

OPTIMIZE ENVIRONMENTAL PRODUCTION CONDITIONS OF EXTRACELLULAR ALKALINE PHOSPHATASE FROM *Bacillus* sp. I

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ABSTRACT

Background: Alkaline phosphatase (ALPase) has vital applications in many aspects of life as molecular biology and genetic engineering applications is used in non-radioactive detection techniques, probing, blotting and sequencing systems and in immunology, diagnosis, linked enzymes in ELISA. Thus it is commercially produced from calf intestine and *Escherichia coli*. Bacterial enzyme located in periplasmic space of *E.coli* cells that require costlier extraction method and complex downstream processes. Whereas *Bacillus spp.* capable to produce Extracellular ALPase which is extracted and purified from crude filtrate and the amount of ALPase is more and easier downstream processes. The present paper aimed to isolate new bacterial

producer for ALPase regarded to *Bacillus sp.* and optimize some environmental conditions of enzyme production. Methodology: We used fifteen *Bacillus* isolates were isolated previously from different soil and waste sources were collected from polluted area, in Advanced Biotechnology and Genetic Engineering Laboratory, College of Science in Babylon University, Iraq. The production of extracellular ALPase enzymes were screened, in liquid media and the best isolate was selected and identified, subsequently it was grown in different cultural conditions as pH, temperature, incubation periods and aeration and agitation to optimize enzyme production. Results: twelve out of fifteen isolates had capable to produce extracellular ALPase enzymes at variant levels. The best one (*Bacillus sp.I*) was selected to produce the enzyme depending for its high value of specific activity of ALPase. After that the environmental conditions for enzyme production were optimized and results revealed that the optimum conditions of extracellular ALPase production in fermentation medium were pH 8.2, 40°C for four days in stand incubator. Conclusion: Most *Bacillus* isolate produce

extracellular ALPase enzymes that encourage more screening of *Bacillus* isolates for ALPase enzymes production especially that isolated from extreme environment sources as alkaliophilic and thermophilic sources.

KEY WORD: Extracellular Alkaline Phosphatase, *Bacillus*, Production, Optimize conditions.

INTRODUCTION

Alkaline phosphatase (ALPase; orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups in the 5- and 3- positions from many types of molecules, including nucleotides, proteins, and alkaloids^[1]. The catalytic activities of the enzyme are metal ion dependent, as Mg^{2+} , Zn^{2+} and Co^{2+} that are the main activators. It has catalytic activity optima at alkaline PH^[1,2,3]. Alkaline phosphatases have been identified in a wide variety of organisms from bacteria to mammals^[3-6], bacterial strains like *Escherichia coli*, *Vibrio sp.*, *Shewanella sp.* and *Bacillus sp.*^[7-11]. Although the actual purpose of the enzyme is still not fully understood, the simple hypothesis, that it is a means for the bacteria to generate free phosphate groups for uptake and use^[4], alkaline phosphatase plays a vital role in phosphate transportation and metabolism and is a most crucial enzyme for the survival of organisms under phosphate starvation^[12]. In bacteria, the enzyme is located in the periplasmic space of Gram negative bacteria. *Bacillus* species produce alkaline phosphatase when phosphate becomes growth limiting as well as during sporulation, when phosphate supplies are abundant^[12, 13]. Alkaline phosphatase of *Bacillus licheniformis* and *Bacillus subtilis* is located intracellularly and extracellularly^[10 11, 13]. It has been shown that culturing conditions significantly affect such as metal ions^[14, 15 ,16] available N and P, temperature and pH^[17,18].

The enzyme has many applications in molecular biology and genetic engineering that used primarily for dephosphorylation of 5'-phosphorylated DNA or RNA end and used in non-radioactive detection techniques, probing, blotting and sequencing systems. Also in immunology, diagnosis, linked enzymes in ELISA^[19- 24]. The aim of this research is screened some *Bacillus sp.* isolates for alkaline phosphatase production and optimization of some production conditions of selected isolate.

MATERIALS AND METHODS

Bacterial Isolates

The isolates were isolated previously in our laboratory from soil samples contaminated with gypsum and decomposed waste feather samples and soil of chicken cage samples. The isolates were preliminary characterized depending on microbiological, cultural characteristics and their reactions with catalase and oxidase tests. The microorganisms were maintained on nutrient agar slants at 4°C and were subcultured every 4 weeks.

