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PURIFICATION AND CHARACTERIZATION OF ALKALINE PHOSPHATASE FROM A HALOTOLERANT FACULTATIVE ALKALIPHILE *BACILLUS FLEXUS* FPB17

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ABSTRACT: The extracellular alkaline phosphatase from halotolerant facultative alkaliphile *Bacillus flexus* FPB17 was purified through precipitation / dialysis / chromatography and resulted in 28 fold of purification. The molecular weight of this enzyme through SDS-PAGE gel is 63 ± 2 kDa. Optimum conditions for purified ALP activity were pH 9.0 and 35°C. The enzyme proved to be thermolabile and gets completely denatured at 60°C in 10 min. p-NPP was found to be a preferred substrate and Km (0.33 mM) and V_{max} (0.2 mM mg-1) determined using p-NPP as a substrate. Inorganic phosphate proved to be a competitive inhibitor of ALP. Mg^{+2} and Ca^{+2} increased the ALP activity by 47% and 20% respectively whereas Sodium fluoride, Sodium arsenate and EDTA decreased the activity by 47%, 65% and 77% respectively. The superiority of dephosphorylation of λ phage DNA by this newly discovered alkaline phosphatase over that of calf intestinal alkaline phosphatase of M/s. HiMedia Laboratories, Mumbai opens up possibility of its future use in molecular biology as a replacement of alkaline phosphatases from *Escherichia coli*, calf intestine and shrimp.

INTRODUCTION: Alkaline phosphatases (ALPs, EC 3.1.3.1) are reported from several organisms ranging from prokaryotes to mammals as stable enzymes removing phosphate groups from diverse molecules, including nucleotides, proteins, and alkaloids [1]. ALPs find potential application in molecular biology and medical research. The commercially available calf intestinal alkaline phosphatase (CIAP) and *Escherichia coli* alkaline phosphatase (ECAP) are resistant to heat inactivation and difficult to remove after use.

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The psychrophilic ALPs are unstable at high temperatures [2] and, therefore are widely used in research requiring post treatment heat inactivation. Shrimp alkaline phosphatase (SAP) is the most widely used psychrophilic ALP because of irreversible inactivation at 65°C. Relative high extraction cost and problems in expression of recombinant enzyme have hampered the large scale production of SAP [3].

Several attempts have been made to identify novel ALP substitutes for SAP from other sources. Antarctic bacterium strain TAB5 alkaline phosphatase (TAP) was cloned and expressed in *E. coli* and improved yields of TAP by 21 times while using a two step chromatography method. The enzyme was compared with ECAP and found Mg^{+2} playing an important role in the dephosphorylation step [4].

The present work deals with the purification and characterization of extracellular ALP by a halotolerant facultative alkaliphile *Bacillus flexus* FPB17 isolated from alkaline lake sediments from North Gujarat, India.

MATERIALS AND METHODS:

Purification of ALP from FPB17:

The cell free supernatant of *Bacillus flexus* FPB17 identified on the basis of 16S rRNA sequencing (NCBI GenBank accession no. JN415115) obtained after centrifugation of the culture at 7000 g for 15 min at 4°C grown in the optimized medium for 48 h at 35°C at 140 rpm, was used as a crude enzyme preparation. The crude enzyme was precipitated by adding a solution of 60% w/v of Ammonium sulfate to the cell free supernatant. The precipitates stored overnight at 4°C were recovered by centrifugation at 7000 g for 15 min. The supernatant was again precipitated by addition of 15% w/v of Ammonium sulfate. All the precipitates pooled and re-suspended in 50 mM Tris-HCl buffer, pH 9.0 (reaction buffer). This suspension of protein precipitates was dialyzed by keeping the dialysis bag in reaction buffer at 4°C kept overnight. After this, the dialysis bag then dipped in sucrose solution for 1 h to remove salt, metal ions and water to get the proteins concentrate only.

