for overnight with continuous shaking at 120 rpm. The crude extracellular ALP extracts were prepared as described already. The activity of ALP produced under different carbon sources was measured spectrophotometrically at room temperature and at pH 8.5(Glycin buffer) by the method as already described.

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3. RESULTS AND DISCUSSION

Table 1: Collection of the sample:

Sample	Spot of collection	Sample No.
RHIZOSPHERIC SOIL SAMPLE	POTATO FIELD-1	RS-1
		RS-2
	POTATO FIELD-2	RS-3
		RS-4

Table 2: Isolation of bacterial flora from the soil samples and colony features of the selected isolates:

Sample No.	Isolate No.	Colony color	Colony shape	Colony size	Colony consistency	Colony margin	Colony elevation	Opacity
RS-1	PB-1	White	Circular	0.2mm	Sticky	Entire	Flat	Translucent
RS-2	PB-2	Off-white	Circular	0.3mm	Mucoidal	Entire	Flat	Translucent
RS-3	PB-3	Yellowish	Circular	0.2mm	Rough	Entire	Raised	Opaque
RS-4	PB-4	Off-white	Circular	0.3mm	Mucoidal	Entire	Flat	Translucent

Table 3: Observations for Gram Staining and Endospore Staining:

Isolate No.	Gram Nature	Cell shape	Presence of Endospore	Position of the spore
PB-1	Gram positive	Straight rods	Yes	Subterminal
PB-2	Gram positive	Coccobacilli	No	----
PB-3	Gram positive	Coccus	No	----
PB-4	Gram positive	Straight rods	Yes	Subterminal

Since, the PB-2 isolate was a coccobacillus and did not show the existence of endospores and the isolate PB-3 was a coccus, they were omitted from the further set of experiments. Only the isolates PB-1 and PB-4 seemed to be the best possible candidates to be *Bacillus* and therefore, they were subjected next to the phosphatase screening assay.

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Table 4: Observations for Phosphatase Screening Assay:

Medium Used	Isolate No.	*Appearance of Clear Hallow
PIKOVSKAYA'S AGAR MEDIUM	PB-1	++
	PB-2	++

*After 48 hrs. of incubation; ++ indicates strongly positive

Since both of these isolates exhibited similar results in phosphatase production assay, the isolate PB-1 was chosen randomly for the next course of the study i.e. for the biochemical and physiological characterization.

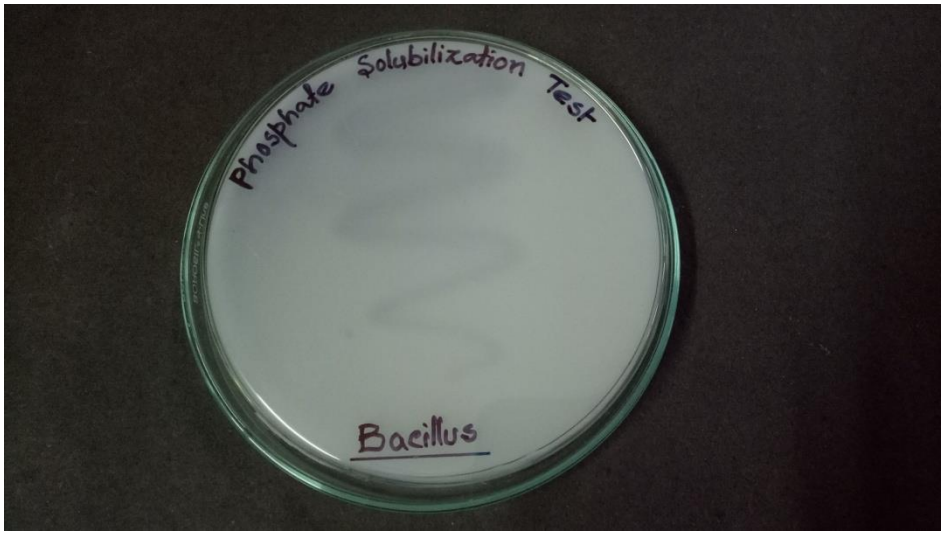

Fig 1: Culture of PB-1 isolate on Pikovskaya's Agar Medium showing clear hallow around the growth.

