



Effect of Carbon and Nitrogen Source for the Production of Alkaline Phosphatase from *Proteus vulgaris*

Mahesh. M¹, Somashekhar. R¹, Puttaiah.E.T²

¹Azyme Biosciences Pvt. Ltd, Bangalore, Karnataka, India.

²Kuvempu University, Shimoga, Karnataka, India.

*Corresponding author's E-mail: Mahesh@azymbio.com

Accepted on: 05-10-2013; Finalized on: 31-10-2013.

ABSTRACT

The 35 bacterial strains were isolated from water and soil sample from Karnataka and boarding states of India and screened for their ability to secrete extracellular Alkaline Phosphatase (ALP). Among them, a potent strain *Proteus vulgaris* up to 99% *Proteus vulgaris* strain LSRC-158 was confirmed by 16S rRNA gene sequencing was selected for the enzyme production. The pNPP was the best substrate for the ALP production and production was stimulated by using starch and casein as sources of carbon and nitrogen respectively. After purification by Ammonium sulphate precipitation, gel filtration and ion exchange chromatography, the fold purification was increase up to 11.3. The optimum temperature and pH of the enzyme were 37 °C and 8-9, respectively with Vmax of 16.3 μmol/mg/min and Km of 3.03 mM. The molar mass was estimated by 12% SDS PAGE and was found to be approximately 64kDa and also confirmed by gel filtration studies. The study holds significance as there are only few reports available on the optimisation protocols and enumeration of microorganism for production of extracellular ALP.

Keywords: Alkaline Phosphatase, Chromatography, Optimisation, Phylogeny, SDS-PAGE, Sequencing.

INTRODUCTION

Alkaline phosphatase (ALP) (orthophosphate monoester phosphohydrolases E. C. 3.1.3.1.) are metalloenzymes, nonspecific, phosphor monoesterases that present in various from bacteria to mammals.¹⁻³ ALP is a metallo dependent enzyme which shows its catalytic activity optima at alkaline pH.⁴ ALP hydrolyzes a wide variety of phosphate esters and is classified as ALP according to its optimum pH ranging from 7.5 to 11.0.⁵ In wide variety of the organism ALP playing very important role in the metabolism and ALP is crucial enzyme for the survival of organisms under the starvation of the phosphate.

The enzyme widely used as biochemical marker in molecular biology, immunological assay, diagnosis of disease and used in non radioactive detection techniques, probing, blotting and sequencing systems.^{6,7} It is very important to identify and/or determine each isozyme in clinical practice because any change in the catalytic activity of alkaline phosphatase isozymes (for instance, in blood, tissues) is accomplished by various pathological states (hepatitis, different diseases of bones, heavy hereditary diseases, prostate cancer etc.). ALP has the biggest market volume share of \$20 million.⁸ The global market for biosensors and other bioelectronics is projected to grow from \$6.1 billion in 2004 to \$8.2 billion in 2009, at an AAGR (average annual growth rate) of 6.3%.⁹ Because of the more clinical application and high commercial value, more research work has to be done in this area. So far, the commonly used and commercial available ALP obtained from *Escherichia coli* ALP and calf intestine ALP.¹⁰

Studies have been carried out for production, purification and characterization of ALP from variety of microorganisms including *E.Coli*, *Pseudomonas*, *Aerobactor* and *Bacillus* species.¹¹⁻¹⁴ In all bacteria, ALP found in the periplasmic membrane which is external to the cell membrane of bacteria.¹⁵ Very little work has been done with respect to extracellular production of ALP. Intracellular production of ALP is quite tedious and expensive process in contrast to extracellular. This statement is supported by the study of which explained that extracellular ALP gave higher specific activity than intracellular ALP is because of short and simple steps of purification.^{16,17} In the present study optimisation of significant concentration of Carbon and nitrogen (Nutrition) are taken into consideration as essential components required for increase microbial growth and enzyme production.

