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MICROBIAL DIVERSITY OF ALKALINE PHOSPHATASE PRODUCERS FROM LAKE ECOSYSTEM IN NORTH GUJARAT REGION, INDIA

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ABSTRACT:

Biodiversity of Alkaline phosphatase producing microbes in soil samples from water bodies and lake ecosystems of North Gujarat region was studied. 57 types of microbes were isolated through enrichment. High alkaline phosphatase producers were screened on Methyl Green dye and Phenolphthalein diphosphate supplemented MG-PDP / Modified Dermatophyte test agar (MDTA) media. Primary characterization of the isolates was carried out. 32 bacteria and 2 fungi produced alkaline phosphatase (ALP). The best six ALP producing bacteria were further characterized by morphological-biochemical tests. All the 6 bacteria produced ALP and other hydrolytic enzymes. ALP production was significant in liquid medium in the alkaline pH range. ALP production was induced by organophosphorous insecticide Acephate in *Bacillus* sp. FPB17 which can detoxify and mineralize a xenobiotic environmental contaminant like organophosphate. Incorporation of PDP and Methyl Green indicator dye can be utilized in the detection of ALP production.

***KEY WORDS*:** *Lake ecosystem; Alkaline phosphatase; Organophosphorous insecticide; Methyl Green; Phenolphthalein diphosphate*

INTRODUCTION:

The insoluble precipitated inorganic phosphates in soil and water is solubilized by the action of mineral and organic acids produced by bacteria and made available to the crops /plants (Fleischer *et al.*, 1988). The organic phosphorus compounds are also decomposed and mineralised by enzymatic complexes of heterotrophic bacteria (Aaronson and Patni, 1976). Phosphatases promote the degradation of complex phosphorous compounds into orthophosphates and have an essential function in the nutrient dynamics of lakes as there is no external natural phosphorus input process (Jansson *et al.*, 1988). The Phosphatases are

typically classified into alkaline and acid phosphatases according to their maximum hydrolysing capacity at different pH values (Jansson, 1988). The Alkaline Phosphatases (ALPs) are by far the most important group in the aquatic environment, since the conditions favouring the acid phosphatases are quite uncommon in aquatic environment and the algae and bacteria in lakes are mostly favoured by alkaline environment and generally produce more alkaline than acid phosphatases (Cembella *et al.*, 1984). Lo´pez *et al.* (2006) assigned ALPs activities contributing significantly to the phosphate pool in the lake water samples.

Phosphorus containing insecticides, herbicides and fungicides enter aquatic environments either by direct application spills, flooding water and/or by aerial deposition and exert diverse effects (direct or indirect) on microbiota. The phosphatases play an important role in the dynamics of the aquatic population of surface waters (Berman *et al.*, 1990). As these ecosystems are often P-limited, bacteria and other microorganisms need phosphatase enzyme activity for mobilising organic phosphorous to soluble inorganic forms need for their metabolism.

The other applications of ALPs in diagnostics, immunology, clinical medicine and molecular biology has made them popular in scientific studies and commercial utility (Chen *et al.*, 2006; Engvall and Perlman, 1997; Ishii and Ghosh, 1993; Jablonski *et al.*, 1986; Muginova *et al.*, 2007; Nilgiriwala *et al.*, 2008; Plebani *et al.*, 1996; Sun *et al.*, 2007; Suzuki *et al.*, 1999). It has been conventionally used as an index of adequate pasteurization, and the detection of ALP activity of thermally treated liquid milk products has become a common procedure for milk quality control (International Dairy Federation, 1991).

The purpose of the present study was to examine, under laboratory conditions, the ALP activities of the microorganisms in sediment samples from lake ecosystem in North Gujarat region, India. Selected microorganisms were also evaluated for their contribution in the biological status of the lake system.

METHODS & MATERIALS:

Soil Samples and analysis:

The sediment soil samples were collected in the monsoon season, August 2009 from different lake sites and waterbodies of Patan district *viz.* A from inside the Lodra lake, B from bank of Lodra lake, C from inside the Bhilot lake, D from bank of Bhilot lake, E from inside the Santalpur lake, F from bank of Santalpur lake, G from inside the water body at Radhanpur, H from inside the Sami water body and I from bank of Sami water body. All soil samples were serially diluted and checked for microbial counts (colony forming units per ml = CFU/ml) by standard plate count (SPC) technique. The soils were also tested for their pH, Electrical conductivity (E.C.) and Phosphorous content.