The concentrated protein solution was then loaded on a DEAE cellulose column (300 mm x 18 mm) pre-equilibrated with reaction buffer. The elution was carried out with 0.1 - 0.5 M NaCl in reaction buffer at a flow rate 0.2 ml/min. The protein content of fractions was determined by measuring O. D. at 280 nm. The protein containing fractions were assayed for ALP activity by *p*-nitrophenyl phosphate disodium salt (*p*-NPP) method at 400 nm[5]. The maximum ALP activity containing eluate fraction was further purified by affinity chromatography. The stationary phase was prepared by coupling diazonium salt of 4-(*p*-aminophenylazo) phenyl arsenic acid to Sephacryl-200 HR[6]. The fraction was loaded on a Sephacryl-200 HR column (200 mm x 10 mm) pre-equilibrated with reaction buffer. The enzyme was eluted with a linear gradient of 0.0 - 0.2 M KH_2PO_4 in reaction buffer at a flow rate of 0.9 ml/min. The protein content and ALP activity of fractions were measured as described earlier.

Characterization of *Bacillus flexus* FPB17 ALP

Determination of Molecular weight (M.W.) of ALP from FPB17:

M.W. of ALP protein was determined by SDS-PAGE electrophoresis [7] on a vertical slab 12% w/v separating polyacrylamide gels at a constant voltage of 50-80 V for 5 h and staining with 0.2% silver nitrate. Standard protein molecular weight markers 10-225 kDa from Novagen were used.

Studies on pH & temperature optima and thermostability of ALP from FPB17:

The effect of pH on ALP activity was studied by incubating the purified enzyme with 1.35 mM *p*-NPP substrate prepared in Citrate-Phosphate buffer (pH 6-7), Tris-HCl buffer (pH 7-9), Carbonate-Bicarbonate buffer (pH 9-11), Na_2HPO_4-NaOH buffer (pH 11-12) and KCl-NaOH buffer (pH 12-13) kept at 35°C. The temperature optima and stability of ALP enzyme was determined in the range 0-60°C by incubating the enzyme in 1.35 mM *p*-NPP in reaction buffer for 10 min, followed by the estimation of ALP activity at 35°C. The experiments were conducted in triplicate and average values are presented**.**

Substrate spectrum and kinetics of ALP from FPB17:

1.35 mM solutions of different phosphate containing substrates including phenyl phosphate, β-glycerophosphate, 2-napthyl phosphate, Adenosine monophosphate, D-Glucose-6-phosphate, D-Fructose-1, 6-diphosphate, Nicotinamide adenine dinucleotide and Phenolphthelein diphosphate were prepared in 50 mM Tris-HCl buffer at pH 9.0 and checked for relative ALP activity in comparison with *p*-NPP. The effect of *p*-NPP on ALP activity was studied by incubating enzyme with different concentrations of *p*-NPP (0.054, 0.081, 0.108, 0.162, 0.270, 0.540 and 1.080 mM) in reaction buffer at 35°C for 10 min. The K_m and V_{max} were determined from Lineweaver-Burk plots.

Effect of inorganic phosphate, metal ions and EDTA on ALP from FPB17:

The effect of inorganic phosphate on ALP was determined by estimation of the ALP activity in presence of 0.00, 0.10, 0.25 and 0.50 mM KH_2PO_4. The inhibition constant (K_i) was determined from

Lineweaver-Burk plot. The different metal ions *viz.* Mg^{+2}, Zn^{+2}, Ca^{+2}, K^{+}, Mn^{+2}, Co^{+2}, Sodium fluoride, Sodium arsenate and EDTA in 0.1 and 1 mM concentration were incorporated in reaction buffer and relative ALP activity was estimated compared to control (without any metal ions).

Dephosphorylation of λ phage DNA:

The 5' ends of *Eco*RI / $M_1\mu$ generated fragments of λ phage DNA were dephosphorylated by this newly discovered ALP as compared to the CIAP obtained from HiMedia Laboratories, Mumbai. The samples were then loaded onto 1% Agarose gel and electrophoresed at 100 V for 2 h and visualized under UV-Transilluminator for locating the dephosphorylated DNA of λ phage [8].

