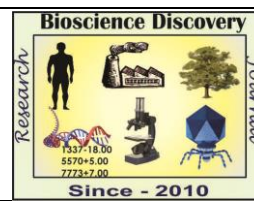


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**Research Article**



## Partial Purification and Characterization of Periplasmic Alkaline Phosphatase from *E.coli* isolated from Water Sample

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### Abstract

*E.coli* strain was isolated from a pond water sample and was identified by its colony characteristics on selective and differential media. Morphological and biochemical features were studied. Investigation of the partially purified periplasmic alkaline phosphatase (ALP) enzyme from the cell lysate of the test strain was carried out. The work reveals that like *Bacillus* sp, the *E.coli* isolate EC4 can also produce typical periplasmic ALP enzyme that has a pH optima of 8.0 and temperature optima of 40°C. The enzyme retained significant functionality at temperatures as high as 60°C indicating its partial thermostable nature that could be of industrial importance. In addition, the present experiment indicates that the enzyme activity increased proportionately with increase in the phosphate concentration in the medium indicating the positive stimulatory effect of inorganic phosphate in the biosynthesis of the periplasmic ALP by the test organism. Interestingly, as far as the effect of carbon source on the ALP activity is concerned, the ALP activity was maximum when glucose was used as the sole carbon source and was the least when sucrose was used as the sole carbon source in the fermentation medium.

### INTRODUCTION

Alkaline phosphatase (Ortho-phosphoric Monoester Phosphohydrolase; E.C. No.3.1.3.1); also known as ALP or ALPase is an enzyme that is widely distributed in all groups of bacteria and is primarily involved in hydrolyzing phosphomonoester bonds from various biological macromolecules like de-oxy ribonucleotides, ribonucleotides, proteins, alkaloids and other tri-phosphate esters. Because of its ubiquitous presence from prokaryotes to higher mammals (including humans), it could be understood that the enzyme is

involved in fundamental biochemical processes of all living organisms. It has been successfully isolated from species of *Bacillus*, *Aerobacter*, *Pseudomonas* and *E. coli*. *E.coli* alkaline phosphatase is a metallo-enzyme with two Zn<sup>+2</sup> ions at its active site although Mg<sup>+2</sup> ions are also found to be present at the divalent metal ions in some ALP enzymes which is essential for ALP activity by providing structural integrity. The mesophilic *E.coli* ALP is found to be a thermostable enzyme, active at temperature as high as 80°C (Janeway *et al.*, 1993).

The ALP enzyme of *E.coli* has been studied extensively in both structural and functional aspects as a model ALP enzyme (Hull *et al.*, 1976; Coleman 1992; Holtz *et al.*, 1999; Stec *et al.*, 2000). The pH optima for the activity of ALP is above neutral range (i.e.pH 7.0); at around pH 8.0 or above. The rate-limiting step of the ALP-catalyzed reaction is the release of the non-covalent product phosphate at optimal pH (Hull *et al.*; 1976). In bacterial cells, ALP plays a pivotal role in phosphate-metabolism that is required for the supply of inorganic phosphates (Pi) to be utilized in various biosynthetic reactions in the cells although the exact physiological role of the enzyme has not been clearly determined as yet. In bacteria, the Pi are transported to the periplasmic space through the phosphate specific transporter (Rao and Torriani, 1990). The enzymatically active alkaline phosphatase is structurally composed of two identical subunits (polypeptides) encoded by a single structural gene (Bradshaw *et al.*, 1981)

In all bacteria, the enzyme is found to be located in the periplasmic space, i.e. the space in between the cell membrane and cell wall of bacteria. Production and characterization of extracellular alkaline phosphatase from *Bacillus* sp by Kannaiyram S. *et al.*, 2015 has found that the bacterium can produce and secrete the ALP enzyme outside the cell as well indicating that the enzyme is not restricted to the periplasmic space only. Purification of extracellular ALP enzyme produced by *Bacillus* sp. isolated from agricultural soil has also been successfully carried out by Parhamfar M.*et al.*, 2016. Similar work done by Mahesh M *et al.*, 2010 isolated and characterized thermostable ALP produced by a strain of *Bacillus* isolated from soil sample again. The enzyme has also been isolated and purified from the periplasmic space of *E.coli* strain C90 in a study by Ali A.F.*et al.*, 2012. The present study aims to partially purify and characterize the ALP enzyme in *E.coli* that is located in the periplasmic space of the bacterium. The test *E.coli* strain was isolated from pond water sample on an enrichment medium and was identified/characterized on the basis of a battery of morphological and biochemical tests as well as by studying the colony features. The characterization of the enzyme was done on the basis of the parameters like temperature, pH, phosphate concentration and carbon source variations. With respect to carbon source, no elaborate mechanism is established about how exactly carbohydrates regulate the production of ALP activity, but