Isolation of microbes and identification of ALP producers:

Thornton's medium (Bajpai *et al.*, 1964) and Martin's Rose Bengal Chloramphenicol agar medium (Atlas, 2006) were used for isolation of microbes for bacteria and fungi respectively. The isolates were purified by repeated streak-plate method on Nutreint agar medium / Potato Dextrose agar medium respectively (Atlas, 2006). The pure isolates of bacteria were streaked on plates with MG-PDP medium and those of fungi were subcultured on Modified Dermatophyte test agar medium to check the ALP production. The MG-PDP medium as well as the modified Dermatophyte test agar medium with variable pH (7.5, 8.0, 8.5, 9.0) were supplemented with Methyl Green dye 50 mg/ml and Phenolphthalein diphosphate tetra sodium salt (PDP) 1 g/l. The colonies producing ALP got stained with deep green color, whereas the ALP non producing colonies remained colorless. The intensity of the green color for each isolate was noted and the isolates were graded according to the green color intensity. Six bacterial cultures with intense green color (FPB17, FPB23, FPB28, FPB34, FPB37 and FPB39) were taken up for the further work.

Characterization of the bacterial isolates:

Characterization of the bacterial isolates was carried out on the basis of Gram's Staining, Spore staining and motility testing by Hanging drop technique. Biochemical characterization of the best isolates were done by using HiAssorted biochemical test kit and HiBacillus^{TM} identification kit of Himedia Laboratories, Mumbai, India and Staph Identification kit and Listeria Identification kit of Tulip Diagnostics (P) Ltd., Goa, India.

Enzyme spectrum of ALP producers:

The 6 best ALP producing isolates were also checked for the production of other enzymes *viz.* Amylase, Protease, Lipase, DNAase, Lecithinase, Gelatinase and Oxidase on different solid media e.g. Starch agar, Casein agar, Tributyrin agar, DNAse test agar, Egg yolk agar, Gelatin agar and Nutrient agar respectively. The isolates exhibiting clearance zones indicated the production of these enzymes.

ALP Production in liquid medium:

The pure cultures of the best ALP producing bacteria were maintained on nutrient agar slants at around 4±1 ^{0}C. Inoculum preparation was carried out in Nutrient broth by transferring single colony from the grown culture. Inoculum was developed in 25 ml Nutrient broth with initial pH 9.0 in 100 ml Erlenmeyer flask, incubated on an orbital shaker at 35 °C and 120 rpm for 6 h to achieve optical density in the range of 0.8-1.2 at 600 nm. 2% v/v inoculum was transferred to 50 ml of medium (composition: Glucose 1 g/l, Peptone 10 g/l, Yeast extract 5

g/l, NaCl 10 g/l and PDP 1 g/l) in 250 ml Erlenmeyer flasks and incubated at 35 °C, 120 rpm for 24 h.

Analysis of ALP:

ALP activity was measured spectrophotometrically by determining the release of p-nitrophenol (*p*-NP) from p-nitrophenyl phosphate disodium salt (*p*-NPP) at 400 nm (Robert and Evan, 2003; Garen and Levinthal, 1960; Zappa *et al.*, 2001). 100 µl cell free supernatant was added to 1000 µl of *p*-NPP solution (1.35 mM in 50 mM Tris-HCl buffer at pH 9.0) and the mixture was incubated at 35 ˚C for 10 min. One unit of enzyme activity is the amount of the ALP catalyzing the liberation of 1µmol of *p*-NP per min.

Organophosphorus insecticide utilization by ALP producers :

Utilization of Organophosphorus insecticide by the best 6 ALP producer bacteria was studied by growing the isolates in the medium containing 1 mg/ml organophosphate insecticide Acephate as a sole Carbon source, Peptone 10 g/l, NaCl 2.5 g/l, $MgSO_4$ 0.2 g/l, $MnSO_4$ 0.1 g/l and $CaCl_2$ 0.1 g/l. The degradation of Acephate was checked by means of removal of phosphate from it by Ascorbic acid method (results not shown) and ALP activity was determined by the *p*-NPP method described earlier.