MATERIALS AND METHODS

Collection of samples

Forty soil sample and twenty water samples were collected from different places of Karnataka and bordering states of Karnataka.

Isolation of Organism

The organism was isolated using serial dilution by pore plate method using modified Minimal Media (MM) Sodium chloride 5g, Ammonium chloride 2g, Magnesium chloride 0.1g, Glucose 10g, and Agar 20g per litre along with Para-nitrophenol phosphate (pNPP) 1g.

Screening the ALP

The isolates were screened by Streak plate on the MM media along with pNPP and the selected isolates were

separately confirmed for their abilities to produce ALP with the following procedure: A loopfull of the selected ALP-producing strain was inoculated into 50 ml MM media along with pNPP of broth in duplicate and incubated on a rotary shaker at 37°C (150 rpm) for 24hrs. Crude supernatant obtained after centrifugation of the cultures at 10,000 rpm, 4°C for 10 min was used as crude enzyme for ALP activity & detection by Spectrophotometry analysis.¹⁸

Identification of strains

The isolates showed more yellow colour surrounding the organism and more activity based on the bioassay method. The isolate was identified on the basis of morphology, chemo-taxonomical characteristics and 16S rRNA sequencing. Results were interpreted according to Bergey's Manual of Determinative Bacteriology, 8th edition and NCBI database.¹⁹ The organism is considered as *Proteus vulgaris*

Production Of enzyme

Proteus vulgaris used for enzyme production was grown at 37°C for 24 hrs shaking (180 rpm) in modified minimal media (MM) along with pNPP as substrates for phosphatase synthesis. ALP activity was measured by using Stopped Spectrophotometric Rate Determination method.¹⁸ The absorbance of released para-nitrophenol phosphate (pNPP) was determined at 410nm. Protein concentrations were estimated by Lowery's method and absorbance determined at 660nm by using bovine serum albumin (BSA) as standard.²⁰

Effect of Phosphates on enzyme production

In the present study, we aim to detect the appropriate source for ALP production by the organic phosphates like adenosine triphosphates, para nitrophenyl phosphates and inorganic phosphates like sodium phosphate, potassium phosphate and calcium phosphates of different concentration like 0.01% to 0.1% were used for regulation of phosphatase synthesis along with 50ml minimal essential media. 1 ml of seed culture was inoculated and incubated in a rotary shaker at the end of the incubation cell free extract was used for enzyme assay.

Effect of carbon sources on production of enzyme

The effect of different carbohydrates on various concentrations was studied. Glucose, sucrose, lactose, maltose, cellulose and starch of different concentrations starting from 0.5% to 3.0% were used along with 50ml minimal essential media with 0.15% pNPP. 1ml of seed culture was inoculated and incubated at 37°C for 24hr.

Effect of nitrogen sources on production of enzyme

The effect of different proteins sources such as Tryptone, Casein, Gelatin, Egg albumin and bovine Serum albumin (BSA) of different concentrations i.e. 0.5% to 3.0% were used to influence the production ALP was tested.

Comparatives studies on cell membrane bound and extracellular alkaline phosphatase

Cell cultivated for the ALP production were harvested by centrifugation at 10000 rpm for 10 minutes at 4°C, washed once with normal saline and resuspended to $A_{600}=1.0$ in the same saline and broken with ultra sonicator for 1 min. The extract was centrifuged for 15000 rpm for 10 min and cell free supernatant was subjected to enzyme assay.

Purification of Extracellular ALP

The cell free media collected was subjected to different steps of purification including ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysis, Gel Filtration and DEAE-cellulose ion-exchange chromatography by using gradient elution buffer.