RESULTS AND DISCUSSION:

Purification of ALP:

Following the protocols of purification of ALP used by Kim et al., 1997 [9] and Ishida et al., 1998 [10] for the extracellular ALP from *Bacillus flexus* FPB17, ammonium sulfate precipitation resulted in 1.57 fold purification, with a rise in specific activity as observed by Ishida et al., 1998 [10] in *Shewanella* sp. This ALP remained stable and got further purified by dialysis at 4°C as in case of the phosphatase from *Shewanella* sp.[10].

The purification with DEAE-cellulose anion exchange column increased the specific activity of this ALP 3.69 fold using the elution procedure followed by Kim et al., 1997 [9]. DEAE-cellulose chromatography of *Bacillus flexus* FPB17 resulted in a peak matching to the single and well defined peak reported in most of the ALPs along with 2 other proteins.

The further purification was done by affinity chromatography using phosphate analog arsenate moiety having specific affinity to ALP [6]. The arsenate is a competitive inhibitor of ALP and has been used commonly as a ligand in the affinity chromatography purification of ALP from marine *Vibrio* sp. [11]. The affinity chromatography on Sephacryl-200HR step resulted in remarkable purification and raised the concentration to 27.88 fold from original 3.64 U mg^{-1} to 101.5 U mg^{-1}. However the yields were very low (16%). The total protein content came down from 350 mg to 2 mg by the recovery steps **Table 1**.

TABLE 1: IMPACT OF PURIFICATION STEPS ON THE RECOVERY OF ALP FROM *B. FLEXUS* FPB17 BROTH.

Purification Step	Total protein (mg)	ALP Total activity (U)	Specific ALP activity (U/mg protein)	Purification (fold)	Recovery (%)
Culture supernatant	350	1275	3.64	1.00	100.00
Ammonium sulfate precipitation	159	911	5.73	1.57	71.45
DEAE-cellulose fraction	40	537	13.43	3.69	42.12
Sephacryl-200 HR fraction	2	203	101.50	27.88	15.92

Characterization of ALP from *Bacillus flexus* FPB17:

The extracellular ALP of *Bacillus flexus* FPB17 got purified to homogeneity as evident from SDS-PAGE analysis, appearing as a monomer of 63 ± 2 kDa (**Fig. 1**). Most ALPs are homodimers as in *B. licheniformis* MC14 and *Serratia marcescens* [12, 13], many monomeric forms also exist. 59.5 kDa monomer ALP from *Bacillus subtilis* KIBGE-HAS [14], 60 kDa monomer ALP from *V. Cholera* [15] and 46 kDa monomer ALP from *B. Intermedius* [16] have been earlier reported.

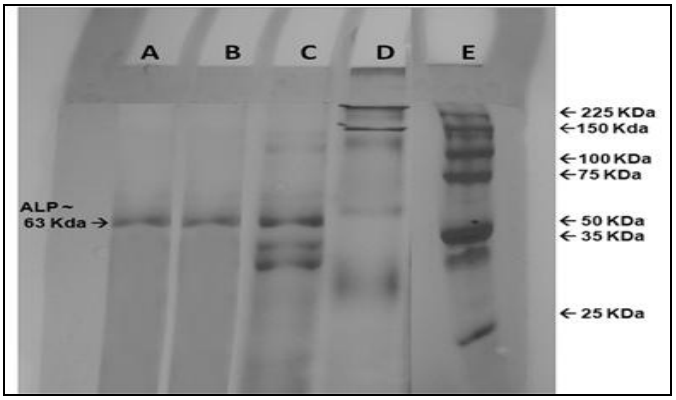

FIG. 1: SDS-PAGE GEL SHOWING SINGLE BAND OF PURIFIED ALP. Lane A, Affinity chromatography purified ALP; Lane B**,** Affinity chromatography purified ALP; Lane C, ALP with other proteins after dialysis; Lane D, ALP without SDS and heat treatment; Lane E, M.W. Markers.