according to a study by Guimaraes *et al.*, 2003 the production of alkaline phosphatase was reduced by a thermotolerant strain of the fungus *Aspergillus caespitosus* when it was cultivated in glucose-supplemented medium. On the contrary, in a study by Sharipova M.R, *et al.*, 2000 1% glucose was found to stimulate the production of extracellular alkaline phosphatase by *Bacillus intermedius*. As far as the effect of temperature is in question, many microorganisms produce thermostable enzymes that may find wide applications in industries. In a recent study by Tambekar DH *et al.*, 2017, a thermostable and alkaline  $\alpha$ -amylase has been obtained from a strain of the bacterium *Bacillus oryzaecorticis* that has immense scope in food, pharmaceutical and textile industries. In a somewhat similar study by Tambekar DH *et al.*, 2017, a thermophilic and alkaliphilic protease enzyme has been reported to be extracted from a strain of *Bacillus aerophilus* isolated from Lonar soda Lake. The enzyme of interest for the current work i.e. alkaline phosphatase has also been obtained from Antarctic yeasts ( Yuivar Y. *et al.*, 2017) indicating its omnipresent nature in both prokaryotic and eukaryotic microorganisms that too has potential industrial applications.

## MATERIALS AND METHODS

### Collection of Water Sample

Pond water sample was collected from a local pond located near Panagarh Bazar, West Bardhaman District, West Bengal, India in September 2016 (Latitude 23.4453 North, Longitude 87.4703 East). About 20 ml of water was collected in a pre-sterilized Erlenmeyer flask and the mouth of the flask was sealed by cotton plugging with paper wrapping during transport from the site of collection to the laboratory to prevent any type of contamination. The water sample was used for bacterial isolation within 2 hrs.of collection.

### Preparation of Media and Isolation, Characterization and Identification of *E.coli*

In order to isolate Gram negative coliforms, Eosin Methylene Blue Agar ( EMB Agar; purchased from Hi Media, India) was used. The pond water sample was serially diluted in sterile test tubes upto  $10^{-3}$  dilution factor using sterile distilled water by using standard Serial Dilution Technique.1 ml.of the sample from the  $10^{-3}$  dilution factor was inoculated onto the EMB agar plates(in duplicates) by simple streaking under aseptic condition. The plates were incubated at 37°C for a period of 18-24 hrs. The plates were then observed for the presence of

colonies with green metallic sheen as *E.coli* produce such type of colonies on EMB agar that distinguishes it from other coliforms. The greenish metallic sheen is formed due to the formation of a dye-conjugate produced by the linkage of Eosin and Methylene Blue by an amide bond at acidic pH. The greenish colonies produced, if any, were then inoculated onto MacConkey Agar (MA; purchased from Hi Media, India) aseptically by simple streaking method to distinguish between the lactose-fermenters (that produce pink colonies) and non-lactose fermenters (that produce white colonies) on the MA surface (It is to be noted that *E.coli* is a strong lactose-fermenter). Among the six numbers of promising colonies that showed positive results for *E.coli* in both of these selective and differential media, one isolate (colony) was chosen randomly and was then transferred to fresh Nutrient Agar (NA; the ingredients for nutrient agar viz. beef extract, peptone, NaCl and agar-agar were all purchased from Merck®, India) slants in order to make pure culture of the isolate which is one of the most likely candidates to be *E.coli*. Further confirmation was done by Gram Staining followed by microscopic observation to find out the presence of Gram-negative rods. The pure culture of the selected isolate was next confirmed to be *E.coli* by performing a series of biochemical tests including IMViC Tests (Indole, Methyl Red, Vogues-Proskauer and Citrate Utilization Test), Catalase Test, Coagulase Test, Nitrate Reduction Test and Carbohydrate Fermentation Tests utilizing glucose and lactose as the sole carbon source. All the reagents required for these tests are either purchased from Merck®, India or SRL®, India.