RESULTS AND DISCUSSION:

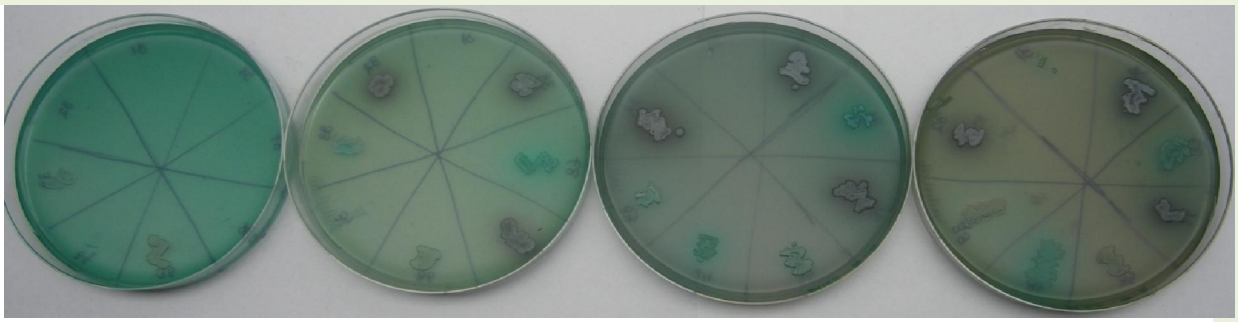
The population of microbes, number of types of bacteria and fungi in samples and the results of soil testing are presented in Table 1.

Table 1: Microbial Population, Bacterial and Fungal Types, pH, Electrical Conductivity and Phosphorus content of soil samples from Lake Ecosystem sediment soils.

Soil sample	pH	E.C. (mili mho/cm)	Phosphorous (kg/hector)	CFU/ml	Bacterial Isolate Nos. (FPB)	Fungal Isolate Nos. (FPF)
A	7.97	9.3	45.96	3×10^5	1-7, 38, 39	13
B	7.80	6.3	11.40	9×10^5	8,10,11,40-42	9-12, 14, 15
C	7.69	8.4	22.98	6×10^5	12-16	1, 2
D	7.95	4.6	11.24	8×10^5	9,17-20	-
E	8.02	1.8	45.90	5×10^5	21,22,35-37	5, 6
G	8.33	4.8	11.49	4×10^5	23-25	7, 8
H	8.31	4.2	05.75	4×10^5	26-32	4
I	7.91	1.5	22.90	12×10^5	33,34	3

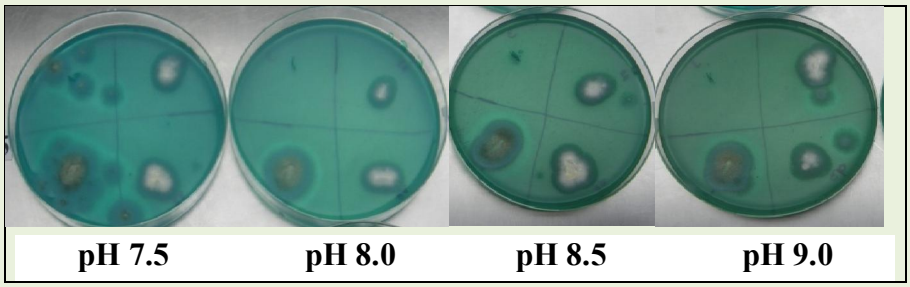
The microbial population was found to be in the range of 3×10^5 to 12×10^5 CFU/ml. On the basis of the colony characterization, a total of 42 bacterial and 15 fungal cultures were isolated from the sediment soil samples of lake ecosystem. The pH of these samples was mostly around 8.0. The soil factors do not exhibit any inter-relationship nor do they correlate with population and microbial types.