pH / Temperature optima and thermolability of ALP:

ALPs have been reported to be active under a wide alkaline pH range from 7.5-11.0 [17]. The optimum pH for *Bacillus flexus* FPB17 ALP was 9.0 similar to that of extracellular ALPs from *Bacillus* sp. P9 [17]. The enzyme activity was completely inhibited at pH below 6.0, and was inhibited about 47% at pH 11 (**Fig. 2**). It is known that under alkaline pH, the rate-limiting step for ALPs is the phosphate-dissociation while under acid pH the rate-limiting step was dephosphorylation of enzyme phosphate complex. Alkaline pH range ALPs possibly allow more E-R_2OP complex formation[1] resulting into the highest enzyme activity around pH 9.0.

The temperature optima for ALP activity was found to be 35°C (**Fig. 3**), as reported in ALP from *Rhizobium* sp.[18] and very close to 37°C in case of ALP from *Bacillus subtilis* KIBGE-HAS [14]. The thermostability of proteins depends upon hydrogen bonds, salt bridges, Vander-Waals and hydrophobic interactions between polypeptide backbones and side chains of amino acids. The ALP from this *Bacillus flexus* is thermolabile and gets completely denatured at 60°C in 10 min (**Fig. 4**) similar to the heatlabile ALP from *Bacillus* sp. P9 [17] completely inactivated at 60°C in 50 min. However the activity of *Bacillus intermedius* S3-19 ALP is stable upto 60°C at pH 8-10 [16]. Heat lability is an important characteristic in the application of ALP for the dephosphorylation activity. ALPs from bacteria other than *Bacillus* e.g. *Rhizobium* sp. and marine bacterium *Cobetia* sp. have been reported to be stable up to 55°C [18] but get completely inactivated in 15 min at 55°C to 60°C in the presence of 2-Mercaptoethanol, Dithiothreitol [19] respectively. Heat stability of ALP from *E. coli* and other mesophilic organisms is very high [20].

