PRODUCTION OF ALKALINE PHOSPHATASE FROM A FACULTATIVE PSYCHROPHILIC *PSEUDOMONAS SP.* MRLBA1 ISOLATED FROM PASSU GLACIER, PAKISTAN

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Abstract. Among our collection of psychrophilic bacteria from various non-polar glaciers, the present study involves the study of the ability of the psychrophilic bacterial strains, isolated from Passu Glacier, Pakistan, to produce thermolabile alkaline phosphatase extracellularly as well as intracellularly. The biophysio-chemical characteristics and 16S rRNA signatures of the isolate MRLBA1 were similar to the members of genus *Pseudomonas.* This rod shaped MRLBA1 isolated from glacial ice exhibited aerobic growth cycle with cardinal limits of pH; 4-11 and temperature; 2-30°C. The strain showed highest extracellular alkaline phosphatase activity at pH 8.0 and 18°C when inoculated with 24 h old inoculum (5%), after 48 h of incubation in shake flask at 150 rpm. The strain's targeting mechanism of alkaline phosphatase at cell membrane as well as in the extracellular medium is interesting. The results demonstrated that alkaline phosphatase being thermolabile, acts as a stress protein also in addition to harvesting the energy directly in carbon deficient ice.
Keywords: *psychrotrophs, thermolabile activity, mountain glaciers, phosphomonoesterase*

Introduction

Non-polar glaciers are analogous to other frozen bodies of the crust and harbor simple life entombed since ages. Facultatively psychrophilic bacteria have been reported in normal flora of glaciers which play major role in harvesting energy through re-designing their metabolic machinery (Morita and Moyer, 2001). Glacial ice represents a unique niche with variant aspects for physiology of bacteria including scarce nutrients, pH, temperature, properties of water and other physico-chemical parameters. A long term exposure and adaptability of these bacteria provide with modified pattern of physiology though with similar biochemistry. Psychrophilic microorganisms produce enzymes which are adapted to be active at low temperatures. As compared to their mesophilic counterparts, they display an increased catalytic efficiency over a temperature range of about 0-30°C and a high thermosensitivity. These enzymes have a great potential to be used in biotechnology industry, e.g. in the detergent and food industries, production of fine chemicals and in bioremediation processes (Gerday et al., 1997). Contrary to primary metabolites (proteases, lipases, nucleases), phosphate cannot be synthesized and must be obtained from nucleic acids, phosphorylated sugars and proteins etc. The nutrient deficient ecosystems stipulate the

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release of Pi through hydrolysis of phosphate esters using phosphatises (De Prada et al., 1996).

In an intensive competition for phosphate, bacteria express enhanced activities of phosphatases. We propose that the same phosphatase under various inductions like stress of temperature is processed and targeted differently, as suggested in previous reports (De Prada et al., 1996; Vincent et al., 1992). There is wide variation among phosphatases depending on specificity of substrate, temperature and pH range, and/or requirement of metal ions (Hong et al., 2007). Periplasmic membrane bound (Baoudene-Assali et al., 1993) and extracellular (Glew and Health, 1971) isozymes of phosphoesterase activity have been reported previously. Phosphatases play major role in ability of a psychrophile to compete at low temperature and attribute in its various commercial applications. Alkaline phosphatase (EC 3.1.3.1) is a cellular metabolite widely distributed in nature from microbes to complex animals. In vitro applications of these enzymes span diagnostics (markers, biosensors), molecular biology (dephosphorylation) and immunology (ELISA), Western blot, nucleic acid hybridization and In-situ hybridization. A heat labile phosphatase will be beneficial in terms of time and shortening of protocols; as robust enzyme without prior thawing procedure before use, or activity silencing steps using heat inactivation (Col et al., 2010).

Lee et al. (2015) reported mAP enzyme as the first thermolabile alkaline phosphatase found in cold-adapted marine metagenomes and expected to be useful for efficient dephosphorylation of linearized DNA. The gene encoding a novel AP was isolated from a metagenomic library constructed with ocean-tidal flat sediments from the west coast of Korea. The amino acid sequence of mAP showed a high degree of similarity to other members of the AP family. Phylogenetic analysis showed mAP to be a member of a recently identified family of PhoX that is different from the classical PhoA (Lewenza et al., 2005) family. The crystal structure of a cold-active alkaline phosphatase from a psychrophile, *Shewanella sp.* (SCAP), have been determined by Tsuruta et al. (2010). Psychrophiles synthesize cold-active enzymes to sustain their cell cycle, and these enzymes are already used in many biotechnological applications requiring high activity at mild temperatures or fast heat-inactivation rate. Most psychrophilic enzymes optimize a high activity at low temperature at the expense of substrate affinity, therefore reducing the free energy barrier of the transition state (Struvay and Feller, 2012).