The growth and degree of green staining of bacterial colonies grown on MG-PDP medium and bacterial colonies grown on Modified Dermatophyte test agar medium at different pH levels (7.5, 8.0, 8.5 and 9.0) indicative of ALP activity is presented in Table 2, Fig.1 and Fig.2.



pH 7.5 **pH 8.0** **pH 8.5** **pH 9.0**

Fig. 1: Production of ALP by bacterial isolates from lake ecosystem at various pH levels as indicated by intensity of green colour



pH 7.5 **pH 8.0** **pH 8.5** **pH 9.0**

Fig. 2: Production of ALP by fungal isolates from lake ecosystem at various pH levels as indicated by intensity of green colour

Table 2: Production of ALP by bacterial isolates (on MG-PDP medium) and fungal isolates (on modified Dermatophyte test agar medium) from Lake Ecosystem at various pH levels as indicated by intensity of green colour

Bacterial isolate no.	pH 7.5	pH 8.0	pH 8.5	pH 9.0	Bacterial isolate no.	pH 7.5	pH 8.0	pH 8.5	pH 9.0
FPB1	NG	+++	++	+	FPB22	NG	+++	-	-
FPB2	NG	+++	++	++	FPB23	NG	NG	++	++++
FPB3	NG	NG	NG	NG	FPB24	NG	+++	+	-
FPB4	NG	+++	++	-	FPB25	NG	NG	NG	-
FPB5	NG	+++	++	-	FPB26	NG	+++	-	-
FPB6	NG	NG	NG	NG	FPB27	NG	+++	±	±
FPB7	NG	NG	-	±	FPB28	NG	++	++++	+++
FPB8	NG	+	-	-	FPB29	NG	+++	+	-
FPB9	NG	NG	+	±	FPB30	NG	NG	±	+
FPB10	NG	NG	+	±	FPB31	NG	NG	-	-
FPB11	NG	++	+	±	FPB32	NG	-	-	-
FPB12	NG	++	-	-	FPB33	±	+	++	±
FPB13	NG	+	+++	+	FPB34	NG	NG	++	+++
FPB14	NG	NG	±	-	FPB35	-	±	+	±
FPB15	NG	++++	+++	+	FPB36	NG	-	-	-

Bacterial isolate no.	pH 7.5	pH 8.0	pH 8.5	pH 9.0	Bacterial isolate no.	pH 7.5	pH 8.0	pH 8.5	pH 9.0
FPB16	NG	NG	NG	NG	FPB37	NG	++	+++	+
FPB17	NG	NG	±	++++	FPB38	NG	-	-	-
FPB18	NG	NG	-	-	FPB39	NG	NG	+	+++
FPB19	NG	NG	NG	-	FPB40	NG	NG	NG	+++
FPB20	NG	+++	+	±	FPB41	NG	NG	±	+++
FPB21	NG	+++	-	-	FPB42	NG	NG	NG	+++
Fungal isolate no.	**pH 7.5**	**pH 8.0**	**pH 8.5**	**pH 9.0**	**Fungal isolate no.**	**pH 7.5**	**pH 8.0**	**pH 8.5**	**pH 9.0**
FPF1	+++	+++	++++	++	FPF9	-	-	NG	NG
FPF2	-	-	-	-	FPF10	-	-	-	-
FPF3	-	-	-	-	FPF11	NG	NG	NG	NG
FPF4	NG	NG	NG	NG	FPF12	++	++	+++	++++
FPF5	NG	NG	NG	NG	FPF13	-	-	-	-
FPF6	NG	-	-	-	FPF14	-	-	-	-
FPF7	-	-	-	-	FPF15	NG	-	-	-
FPF8	NG	NG	NG	-					

NG = no growth; - = growth with no green stain; ± = faint green stain; + = light green stain, ++ moderate green stain =, +++= dark green stain, ++++ = very dark green stain

Based on the ALP activity related green pigment / color, bacterial isolates: FPB17, FPB23, FPB28, FPB34, FPB37 and FPB39 and fungal isolates: FPF1 & FPF12 were observed as the best ALP producers. Due to the formation of stained precipitates, bacterial cells have been reported to become stained due to the phosphatase action around the cell membranes resulting in the intense pigmentation of the phosphatase positive colonies, progressive intensity of staining being indicative of phosphatase level (Satta *et al*., 1979: Satta *et al*., 1984).

Bacterial phosphatases have an important role source in the recycling organic phosphorus compounds in freshwater ecosystems (Barik and Purushothaman, 1999). Such a possibility in the Lake ecosystem appears to be in place as 25 out of the 42 bacterial isolates exhibited significant ALP activity at some or the other pH level, whereas only 2 of the 15 fungi exhibited the ALP activity.