Compiling the results of all the tests, the isolate from the pond water sample was identified as *E.coli*. The pure culture of the test *E.coli* isolate was maintained on phosphate-deficient NA slants containing 2% agar and pH 7.0 for further work. This *E.coli* strain was tested for its periplasmic ALP activity as described below.

#### **Production of the Enzyme**

To increase the periplasmic alkaline phosphatase content, the positive isolate was grown on a phosphate deficient Luria Broth (10g/l tryptone, 5 g/l yeast extract, 5g/l NaCl, 0.4% (w/v) glycerol, pH 7.4) until it reaches an exponential phase (as measured spectrophotometrically till the O.D. at 450 nm becomes 0.3-0.4). The enzyme production in the growing culture was carried out in 250 ml. Erlenmeyer flask containing 150 ml. of the fermentation medium. The flask was incubated at

37°C for about 48hrs. with continuous shaking in a shaker incubator and was observed intermittently until the desired O.D. was reached.

#### **Partial purification of the Enzyme and Phosphatase Activity Assay**

The cell-pellets were collected after centrifuging 100 ml. of the *E.coli* broth culture at 15,000 g for a period of 20 minutes at 4°C and the pellets were next dissolved in 30-mM Tris-HCl, 20% sucrose buffer (pH 8.0), 0.8 ml of 10mM EDTA and 100µl lysozyme (10 mg lysozyme/ml of 30mM Tris-HCl buffer of pH 8.5). The whole mixture was then centrifuged at 15,000 g for 15 minutes at 4°C temperature. The supernatant was collected and to that 40% of ammonium sulphate was added and the mixture was again centrifuged at 10,000 g for 10 minutes at 4°C. The suspension was finally adjusted with 65% saturated ammonium sulphate and was incubated at room temperature for overnight. The precipitated protein was then collected by centrifugation of the mixture at 15,000 g for 10 minutes at 4°C and was dissolved in 3 ml. of Glycin buffer (pH 8.5). This was then dialyzed against Glycin buffer (pH 8.5) for 18-24 hrs. and was used as the partially purified enzyme extract for further analysis.

The crude enzyme activity was then determined utilizing p-nitrophenyl phosphate (PNPP) as the substrate in 0.5M Glycine Buffer (pH 8.5). For this assay, 200 µl of the crude enzyme preparation and 500 µl of a solution of 10mM PNPP (in Glycin buffer of pH 8.5) was added with appropriate volume of 0.5M Glycin buffer (pH 8.5) to make the total volume up to 2000 µl. The reaction mixture was then incubated at 40°C temperature for a period of 10 minutes. The reaction was stopped by the adding 0.2 ml of 13% KH<sub>2</sub>PO<sub>4</sub>. The absorbance of the reaction mixture (i.e. the liberated p-nitrophenol or PNP) was measured by using a UV-Visible Spectrophotometer (purchased from Sytronics, India, Model No.-AU-2603) at 405 nm.

#### **Preparation of Standard Curve for Para-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 (pH 8.5) solution. Then the tubes were incubated for 10 min. at room temperature and the absorbance was read at 405 nm wavelength against an appropriate blank using a UV-visible spectrophotometer as mentioned above. PNP is the

end product of alkaline phosphatase reaction when 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 a reference for determining the ALP activity under different parameters in the experiment as follows.