Isolate MRLBA1 exhibited robust phosphatase activity which was inactivated completely at 56°C, suggesting it as a heat labile enzyme. A comprehensive biophysio-chemical characterization and identification of *Pseudomonas sp.* MRLBA1 is presented here. With emphasis on physiological adaptations of this strain under stress conditions, alkaline phosphatase is proposed to be expressed differently and functioning under various inductions, as described here.

Materials and methods

Sample collection

Samples of deep ice and melt water were collected in sterile polypropylene bags and bottles aseptically from Passu Glacier (temperature 0°C, pressure 794 mb, located about 2830 m above sea level) situated at 36° 27'21.9E and 074° 52'32.8N in Karakoram Range of mountains, Northern Areas of Pakistan. The samples were transported to the laboratory in their intact physical conditions and stored at -80°C.

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Isolation and characterization of isolate MRLBA1

Samples of molten ice and water were serially diluted up to ten fold in normal saline. About 100 µl of each dilution was spread on Nutrient agar plates (Oxoid, Basingstoke, UK), which were then, incubated aerobically at 4, 10 and 20°C for 7 days. A few discrete colonies were inoculated in Nutrient broth and incubated at 20°C. The isolates were screened for alkaline phosphatase activity intra and extracellularly and the isolate MRLBA1 was selected for further studies.

For morpho-physiological studies, colony morphology (color, shape, elevation, margins, and odor), Gram's staining, optimum temperature, pH, respiration using Gas Generating Kit (Oxoid, UK), growth rate and carbohydrate assimilation using Analytical Profile Index (API® 50CHB, BioMérieux® France) was examined. For biochemical characteristics, the tests were performed following protocols described by Lanyi (1987) and Simbert and Kreig (1994).

Electron microscopy

Cells grown at log phase under conditions given above were fixed with 3% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.3). After dehydration, the samples were coated with gold in a sputter-coater (SPI-Module, USA) and examined with a scanning electron microscope (JSM5910, JEOL, Japan).

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA extraction and amplification of the 16S rRNA gene were done as mentioned previously (Janarthanan and Vincent, 2007) using the set of universal primers; 27F 5′AGAGTTTGATCCTGGCTCAG3′ and 1492R 5′TACGGTTACCTTGTTACGACTT3′ according to Lane (1991) and Reysenbach et al. (1995). PCR products were sequenced according to the manufacturer's instructions using the ABI PRISM® BigDye™ Terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom). The nucleotide homology search was performed against the partial 16S rRNA sequences of 1047 base pairs using the nucleotide BLAST program "BLASTn" (Altschul et al., 1997) in non-redundant (nr) data base. The sequences having equal or more than 97% sequence homology but validated by IJSEM previously were retrieved from the Gene Bank and aligned using Clustal W program (Thompson et al., 1994) in the Molecular Evolutionary Genetics Analysis Program (MEGA) version 4.0.1 (Tamura et al., 2007). The regions in the sequences corresponding to the isolate MRLBA1 sequence were retained and all non-aligned sequence parts were trimmed. This alignment was used to construct a neighbor joining (NJ) tree and finally the maximum parsimony (MP) tree (Eck and Dayhoff, 1966) using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with random addition of 10 replicates.

Culture conditions for production of phosphatase enzyme

The cultures were routinely maintained on nutrient agar slants at 4°C. The production of enzyme was carried out in modified production medium (0.5% peptone, 0.2% glucose, 0.08 M NaCl, 0.2 mM $CaCl_2$, 0.02 M NH_4Cl, 0.02 M KCl, 1 mM $MgSO_4$ and 0.004 mM $ZnCl_2$). Sodium phosphate (0-200 µM) and calcium phosphate (0-50 mM) were added in the basal medium to study the regulation of alkaline phosphatase

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production as given below. The effect of pH on growth and enzyme production was studied by growing cells in basal medium. The pH of the medium was adjusted to 5-13 using appropriate buffers (10 mM; Dhaked et al., 2005). Growth was measured spectrophotometrically at 600 nm (Agilent 8354).

