ISOLATION AND CHARACTERIZATION OF EXTRACELLULAR THERMOSTABLE ALKALINE PHOSPHATASE ENZYME FROM *BACILLUS* SPP.

Mahesh.M[1], Guleria Neha[1], Rajesh.T.S[1], Somashekhar.R[1]*, Puttaiah.E.T[2]
[1]Azyme Biosciences Private Limited, Jayanagar, Bangalore, Karnataka-560069.
[2]Kuvempu University, Jnana Sahyadri, Shankaraghatta, Karnataka- 577 451

ABSTRACT: Alkaline phosphatase (E C 3.1.3.1) belongs to the class of hydrolases and catalyzes the alkaline hydrolysis of a number of phosphoric acid esters, nucleotides etc. Alkaline phosphatase was produced from *Bacillus* spp, isolated from soil samples. The *Bacillus* spp. was identified by staining and standard biochemical tests after which screening was done using modified Pikovoskaya's agar method. Production of alkaline phosphatase using different substrates like calcium phosphate along with casein, starch, glucose and glutamic acid was carried out. High activity was found in calcium phosphate along with the casein. The specific activity of the crude extract was found to be 0.825U and it was subjected to purification by DEAE-Cellulose ion exchange chromatography. Finally,36% recovery was obtained. The molar mass was estimated by using 10% SDS-PAGE and was found to be approximately 84 KD. The optimum activity was at pH 8.8 and temperature of 65^0C. Alkaline phosphatase activity was enhanced by $Mg2^{++}$ upto 66% and 80% activity was inhibited by EDTA. Alkaline Phosphatase activity was also confirmed by zymography using malachite green staining method.

KEY WORDS: Alkaline phosphatase, Pikovoskaya's agar, DEAE-Cellulose, SDS-PAGE, Malachite green.

Abbreviations used: ALPase – Alkaline Phosphatase, SDS-PAGE—Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis, pNPP - para-Nitrophenol phosphate, DEAE-cellulose-Diethylaminoethylcellulose.

INTRODUCTION

Alkaline Phosphatase (EC 3.1.3.1) enzyme hydrolyzes the phosphomonoesters from number of organic molecules like ribonucleotides, deoxy-ribonucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid (Holander, 1971). Alkaline Phosphatase is a metallo-dependent enzyme (Mori et al, 1999) which shows its catalytic activity optima at alkaline pH (Rina et al, 2000). Alkaline Phosphatase can be isolated from variety of microorganisms including *E.Coli* (Torriani, 1968), *Pseudomonas* (Friedberg and Avigad 1967), *Aerobactor* (Wolfenden and Spence, 1967) and *Bacillus* species (Takeda and Tsugita, 1967). In all bacteria, ALPase found in the periplasmic membrane which is external to the cell membrane of bacteria (Mickaelis and Beckwith,1982). Usually the ALPase is produced at commercial level from *E.coli* (Seeburg et al 1977) or Calf intestine (Pozidis and Bouriotis,1995).

Studies have been carried out for production, purification and characterization of ALPase from genus *Bacillus*. Like *B.subtilis*,(Glen and Coote,1975), *B.subtilis JH646MS* strain (Hulett et al, 1990), they purified the two ALPase producing proteins. *B.cereus* (Bursik and Nemec, 1999), *B.subtilis KIBGE-HAS* strain newly synthesized by Quader et al (2009). All these studies based on intracellular production of ALPase.

Very little work has been done with respect to extracellular production of ALPase in genus *Bacillus*. Hulett et al (1986) investigated the extracellular production of ALPase in *Bacillus licheniformis* which shows that it synthesizes 10 times more ALPase activity than is reported for other *Bacillus* species (Hydrean et al, 1977). However, the extracellular production of ALPase have been studied in *Micrococcus sodonensis* (Glew and Heath, 1971), *Pseudomonas* spp (Kobori and Taga, 1980), Alkalophilic bacterium (Nomoto et al, 1988), *Haloarcula marismortui* (Goldman et al, 1990) and *Arthobacter* (Prada et al, 1996).

Intracellular production of ALPase is quite tedius and expensive process in comparison to extracellular. This statement is supported by the study of Hulett et al (1986) which explained that extracellular ALPase gave higher specific activity than intracellular ALPase is because of short and simple steps of purification. Thus, the present studies are proposed to be conducted for the isolation and characterization of extracellular alkaline phosphatase from *Bacillus* specie.