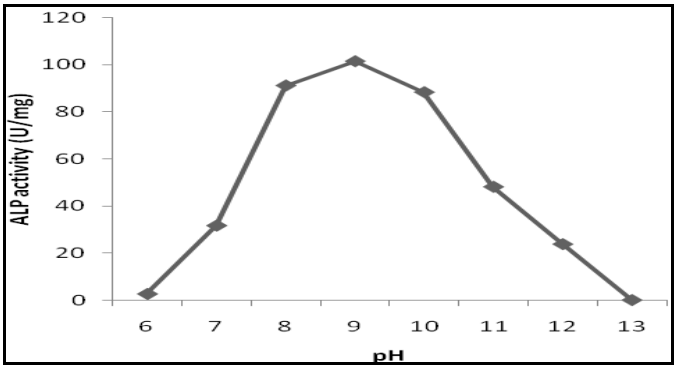

FIG. 2: EFFECT OF PH ON ACTIVITY OF ALP FROM *BACILLUS FLEXUS* FPB17

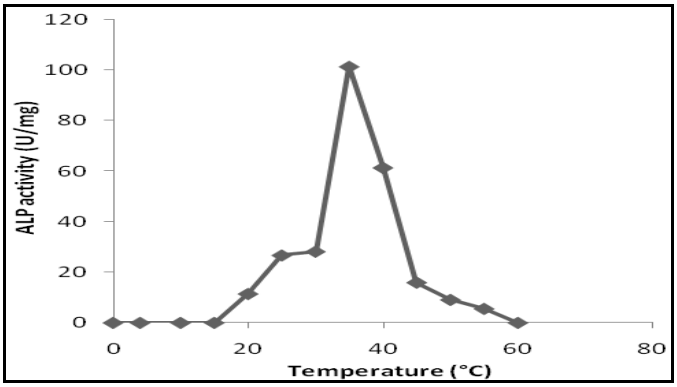

FIG. 3: EFFECT OF TEMPERATURE ON ACTIVITY OF ALP FROM *BACILLUS FLEXUS* FPB17

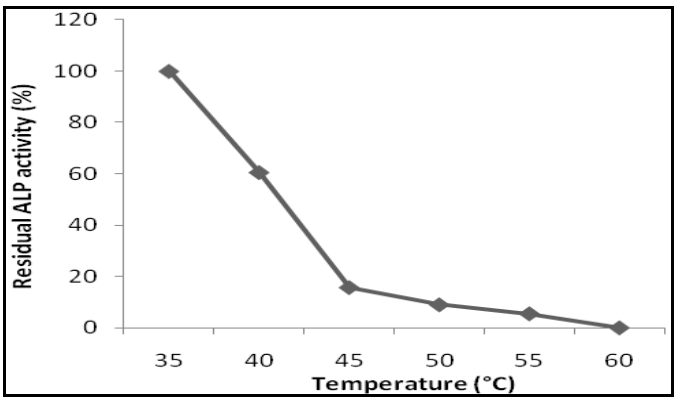

FIG. 4: THERMAL STABILITY OF ALP FROM *BACILLUS FLEXUS* FPB17

Substrate specificity, Kinetics and effect of inorganic phosphate (Pi) on ALP activity:

The ALP is a nonspecific phosphomonoesterase enzyme and thus can utilize a wide variety of phosphorylated substrates. Most of the ALPs prefer the synthetic substrate *p*-NPP in ALP from *Cobetia marina* [19] and ALP from *Aspergillus caespitosus* [21]. ALPs from thermophiles like *Thermus caldophilus* GK24 [9] prefer glycerophosphate. ALPs from psychrophiles like *Vibrio cholera* [15] showed preference towards glucose-1-phosphate. ALP of FPB17 was able to hydrolyze all the tested substrates but preferred *p*-NPP (**Table 2**).

TABLE 2: EFFECT OF DIFFERENT PHOSPHATE SUBSTRATES ON ALP ACTIVITY OF *B. FLEXUS* FPB 17.

Substrate	Relative activity
p-NPP	100.00
Phenyl phosphate	88.43
β-glycerophosphate	86.34
2-napthyl phosphate	83.19
Adenosine mono phosphate	51.84
D-Glucose-6-phosphate	42.94
D-Fructose 1,6-diphosphate	24.21
Nicotinamine adenine dinucleotide	18.15
Phenolphthelein diphosphate	14.66

Michaelis constants (K_m) and V_{max} for ALP from FPB17 were found to be 0.33 mM and 0.2 mM mg^{-1} respectively (**Fig. 5**). *Bacillus flexus* ALP presents K_m values which are identical to the ALPs reported by von Tigerstrom, 1984 [22] and Brenna et al., 1975 [6]. One of the characteristics of nonspecific ALPs is their high sensitivity to the final end product Pi. Increasing the concentration of Pi causes repression of ALPs in this *Bacillus flexus* ALP as reported in other bacterial ALP [23]. Same V_{max} and increased K_m (from 0.33 to 1 mM) in case of ALP from *Bacillus flexus* FPB17 in presence of the Pi (Fig. 6) is an assignable characteristic of competitive inhibition of the enzyme [24].

This ALP enzyme showed a high K_i value of 1 mM for competitive inhibition by inorganic phosphate indicating a low binding affinity for phosphate groups in comparison to calf intestinal ALP (K_i of 0.22 mM), which got bound to the affinity column. Low phosphate affinity may be a general characteristic of heat labile ALPs [25]. Thermolability of ALP from *Bacillus flexus* FPB17 is also associated with low binding affinity for phosphate groups. Competitive inhibition by phosphate was reported for the ALP from *Bacillus licheniformis* MC14 [13], with the inhibition constant (K_i of 0.037M).