Enzyme Characterization

The optimum temperature needed for maximum activity of the purified ALP from *Proteus vulgaris* was determined by varying the reaction temperature from 25°C – 70°C. The thermal stability was determined by measuring the residual activity after incubating the enzyme at temperature ranging from 25°C to 70°C for 4hrs. The effect of pH on the activity of ALP was observed by incubating the purified enzyme in the various buffers used for 50mM sodium acetate (4-6), 50mM Tris-HCL (7-9), and 50mM Glycine-NaOH (10-11). The stability was determined after incubating the enzymes at a pH of 4-11 at room temperature for 4hrs and residual activity was determined under standard assay conditions. The effect substrate on the activity of alkaline phosphatase was observed by incubated with different concentration of pNPP. Kinetic parameters K_m and V_{max} were calculated. Molecular weight SDS PAGE was performed according to the Laemmli with the 4% Acrylamide stacking gel and 10% Acrylamide separating gel to determine the molecular mass and purity of protein. Staining was carried out with Comassive Brilliant blue.²¹

RESULTS AND DISCUSSION

Identification of the organism

The organism showing more activity was identified by using 16SrRNA sequencing. The universal primers (27F' and 1492R') for the amplification of 16S rRNA were able to amplify the region giving ~1.4 kb size fragment in isolated strain. Amplicons visualized on 1% agarose gel with 1 X Tris acetate EDTA buffer at constant voltage of 80V. The absorbance ratios (A_{260}/A_{280}) of the preparations were in range of 1.80-1.85 by spectrophotometer. Based on 16S rRNA sequence data, BLAST search showed that *Proteus sp.* highly resembles *Proteus vulgaris* (similarity upto 99%, *Proteus vulgaris* strain LSRC-158). Differentiation is considered based on biochemical test on the species level, Indole test is considered reliable, as it is negative for *Proteus vulgaris*. One of the best advanced processes to identification of

bacteria analysis by 16S rRNA sequences is the single best method.^{22, 23}

Effect of Phosphates on enzyme production

Different concentration organic phosphates like adenosine tri phosphate, pNPP and inorganic phosphates like sodium phosphate, potassium phosphate and calcium phosphates of concentration like 0.01% to 0.1% were used for regulation of phosphatase synthesis along with 50ml MM. Maximum ALP activity obtained from pNPP and using the inorganic phosphate the activity was very low (Table 1). Previous studies reveal the inorganic phosphates decreases the activity by increasing the concentration in *E.coli*.²⁴ The optimum concentration of pNPP 0.15% to 0.18% was given by the organism. The active sites have a particular orientation of specific amino acid side chains (and their respective chemical properties) there is usually only one molecule or at most a few types of molecules that can bind to the active site for a long enough period of time for a chemical reaction to take place. Thus most enzymes show a very high degree of specificity - they bind specific substrates, catalyze specific reactions involving those substrates, and thus produce specific products.

Table 1: Effect of Phosphates on enzyme production

Substrate	Activity	Concentration pNPP	Activity
Adenosine Tri Phosphate	3.6±0.56	0.02%	4.2±0.23
(pNPP)	5.6±0.46	0.06%	4.6±0.15
Sodium Phosphate	1.8±0.5	0.10%	5.2±0.72
Calcium Phosphate	2±0.45	0.14%	5.9±0.46
Potassium Phosphate	1.8±0.25	0.18%	6.5±0.4
-	-	0.20%	6.4±0.23

Effect of carbon sources on production of enzyme

The effect of different carbohydrates on various concentrations was studied. Glucose, sucrose, lactose, maltose, cellulose and starch at 0.1% level were used along with 50ml minimal essential media with 0.15% pNPP. Starch gives optimum production of enzymes (Table 2). The different concentration of starch was used 0.5% to 3%. The optimum activity shown in starch the optimum activity was shown 1% to 1.5%, but increase the concentration activity was decreased. Organic carbons are the common substrates i.e. usually referred to heterotrophic microorganisms for the main energy sources. The similar results reveals the production of alkaline phosphatase from *Neurospora crassa* was also stimulated by sucrose in combination with acetate.²⁵ Increase the concentration activity was reduced. High concentration of substrates or products often lead to inhibitory effect which results in poor utilization of the

substrates which in turn decrease both product yields and fermentation rates.