MATERIALS AND METHODS

Microorganism Preparation: Microorganism used was isolated from soil sample and identified by standard biochemical tests and staining techniques. Pure cultures were maintained on nutrient agar slants containing 2% agar and pH was adjusted to 7. Screening test for ALPase production was done by using modified Pikovskaya's Agar method.

Growth Conditions: Cultures used for enzyme production were grown at 37^0C for 24 hrs shaking (180 rpm) in modified minimal essential media (MEM). Calcium phosphate along with casein, starch and glucose as substrates in different concentrations to study the regulation of phosphatase synthesis. Bacterial growth was assayed by spectrophotometrically at 600nm.

Enzyme Assay: ALPase activity was measured by using Stopped Spectrophotometric Rate Determination method (Bernt, 1974). The absorbance of released para-nitrophenol phosphate (pNPP) was determined at 410nm.

Protein Determination: Protein concentrations were estimated by Lowery et al (1951) method and absorbance determined at 660nm by using bovine serium albumin (BSA) as standard.

Purification of Extracellular ALPase: The cell free media collected was subjected to different steps of purification including ammonium sulphate $(NH_4)_2SO_4$ precipitation, dialysis and DEAE-cellulose ion–exchange chromatography by using gradient elution buffer.

Enzyme Characterization: SDS PAGE was performed according to the Laemmli (1970) 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 silver nitrate solution (Blum et al, 1987) and Ezee blue. The optimum pH, temperature were measured. The effect of substrate concentration, thermal stability of enzyme, activatiors, inhibitors and time course of intervals on enzyme activity were also studied.

Zymography: PAGE was executed according to the method of Laemmli (1970). Electrophoresis was done under non-denaturing conditions. Gels were incubated at 22°C in 50 mM NaCl (pH 9.6) and 2mM $CaCl_2$ with pNPP as substrate. Phosphatase release was detected by Malachite green staining procedure (Queiroz-Claret and Meunier, 1993).

RESULTS AND DISCUSSION

In our study, we have isolated *Bacillus* specie from soil and we reasoned that the genetic diversity of soil bacteria is high (Clegg et al , 1998; Oyreas and Torsvik, 1998; Torsvik et al, 1990) or soil contain great diversity of bacteria, with many of organisms belonging to groups for which no cultivated representatives are known. From soil we can isolate some members of groups that to date have not been grown in pure culture. Thus, soil is an excellent source for unknown microorganisms and it has been studied that *Bacillus* genus is most frequently isolated from soil (Steubing, 1993). As the microorganisms have been known to produce specific enzymes, secondary metabolites, single cell proteins of economic benefits. w.r.t. production of enzyme, although, the earliest reports concerning exploitation of enzymes were documented in the late 1800,true industrial applications of enzymes only began in earnest in the late 1960s.And the majority of enzyme used in industrial/ biotechnological applications are derived from particular bacteria (*Bacillus)* and fungi (*Aspergillus)* (odofine.com.2004). Hence, safe organisms must be used for consumer related applications.

Purification of ALPase: Extracellular ALPase was eluted on DEAE-cellulose, (Reid and Wilson, 1971) after elution the specific activity was increased up to 11 U/mg and purification % recovery was found to be 36 % (Table 1) with fold purification of 14. While, in contrast to our results, a 135 fold purification and 50% yield of extracellular ALPase was obtained in *Lysobacter enzymogenes* (Richard and Tigerstrom, 1984) and in case of *Bacillus licheniformis* studied by Hulett et al (1986), fold purification and yield were found to be 2.4 and 10%, respectively. Yield of ALPase are dependent on the strain of bacteria used (Sayer, 1968). This may be the reason for the difference between our results and those reported earlier. One unit of phosphatase is the amount which hydrolyzes 1µmol of substrate per minute at pH 8.8 and temperature 37°C.

Table 1. Purification of Alkaline Phosphatase

Sample	Enzyme Activity (U/ml)	Specific Activity (U/mg)	Total Activity (U)	% Recovery	Fold Purification
Crude Enzyme	0.33	0.825	33	-	3.02
Pure Enzyme	0.109	11	0.545	36	14

Molecular Mass Determination: Electrophoretic analysis of extracellular ALPase from *Bacillus* specie has been carried out. As ALPase found in both diameric and monomeric forms, we found a single band with approximate molecular mass of 84KD. The homodiameric forms of ALPse, with a molecular mass of 54KD have been previously reported *E.pyrococcus abyssi* (Robert and Evan, 2003; Hulett et al, 1990; Hulett et al, 1991). The SDS-PAGE of *B. stearothermophilus* alkaline phosphatase showed a single protein band of 32KD (Mori et al, 1999).