Enzyme assay conditions

Activity of alkaline phosphatase was measured by the method of Dhaked et al. (2005) by measuring the absorbance (OD_{405}) to monitor the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP) as mentioned. Reaction mixture contained 300 µl of enzyme diluted in 1 M diethanolamine buffer (pH 9.8), 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 150 mM *p*-nitrophenyl phosphate (pNPP), in a final volume of 3 ml.

The reaction performed at 37°C for 30 min was stopped by addition of 50 µl of 4 M sodium hydroxide. One unit was defined as the amount of alkaline phosphatase which hydrolysed 1 µmol of *p*-nitrophenyl phosphate to *p*-nitrophenol in 1 min at pH 9.8 and 37°C. The quantification of enzyme activity (OD_{405}/OD_{600}) was done by standard curve of *p*-nitrophenol (0-500 µM) at 405 nm.

Results and discussion

Isolation and characterization of isolate MRLBA1

The bacterial colonies appeared on Nutrient agar (pH 8.0) after incubation at 4°C for 16 days. The isolate MRLBA1 formed white, smooth circular colonies. The cells were Gram negative short rods (*Fig. 1*).

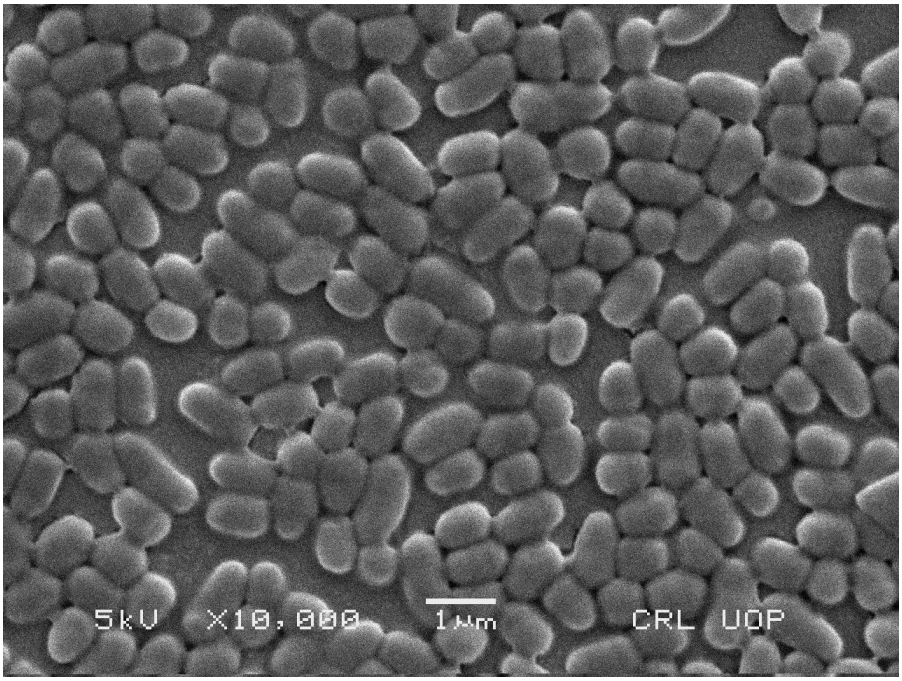

Figure 1. *Scanning electron micrograph of cells of Pseudomonas sp. MRLBA1 (10000× at 5 kV). The bacteria were harvested in log phase and i). Fixed: (3% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.3), incubated at 4°C), Rinsed: (0.1 M phosphate pH 7.3), Dehydrated: (50-90% gradients of acetone for 10 min each), and dried using $CaCl_2$ (Stadtländer, 2007)*

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Obligatory aerobic isolate MRLBA1 was positive for catalase, urease, and Voges Proskauer tests. The isolate exhibited facultatively psychrophilic adjustment of temperature and exhibited respective cardinal and optimum limits of temperatures as: 2-37°C; 25°C, and pH: 4-11; 9 (*Fig. 2*). The physiological pattern of metabolism and carbon assimilation is given in *Tables 1* and *2*, respectively.