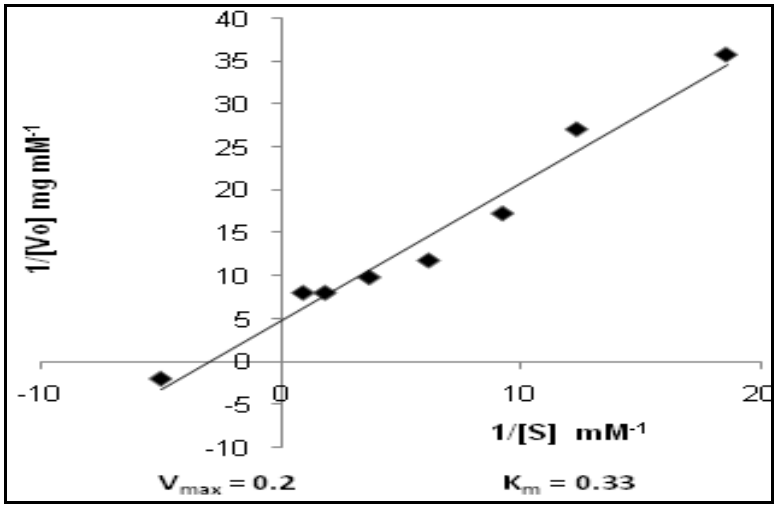

FIG. 5: EFFECT OF SUBSTRATE CONCENTRATION ON ALP ACTIVITY FROM *BACILLUS FLEXUS* FPB17 (LINEWEAVER-BURK PLOT)

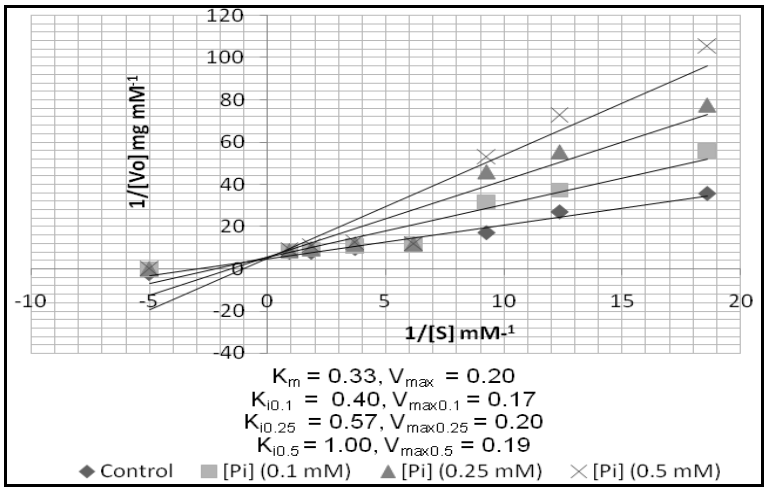

FIG. 6: EFFECT OF DIFFERENT INORGANIC PHOSPHATE ION CONCENTRATION [PI] ADDITION ON ALP ACTIVITY FROM *BACILLUS FLEXUS* FPB17

Effect of metals ions and enzyme inhibitors on ALP activity:

ALPs are metalloenzymes [26], often inhibited by metal ion chelators like EDTA [27]. They are classically considered to be Zn^{+2} and Mg^{+2}-dependent enzymes, especially *E. coli* and mammalian ALPs [28]. The *Bacillus flexus* FPB17 ALP similarly got activated by increasing Mg^{+2} and

Ca^{+2}, while Zn^{+2} showed such effects only at its lower concentrations (**Table 3**). ALP from psychrophilic bacterium *Bacillus* sp. P9 also exhibits the requirement of same metal ions as activators [17]. 47%, 65% and 77% decrease of the *Bacillus flexus* ALP activity was observed with Sodium fluoride, Sodium arsenate and EDTA respectively, similar to the reports for ALP enzyme from *Rhizobium* sp. by Kumar et al., 2008[18].

TABLE 3: EFFECT OF DIFFERENT METAL IONS AND EDTA ON ALP ACTIVITY FROM *B. FLEXUS* FPB17.