Effect of Temperature: Effect of temperature was studied on enzyme activity by incubating on different temperatures 10°C- 70°C per 10 minute. The observation showed that the enzyme is thermostable, as the temperature increased the activity of enzyme also increased and enzyme showed its optimum activity at 65°C and became inactivated at 70°C (Figure 1), similar to the values reported for *A. caespitosus* (Guimaraes et al, 2003), *Humicola grisea* (Buainain et al, 1998) and *Rhizopus microspores* (Junior et al, 2008). 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 (Bryan and Keith, 1981). A thermostable ALPase isolated from *Geobacillus thermodenitrificans* T2 by Zhang et al, (2008) with optimal pH 9 and temperature 65^0C which showed that these properties make this enzyme suitable for applications in molecular cloning or amplifications.

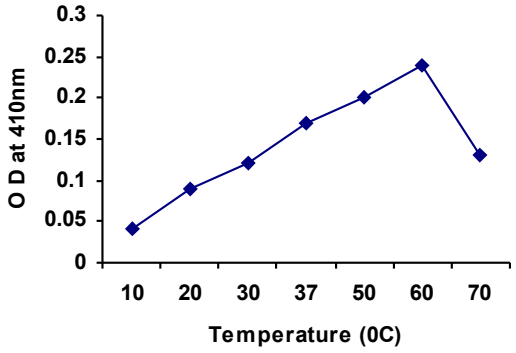

Figure1. Effect of temperature on alkaline phosphatase activity.

Effect of pH: Activity of enzyme was studied on different pH ranges 3-11. In our study**,** the optimum enzyme activity was found at pH 8.8 (Figure 2). This result is supported by (Prada et al 1996) who studied *Anthrobactor* Strain for extracellular production of ALPase. As the pH increases the decrease in enzyme activity was observed and only 30% activity was noted at pH 11. Increase in pH effect the charges on the amino acids with in the active site such that the enzyme is not to be able to form enzyme-substrate complex. Thus, there is decrease in enzyme activity (Hulett et al, 1971).

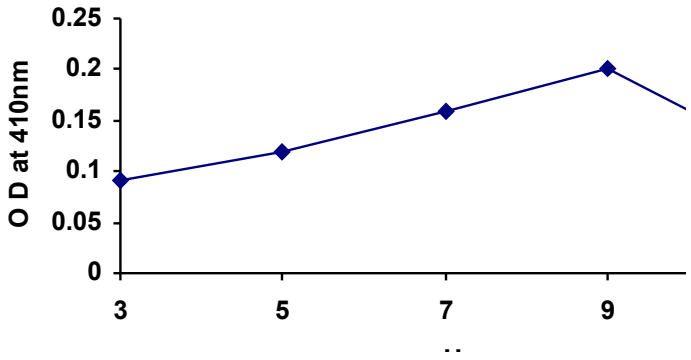

Figure 2. Effect of pH on alkaline phosphatase activity.

Effect of Time Course of Reaction: Effect of time was studied on enzyme activity by incubating enzyme-substrate complex at 37°C for different time intervals. The optimum activity was obtained when incubation was done for about 30 minutes. After this as the time period increased the activity became constant (Figure 3). This may be because of the thermal liable nature of the enzyme, with the increase in time period the temperature starts breaking the bonds between two amino acids. (Hulett and Campbell, 1971).

Effect of Substrate Concentration: ALPase shows higher substrate specificity to pNPP (Robert and Evan, 2003; Sebastein et al, 2001). 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 (Figure 4). Further the activity was constant. The Michealis constant for pNPP at pH 8.8 was 0.26mM and Vmax was 0.28mM. Some scientists reported that Km value for ALPase was 1.11mM in *B. stearothermophilus* (Mori et al, 1999)