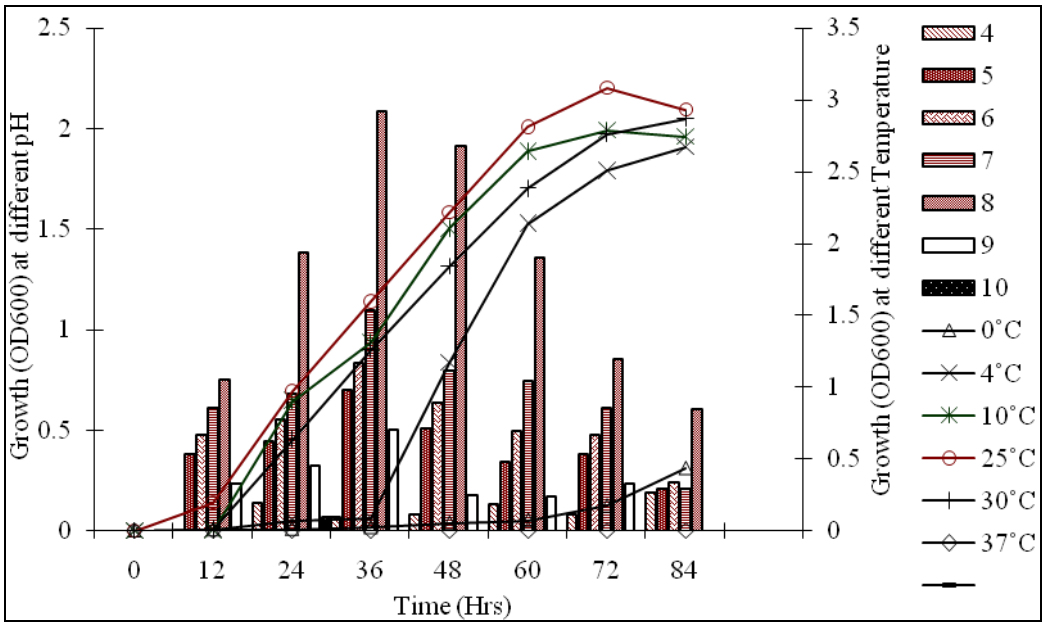

Figure 2. *Effect of pH and temperature on the growth of Pseudomonas sp. MRLBA1. The isolate exhibited maximum growth at pH 8 and 25°C*

Table 1. *Morpho-physiological and biochemical characteristics of the MRLBA1 isolate*

Colony	
Color	White
Margin	Smooth
Gram's staining	-
Cells	
Shape	Rod
Spore	-
Motility	-
Oxygen utilization	Aerobic
Growth Cardinals	
Lower limit	4
Upper limit	11
Lower limit (°C)	2
Upper limit (°C)	30
Biochemical	
Alk. Phosphatase	+
Amylase	+
Catalase	+
Gelatinase	-

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Methyl Red	-
Nitrate Reductase	-
Simmon citrate	-
Triple sugar Iron	-
Urease	+
Voges-Proskauer	+

Table 2. *Carbon source utilization of the isolate MRLBA1*

Carbon source	Growth	Carbon source	Growth	Carbon source	Growth	Carbon source	Growth
Glycerol	+	D-Fructose	+	N-Acetyl Glucosamine	-	Glycogen	-
Erythritol	-	D-Mannose	+	Asculin	-	Xylitol	-
D-Arabinose	+	Methyl α-D Glucopyranoside	-	Salicin	-	Gentiobiose	+
L-Arabinose	+	L-Sarbose	-	D-Cellobiose	+	D-Turanose	-
D-Ribose	+	L-Ramnose	-	D-Maltose	+	D-Lyrose	-
D-Xylose	+	Dulcitol	-	D-Lactose	+	D-Tagatose	-
L-Xylose	-	Inositol	+	D-Mallobiose	+	D-Fucose	+
D-Addonitol	-	D-Mannitol	+	D-Sucrose	+	L-Fucose	+
Methyl β-Dxylpyranoside	-	D-Sorbitol	-	D-Trehalose	+	D-Arabitol	+
D-Glactose	+	Methyl α-D Mannpyrunoside	-	Inuline	-	L-Arabitol	+
D-Glucose	+	Amygdaline	-	D-Melezitose	-	Potassium Gluconate	+
Glycerol	+	Arabutin	-	D-Rafinose	+	Potassium 2-Ketoglutarate	+
Erythritol	-	L-Arabinose	+	Amidon	-	Potassium 5-Ketogluconate	+
D-Arabinose	+	D-Ribose	+	D-Addonitol	-	D-Xylose	+
D-Xylose	+	Methyl β-Dxylpyranoside	-	L-Arabinose	+	L-Xylose	-
L-Xylose	-	D-Glactose	+	D-Ribose	+	D-Addonitol	-
Methyl β-Dxylpyranoside	-	D-Melezitose	-	D-Turanose	-		
D-Glactose	+	D-Rafinose	+	D-Lyrose	-		