#### Effect of Temperature

Effect of different temperatures (25<sup>o</sup>C, 40<sup>o</sup>C, 60<sup>o</sup>C and 80<sup>o</sup>C) on partially purified alkaline phosphatase enzyme of *E.coli* was investigated. In each tube, an aliquot of 200 $\mu$ l of the crude enzyme source was mixed with 1500  $\mu$ l of the 10mM PNPP solution and the total volume was made upto 2000 $\mu$ l by

adding 0.5 M of glycine 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-

$$\text{ALP activity} = \frac{\text{Amount of PNP released (in } \mu\text{g)}}{\text{Mol.wt.of PNP(gm.)} \times \text{vol.of enzyme source (ml.)} \times \text{Reaction Time (in minutes)}} \mu\text{mol/ml/min}$$

#### Effect of pH

Effect of different pH values (pH 4.0, 6.0, 8.0, 10.5 and 11.0) on the partially purified ALP enzyme of *E.coli* was investigated by following the method as described above. The total volume in each tube was made up to 2000  $\mu$ l by adding buffers of respective pH values-acetate buffer for pH 4.0, citrate buffer for pH 6.0, sodium phosphate buffer for pH 8.0, Glycin buffer for pH 10.5 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% and 4.0% of sodium di-hydrogen phosphate, purchased from SRL<sup>®</sup>, India) on production of partially purified periplasmic alkaline phosphatase enzyme by *E.coli* was determined. The appropriate amounts of phosphates were added in Nutrient Broth (NB) basal medium in Erlenmeyer flasks. The pH of the broth was adjusted to 7.0 in each flask. All the NB media with different concentrations of phosphates were inoculated with

1.0ml of pure broth culture of the isolated *E.coli* strain and the flasks were incubated at 37<sup>o</sup>C for overnight with continuous shaking. The crude periplasmic ALP extracts were prepared from the culture broths as described already. The activity of ALP produced under different phosphate concentrations was measured spectrophotometrically at room temperature and at pH 8.0(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 from Merck<sup>®</sup>, India) on partially purified periplasmic alkaline phosphatase enzymes produced by *E.coli*. was studied. 1% of each of the carbon source mentioned was added in Nutrient Broth (NB) 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 *E.coli* strain and the flasks were then incubated at 37<sup>o</sup>C for overnight with continuous shaking. The crude periplasmic 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 8.0(Glycin buffer) by the method as already described.

**RESULTS AND DISCUSSION**

The colonies that did not exhibit any green metallic sheen on the EMB Agar and did not produce pink colonies on MA were not chosen for the Gram-staining and further characterization since they lack the basic signature features of typical *E.coli*

colonies on the selective (EMB Agar) and Differential (MA) media. Isolate number EC4 was selected randomly and subjected to next confirmatory tests to ensure that the isolate is *E.coli* only

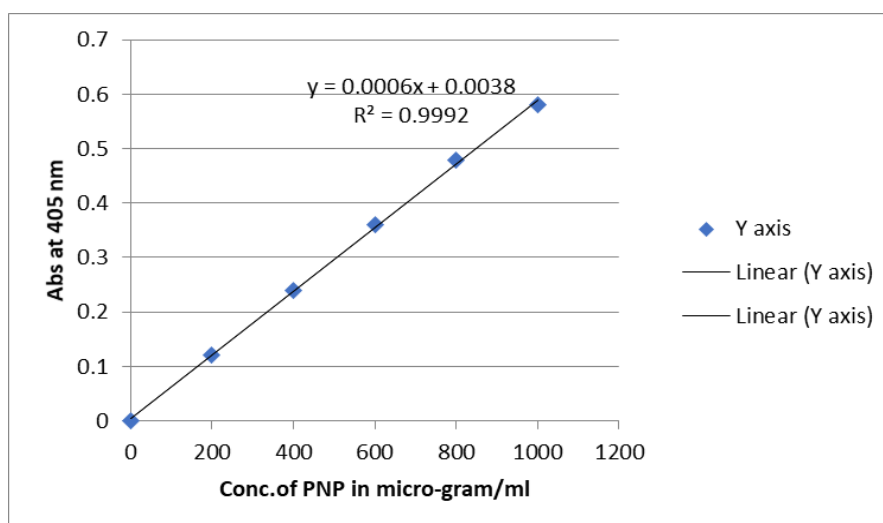
**Table 1: Characterization and Identification of *E.coli* from Pond Water Sample**

