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Optimization for the Production of Extracellular Alkaline Phosphatase from *Proteus mirabilis*

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Abstract

The 35 bacterial strains were isolated from water and soil sample from Karnataka (India) and screened for their ability to secrete extracellular Alkaline Phosphatase (ALP). Among them, a potent strain *Proteus mirabilis* resembling to *Proteus mirabilis* strain M54 was confirmed by 16S rRNA gene sequencing, was selected for the optimization of enzyme production. The optimum production was at pH 7-8 and at temperature 35°C for 28 hrs incubation. The pNPP was the best substrate for the ALP production. This 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 9.7. The molar mass was estimated by 12% SDS PAGE and was found to be approximately 56 kDa and also confirmed by gel filtration studies (data not shown). The study holds significance as there are only few reports available on the optimisation protocols.

Keywords: Alkaline phosphatase; Optimisation; Chromatography; SDS-PAGE; Phylogeny; Sequencing

Abbreviations: ALP: Alkaline Phosphatase; SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; pNPP: para-Nitrophenol phosphate; NCBI: National Centre for Biotechnology Information; BLAST: Basic Local Alignment Search Tool

Introduction

Alkaline phosphatases (ALP) (EC 3.1.3.1) belong to the class of hydrolases and catalyze alkaline hydrolysis of more number of different phosphoric acid esters. Now a day, 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.

ALPs may potentially be employed as therapeutic agents and therapeutic targets and show several uses in clinical medicine and in biotechnology. An example of its use in clinical medicine was described in a technique for labelling monoclonal antibodies. ALP has the biggest market volume [1] share of $20 million. 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% [2].

Phosphatases also vary widely in their substrate specificities, pH ranges, and metal ion requirements. Some, such as the one from *E. coli*, are periplasmic, some are membrane associated [3] and some are extracellular [4,5]. The purification of intracellular enzymes is a tedious process. This picture of vast biochemical diversity is emerging from limited studies with a few microorganisms. These preliminary results suggest that the characterization of new phosphatases will be particularly fruitful, because there is a high probability of discovering new types with novel properties.

Materials and Methods

Collection of samples

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

Isolation of organism

The organism was isolated using serial dilution by pour plate method using modified minimal media (MM): (Sodium chloride -5 g, Ammonium chloride-2 g, Magnesium chloride-0.1 g, Glucose-10 g, and Agar-20 g per litre along with pNPP-1 g).

Screening the ALP

The isolates were screened by streak plate on the MM along with pNPP and the selected isolates were separately confirmed for their abilities to produce ALP with the following procedure**:** A loop full of the selected ALP-producing strain was inoculated into 50 ml MM along with pNPP of broth in duplicate and incubated on a rotary shaker at 35°C 150 at rpm for 24 hrs. 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 [6].

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, chemotaxonomical characteristics and 16S rRNA sequencing. Results were interpreted according to Bergey's Manual of Determinative Bacteriology, 8th edition [7] and NCBI database. The organism was identified as *Proteus mirabilis.*

Production of enzyme

Proteus mirabilis used for enzyme production was grown at 37°C for 24 hr in a rotary shaker at 150 rpm in modified MM along with pNPP as substrates for phosphatase synthesis. ALP activity was measured by using Stopped Spectrophotometric Rate Determination method [6]. The absorbance of released pNPP was determined at

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410 nm. Protein concentrations were estimated by [8] method and absorbance determined at 660 nm by using Bovine Serum Albumin (BSA) as standard.

Optimization of culture conditions for enzyme production

The protocol adopted for optimization of various parameters influencing ALP yield was to evaluate the effect of individual parameter, and to incorporate it at the standard level before optimization of the next parameter.

Effect of pH on enzyme production: To determine optimal pH, *Proteus mirabilis* was cultivated in a 250 ml flask containing 100 ml MM along pNPP with different pH ranges from 4.0 to 10.0. 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 24 hr of cultivation. After incubation the cell free filtrate was used for enzyme assay.