Expression of proteins from stressed microorganisms entombed away from natural exposure of communities may provide clues of resistance mechanism other than acquired through exposure to antibiotics. Out of seven β-lactam antibiotics (cell wall synthesis inhibitors and protein synthesis inhibitors), isolate MRLBA1 exhibited sensitivity to neomycin (18 mm), streptomycin (22 mm); vancomycin (11 mm), aztreonam (24 mm) and tetracycline (25 mm) but was resistant to penicillin and fosphomycin (*Table 3*). The additional antibiotic resistance mechanisms regulated through Mg^{++} (Lewenza, 2005), also regulate the secretion and/or activity of alkaline phosphatase.

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Table 3. *Antibiotic sensitivity of Pseudomonas sp. MRLBA1*

Class	Antibiotics (groups)	Zone of inhibition (MRLBA1)
Protein synthesis inhibitors	Streptomycin (aminoglycoside)	S (22)
	Neomycin (aminoglycoside)	S (18)
Cell wall synthesis inhibitors	Vancomycin (glycopeptide)	S (11)
	Tetracycline (glycopeptide)	S (25)
	Penicillin (β-lactam)	R
	Aztreonam (monocyclicβ-lactam)	S (24)
	Fosphomycin (phosphonomycin)	R

R = resistant, S = susceptible

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequence and relevant phylogenetic analysis revealed that bacterium MRLBA1 closely resembles genus *Pseudomonas* (*Fig. 3*).

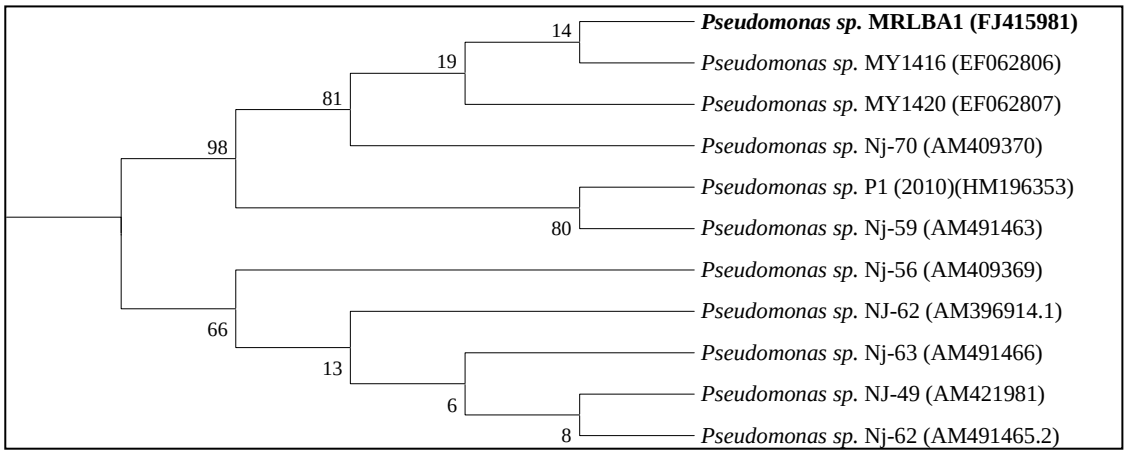

Figure 3. *Maximum parsimony phenogram; showing phylogenetic relationship between Pseudomonas sp. MRLBA1 and other related species of the genus Pseudomonas and related reference microorganisms based on the 16s rRNA gene sequence analysis. Bootstrap values (500 replicates) are given at the nods. Tree was generated with 10 replicates using MEGA4 (Close-Neighbor-Interchange algorithm)*