The ability of extracellular alkaline phosphatase production from these isolates was screened using production liquid medium according to Pandey and Banik method ^[25]. The production medium composed of (g/L) glucose 0.2%; peptone 0.5% , (NH₄)₂SO₄ 3.0 g/L, CaCl₂ 0.2 mmol , NaCl 0.08 mol , KCl 0.02 mol, NH₄Cl 0.02 mol ,MgSO₄ 1 mmol, ZnCl₂ 0.004 mmol, Na₃PO₄ 200 µmol, Ca(NO)₃ 50 mmol ^[26]. 10 ml of culture medium was taken in 50 ml Erlenmeyer flask with an initial pH maintained at 8, and Flasks were autoclaved at 121 °C for 20 min. The sterilized media was cooled at room temperature. 5% of *Bacillus sp.* suspension culture was inoculated in each flask and the flasks were incubated at 37°C for 4d. Subsequently the culture broths were centrifuged at 6000 rpm for 15 min at 4°C; the biomass was separated while the supernatant that containing extracellular ALPase was stored at -18°C for further analysis. The selected isolate was confirmed identification according to microbiological, cultural and biochemical characteristics ^[27]

Assay of Alkaline Phosphatase

ALPase activity was measured by spectrophotometrically by monitoring the release of p-nitrophenol from p-nitrophenyl phosphate (pNPP) at 405nm according to modified method ^[26]. A typical reaction mixture contained 1.9 ml of 20 mM p-nitrophenyl phosphate (pNPP) diluted in 1 M diethanolamine buffer (pH 9.8) and 0.1ml of enzyme filtrate. The reaction was performed at 37°C for 5 min, and then was stopped by adding 50 µl of 4 M sodium hydroxide solution. The color intensity was measured spectrophotometrically at 405 nm against blank. Blank was prepared by replacing the enzyme with 0.1 ml distilled water. One unit of enzyme defined as the amount of enzyme required to liberate 1 µg orthophosphoric acid under assay conditions.

Preparation of Substrate

0.219 gm of para nitro phenyl phosphate (PNPP) were dissolved in 50ml of 1 M diethanolamine buffer (pH 9.8) as a substrate ^[26].

Protein Concentration Determination

The protein concentration was determined using Bradford method ^[28] with bovine serum albumin as the standard protein.

Effect of Some Environmental Conditions on Extracellular Alkaline Phosphatase Productions

The effect of initial pH on alkaline phosphatase production was observed by adjusting initial pH of fermentation medium in the range 5-12 by using available buffers and incubation at temperature 37°C for 4d

The effect of temperature on productivity of alkaline phosphatase was checked by incubation fermentation media of *Bacillus sp.I* in temperature ranging from 20 to 55°C for 4d.

The optimal incubation period of alkaline phosphatase was determined by incubation of fermentation media of *Bacillus sp.I* for different periods ranging 1-7d under optimal conditions.

The effect of aeration and agitation on productivity of alkaline phosphatase was checked by incubation of fermentation media of *Bacillus sp.I* in shaker incubator at agitation speed 120 rpm, also it was incubated in stand incubator under optimal conditions.

RESULTS AND DISCUSSION

Bacillus isolates were subjected to screening of extracellular ALPase production were isolated previously from different sources (Table 1) in Biotechnology and Genetic engineering Laboratory / College of Science / Babylon University, twelve out of fifteen isolate appeared variable abilities to produce the extracellular ALPase enzyme in liquid medium, this variation may be due to the differences among bacterial species and the sources of bacterial isolation as well as the genetic content of these isolates, cultural media and environmental conditions of screening ^[10,11,13]. Because of the presence many members or types of alkaline phosphatase group such as bound ALPase enzymes with bacterial cell membrane or Free enzyme that secreted to extracellular in Gram positive bacteria or located in periplasmic space of Gram negative bacteria or located intracellularly ^[10-13]. In the present study, liquid production medium was used in the screening to produce extracellular ALPase from *Bacillus* isolates instead of primary screening solid media that cannot be differentiate between the types of ALPases ^[26]

Table (1): Screening of extracellular alkaline phosphatase from *Bacillus* isolates