Sample	Dilution Factor	Isolate No.	Observation for EMB plates	Observation for MA plates	Microscopic observation for the pink colony isolates (under 100X)	Inference
POND WATER	10 <sup>-3</sup>	EC1	Colony with green metallic sheen	Pink Colony	Gram-Negative rods	May be <i>E.coli</i>
		EC2	Colony with green metallic sheen	Pink Colony	Gram-Negative rods	May be <i>E.coli</i>
		EC3	Colony with green metallic sheen	Pink Colony	Gram-Negative rods	May be <i>E.coli</i>
		EC4	Colony with green metallic sheen	Pink Colony	Gram-Negative rods	May be <i>E.coli</i>
		EC5	Colony with green metallic sheen	Pink Colony	Gram-Negative rods	May be <i>E.coli</i>
		EC6	Colony with green metallic sheen	Pink Colony	Gram-Negative rods	May be <i>E.coli</i>

By compiling the results of the biochemical tests and the colony features (on the EMB Agar and MA media) and Gram character of the selected isolate EC4, it was confirmed that the test isolate is *E.coli*.

**Preparation of Standard Curve for Para-Nitro-Phenol (PNP):**

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



**Fig 1: Standard Curve of Para-nitro-Phenol**

Table 2: Biochemical and physiological analysis of *E.coli* samples:

Isolate No.	Indole Test	MR Test	V-P test	Citrate Utilization Test	Nitrate Reduction Test*	Lactose Fermentation Test	Catalase test	Coagulase Test	Glucose Fermentation Test	Inference
EC4	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	<i>E. coli</i>
	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	
	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	
	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	
	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	
	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	
	+	+	--	--	--	Positive with both Acid and Gas	+	--	Positive with both Acid and Gas	

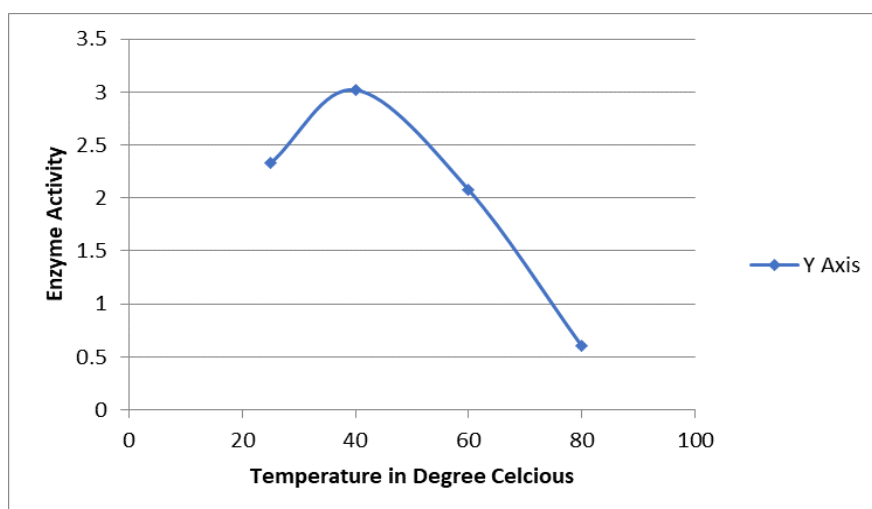
#### Determination of Enzyme activity of the partially purified alkaline phosphatase enzyme obtained from cell-lysate of the *E.coli* isolate:

The partially purified alkaline phosphatase enzyme extracted from the cell-lysate of the test *E.coli* strain showed an activity of 2.08 micro-mole/ml/min.at room temperature (i.e.33°C, as measured by a thermometer) and at a pH of 8.5. The high activity of periplasmic ALP enzyme may be contributed to

its folding after being transported from cytoplasm into the periplasmic space that is not possible in cytoplasm where the formation of di-sulphide bond is prevented which is necessary for the proper folding of the protein (Sone *et al.*, 1997).