The closest relative appears to be *Pseudomonas sp.* MY1416 with a similarity of 97% in BLAST analysis. The isolates from polar (Antarctica) and non- polar glaciers like mountain glaciers of Pakistan-China border mostly show novel characteristics, since most of the flora has not been studied in these ecosystems. A small number of similar isolates have been characterized as psychrophilic (Miteva, 2008) including some species

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from *Methanococcoides*, *M. burtonii* (Franzmann et al., 1992). Along with other nearby glaciers in the region, like Batura Glacier in Hindu Kush (West Pakistan), Mingyong Glacier in Tibet (North China), Siachen in Himalaya (North-East Kashmir) and the Karakoram glaciers present a huge frozen ancient ecosystem out of pole harboring psychrophilic mode of life. From this isolated cold niche, contrary to other *Pseudomonas* species under study, the isolate MRLBA1 showed resistance to certain cell wall synthesis inhibitors like penicillin (β-lactam) and fosphomycin (phosphonomycin). The finding points out the strain's spatial induction of genetic assembly and/or cell wall divergence.

Growth and enzyme production

Psychrotolerants with scant energy express alkaline phosphatase as a stress protein to release energy from phosphoanhydrides – the high energy bonds (Seufferheld et al., 2008). Various cold active bacterial species including *Arthrobacter sp.* (De Prada et al., 1996), *Bacillus sphaericus* P9 (Dhaked et al., 2005), *Pseudomonas aeruginosa* (Cheng et al., 1970) and *Pseudomonas fluorescens* E2 and *Pseudomonas sp.* 8E3 (Pratt-Lowe et al., 1988) have been used for the production of alkaline phosphatase. The cell mass was observed as 1.81 cells per hour during exponential phase of the bacterium. A plot of growth (O.D) versus time (hours), yielded a typical growth curve with a prolonged lag phase of about 10 h. The exponential phase appeared as a steep curve after 24 h and lasted up to 36 h followed by a long stationary phase that lasted up to 120 h (*Fig. 4*). The bacterial physiology is not dependent upon the growth rates observed at various temperatures. Also the viable counts have been considered as the crucial parameter to distinguish between psychrophilic and psychrotrophic beings (Feller et al., 1994). A very little activity of alkaline phosphatase (0.396 U/ml) was found associated with periplasmic space during growth curve.

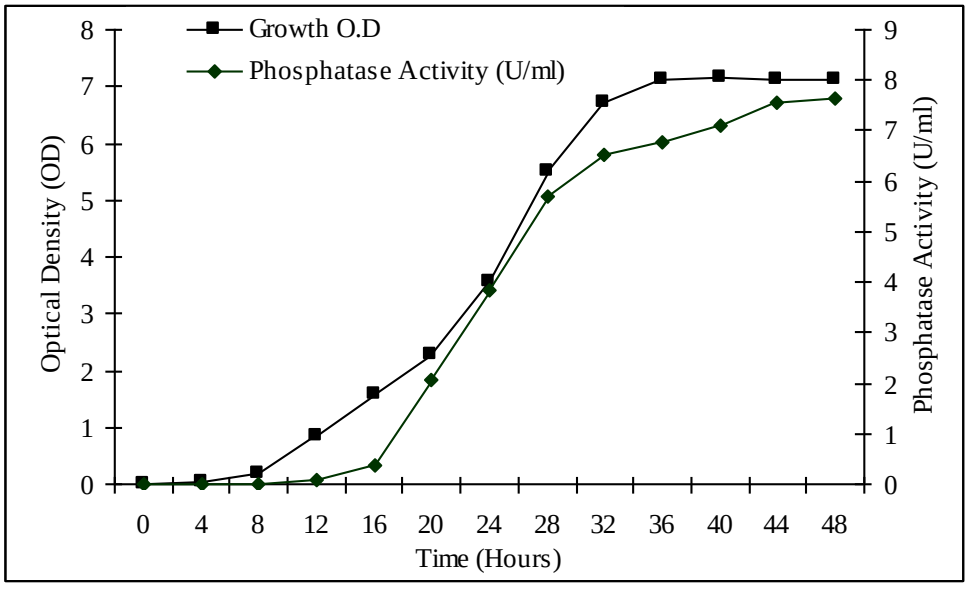

Figure 4. *Production of alkaline phosphatase at different stages of bacterial growth. Cells were grown in the basal medium described in the text and growth was monitored by measuring optical density at 600 nm. Phosphatase activity was estimated in the culture supernatant at different stages of growth*

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Batch culturing in shake flask

Pseudomonas sp. MRLBA1 yielded 16 g of wet cells after 72 h from 1.6 l biomass under optimized conditions.