Effect of temperature on enzyme production: In order to determine the effective temperature for ALP production by *Proteus mirabilis*, 100 ml of standardized media was prepared in different conical flasks and was adjusted to pH 7.5. 1ml of seed culture was inoculated and incubated at different temperature viz., 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C, in a rotary shaker. Enzyme extraction **and** assay procedures were same as described earlier.

Effect of incubation period on enzyme production: 100 ml of standardized media was prepared in different conical flasks and adjusted to pH 7.5. 1 ml of seed culture was inoculated and incubated in a rotary shaker at different time intervals viz., 12 hrs, 18 hrs, 24 hrs, 30 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs at 35°C at 150 RPM. After incubation the cell free filtrate used for enzyme assay.

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, pNPP 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 50 ml minimal MM. 1 ml of seed culture was inoculated and incubated in a rotary shaker at pH 8, at 35°C for 24 hrs. 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 50 ml MM with 0.15% pNPP. 1 ml of seed culture was inoculated rotary shaker at pH 8, at 35°C for 24 hrs. The end of the incubation cell free extract was used for enzyme assay.

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 at optimum condition.

Comparatives studies on cell membrane bound and extracellular alkaline phosphatise

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 re-suspended to A_{600}=1.0 in the same saline and broken with ultrsonicator 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 $(NH_4)_2SO_4$ precipitation, dialysis, gel filtration and DEAE-cellulose ion-exchange chromatography by using gradient elution buffer.

Enzyme characterization

SDS PAGE was performed according to the [9] 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 commassie brilliant blue.

Result and Discussion

Identification of the organism

The organism showing more activity confirmed by the biological activity was identified by using 16S rRNA 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 1X Tris-acetate EDTA buffer at constant voltage of 80V. The absorbance ratios (A 260/280) 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. is highly resembled with *Proteus mirabilis* up to 99% *Proteus mirabilis* strain M54 (Figure 1). Differentiation is considered based on biochemical test on the species level, Indole test is considered reliable, as it is positive for *Proteus mirabilis.* One of the best advanced processes

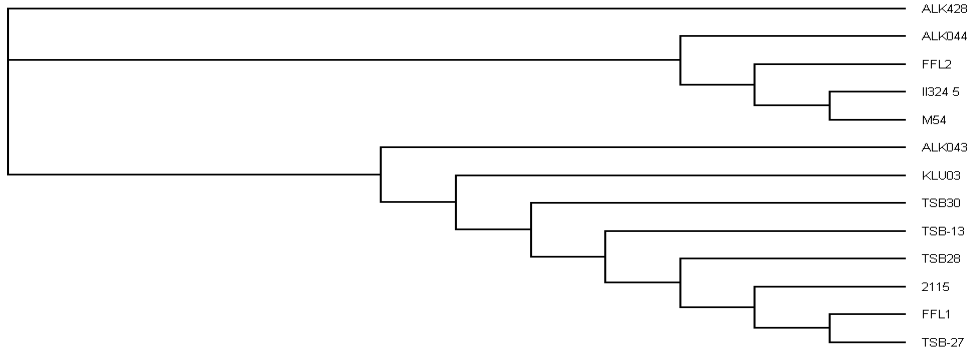

Figure 1: Phylogenetic tree (Cladogram) of *Proteus mirabilis.*

to identification of bacteria analysis by 16S rRNA sequences is the single best method.

Optimization of culture conditions for enzyme production

Effect of pH on production of enzyme: An important factor significantly affecting the production of ALP was the initial pH. The fermentative production of ALP was carried out at different pH at 4, 5, 6, 7, 8, 9 and 10. The Maximum ALP production 3.4 to 4.1 U/mg ($P<0.001$) at pH is 7 to 8. The enzyme activity was very low pH 4 and pH 10 (Figure 2). These results clearly indicated that ALP can produce wide range from pH (4 to 10). 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 [10] in the case of alkaline phosphatase production by *E.coli* at pH 8.3 [11] of alkaline phosphatase production by *Penicillium expansum* at pH 9.5.

