EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 6
338-343
Year: 2018

IMPACT OF MUTATION ON *BACILLUS SP* FOR THE PRODUCTION EXTRACELLULAR ALKALINE PHOSPHATASE (ALPs)

***Somashekhar R.**

Azyme Biosciences Private Limited, Bengaluru, Karnataka, India.

***Corresponding Author: Somashekhar R.**
Azyme Biosciences Private Limited, Bengaluru, Karnataka, India.

Article Received on 29/03/2018 **Article Revised on 18/04/2018** **Article Accepted on 08/05/2018**

ABSTRACT
ALPs used as therapeutic agents and therapeutic targets show several application in clinical medicine and in biotechnology. An example used in a technique for labelling monoclonal antibodies. ALPs has the biggest market volume, to reach the market value we made an attempt to enhance the ALPs production by mutation on organisms. Six organisms were isolated using soil samples collected from different localities of Karnataka. Among the six organisms, one of the organism showed more activity based on the maximum yellow colour zone developed surrounding the organisms on the selective media and also maximum ALPs activity. This organism was identified as *Bacillus sp* based on the morphological, biochemical tests and 16s rRNA sequence. ALPs activity was observed maximum at 35℃, pH 7 and 30 hrs of incubation. This organism was subjected to UV exposure at different time intervals such 5, 10, 15, 20 and 25min with different distance such 20 cm, 40 cm and 60 min at 250 nm. The result reveals that 22% enzyme activity increased at 40 cm with 15 min. In addition to this the organism was subjected X-ray with different greys such as 30, 60 and 90. Result reveals that, 6% of the enzyme activity was increased at 30 greys. After purification by Ammonium sulphate precipitation, dialysis, ion exchange chromatography and gel filtration, Fold purification was increased up to 4.02 and 40% yield. Molecular weight of the enzyme 64kDa was determined by SDS-PAGE.

KEYWORDS: Mutation, X-ray, Ion exchange Gel filtration and SDS –PAGE.

INTRODUCTION
Alkaline phosphatases (EC 3.1.3.1) are hydrolytic enzyme which hydrolyse of more number of different phosphoric acid esters at alkaline condition. ALPs used as therapeutic agents and therapeutic targets and show several application in clinical medicine and in biotechnology. ALPs used in clinical diagnosis as a marker and medicine in therapeutic drug for lipopolysaccharide-mediated diseases. ALP has the biggest market volume share of $20 million. ALP is isolated from various sources such as microorganisms, tissues of different organs, body and connective tissues of invertebrates and vertebrates, animals as well as from human beings.

Alkaline Phosphatase is a metallodependent enzyme (Mori et al, 1999). In all bacteria, ALPs found in the periplasmic membrane which is external to the cell membrane of bacteria (Mickaelis and Beckwith, 1982). Alkaline Phosphatase can be isolated from variety of microorganisms including *E.Coli* (Torriani, 1968), *Pseudomonas* (Friedberg and Avigad 1967) and *Bacillus species* (Takeda and Tsugita, 1967).

UV rays are important inducers for altering the bio catalytic activity. Main effect of this light is to modify the structure of pyrimidine (cytosine and thymine) causing the formation of thymine dimmer which distort the structure of DNA. UV mutation are very harmful but at some time it may lead to better adaptation of an organism to its environment with improved biocatalytic performance. According to Agrawal et al (1999) UV radiation was a potent mutagen. UV irradiation was found to be best for the improvement of strains like *Aspergillus niger* for maximum production of various enzymes.

In the previous studies, isolation of the various type of organisms for the production of ALPs have been reported, but present prospect in biotechnology improve the production of enzymes using mutation on the organisms playing vital role for the large scale production.

2. METHODS AND MATERIALS
2.1. Collection soil samples
Thirty five soil samples were collected from different location of the Karnataka. Soil samples were collected

from 15 cm depth of surface in sterile plastic bags and carried to laboratory for isolation of organisms.