Effect of pH on the production of alkaline phosphatase

The *Pseudomonas sp.* MRLBA1 was capable of growing in the pH range of 4-11 with maximum growth at pH 9.0. However, the strain produced alkaline phosphatase optimally at pH 8.0 after 72 h of incubation at 18^{o}C and 150 rpm with specific activity of 57.56 U/mg protein ($P < 0.0001$). The production of enzyme (specific activity) was reported as 33.05, 44.32, 47.14, 56.28 and 35.39 U/mg at pH 5.0, 6.0, 7.0, 9.0 and 10.0, respectively (*Fig. 5a*).

The production of enzyme significantly decreased on pH other than optimal value. The production of enzyme was targeted in late stationary and death phase of the growth curve. A very little activity was found attributed to the cell bound during growth cycle of the bacterium (*Fig. 5a*).

Effect of temperature on the production of alkaline phosphatase

Optimum alkaline phosphatase was produced (22.41 U/ml) with specific activity (53.72 U/mg) at 18°C after 48 h of incubation ($P < 0.0001$). At lower temperatures i.e. 4, 10 and 15°C, the production of enzyme (6.73, 11.13 and 14.57 U/ml) with specific activities as 28.56, 41.17 and 45.81 U/mg, respectively (*Fig. 5b*). The yield of enzyme was enhanced when harvested cells were temperature shocked at -70°C for 10 min and harvested again after resuspension in 20 mM Tris HCl (pH 8.0; *Fig. 5b*). The same practice has been adapted by Hong et al. (2007) for *E. coli* and Cheng et al. (1970) for *Pseudomonas aeruginosa* to collect the alkaline phosphatase from periplasm.

Effect of incubation period

The inoculated production medium revealed specific activities of 4.42 U/mg (24 h), 54.01 U/mg (48 h) and 59.43 U/mg after 72 h of incubation at pH 8, 18°C for 72 h and 150 rpm (*p* 0.07; *Fig. 5c*).

Effect of size of inoculum on the production of alkaline phosphatase

Optimum alkaline phosphatase (specific activity; 77.02 U/mg) was produced in case of 5% inoculum, whereas, in case of 1, 10, 15 and 20% inoculum size, the specific activities were 38.71, 32.37, 18.85 and 12.7 U/mg, respectively, with significant value ($p > 0.001$; *Fig. 5d*). The pH of the medium increased up to 8.3 starting from initial pH 8.0.

Effect of age of inoculum on the production of alkaline phosphatase

Optimum production of alkaline phosphatase (56.42 U/mg) was observed in production medium inoculated with a 24 h old inoculum after 48 h of incubation at pH 8.0, 18°C, 5% inoculum and 150 rpm ($p < .001$). When 12, 48 and 72 h old inocula were used, specific activities of alkaline phosphatase were recorded as 34.19, 39.53.12 and 16.8 U/mg respectively (*Fig. 5e*).