Metals	Residual ALP activity (%)	
	0.1 mM	1 mM
Control	100	100
Mg^{+2}	128	147
Zn^{+2}	109	96
Ca^{+2}	105	120
KCl	102	84
Mn^{+2}	97	75
Co^{+2}	94	83
Sodium fluoride	78	53
EDTA	66	23
Sodium arsenate	63	35

Dephosphorylation of λ phage DNA by Purified ALP:

ALPs are commonly used in molecular biology for removal of 5'-terminal phosphate from DNA fragments to prevent self-ligation and to enhance the possibilities of exogenous gene insert. Dephosphorylation efficiency of *P. abyssi* ALP under plasmid dephosphorylation conditions using a linear cohesive or blunt-ended pBSK vector was estimated at 93.8 and 84.1%, respectively [5]. ALP from *Cobetia marina* gave much more complete removal of phosphate from 5' ends of *Eco*R1-generated DNA fragments of λ phage [19]. As depicted by sharp bands in Lane F of the Electropherogram (**Fig. 7**) compared to the bands in Lanes G and multiple bands in Lane F compared to single bands in Lanes C and D, it is evident that this new ALP from *Bacillus flexus* dephosphorylated DNA of λ phage more efficiently than the standard CIAP of M/s. HiMedia Laboratories, Mumbai.

Of the several characterized ALPs, only ECAP, SAP and CIAP are routinely used in molecular biology at commercial scale [29]. In comparison with newly discovered *Bacillus flexus* FPB17 ALP, ECAP and CIAP require much higher temperature for after use denaturation, and the SAP is cost prohibitive. The purified ALP from *Bacillus flexus* has shown satisfactory production yields, ambient (35°C and pH 9.0) kinetics, thermolability and desirable dephosphorylation activity, making it suitable for most molecular biology applications as a substitute for the widely used ECAP, CIAP and SAP enzymes. Further studies are necessary to elucidate its potential use and commercial feasibility of such applications.

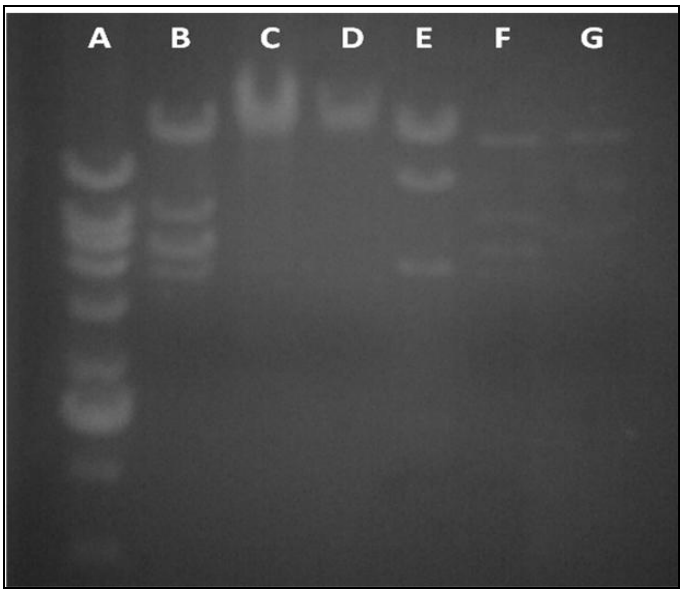

FIG. 7: ELECTROPHEROGRAM OF DEPHOSPHORYLATION OF Λ PHAGE DNA BY ALP OF *B. FLEXUS* FPB17.

Lane A, DNA ladder (10000 base pairs); Lane B, Control (Eco R1 digested λ phage DNA without ALP treatment); Lane C, Control (λ phage DNA); Lane D, Ligated DNA without ALP treatment; Lane E, $M_1\mu$ digested DNA; Lane F, FPB17 ALP treated Eco R1 digested DNA; Lane G, Himedia ALP treated $M_1\mu$ digested DNA

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CONFLICT OF INTEREST: The author declares that there is no conflict of interest.

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