Table 2: Effect of Carbon sources on production of enzyme

Carbon Source	Activity	Concentration Starch	Activity
Glucose	4.8±0.6	0.5%	6.5±0.4
Sucrose	6.9±0.4	1.0%	7.2±0.01
Lactose	5.8±0.5	1.5%	6.6±0.2
Maltose	4±0.45	2.0%	5.8±0.35
Starch	6.8±0.25	2.5%	4.6±0.4
Cellulose	4.8±0.3	3.0%	4.2±0.2

Effect of nitrogen on production of enzymes

The effect of different proteins sources such as Tryptone, Casein, Gelatin, Egg albumin and Bovine Serum albumin of different concentrations i.e., 0.5% to 3.0% were used to influence the production ALP was tested. The activity was increased 22% in casein media (Table 3). Nitrogen is required for protein synthesis which is responsible for enzyme production. The similar works reveals nitrogen is very important source for alkaline phosphatase production.²⁶⁻²⁹

Table 3: Effect of nitrogen for the Production of Enzyme

Nitrogen Source	Activity	Concentration Casein	Activity U/mg
Casein	11±0.35	0.5%	11±0.6
Gelatin	5.8±0.2	1.0%	13.5±0.9
Tryptone	8.8±0.80	1.5%	13±0.45
Egg Albumin	7.0±0.10	2.0%	12.8±0.65
BSA	7.2±0.45	2.5%	12.6±0.0
-	-	3.0%	12±0.45

Comparatives studies on cell membrane bound and extracellular ALP

Cell cultivated for the ALP production were by centrifugation at 10000 rpm for 10 minutes at 4°C, washed once with normal saline and resuspended to A₆₀₀=1.0 in the same saline and broken with ultrasonicator for 1 min. The extract was centrifuged for 15000 rpm for 10 min and cell free supernatant was subjected to enzyme assay. The activity of extracellular ALP was more than 27% contrast intracellular ALP. There are reports on generating osmotic shock through treatment with magnesium ions or sonication.^{30,31} Production of extracellular ALP in *Bacillus licheniformis* which shows that it synthesizes 10 times more ALP activity than is reported for other *Bacillus species*.³² However, the extracellular production of ALP has been studied in *Micrococcus sodonensis*.³³

Purification of Extracellular ALP

The purification of ALP resulted by increase of 2.14 fold purification after ammonium precipitation and after gel



filtration the fold purification was increase up to 6.6. Further purification was done by ion exchange chromatography, the fold purification was increase up to 11.3 (Table. 3.4) Extracellular ALP was eluted on DEAE-cellulose, after elution the specific activity was increased up to 9.2 and 12 with fold purification in contrast to our

results and in case of *Bacillus licheniformis*.³¹ Fold purification and yield were found to be 2.4. Yield of ALP are dependent on the strain of bacteria used.³⁴ This may be the reason for the difference between our results and those reported earlier.

Table 4: Summary of the purification Alp *Proteus vulgaris*

Purification step	Total Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Fold Purification	Yield (%)
Crude Sample	200	2700	272	9.9	1	100
Ammonium sulfate Precipitation (10-70%)	25	2100	100	21	2.14	77.8
Gel filtration	10	1260	19	66.3	6.6	47
Ion Exchange Chromatography	10	785	7	112.1	11.3	29

Characterisation of Purified enzyme

Effect of temperature on the activity and stability of ALP from *Proteus vulgaris*