#### Effect of Temperature

Effect of temperature on the partially purified periplasmic ALP activity of *E.coli* in terms of  $\mu\text{mol/ml/min}$ .is represented in the plot as follows:

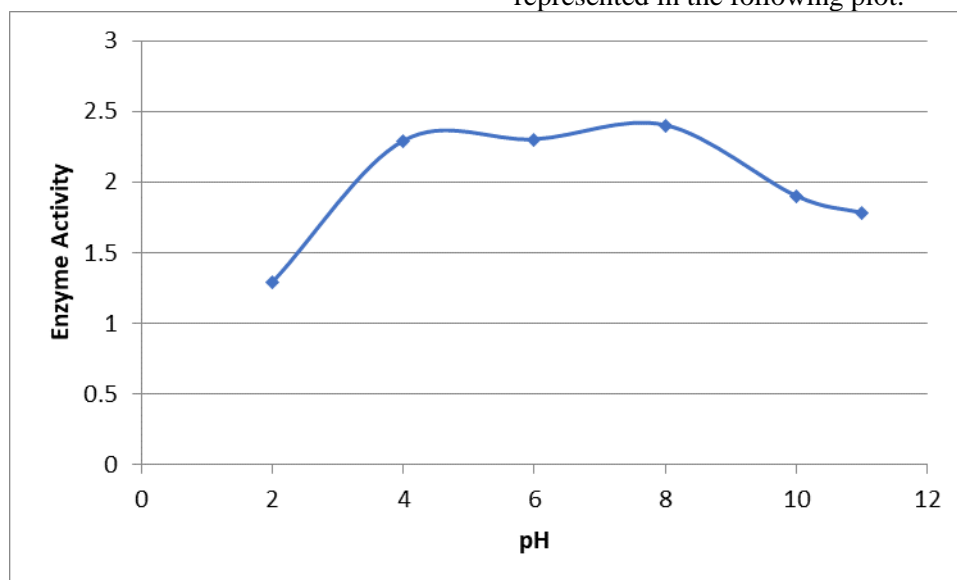
Fig 2: Effect of Temperature on *E.coli* Alkaline phosphatase activity

In accordance with the plot above, it could be inferred that the optimal enzyme activity is exhibited at 40°C temperature although considerable enzyme activity is also observed at temperature as high as 60°C indicating the partial

thermostable nature of the ALP enzyme of the isolated *E.coli* strain.

**Effect of pH**

Effect of pH on the partially purified periplasmic ALP activity of *E.coli* in terms of  $\mu\text{mol/ml/min}$  is represented in the following plot:

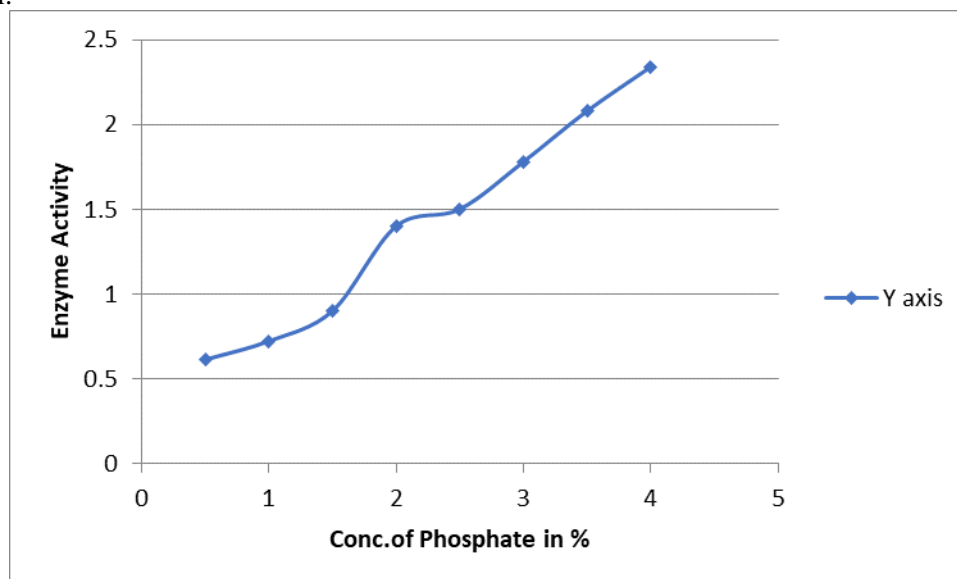


**Fig 3: Effect of pH on *E.coli* Alkaline phosphatase activity**

It is evident from the plot that the partially purified periplasmic ALP enzyme of the isolated *E.coli* strain exhibited maximum activity at pH 8.0 and hence, at this pH the enzyme activity is considered to be optimal.