Effect of temperature on production of enzyme: The production of ALP at different temperature viz., 20°C 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 60°C, in a rotary shaker 150 rpm was examined by keeping the other fermentation condition constant. The maximum production ALP was 3.5 to 4.2 U/mg protein ($P<0.0015$) at 35°C to 40°C. The enzyme activity was very low at 20°C and above 40°C activity was decreased (Figure 3). Temperature influence all the physiological activities in living cell and is one of the important environmental factor to control the growth, microbial activities, normal functioning of enzyme [12].

Effect of incubation on production of enzyme: The amount of ALP produced was studied from the 4 hrs to 48 hrs incubation. There was activity found from the 4 to 32 hrs of incubation, after 32 hrs the activity was decreased considerably. The maximum activity was at 24 to 28 hrs it reaches 4.9 U/mg ($P<0.0025$) (Figure 4). The growth and enzyme yield increased with passage of time and the maximum enzyme secretion was found (0.688 U/ml) after 24 hrs but on prolong

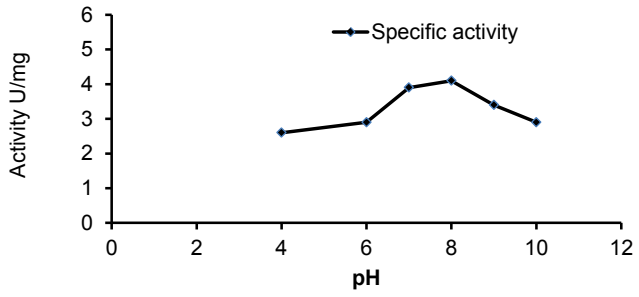

Figure 2: Effect of pH on production of enzyme.

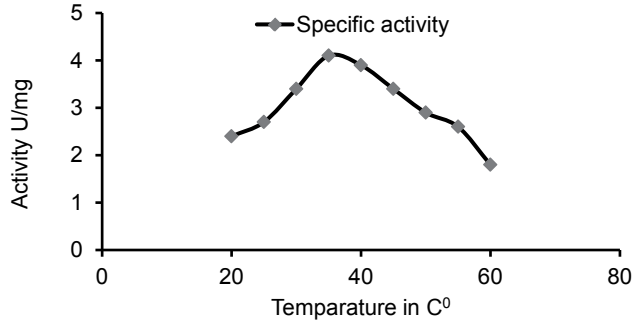

Figure 3: Effect of temperature on production of enzyme.

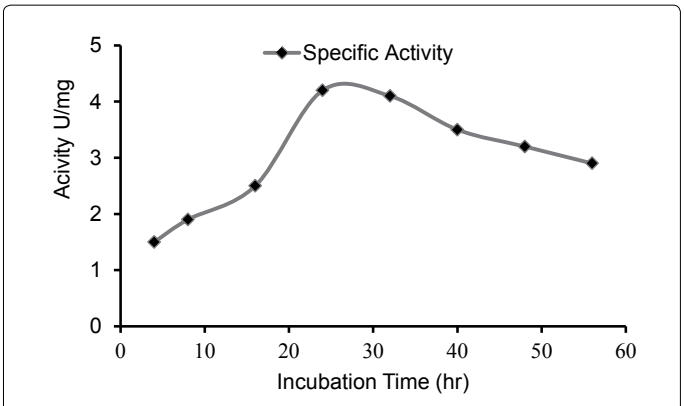

Figure 4: Effect of incubation period on production of enzyme.

Substrate	Activity	Concentration of pNPP	Activity
Adenosine Triphosphate	3.9 ± 0.26	0.02%	3.6 ± 0.1
Para-nitrophenol Phosphate(pNPP)	5.9 ± 0.72	0.06%	4.2 ± 0.1
Sodium Phosphate	2.2 ± 0.2	0.10%	5.9 ± 0.5
Calcium Phosphate	1.8 ± 0.72	0.14%	6.0 ± 0.4
Potassium Phosphate	1.8 ± 0.10	0.18%	6.2 ± 0.2
-	3.6 ± 0.10	0.20%	6.0 ± 0.23

Table 1: The effect of phosphates on production of enzyme.

incubation. Production decreased may be due to change in pH [13].