The optimum temperature of ALP was found to be 35°C to 40°C (Figure 1). At 25°C twenty five percent of the activity was retained and 100% activity retained 35°C. The 90% activity was retained at 45°C and 22% activity was retained at 60°C. After 4hrs incubation the ALP activity is retained 100% at 35°C to 40°. After 4hrs incubation the activity seen was decreasing but after 1 hrs activity was seen decreasing at 50°C and only negligible activity was shown at higher temperature above 70°C. Similar values were reported by *Sphingobacterium*, ALP showed maximum activity at 37°C but it lost completely at 62°C.³⁰ The *Ulva pertusa kjellm* ALP showed maximum activity at pH 9.8 and 37°C, *A.caespitosus*, *Humicola grisea* and *Rhizopus microspores*.^{26, 12, 35, 5} It shows that the higher temperature increase the kinetic energy of molecules which break the bond that holding the active amino group and enzyme gets denatures. Hence, results in the loss of enzyme activity.³⁶

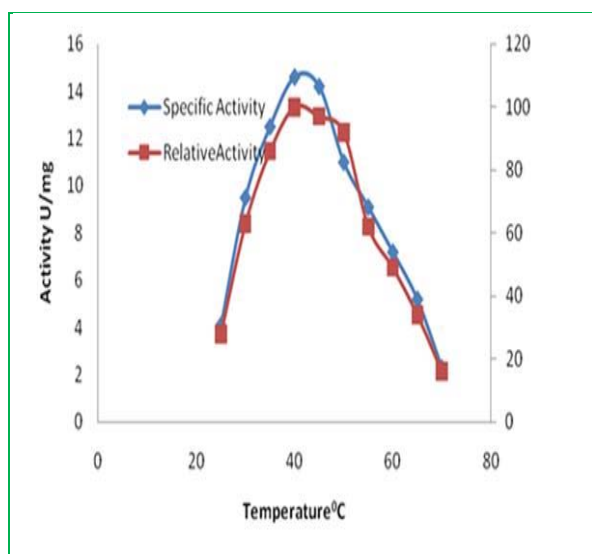


Figure 1: Effect of temperature on the enzyme activity

Effect of pH on the activity and stability of ALP from *Proteus vulgaris*

The activity was studied at different pH ranging from 4 to 11 and it was found to be pH 8 to 9 in 50mM glycine buffer (Figure 2). The organism was incubated at various pH levels 4-11 for 4hrs at 37°C and residual activity was measured 100% activity was retained for 1hr incubation ranging from pH 5-9. More than 50% activity was retained after 4hr of incubation at pH 5-9, it was highly stable at higher pH because its nature. This result is supported by who studied *Anthrobactor* strain for extracellular production of ALPase.³⁷ As the pH increases the decrease in enzyme activity was observed and only 50% activity was noted at pH 11. Increase in pH effect the charges on the amino acids within the active site such that the enzyme is not to be able to form enzyme substrate complex. Thus, there is decrease in enzyme activity.³⁴

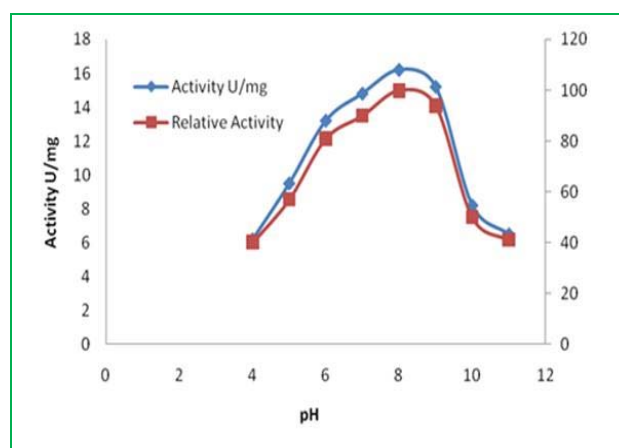


Figure 2: Effect of pH on enzyme activity

Determination of K_m and V_{max}

The K_m and V_{max} of partially purified enzyme were approximately 3.33 mM and 16.3U/mg/min, respectively, as (Figure 3) shows. Related studies shows higher substrate specificity to pNPP.³⁷ It was noted that enzyme activity was increased with the increased in the substrate concentration and optimum activity was obtained at