2.2. Isolation of organisms

The organisms were isolated using suspension culture method. In this process, 1g of soil sample added to 10 ml of saline used as inoculum. 1ml of inoculum was added to sterile petri plate and above the inoculum, 15 ml of sterile M9 media (Na_2HPO_4 - 6 g KH_2PO_4 - 3.0 g NaCl - 0.5 g, NH_4Cl - 1.0 g, $MgSO_4$ - 0.01g, Agar - 20g and distilled water- 100 ml) along with 10 mg of pNPP was poured. Allow solidify and incubated at 37℃ for 24hrs in incubator. After 24hrs incubation, colonies showing zone of yellow colour and sub cultured on LB agar slants.

2.3. Identification of the organisms

Maximum yellow colour zone produced organisms are identified based on the morphological character, biochemical test and 16S RNA sequencing.

2.4. Effect of Incubation time: 200ml M9 media was prepared in 500ml in conical flask and inoculated the loopful organisms and incubated the organisms at 37℃. For every 12hrs till the 60 hrs ALPs assay was carried out by spectrophotometric method by (Bernt 1974). The absorbance of released pNPP was determined at 410 nm.

2.5. Effect of pH: 100ml M9 media along with 10mg pPNP was prepared in 250 ml conical flask and pH was adjusted to 4 to 9. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The culture was incubated in rotary shaker at 35°C for 36 hr of cultivation. After incubation the cell free filtrate was used for enzyme assay.

2.6. Effect of Temperature: Six conical flask of 100ml of m9 media along with 10mg pNPP was prepared. pH was adjusted to 7 and inoculated the loopful organisms. Incubated the conical flasks in various temperature such as 25℃, 30℃, 35℃, 40℃, 45℃ and 50℃ for 36 hrs. After incubation the cell free filtrate was used for enzyme assay.

2.7. Effect of UV on organism: Based on Mcfarland standard, 0.5 ml of the suspension was spread on different LB agar plate uniformly. The organisms exposed to UV wave length 254 nm at the height of 50 cm for 5, 10, 15, 20 and 25 mins**.** After exposure, replicated was done to achieve the survived organism. Loopfull of mutated organism was inoculated into 100ml of M9 media along with maintained standard condition. After incubation the cell free filtrate was used for enzyme assay.

2.8. Effect of X-ray on organism: Based on Mcfarland standard, 0.5 ml of the suspension was spread on different LB agar plate uniformly. The organisms exposed to X- rays with different intensities such as 10 keV, 15keV and 20 Kev. After exposure replicated was done to achieve the survived organism. Loopfull mutated organism was inoculated into 100ml of M9 media along with maintained standard condition. After incubation the cell free filtrate was used for enzyme assay.

2.9. Purification of Extracellular ALPs: Cell free media was used for production of enzyme from mutants strain. In this process, ammonium sulphate precipitation, dialysis, ion exchange chromatography and gel filtration was used to purification of enzyme.

2.10. Enzyme Characterisation: The optimum pH, temperature and substrate concentration, were measured. SDS PAGE was performed according to the Laemmli (1970) with the 4% Acrylamide stacking gel and 12% Acrylamide separating gel to determine the molecular mass and purity of protein. Staining was carried out with Comassive brilliant blue solution.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification organisms: The genetic diversity of soil bacteria is high (Clegg et al 1998) or soil contain great diversity of bacteria. The organism which showed zone of yellow colour (Fig A) surrounding the organism was identified as *Bacillus sp* . Organism is gram positive based on the gram staining and based on the biochemical tests such as oxidase catalase gelatine liquefaction, starch hydrolysis, lipid hydrolysis dextrose, sucrose and nitrogen reductase are positive result, Indole, *voges* proskauer, Methyl rest citrate urease activity are negative result. In addition to this, isolated organism confirmed by the 16S RNA sequencing (Fig B). One of the best advanced processes to identification of bacteria analysis by 16S rRNA sequences is the single best method.