Effect of phosphates on production of enzyme: Different concentration organic phosphates like adenosine triphosphate, 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 50 ml MM. Maximum ALP activity obtained from pNPP and using the inorganic phosphate. The ALP activity was decreased as shown (Table 1). 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 as they bind to specific substrates and catalyze specific reactions involving those substrates resulting in specific products.

Effect of carbon and nitrogen 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 50 ml MM with 0.15% pNPP. Average sucrose and starch gave slightly better production than the other sugar. Different concentration of starch and sucrose used was 0.5% to 3%. The optimum activity of the entire organism shown in sucrose was 0.5% to 1% and increase in the concentration, the activity was reduced but in starch the optimum activity was shown 1% to 1.5%. Comparatively starch showed more activity (Table 2). Organic carbons are the common substrates i.e., usually referred to heterotrophic microorganisms for the main energy sources. The similar results reveal the production of alkaline phosphatase from *Neurospora crassa* was also stimulated by sucrose in combination with acetate [14]. 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 up

to 22% (Table 3). The similar studies reveals tryptone and yeast extract have been reported as suitable nitrogen source for alkaline phosphatase production [3,15-17].

Comparatives studies on cell membrane bound and extracellular ALP

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 re-suspended to A_{600}=1.0 in the same saline and broken with ultrsonicator 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 enzyme was more than 29% in contrast to intracellular enzyme (Figure 5). There are reports on generating osmotic shock through treatment with magnesium ions or sonication [18] only. Hulett et al. [19] investigated the extracellular production of ALP in *Bacillus licheniformis* which showed that it synthesized 10 times more ALP activity than was reported for other *Bacillus* sp. [20]. However, the extracellular production of ALP has been studied in *Micrococcus sodonensis* [4]. Intracellular production of ALP is quite tedious and expensive process in comparison to extracellular. This statement is supported by the study of [19] which explained that extracellular ALP gave higher specific activity than intracellular ALP is because of short and simple steps of purification [21].

Purification of extracellular ALP

After production, the cell free media was taken for 70% ammonium sulphate precipitation. The specific activity was increased up to the 1.5% and fold purification was 1.84. Similar reports 1.92 and 2 folds by [22] and [23] from *Schewanella* sp. and *E. coli* respectively. Specific activity increased from 17.5 U to 24 U after desalting. The sample was subjected to gel filtration. The sample of 5 ml eluted at the rate of 1 ml/min per from G-100 sephadex (V_o=10 ml; V_t=40 ml; Flow rate=1 ml/5min; Fraction volume=2 ml) column exhibited peaks with phosphatase activity in fractions showing absorbance 280 nm spectrophotometrically. Fractions 5, 6, 7, 8, and 9, showed phosphatise activity (0.34, 0.40, 0.67, 0.22, 0.15, 0.043, and 0.025 U/ml), respectively. The specific activity increased 59 U/mg and fold purification 5.17. Previous study [24] used Sephadex G-100 to elute a halophilic alkaline phosphatase from analytical gel filtration G-150 (3 × 70 cm) columns using standard proteins.

Carbon source	Activity	Concentration of starch	Activity
Glucose	4.2 ± 0.6	0.5%	5.7 ± 0.25
Sucrose	5.5 ± 0.1	1.0%	7.2 ± 0.5
Lactose	4.8 ± 0.10	1.5%	7.1 ± 0.25
Maltose	4.2 ± 0.2	2.0%	6.5 ± 0.4
Starch	7.2 ± 0.10	2.5%	6.2 ± 0.2
Cellulose	3.6 ± 0.10	3.0%	5.6 ± 0.23

Table 2: Effect of carbon source on production of enzymes.

Nitrogen Source	Activity	Concentration of Casein	Activity U/mg
Casein	10.5 ± 0.8	0.5%	11 ± 1.2
Gelatin	7.5 ± 0.10	1.0%	13 ± 1.0
Tryptone	8.5 ± 0.2	1.5%	13.5 ± 0
Egg Albumin	6.5 ± 0	2.0%	13. 5 ± 0.15
BSA	7.0 ± 0.15	2.5%	12 ± 0.8
-	-	3.0%	12.5 ± 0.4

Table 3: Effect of Nitrogen source on production of enzymes.