Table 5a: Observations for Biochemical Characterization: IMViC, Starch Hydrolysis, Gelatinase and Urease Tests:

Isolate No.	Indole Test	Methyl Red Test	V-P Test	Citrate Utilization Test	Starch Hydrolysis Test	Gelatinase Test	Urease Test
PB-1	--	+	--	--	++	++	--

- indicates negative, + indicates weakly positive and ++ indicates strongly positive results

Table 5b: Results for carbohydrate Fermentation Profile:

Isolate No.	Glucose	Galactose	Lactose	Sucrose	Fructose	Mannitol
PB-1	++	++	+	+	++	--

- indicates negative (with no acid or gas), + indicates weakly positive (with mild production of acid and no gas) and ++ indicates strongly positive (with both acid and gas) results.

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Table 5c: Observations for Catalase, Oxidase, Cellulase, Nitrate Reductase and Arginine Hydrolysis Tests:

Isolate No.	Catalase Test	Oxidase Test	Cellulase Test	Nitrate Reductse Test	Arginine Dihyrolase Test
PB-1	++	+	--	++	--

- indicates negative, + indicates weakly positive and ++ indicates strongly positive results

Table 6a: Effect of pH on the growth of the PB-1 isolate (O.D.taken at 540 nm):

Isolate No.	O.D.at pH 4.0	O.D.at pH 6.0	O.D.atpH 7.0	O.D.atpH9.0	O.D.atpH 11.0
PB-1	0.09	0.29	0.35	0.31	0.12

This observation infers that the isolated bacterium is mostly a neutrophile; optimum pH for its growth being pH.7.0 although it exhibited significant growth over a broad pH range (pH 6.0-11.0).

Table 6b: Effect of NaCl on the growth of the PB-1 isolate (O.D.taken at 540 nm):

Isolate No.	O.D.at 1% NaCl	O.D.at 2% Nacl	O.D.atpH 3% NaCl	O.D.at 4% NaCl	O.D.at 5% NaCl	O.D.at 6% NaCl
PB-1	0.32	0.25	0.18	0.12	0.09	0.03

This observation had lead to the conclusion that the PB-1 bacterial isolate is a non-osmophilic, non-halotolerant organism exhibiting maximum growth at the least (1%) concentration of NaCl. Higher concentrations of NaCl drastically inhibit its growth. This feature matches with the characteristic of a typical soil-borne *Bacillus* species.

Table 6c: Effect of Temperature on the growth of the PB-1 isolate (O.D.taken at 540 nm):

Isolate No.	O.D.at 10^0C	O.D.at 28^0C	O.D.at 37^0C	O.D.at 45^0C	O.D.at 60^0C	O.D.at 80^0C
PB-1	0.11	0.34	0.31	0.22	0.18	0.13

From this observation, it could be interpreted that the optimum temperature for the growth of the test bacterial strain is 28-37^{0}C (which matches with the characteristics of a soil-borne bacterium) although it can withstand higher temperatures-up to 80^{0}C that might be contributed to its spore-forming ability that enables the organism to be heat-resistant to a considerable extent.

Thus, based on the Gram positive nature, rod shape and endospore forming ability of the PB-1 isolate, it could be concluded that the bacterium belongs to the genus *Bacillus*. This is further supported by all the data compiled from the series of biochemical and physiological tests performed during the study. The organism was found to be an aerobic, mesophilic, neurtophilic and non-halotolerant strain

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of *Bacillus* that has the ability to ferment diverse types of carbohydrates and gives positive results for both oxidase and nitrate reduction tests as well as shows heat resistance nature due to the presence of spores. These confirm the fact that the PB-1 isolate is a strain of *Bacillus* species isolated from rhizospheric soil of potato field.

Preparation of Standard Curve of p-nitro phenol (PNP):

A standard curve for PNP was prepared by the method as described above. The plot is represented as below-