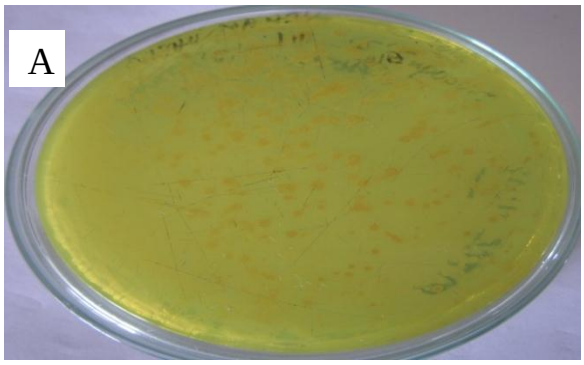
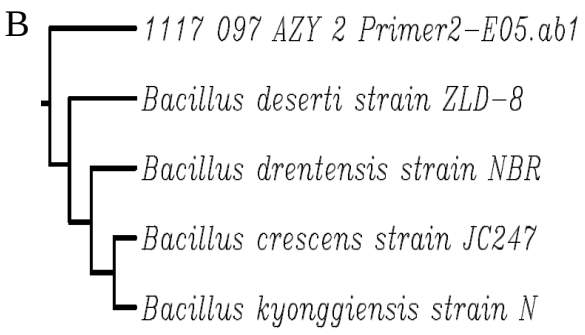


Fig A: Organisms showed Yellow colour.



B: Phylogentici tree Based 16S RNA seuencing.

3.2. Effect of Incubation period: Production of enzyme is required for particular period of time, results revealed that maximum activity was found at 24hrs to 48hrs but maximum activity was at 36hrs up to 96 U/ml (fig 3). The growth and enzyme yield increased with passage of time and the maximum enzyme secretion was found after 24 hrs but on prolong incubation. Production decreased may be due to change in pH (Qureshi et al 2010).

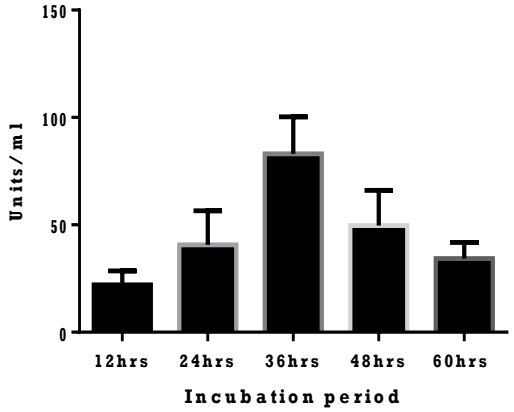


Fig 3: Effect of Incubation period for the production of enzyme.

2.3. Effect of pH: pH playing a very important role in the growth of the organisms and also in the production of ALP enzyme. pH was used 4 to 9 for the production, maximum activity was showed at 7 and 8 pH but very low activity was at pH 4 (Fig 4). During the production of enzyme, there were no much significant changes in the pH of the medium. The results are in the accordance with the reported results of (Danielle and Raymond 1984).

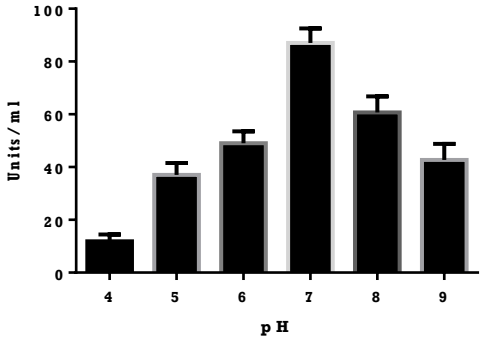


Fig 4: Effect of pH for the production of enzyme.

2.5. Effect of Temperature: Temperature impacts all the functional activities in living cell and is one of the important conservational aspects to control the growth, microbial activities, and normal functioning of enzyme (Demark and Batzing 1987). Production of ALPs was carried out for the different temperature such as 25℃, 30℃, 35℃, 40℃, 45℃ and 50℃, results reveals that maximum enzyme activity showed at 35℃ to 40℃(fig 5).

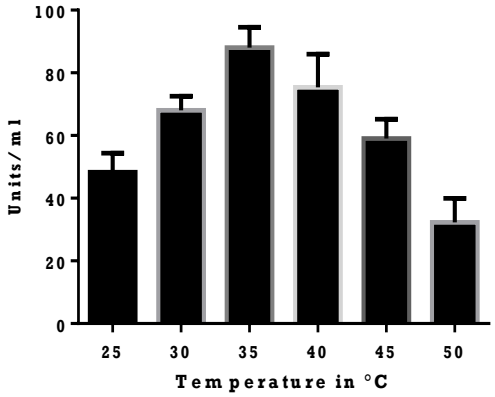


Fig 5: Effect of temperature for the production of enzyme.