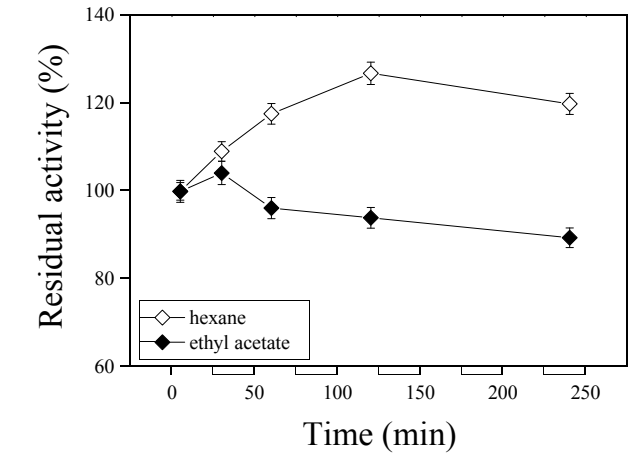

Figure 5: Comparatives studies on cell membrane bound and extracellular ALP.

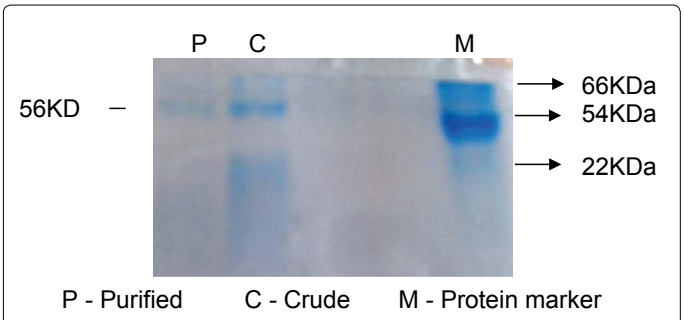

Figure 6: Molecular mass of ALP as determined by SDS-PAGE.

The pooled fractions from gel chromatography were eluted from DEAE cellulose column by linear gradient of NaCl (0.05-0.2 mol). The phosphatase activity in fractions (3 and 4) showed absorbance at 280 nm. The specific activity of was increased up to 105 U/mg and fold purification 9.7 (Table 4) in contrast to our results, a 135 fold purification and 50% yield of extracellular ALP was obtained in *Lysobacter enzymogenes* [25] and in case of *Bacillus licheniformis* studied by [26] fold purification and yield were found to be 2.4 and 10%, respectively. Yield of ALP is dependent on the strain of bacteria used [25]. This may be the reason for the difference between our results and those reported earlier.

Molecular weight determination

A single band of monomer protein was obtained on 13% of the SDS PAGE. Molecular weight is 56 kDa approx. (Figure 6) and also

Purification step	TotalVol. (ml)	TotalActivity(U)	TotalProtein (mg)	Specific activity(U/mg)	Fold Purification	Yield(%)
Crude Sample	200	3000	260	11.5	1	100
Ammonium sulfate Precipitation	25	2160	102	21.2	1.84	72
Gel filtration	10	1257	21	59.9	5.17	42
Ion Exchange Chromatography	10	740	7	105.5	9.7	25

Table 4: Summary of purification of ALP.

confirmed by Gel Filtration. The homodimeric forms of ALP, with a molecular mass of 54 kDa have been previously reported in *E. pyrococcus obyssi* [27-29]. The SDS-PAGE of *B. stearothermophilus* ALP showed a single protein band of 32 kDa [21]. HK47 ALP [9] and *P. aeruginosa* ALP [30] appeared as 68 kDa while T59R ALP [23] appeared as a single band of 44 kDa against staining with coomassie brilliant blue after SDS-PAGE [31-33] purified a hemophilic 54kDa phosphatase monomer from *Pyrococcus abysii*. Hulett [29] calculated the molecular weight of *Pho AIII* amino acids as 45.9 kDa and *Pho AIV* amino acids as 47 kDa from *Bacillus subtilis*. Ishida [22] purified a 41.8 kDa phosphatase from *Schewnella* sp.

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.

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