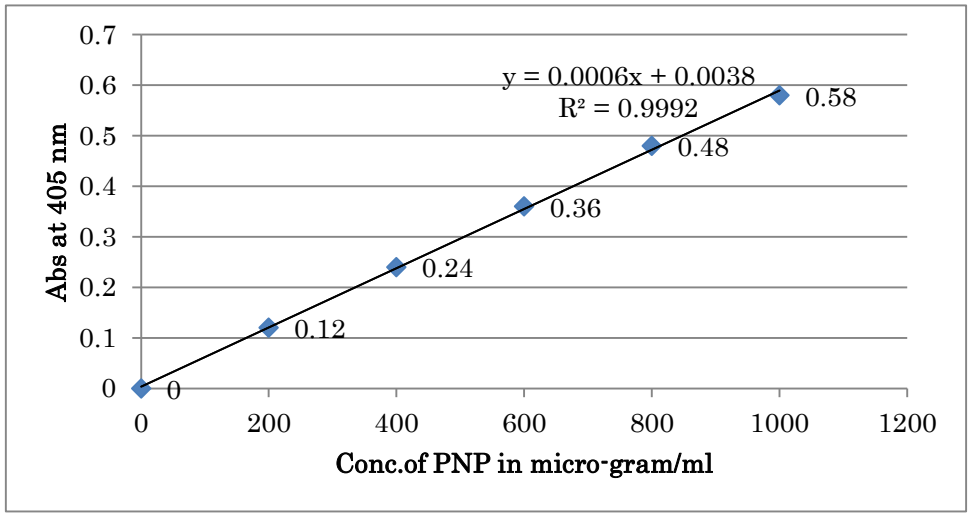

Fig 2: Standard Curve of Para-nitro-Phenol

Activity of the partially purified ALP enzyme from the PB-1 isolate: Based on the standard curve, it was measured that the activity of the ALP enzyme as estimated from the partially purified protein concentrate obtained from the PB-1 isolate at room temperature (30^0C) and at pH 8.5 was 2.4 µmol/ml/min.

Effect of temperature variation assay on the extracellular ALP enzyme activity:

The partially purified ALP enzyme activity (in terms of µmol/ml/min) of the PB-1 isolate was affected by the temperature treatments as depicted in the following plot-

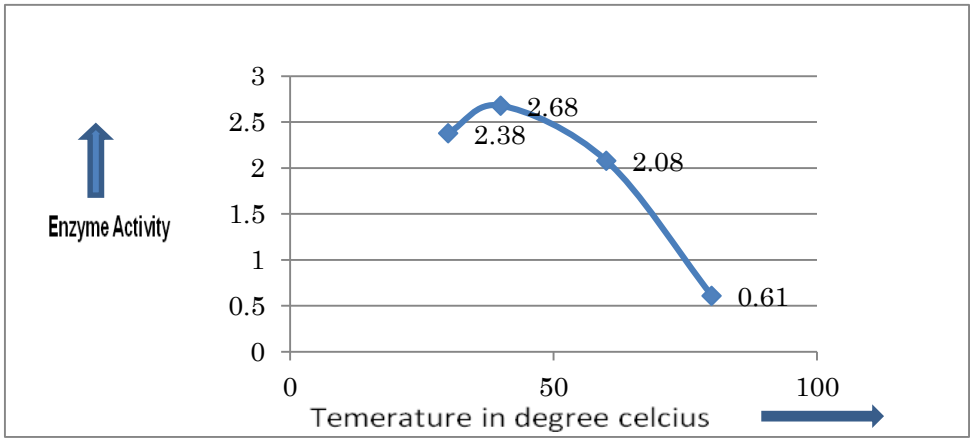

Fig 3: Effect of temperature treatment on *Bacillus* ALP enzyme activity:

The plot revealed that the enzyme under study acted optimally at 40^0C as understood by its highest

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activity at this particular temperature. Temperatures higher than this inactivated the enzyme as exhibited by a steep decrease in its activity.

Effect of pH variation assay on the extracellular ALP enzyme activity:

Effect of pH on the partially purified extracellular ALP activity of the *Bacillus* isolate PB-1 in terms of µmol/ml/min. is represented in the plot as follows:

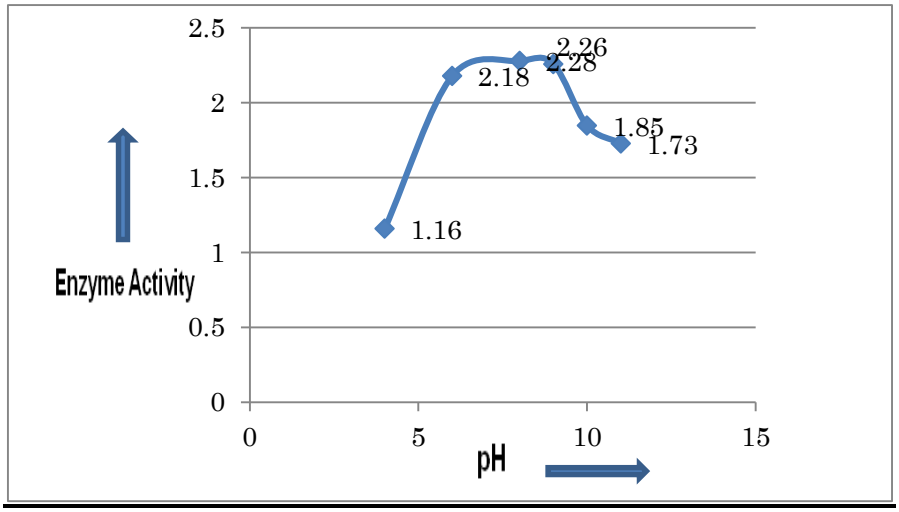

Fig 4: Effect of pH variation on *Bacillus* ALP enzyme activity:

The plot showed that the extracellular ALP enzyme of the PB-1 isolate shows optimum activity at pH 8.0-9.0 that matches with the criterion of a typical alkaline phosphatase enzyme. Its activity became lower at pH higher than this range possibly because of the partial denaturation of the enzyme.

Effect of Phosphate concentration variation assay on the extracellular ALP enzyme activity:

The effect of increasing concentrations of phosphates in the growth medium on the activity of the ALPase enzyme under study (in µmol/ml/min) is represented in the plot below-

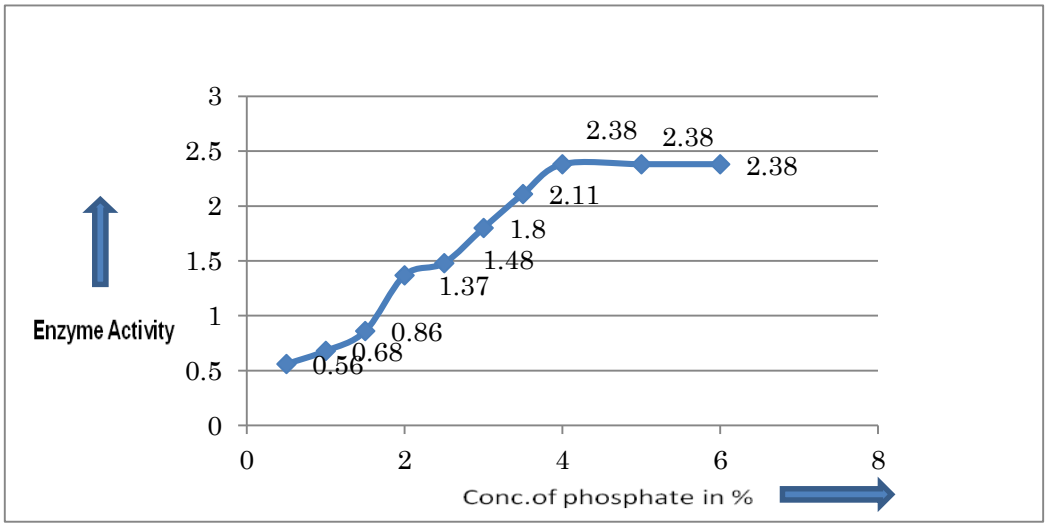

Fig 5: Effect of phosphatase concentration on *Bacillus* ALP enzyme activity:

From the plot, it could be speculated that the enzyme activity increased consistently with higher concentrations of the inorganic phosphate source (NaH_2PO_4) up to 4.0% after which no further

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increase in the enzyme activity was noted. It suggested that the enzyme became saturated at 4.0% substrate concentration and concentrations of phosphates higher than that did not affect the activity of the partially purified ALPase of the test *Bacillus* isolate designated as PB-1. This could be a crucial aspect for studying the kinetics of the enzyme.