2.6. Effect of UV exposure on the organism

Ultraviolet radiation may alter enzymes in numerous biologic systems by producing alterations in proteins. Peptide bonds may be split, photochemical oxidations may occur, sulfide and disulfide bonds may be altered (Parrish et al 1982). In our study, results reveals the same, the production ALPs enzyme increased up to 22% after exposure to UV radiation for 20 min at 254 nm and also activity was decreased in 25 min.

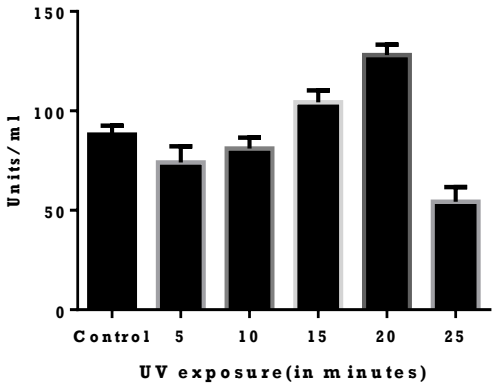


Fig 6: Effect of UV exposure for the production of enzyme.

2.7. Effect of X-rays on the organism: Many investigators have suggested on the basis of general observations that small doses of X-rays may stimulate cellular activity and growth, but convincing proof of such action has been wanting (Charles et al., 1933). We carried out the effect of x-ray in different intensity, but results reveals that there is no significant increase in the production (fig 7).

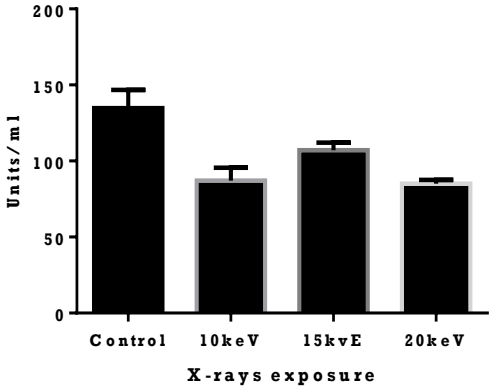


Fig 7: Effect of X-rays for the production of enzyme.

2.8. Purification of ALPs enzyme: Production of enzyme in standard condition such as incubation period 36hrs, pH at 7 and temperature 35℃. After production, cell free media was used for the purification 70% ammonium sulphate precipitation, dialysis, ion exchange and gel filtration chromatography, the fold purification was increased up to 4.02 and 40.5 yield (Table 1) was obtained. In contrast to our results, a 135 fold purification and 50% yield of extracellular ALP was obtained in *Lysobacter enzymogenes* (Sayer., 1968) and in case of *Bacillus licheniformis* studied by (Von Tigerstrom., 1984) fold purification and yield were found to be 2.4 and 10%, respectively. Yield of ALP is dependent on the strain of bacteria used (Sayer., 1968). This may be the reason for the difference between our results and those reported earlier

Table1: Summary of purification ALPs enzyme.

Samples	Enzyme Activity(U/ml)	Protein (mg/ml)	Specific activity(U/mg)	Fold Purification	% of Yield
Crude	87	1.16	75	1	100
Ammonium sulphate PPT	92	0.96	95.8	1.27	82.7
Dialysis	96	0.72	133	17.7	62
Ion Exchange Chromatography	137	0.56	244.6	3.26	48.2
Gel Filtration Chromatography	142	0.47	302.12	4.02	40.5

2.9. Characterisation of Enzyme: Activity of ALPs was carried out for different pH, maximum activity was showed at pH 8, and activity was decreased at extreme pH such as pH 4 and pH 10. As the pH increased decrease in enzyme activity was observed. 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-cowling and Campbell 1971). Also enzyme activity was determined at different temperatures. Result reveals that 35℃ was best temperature for the ALPs activity. 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). The maximum activity was showed at 100µg of pPNP substrate similarly ALPs shows higher substrate specificity to pNPP (Robert and Evan, 2003; Sebastein et al, 2001).