3.04mM substrate concentration. Km value for ALP was 1.11mM in *B. stearothermophilus*.¹⁴

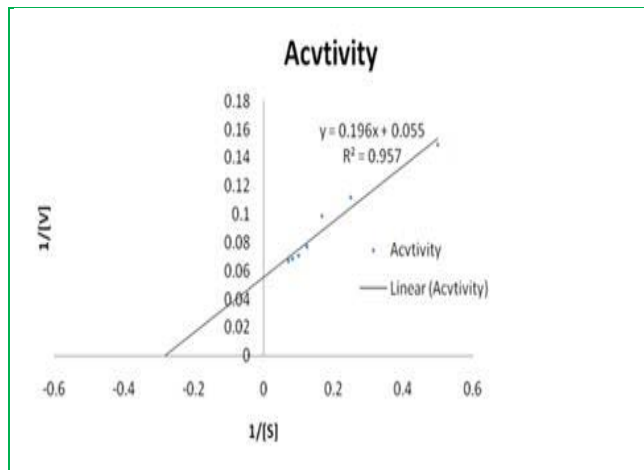


Figure 3: Line-Weaver Burk plot of ALP

Determination of Molecular Mass

The molar mass of purified ALP, as determined by SDS-PAGE, was found to be the monomer band of 64 kDa and confirm by gelfiltration by using Sephadex 100 G (Figure 4). The related studies, homodimeric forms of ALPse, with a molecular mass of 54kD have been previously reported *E. pyrococcus abyssi*.^{30,38,39} The SDS-PAGE of *B. stearothermophilus* alkaline phosphatase showed a single protein band of 32kDa.¹⁴

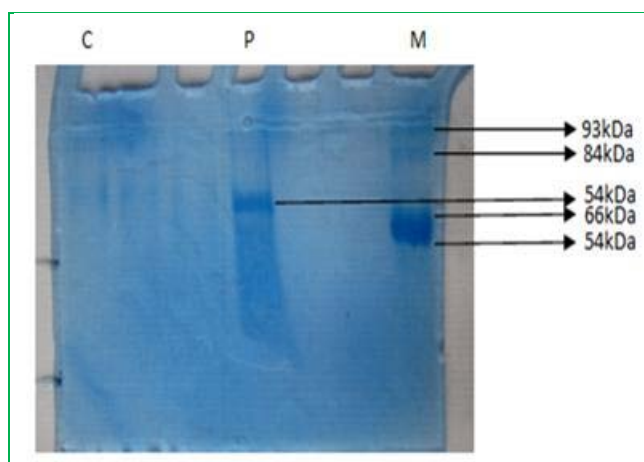


Figure 4: Molecular mass determination by SDS-PAGE

CONCLUSION

In this study, the organism that produces the extracellular ALP was identified and optimized for its maximum production subjecting to different physiological conditions. Further investigations are required to make use of the full potential of these organisms for the production of ALP by genetic engineering techniques.