No.	The isolates	ALPase activity (U/ml)	Protein concn. (mg /ml)	Specific activity U/mg of protein	Sample source and date of isolation
1	<i>Bacillus sp.</i> Fea 89	8	42.2	0.19	decomposed waste feather, 2012
2	<i>Bacillus sp.</i> Fea 8b	19	32.75	0.58	decomposed waste feather, 2012
3	<i>Bacillus sp.</i> Fea ab	0	150	0	decomposed waste feather, 2012
4	<i>Bacillus sp.</i> R18	170	150	1.13	soil of chicken cage, 2012
5	<i>Bacillus sp.</i> R19	160	105.8	1.5	soil of chicken cage, 2012
6	<i>Bacillus sp.</i> R20	0	101.05	0	soil of chicken cage, 2012
8	<i>Bacillus sp.</i> S14	68	102.6	0.66	soil of chicken cage, 2012
9	<i>Bacillus sp.</i> S20	0	103.3	0	soil of chicken cage, 2011
10	<i>Bacillus sp.</i> S25	100	150	0.66	soil of chicken cage, 2011
11	<i>Bacillus sp.</i> S24	108	150	0.72	soil of chicken cage, 2011
12	<i>Bacillus sp.</i> 1	1166	97.5	11.95	Soil contaminated with gypsum, 2010
13	<i>Bacillus sp.</i> 40A	1123.85	118.3	9.5	Soil contaminated with gypsum, 2010
14	<i>Bacillus sp.</i> 281	162	150	1.08	Soil contaminated with gypsum, 2010
15	<i>Bacillus sp.</i> 282	618	150	4.12	Soil contaminated with gypsum, 2010

Depending on the value of specific activity of producing ALPase from the isolates, the isolate has the higher one (*Bacillus sp.* 1) was selected to produce ALPase.

Bacillus sp. 1 was re-identified depending on morphological and biochemical characteristics according to Bergey's Manual of determinative Bacteriology^[27]. The isolate is Gram positive bacilli and arrange in single cells, pair or short chain. It form ellipsoidal spore located in center or sub-center of the cell. It has circular white to creamy color colonies with smooth texture and regular edge, its diameter 4-5mm when it was grown on nutrient agar medium for 24h. the isolate is motile and showed positive reactions to catalase and oxidase tests and it produced protease and amylase and appeared ability to gelatin liquefaction, citrate utilization and ferment carbohydrates and sugar as Glucose, Fructose, Maltose, Xylose, Sucrose and

Arabinose, whereas it cannot ferment Lactose, Melezitose and Rhamnose. *Bacillus sp. I* has ability to grow in different temperatures (15-55°C), pHs (5-12) and in the presence different concentrations of sodium chloride ranged from 0 to 20%. The optimal conditions for growing are 37°C, pH8.5 and 2% NaCl.

Some optimal conditions for ALPase production were studied and the results revealed that the optimum initial pH of production medium was approximately 8.2 to 8.3 that get maximal production and the enzyme produced in the alkaline medium ranged from 8 to 9 at 37°C for 4d (Fig.1). The level of enzyme production became limited at acid and neutral pHs (5-7) and extreme alkaline pH (10-12). *Bacillus sp. I* showed optimum productivity of alkaline phosphatase at pH 8.2 and this result was similar to that of optimum pH of alkaline Phosphatases produced from *E.coli* (pH 8.3) [29] and *B. licheniformis* MTCC1483 (pH8) [27]. The effects of pH on solubility of nutritional medium compositions that available for bacteria growth, as well as ion state and stability of biological compounds that produced from fermentation processes [30].

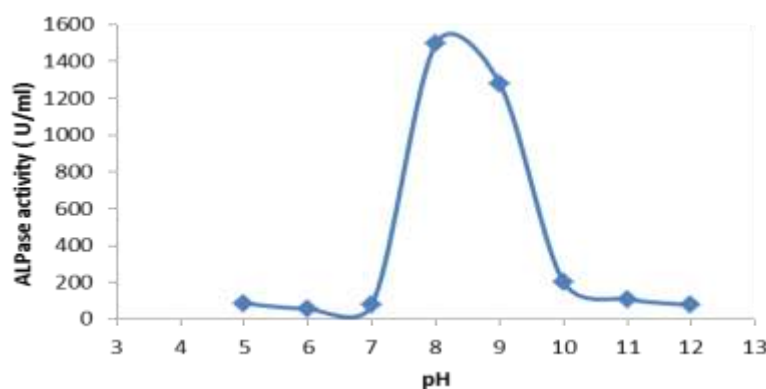


Fig.1. The effect of pH on ALPase production from *Bacillus sp. I*

The bacterial isolate was grown in production medium at 37°C for 4d.

The optimal temperature for maximal production at 40°C and the enzyme produced in the range from 30 to 40 °C at pH8 for 4d (Fig.2). The level of ALPase production became limited at the temperature less than 30°C and above 40°C but the production stopped at 55°C. *Bacillus sp* showed optimum productivity of alkaline phosphatase at temperature 40°C and this result was similar to that of alkaline phosphatase from Psychrophilic bacteria [31] and *E. coli* [32]. Temperature plays a vital role in producing of enzyme from microorganisms by effect in solubility of oxygen in nutritional medium , increasing of kinetic energy of

molecules and speed of enzymatic reaction in the cell, bacterial growth and its metabolism [30].