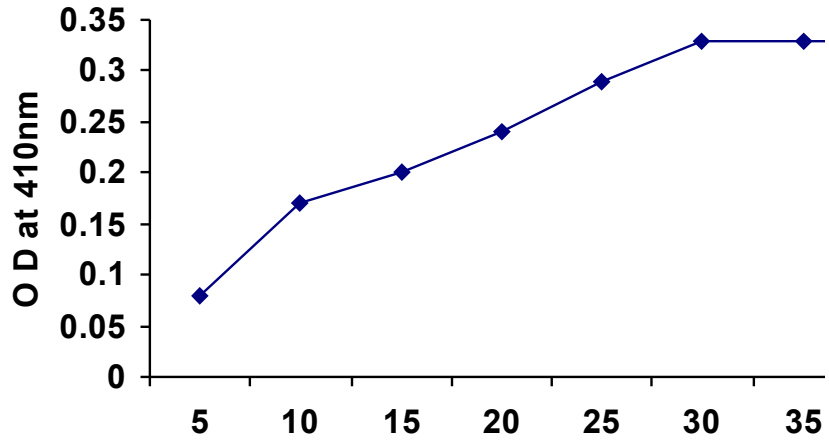

Figure 3. Effect of time on alkaline phosphatase activity.

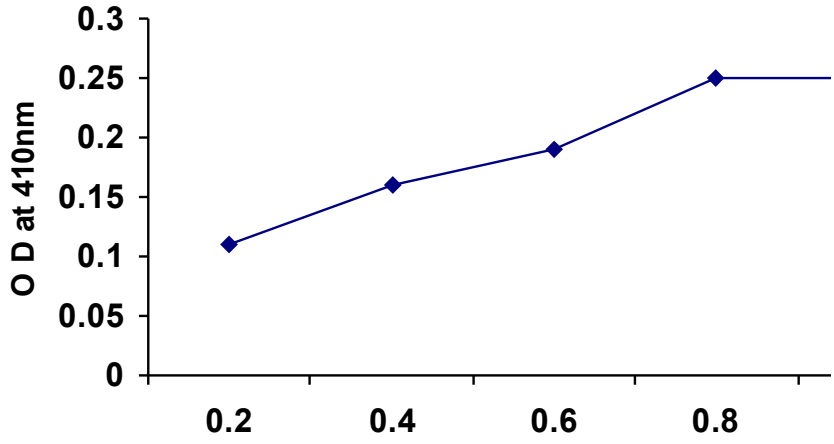

Figure 4. Effect of substrate concentration on alkaline phosphatase activity.

Effect of Inhibitors and Metal ions: The activity of ALPase enzyme was strongly inhibited by diavalent ions i.e. EDTA (upto 80%), a similar situation has been described in other bacterial phosphatases which are Zn^{2+} and Ca^{2+} dependent (Glew and Heath, 1971; McComb et al, 1979; Reichenbach and Dworkin, 1981) and are strongly inhibited by EDTA, while the reverse is true in case of extracellular enzyme produced by *Lysobacter enzymogenes* (Richard and Tigerstrom, 1984). And NH_4^{2+}, Cu^{2+} showed very little inhibitory effect. On the other hand, the enzyme activity was enhanced by Mg^{2+} upto 66% and Mn^{2+}, Zn^{2+}, Ca^{2+} also stimulate the activity near to 34%,which indicate that the enzyme is metallo-dependent (Posen, 1967). ALPase classically considered to be Zn^{2+} and Mg^{2+} dependent, especially, in *E.coli* and mammalian ALPase (Kim and Wyckoff, 1991). The studies of effect of metal ions on ALPase activities in *Bacillus* sp. suggested that there is strong interaction between metal ions and enzyme as ALPase may have several metal binding sites. These results are consistent to those of reported earlier (Goldman et al, 1990; Spencer et al, 1981) and enzyme may be Mg^{2+} dependent.

Conclusion: In our studies, the ALPase shows its optimum activity at pH 8.8 and temperature 65^0C, which indicate that the enzyme is thermostable. Apart from higher temperature, the thermostable enzymes are also known to withstand denaturants of extremely acidic and alkaline conditions. These enzymes are highly specific with inherent stability and thus, have considerable potential for many industrial and commercial applications.

This present investigation emphasized on the need of more research to be carried on *Bacillus* species to produce extracellular alkaline phosphatase, so that some good strains of *Bacillus* can be harvested for the commercial production of ALPase apart from its other intracellular sources such as *E.coli* and calf intestine which are comparatively costlier and have very complex downstream processes.