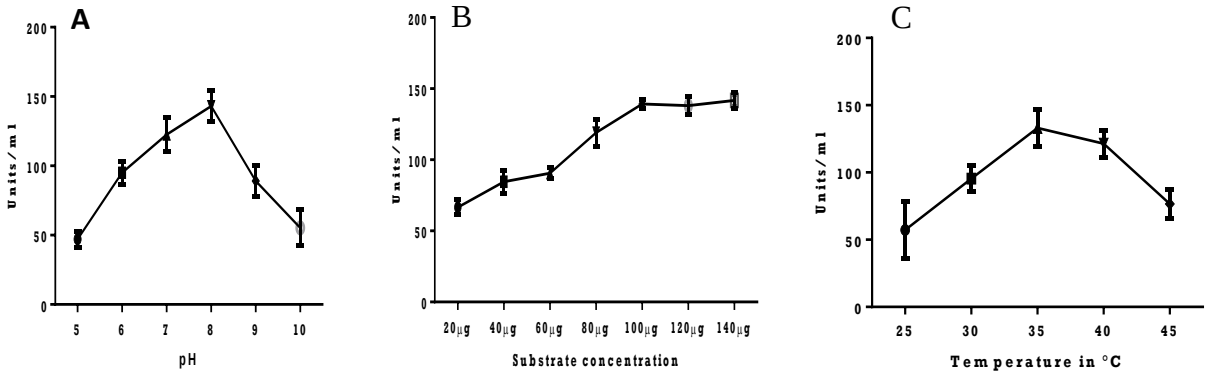


Fig 8 A: Effect of pH. **Fig 8 B: Substrate Concentration.** **Fig 8 C: Temperature on Enzyme Activity.**

2.10. Molecular weight Determination: Molecular weight of enzyme ALPs was determined by SDS PAGE. Based on the molecular weight of partial purified enzyme was 64 kDa. The SDS-PAGE of *B. stearothermophilus* ALP showed a single protein band of 32 kDa (Mahesh et al 2010), *P. aeruginosa* ALP appeared as 68 kDa 30). Hulett et al (1991) found 47 kDa from *Bacillus subtilis* and (Ishida et al 1998)

purified a 41.8 kDa phosphatase from *Schewnella sp*, review reveals that molecular weight varies in organisms.

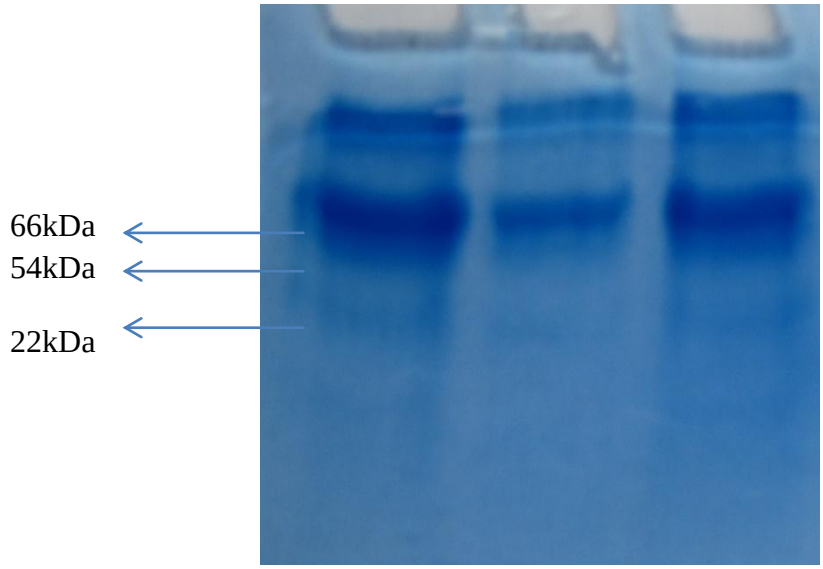


Fig 9: Molecular weight of ALPs by SDS-PAGE.

CONCLUSION

Based on this study mutation on the organism will be the future aspect for researcher to develop new concept of strain improvement for increase the production of enzyme in large scale. Our results concluded that UV exposure is best method to obtain mutant strain for the production of ALPs.