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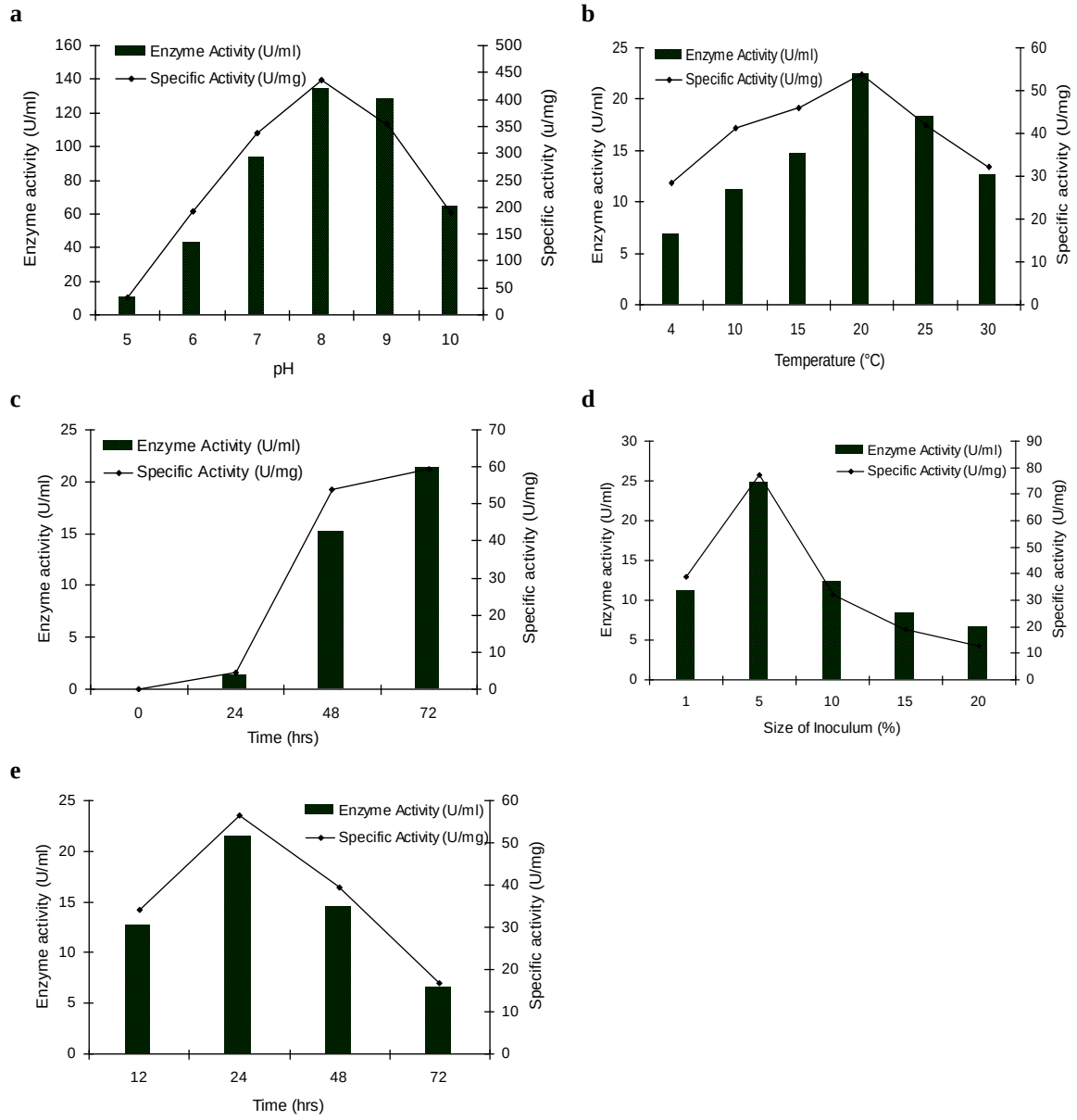

Figure 5. *Effect of various parameters (a-e) on the production of alkaline phosphatase. a Effect of pH on the production of alkaline phosphatase. b Effect of temperature on the production of alkaline phosphatase. c Effect of incubation period on the production of alkaline phosphatase. d Effect of size of inoculum on production of alkaline phosphatase. e Effect of age of inoculum on the production of alkaline phosphatase*

Conclusion

Out of pole, the mountain glaciers are a big resource of psychrophilic microbial diversity and hence and hence for industrial biotechnology. Cold active bacteria from depth of glacial ice could be potential source of enzymes capable of catalysis at lower temperatures. Bacterial metabolism at psychrophilic and/or psychrotrophic range of temperatures depends on the adjustment of their enzymes to work in cold conditions. Hence their enzymes drive the evolutionary adaptations. Psychrophilic enzymes produced by cold-adapted microorganisms display a high catalytic efficiency and may

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be associated with high thermosensitivity. The alkaline phosphatase from *Pseudomonas sp.* MRLBA1 was produced in acceptable range of physiological parameters in accordance with previous reports and implies an uncomplicated commercial source. The characterized production in this investigation is a valuable distinguished information to proceed for pilot scale production of low temperature active alkaline phosphatase.

We want to further this study for further purification and characterization of the heat sensitive activity.

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