REFERENCES

Bernt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed) 2nd ed., Volume II, pp 868-870, Academic Press, Inc., New York, NY

Bursik M and Nemec M. (1999). Alkaline phosphatase production during sporulation of *Bacillus cereus.* Folia Microbiology. **44**(1): 90-92

Biotechnology: The food solutions. (2004). http://www.odofine.com

Blum H, Beier H and Gross H J. (1987). Improved silver staining of plant protein, RNA and DNA in polyacrylamide gels. Electrophoresis. **81**: 93-99

Bryan L W and Keith W (1981). General principles of biochemical investigation. Principles and techniques of practical biochemistry.(2nd ed) .32-34p

Buainain l B, Kadowaki M K, Polizeli M L T M, Terenzei H F and Jorge J A. (1998). Characterization of conidial alkaline phosphatase from thermophilic fugus *Humicola grisea* var. *thermoidea.* Journal of Basic Microbiology. **38**: 85-94

Clegg C D, Ritz K and Griffith B S. 1998. Broad-scale analysis of soil microbial community DNA from upland grasslands. Antonie Leeuwenhoek **73:**9-14

Friedberg I and Avigad G. (1967). Some properties of alkaline phosphatase of Ps. flourescens. Europian Journal Biochemistry. **1**: 193-198

Glenn A R and Coote J G. (1975). Cytochemical studies on alkaline phosphatase production during sporulation in *Bacillus subtilis.* Journal of Biochemistry. **152:**85-89

Glew R H and Heath E C. (1971). Studies on the extracellular alkaline phosphatase of *Micrococcus sodonensis* : Isolation and characterization. J. Biol. Chem. **246:**1556–1565 Guimaraes L H S, Terenzi H F, Jorge J A, Leone F A and Plizeli M L T M. (2003). Extracellur alkaline phosphatase from the filamentous fungus *Asperigillus caespitosus*: Purification and Biochemical characterization. Folia Microbiology.**48**:627-632.

Holander V. P. (1971). Acid Phosphatases. **In**: Enzymes. P.D. Boyer (Ed.) Academic Press, NewYork pp 450-498

Hulett F M, Bookstein C and Jensen K. (1990). Evidence for two structural gene for alkaline phosphatase in *B. subtilis*. Journal of Bacteriology. **172**: 735-740

Hulett F M, Stuckmann K, Spencer D B and Sanopoulou T. (1986). Purification and Characterization of the Secreted Alkaline Phosphatase of *Bacillus licheniformis* MC14: Identification of a Possible Precursor. Journal of General Microbiology. **132:** 2387-2395

Hydrean C, Ghosh A, Nallin M and Ghosh B K. (1977). Interrelationship of carbohydrate metabolism and APase synthesis in *Bacillus licheniformis* 759/C. Journal of Biological Chemistry. 252: 6806-6812

Hulett F M, Kim E E , Bookstein C, Kapp N V, Edward C W and Wyckoff H W. (1991). *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**: 1077-1084

Hulett-cowling F M and Campbell L. (1971). Purification and properties of an alkaline phosphate of *B. licheniformis*. J. Biochem. **10**: 1364-1371

Junior A B, Guimaraes L H S, Terenzi H F, Jorge J A, Leone F A and Plizeli M L T M. (2008). Purification by chemical characterization of thermostable alkaline phosphatases produced by *Rhizopus microspores* var. *rhizopodiformis*. Folia Microbiology. **53**(6):509-516

Kim E E and Wyckoff H W. (1991). Reaction mechanisn of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. Journal of Molecular Biology. **218**: 449-464

Kobori H and Taga N. (1980). Extracellular alkaline phosphatase from marine bacteria: purification and properties of extracellular phosphatase from a marine *Pseudomonas* sp. Can. Journal of Microbiology. **26:**833–838

Laemmli U K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature **227:** 680-685

Lowry O H, Rosebrough N J , Farr A L and Randall R J. (1951). Protein measurement with the Folin-Phenol reagents. J. Biol. Chem. **193:** 265-275

Mc Comb R B, Bowers G N and Posen S. (1979). Alkaline phosphatase. Plenum press, New York

Michaeliss and Beckwith J . (1982)**.** Mechanism of incorporation of cell envelope proteins in *Escherichia coli.* Annual Review of Microbiology. **36:** 435-465

Mori S, Okamoto M, Nishibori M, Ichimura M, Sakayama J and Endo H. (1999). Purification and characterization of alkaline phosphatase from *Bacillus stearothermaphilus.* Biotechnol. Applied Biochem., **29:** 235-239