REFERENCES

1. Agrawal R, Deepika NU and Joseph R. Strain improvement of *Aspergillus sp*. and *Penicillium sp*. by induced mutation for biotransformation of alpha-penine to verbenol. Biotech. Bioeng, 1999; 63: 249-252.
2. Bernt E. Methods of Enzymatic Analysis (Bergmeyer HU, editor) 2nd edition, Volume II, 1974; 868-870.
3. Bryan LW and Keith W. General principles of biochemical investigation. Principles and techniques of practical biochemistry.(2nd ed), 1981; 32-34.
4. Charles A. Shiull and John W. Mitchell. stimulative effects of x-rays on plant growth' plant physiology fourth edition, 1933.
5. Danielle A, Raymond P. Optimized extracellular production of alkaline phosphatase by lky mutants of *Escherichia coli* K12. Appl Microbiol Biotechnol, 1984; 19: 5-12.
6. Demark PJV, Batzing BL. The microbes, an introduction to their nature and importance. (1stedn). San Francisco, USA: Benjamin/Cummings, 1987.
7. Friedberg I and Avigad G. Some properties of alkaline phosphatase of Ps. flourescens. Europian Journal Biochemistry, 1967; 1: 193-198.
8. Hulett FM, Kim EE, Bookstein C, Kapp NV, Edwards CW, et al. Bacillus subtilis alkaline phosphatases III and IV. Cloning, sequencing, and comparisons of deduced amino acid sequence with *Escherichia coli* alkaline phosphatase three-dimensional structure. J Biol Chem, 1991; 266: 1077-1084.
9. Hulett-cowling FM and Campbell L. Purification and properties of an alkaline phosphate of *B. licheniformis*. J. Biochem, 1971; 10: 1364-1371.
10. Ishida Y, Tsuruta H, Tsuneta S, Uno T, Watanabe K, et al. Characteristics of psychrophilic alkaline phosphatase. Biosci Biotechnol Biochem, 1998l 62: 2246- 2250.
11. Laemmli U K. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature, 1970; 227: 680-685.
12. Mahesh M, Guleria N, Rajesh TS, Somashekhar R, Puttaiah ET. Isolation and Characterization of Extracellular Thermostable Alkaline Phosphatase Enzyme from *Bacillus Spp*. International Journal of Applied Biology and Pharmaceutical Technology, 2010**;** 1: 21-33
13. Michaeliss and Beckwith J. Mechanism of incorporation of cell envelope proteins in Escherichia coli. Annual Review of Microbiology, 1982; 36: 435-465.
14. Mori S, Okamoto M, Nishibori M, Ichimura M, Sakiyama J, et al. Purification and characterization of alkaline phosphatase from *Bacillus stearothermophilus*. Biotechnol Appl Biochem, 1999; 29: 235-239.
15. Parrish JA, Jaenicke KF and Anderson RR. Erythema and melanogenesis action spectra of normal human skin *Photochem. Photobiol*, 1982; 36: 187-91.

16. Qureshi AS, Dahot MU, Panhwar SI. Biosysnthesis of Alkaline Phosphatase by Escherichia coli Efrl 13 in Submerged Fermentation. World Applied Sciences Journal (8 Special Issues of Biotechnology & Genetic Engineering), 2010; 50-56.
17. Robert RB and Evan RK. Characterization of a monomeric *E.coli* alkaline phosphatase formed upon a single amino acid substitution. J. Biol. Chem, 2003; 278: 23497-23501
18. Sayer PD. Production of alkaline phosphatase from *Escherichia coli* in continuous culture. Appl Microbiol, 1968; 16: 326-329.
19. Sebastien Z, Jean LR, Didier H, Yannick G, Joseph B and Jacques D. Characterization of highly thermostable alkaline phosphatase from the *Euryarchaeon pyrococus abyssi*. J. Appl. Env. Microbiol., 2001; 67: 4504-4511
20. Takeda K and Tsugita A. Phosphoesterase of B. subtilis. II crystallization and properties of alkaline phosphatase. Journal of Biochemistry, 1967; 61: 231-241.
21. Torriani A. Alkaline phosphatase subunit and their dimerization in vivo. Journal Bacteriology, 1968**;** 96: 1200- 1207.
22. Von Tigerstrom RG. Production of two phosphatases by *Lysobacter enzymogenes* and purification and characterization of the extracellular enzyme. Appl Environ Microbiol, 1984; 47: 693-698