**Effect of phosphate source**

The increasing concentrations of phosphate lead to the enhanced enzyme activity in a proportional manner as shown by the following plot:



**Fig 4: Effect of phosphate concentration on *E.coli* Alkaline phosphatase activity**

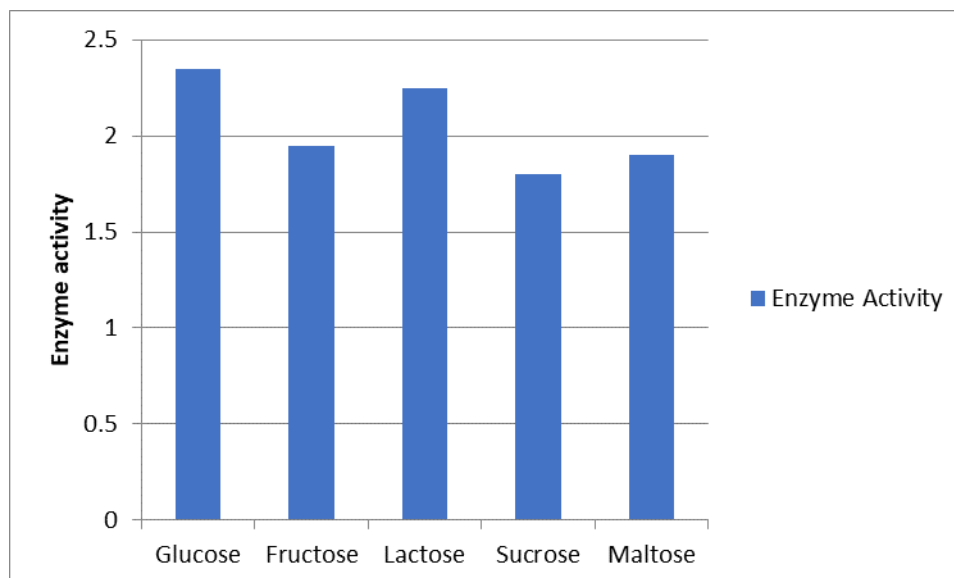
From the above plot, it is evident that the more will be the concentration of phosphate in the environment, the greater will the production of the

periplasmic ALP enzyme by the test bacterial strain which is consistent with the role of the enzyme as speculated.

**Effect of carbon source**

The influence of various carbon sources (in the form of carbohydrates) in the production of periplasmic ALP enzyme of the isolated *E.coli* strain is represented below in the form of a bar plot:

The plot shows that activity of periplasmic ALP enzyme by the test *E.coli* strain is at maximum when they utilize glucose as the sole carbon source and becomes the least when sucrose is utilized as the sole source of carbon.



**Fig 5: Effect of carbon source on *E.coli* Alkaline phosphatase activity**

**DISCUSSION**

The current study explored that like the *Bacillus* sp, the organism *E.coli* isolated from natural pond water is also a potent producer of periplasmic alkaline phosphatase enzyme. The enzyme functions optimally at 40°C temperature and at a pH of 8.0 which is much below the pH optima (pH 10.5-11.0) of extracellular ALP obtained from a soil borne *Bacillus* sp (Suganya Kannaiyram *et al.*, 2015). The test ALP enzyme, like extracellular *Bacillus* ALP (Mahesh M *et al.*, 2010), also exhibited thermostability up to a temperature of 60°C that makes it useful for industrial applications where enzyme functionality at higher temperatures is required. The production of periplasmic ALP in the test *E.coli* strain increased proportionately as evident by consistently higher enzyme activity with increased phosphate concentration in the external environment (media) suggesting that inorganic phosphate acts as a positive inducer for the enzyme synthesized by the test bacterial strain. The study also revealed that the synthesis of periplasmic ALP by the *E.coli* strain maximized in presence of glucose and was minimum when the test bacterium utilized sucrose as the sole source of carbon. Further characterization of the enzyme and proper

identification of the *E.coli* isolate by 16s rRNA sequencing remain to be done. The work explored the fact that like the *Bacillus* sp, *E.coli*, a common water borne bacterium can also be exploited as a potential source of active periplasmic ALP with thermostable nature that may have a promising scope as far as large scale industrial production and applications are concerned.

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