Nomoto M, Ohsawa M, Wang H-L, Chen C-C and Yeh K-W. (1988). Purification and characterization of extracellular alkaline phosphatase from an alkalophilic bacterium. Agric. Biol. Chem. **52:**1643–1647

Ovreas L and Torsvik V. (1998). Microbial diversity and community structure in two different agricultural soil communities. Microb. Ecol. **36:**303-315

Posen S. (1967). Alkaline phosphatase. Ann. Int. Med. **67**:183-203

Pozidis C, Bouriotis V. (1995). Preparation of high purity alkaline phosphatase from calf intestine using dye-ligand chromatography. Bio-separation. **5:**89-93

Prada Paloma De, Jennifer Loveland-Curtze, and Jean E. Brenchley. (1996). Production of Two Extracellular Alkaline Phosphatases by a Psychrophilic *Arthrobacter* Strain. Applied And Environmental Microbiology. **62** (10):3732–3738

Queiroz-Claret C and Meunier J C. (1993). Staining technique for phosphatases in polyacrylamide gels. Anal. Biochem. **209:**228–231

Reid, T W and Wilson I B. (1971). E. coli alkaline phosphatase. Enzymes **4**:373-415

Reichenbach H and Dworkin M. (1981). The order Cytophagales (with addenda on the genera Herpetosiphon, Saprospira, and Flexithrix). **In**: The prokaryotes. Starr M P, Stolp H,

Truper H G, Bolows A, and Schlegel H G (eds.) Springer Verlag, New York. 356-379 p

Richard G and Tigerstrom Von. (1984).Production of Two Phosphatases by *Lysobacter enzymogenes* and purification and characterization of the extracellular enzyme. Applied and Environmental Microbiology. **47:** 693-698

Rina M, Pozidis C, Mavromatis K, Tzanodaskalaki M, Kokkinidis M and Bouriotis V. (2000). Alkaline phosphatase from the Antarctic strain TAB5. Properties and psychrophilic adaptations. Europian Journal of Biochemistry. **267:** 1230-1238

Robert R B and Evan R K. (2003). Characterization of a monomeric *E.coli* alkaline phosphatase formed upon a single amino acid substitution. J. Biol. Chem. **278:** 23497-23501

Sayer P D.(1968).Production of alkaline phosphates from *E.coli* in continuous culture. Applied Microbiology. **16**(2):326-329

Seeburg PH, Shine J, Martial J A, Baxter J D, Goodman H M. (1977). Nucleotide sequence and amplification in bacteria of structural gene of rat growth hormone. Nature**. 270**:486-494

Sebastien Z, Jean L R, Didier H, Yannick G, Joseph B and Jacques D. (2001). Characterization of highly thermo-stable alkaline phosphatase from the *Euryarchaeon pyrococus abyssi*. J. Appl. Env. Microbiol. **67**: 4504-4511

Spencer D B, Chen C P and Hulett F M. (1981). Effect of cobalt on synthesis and activation of *Bacillus licheniformis* alkaline phosphatase. Journal of Bacteriology. **145**:926-933

Steubing, P. M. (1993). Isolation of an unknown bacterium from soil. **In:** Tested studies for laboratory teaching. C. A. Goldman (Ed). **14**: 81-114. Proceedings of the 14th Workshop/Conference of the Association for Biology Laboratory Education (ABLE). 240 p

Takeda K and Tsugita A. (1967). Phosphoesterase of *B. subtilis*. II crystallization and properties of alkaline phosphatase. Journal of Biochemistry. **61**: 231-241

Torriani A. (1968). Alkaline phosphatase subunit and their dimerization *in vivo*. Journal Bacteriology. **96**: 1200-1207.

Torsvik V, Goksøyr J and Daae F L**.** (1990). High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782-787

Wolfenden R and Spence G. (1967). Depression of phosphomonoesterase and phosphodiesterase activities in *Aerobacter aerogenes*. J. Biochim. Biophys. Acta. **46**: 296-298

Zhang Yong,Chaoneng Ji, Zhang X, Yang Z, Peng J, Qiu R, Xie Y and Mao Y. (2008). A moderately thermostable alkaline phosphatase from *Geobacillus thermodenitrificans* T2: Cloning, expression and biochemical characterization. Appl Biochem Biotechnol. **151:**81-92

****