Effect of Carbon source variation on the extracellular ALP enzyme activity: The effect of the carbon source variation on the secretion/activity of the ALP enzyme from the *Bacillus* PB-1 isolate is depicted in the plot below-

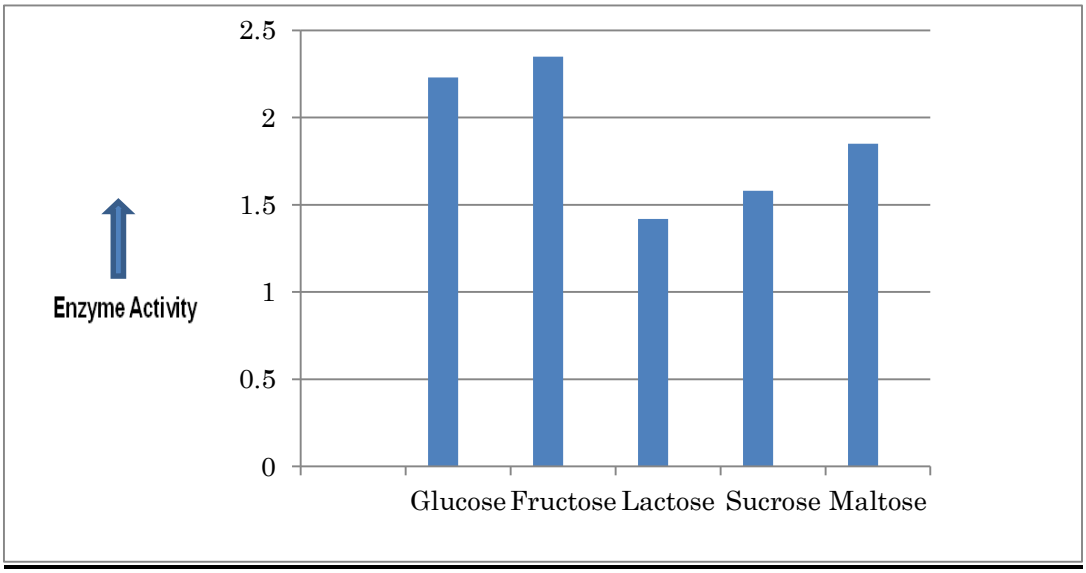

Fig 6: Effect of carbon source variation on *Bacillus* ALP enzyme activity:

As evident from the plot, the secretion (and hence, activity) of ALPase enzyme in the growth medium was maximum in presence of fructose as compared to other carbon sources, including glucose which is used as the carbon source in the conventional Pikovskaya's medium. The PB-1 *Bacillus* isolate under study secretes maximum amount of the ALP when it utilizes fructose as the primary carbon source. How exactly fructose in particular stimulates the production of the ALP enzyme by the *Bacillus* PB-1 isolate remains an interesting area of future research. However, a study by Pandey S.K. et al.(2012) [9] has found that glucose was the most suitable carbon source as far as ALP production by a strain of *Bacillus licheniformis* is in question. On the contrary, Mahesh M. et al.(2013) [10] reports that the production of extracellular ALP production by a strain of *Proteus vulgaris* was maximum when starch was used as the source of organic carbon. In addition, Priya D.et al. (2014) [11] has reported that maltose stimulated the production of extracellular alkaline phosphatase enzyme in comparison to other carbon sources by a strain of *Bacillus megaterium*. Thus, it might be proposed that the carbon source inducing maximum production of the alkaline phosphatase enzyme varies with the species of the bacterium. In the present context of study, the identification of the PB-1 isolate that belongs to the genus *Bacillus* up to species level by 16srRNA sequencing needs to performed for better understanding of its characteristics at the molecular level as well as for exploring its potential role as a PGPR owing to its phosphatase production activity

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which is important in solubilization of the bound phosphate in soil.

4. CONCLUSION

The current study involves isolation and identification (up to genus level) of a novel bacterial strain from potato field rhizospheric soil that belongs to the genus *Bacillus*. In the next phase of the study, the partial purification and characterization of extracellular alkaline phosphatase enzyme by the isolate (designated as PB-1) was studied that might play a pivotal role in its function as PGPR by de-mineralizing the bound phosphate. The enzyme was found to have an optimum temperature of 30-40^{0}C and an optimum pH of 8.0-9.0 for its activity. The enzyme activity increased proportionately with the increase in phosphate concentration in the growth medium up to a level of 4.0% after which the substrate-binding site of the enzyme might have become saturated and resulted in no further increase in the enzyme activity. Fructose was found to be the best carbon source for inducing the highest production of extracellular ALPase enzyme under controlled conditions.

5.ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors hereby declare no conflict of interest in context to the present work.

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