REFERENCES

- Oh WS, Im YS, Yeon KY, Yoon YJ, Kim JW, Phosphate and carbon source regulation of alkaline phosphatase and phospholipase in *Vibrio vulnificus*, *J. Microbiology*, 45, 2007, 311-317.
- Simão AMS, Beloti MM, Cezarino RM, Rosa AL, Pizauro JM, Ciancaglini P, Membrane-bound alkaline phosphatase from ectopic mineralization and rat bone marrow cell culture, *Comp Biochem Physiol A: Mol Integrat Physiol*, 146, 2007, 679-87.
- Junior AB, Guimaraes LHS, Terenzi HF, Jorge JA, Leone FA, Plizeli MLTM. Purification by chemical characterization of thermostable alkaline phosphatases produced by *Rhizopus microspores* var. *rhizopodiformis*, *Folia Microbiolog*, 53(6), 2008, 509-516.
- Mori S, Okamoto M, Nishibori M, Ichimura M, Sakayama J, Endo H, Purification and characterization of alkaline phosphatase from *Bacillus stearothermophilus*, *Biotechnol. Applied Biochem*, 29, 1999, 235-239.
- Dhaked RK, Alam SI, Dixit A, Singh L, Purification and characterization of thermo-labile alkaline phosphatase from an Antarctic psychrotolerant *Bacillus* sp. P9, *Enzyme and Microbial Technology*, 36, 2005, 855-861.
- Chen CC, Tai YC, Shen SC, Tu YY, Wu MC, Chang HM, Detection of alkaline phosphatase by competitive indirect ELISA using immunoglobulin in yolk (IgY) specific against bovine milk alkaline phosphatase, *Food Chemistry*, 95, 2006, 213-220.
- Nilgiriwala KS, A Alahari, AS Rao, SK Apte, Cloning and overexpression of alkaline phosphatase pho K from *Sphingomonas* sp. Strain BSAR-1 for bioprecipitation of uranium from alkaline solutions, *Applied and Environmental Microbiology*, 74, 2008, 5516-5523.
- (Enzymes for clinical purpose, 2004).
- Biosensor/Bioelectronics Market, 2005, Last visited 21/11/2009 http://www.nanotechbriefs.com/auth/biz/biz_2_0205.html
- Suzuki Y, Mizutani Y, Tsuji T, Ohtani N, Takano K, Haruki M, Morikawa M, Kanaya S, Gene cloning, overproduction, and characterization of thermolabile alkaline phosphatase from a psychrotrophic bacterium, *Biosci Biotechnol Biochem*, 69, 2005, 364-73.
- Torriani A, Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*, *Biochem Biophys Acta*, 38, 1960, 460-469.
- Guimaraes LHS, Jorge JA, Terenzi HF, Jamus MC, Oliver C, Polizelli MLTM, Effect of carbon source on alkaline phosphatase production and excretion in *Aspergillus caespitosus*, *J Basic Microbiol*, 43, 2003, 210-217.
- Wolfenden R, Spence G, Depression of phosphor monoesterase and phosphodiesterase activities in *Aerobacter aerogenes*, *J. Biochim. Biophys. Acta*, 46, 1967, 296-298.
- Takeda K, Tsugita A, Phosphoesterase of *B. subtilis*. II crystallization and properties of alkaline phosphatase, *Journal of Biochemistry*, 61, 1967, 231-241.
- Michaeliss, Beckwith J, Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. *Annual Review of Microbiology*, 36, 1982, 435-465.
- Hulett FM, Stuckmann K, Spencer DB, Sanopoulou T, Purification and Characterization of the secreted Alkaline Phosphatase of *Bacillus licheniformis* MC14: Identification