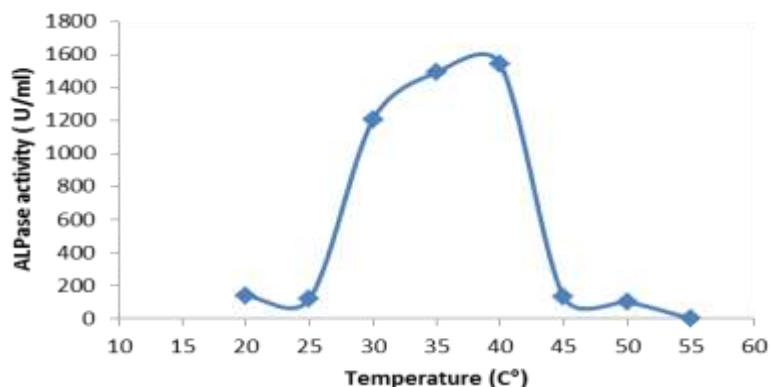


Fig.2. The effect of temperature on ALPase production from *Bacillus sp. I*

The bacterial isolate was grown in production medium at pH8.2 for 4d.

The bacterial cells produced the ALPase during sporulation stage at stationary phase after two days (Fig.3) and the production was reaching maximum level after four days, subsequently the production was reduced that indicates the optimal incubation period was four days under optimal conditions. *Bacillus* species produce alkaline phosphatase when phosphate becomes growth limiting as well as during sporulation, when phosphate supplies are abundant [1, 25]

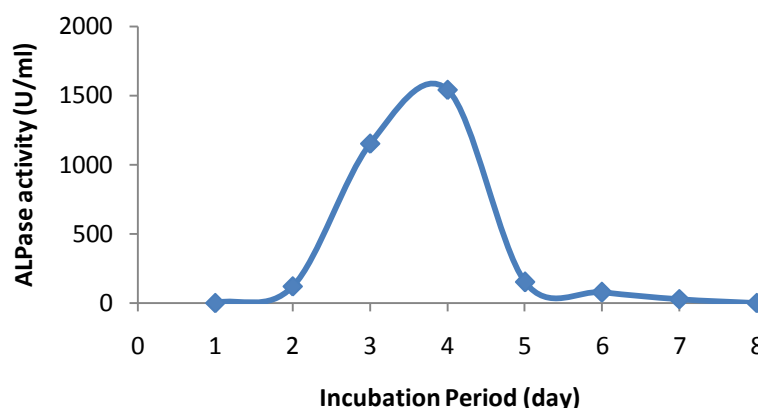


Fig.3. The effect of incubation period on ALPase production from *Bacillus sp. I*

The bacterial isolate was grown in production medium at pH8.2, 40°C.

The effect of aeration and agitation was checked on enzyme production by incubating bacterial culture in the shaker incubator at 120 rpm under optimal conditions and in stand incubator, the results revealed that ALPase produced in stand conditions higher than in shaker

conditions (Fig.4) that may be due to the sensitivity of enzyme protein to agitation or oxidation^[25].

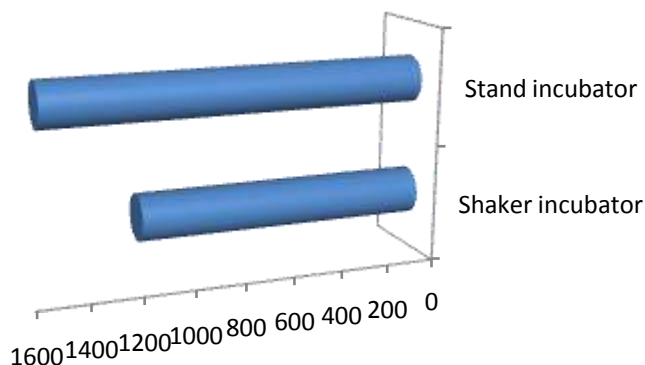


Fig.4. The effect of aeration and agitation on ALPase production from *Bacillus sp. I*

The bacterial isolate was grown in production medium at pH8, 40°C for 4d.

CONCLUSION

The genus of *Bacillus* is mostly produced extracellular ALPase enzymes that can be harvested from the commercial production medium in comparison with other sources of alkaline phosphatase that located intracellular such as *E.coli* and calf intestine which are comparatively costlier and have very complex downstream processes.

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