- of a Possible Precursor, Journal of General Microbiology, 132, 1981, 1389-1395.
17. Somashekhar R, Mahesh M, Guleria N, Rajesh TS, Puttaiah ET. Isolation and Characterization of Extracellular Thermostable Alkaline Phosphatase Enzyme From *Bacillus Spp*, International Journal of Applied Biology and Pharmaceutical Technology, 1(1), 2010, 21-33.
 18. Bernt E, Methods of Enzymatic Analysis (Bergmeyer, H.U., ed) 2nd ed., Volume II, 1974, 868-870.
 19. Buchanan RE, Gibbons NE, Bergey's Manual of Determinative Bacteriology, 8th edition. The Williams and Willikans Company, Baltimore, Maryland, 1974.
 20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the Folin-Phenol reagents, J. Biol. Chem, 193, 1951, 265-275.
 21. Laemmli UK, Cleavage of structural proteins during the assembly of the head bacteriophage T4, Nature, 227, 1970 680-685.
 22. Kolbert KP, Persing DH, DNA sequencing as a tool for identification of bacterial pathogens, Curr. Opin. Microbiol 2, 1999, 299-305.
 23. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D, 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates, J. Clin. Microbiol, 38, 2000, 3623-3630.
 24. Horiuchi T, Horiuchi S, Mizuno D, Possible negative feedback phenomenon controlling formation of alkaline phosphomonoesterase in *Escherichia coli*, Nature, 183, 1959, 1529-1530.
 25. Nahas E, Terenzi HF, Rossi A, Journal of General Microbiology, 128, 1982, 2017-2021.
 26. Yang D, Wang J, Bao Y, An L, Purification and characterization of *Ulva pertusa* Kjellm alkaline phosphatase, 33, 2003, 113-123.
 27. Nomoto M, Ohsawa M, Wang HL, Chen CC, Yeh KW, Purification and characterization of extracellular alkaline phosphatase from an alkalophilic bacterium, Agric. Biol. Chem, 25, 1988, 1643-1647.
 28. Prada DP, Curtze JL, Brenchley JE, Production of Two Extracellular Alkaline Phosphatases by a Psychrophilic *Arthrobacter* Strain, Applied and Environmental Microbiology, 62(10), 1996, 3732-3738.
 29. Yamane K, Maruo B, Purification and characterization of extracellular soluble and membrane-bound insoluble alkaline phosphatases possessing phosphodiesterase activities in *Bacillus subtilis*, J Bacteriol, 134, 1978, 100-107.
 30. Chattopadhyay MK, Devi UK, Gopisankar Y, Shivaji S, Thermolabile alkaline phosphatase from *Sphingobacterium antarcticus*, a psychrophilic bacterium from Antarctica, Polar Biol, 15, 1995, 215-219.
 31. Hulett FM, Stuckmann K, Spencer DB, Sanopoulou T, Purification and Characterization of the secreted Alkaline Phosphatase of *Bacillus licheniformis* MC14: Identification of a Possible Precursor, Journal of General Microbiology, 132, 1986, 2387-2395.
 32. Hydrean C, Ghosh A, Nallin M, Ghosh BK, Interrelationship of carbohydrate metabolism and ALP synthesis in *Bacillus licheniformis* 759/C. Journal of Biological Chemistry, 252, 1977, 6806-6812.
 33. Glew RH, Heath EC, Studies on the extracellular alkaline phosphatase of *Micrococcus sodonensis*, Isolation and characterization, J. Biol. Chem, 246, 1971, 1556-1565.
 34. Sayer PD, Production of alkaline phosphates from *E.coli* in continuous culture. Applied Microbiology, 16(2), 1968, 326-329.
 35. Buainain IB, Kadowaki MK, Polizeli MLTM, Terenzi HF, Jorge JA, Characterization of conidial alkaline phosphatase from thermophilic fungus *Humicola grisea* var. *thermoidea*, Journal of Basic Microbiology, 38, 1998, 85-94.
 36. Bryan L W, Keith W, General principles of biochemical investigation. Principles and techniques of practical biochemistry, 2, 1981, 32-34.
 37. Robert RB, Evan RK, Characterization of a monomeric *E.coli* alkaline phosphatase formed upon a single amino acid substitution, J. Biol. Chem, 278, 2003, 23497-23501.
 38. Hulett FM, Kim EE, Bookstein C, Kapp NV, Edward CW, Wyckoff HW, *B. subtilis* alkaline phosphatase III and IV cloning sequencing and comparison of deduced amino acid sequence with *E.coli* alkaline phosphatase three dimension structure, J. Biol. Chem, 266, 1991, 1077-1084.
 39. Hulett FM, Bookstein C, Jensen K, Evidence for two structural gene for alkaline phosphatase in *B.subtilis*. Journal of Bacteriology, 172, 1990, 735-740.

Source of Support: Nil, Conflict of Interest: None.