It has been opined by Sayler *et al*. (1979) and Sinke *et al*. (1991) that the variations in the activity of the freshwater sediment systems are related to microbial biomass and rich organic matter as by that the change in ALP activity is a function of microbial biomass in sediment systems. However, all the activity present in the sediment might not be due to bacterial origin only as the flora and fauna may also contribute to the phosphatase pool. Such a correlation was not evident in our studies.

Evaluation of Screening ALP production methods:

The use of chromophore *p*-NPP as substrate for screening of the phosphatase activity of bacterial colonies on plates was initiated by Echols *et al.* in 1961. The flooding of the assay plates a solution containing Tris-HCl buffer (pH 8.5), $MgCl_2$, and *p*-NPP resulted in intense yellow color in the colonies displaying the wild type ALP activity, while the colonies of putative mutants for ALP activity remained white (Haugland *et al.*, 1994). The flooding had

the disadvantage of introduction of contaminants to the plate or cross-contamination of colonies. Wolf *et al.*, 1973 described the detection of enzyme production through measurement of development of an indigo blue color formed by conversion of indolyl phosphate substrate. al-Niemi *et al.*, 1997 used a precipitating fluorescent probe 2-(5'-chloro-2'-phosphoryloxyphenyl)-4-[3H]-quinazolinone (CPQP) to screen phosphatase producing colonies. Based on the techniques used by Satta *et al.*, 1979; Riccio *et al.*,1997; Nilgiriwala *et al.*, 2008, the method utilized in this study used PDP as the substrate and Methyl Green as an indicator dye for detection of ALP production. Colonies producing ALP get stained with deep green color, whereas the other colonies remained colorless. This method is advantageous as there is no chance of contamination as compared to *p*-NPP containing medium, preparation of medium is easy compared to CPQP containing medium and it has additional benefit of distinguishing between excreted and cell associated phosphatase.

Characterization of the good ALP producers:

The morphological and the Biochemical characteristics of the 6 good ALP producers are presented in Tables 4 & 5. Most of these isolates are aerobic, sporulating, motile Gram positive bacilli (Table 4). The biochemical and enzymatic characters of the good ALP producing bacteria confirm that these belong to genus *Bacillus* being catalase and oxidase positive. These isolates exhibited important hydrolytic enzymes including amylase, protease, lipase (Table 6). *Bacillus* spp. have been found to be the dominant phosphatase producing bacteria (Barik and Purushothaman, 1999).

Table 4: Morphological characteristics of the best bacterial ALP producers from Patan Lake Ecosystem

Bacterial isolate	Gram's Reaction	Cell Morphology	Capsule staining	Spore staining	Motility testing
FPB17	+ve	Thick, large rods	Noncapsulated	Sporulating	Motile
FPB23	+ve	Thick, medium rods	Noncapsulated	Sporulating	Motile
FPB28	+ve	Thin, large rods	Capsulated	Sporulating	Motile
FPB34	+ve	Thick, medium rods	Noncapsulated	Sporulating	Motile
FPB37	+ve	Thick, small rods	Noncapsulated	Sporulating	Motile
FPB39	+ve	Thick, small rods	Noncapsulated	Sporulating	Motile

Table 5: Biochemical characteristics of the best bacterial ALP producers from Patan Lake Ecosystem

S.N.	Biochemical test	Good ALP producing Bacterial Isolates					
		FPB17	FPB23	FPB28	FPB34	FPB37	FPB39
1	Glucose Utilization	+ve	+ve	+ve	+ve	+ve	+ve
2	Xylose Utilization	-ve	-ve	+ve	-ve	+ve	-ve
3	Lactose Utilization	-ve	-ve	-ve	-ve	-ve	-ve
4	Mannitol Utilization	+ve	+ve	+ve	+ve	+ve	+ve
5	Rhamnose Utilization	-ve	-ve	-ve	-ve	-ve	-ve
6	α-Methyl-D-Mannoside Utilization	-ve	-ve	-ve	-ve	-ve	-ve
7	Ribose Utilization	+ve	+ve	-ve	+ve	+ve	+ve

S.N.	Biochemical test	Good ALP producing Bacterial Isolates					
		FPB17	FPB23	FPB28	FPB34	FPB37	FPB39
8	Arabinose Utilization	-ve	-ve	+ve	-ve	-ve	-ve
9	Sucrose Utilization	+ve	+ve	+ve	+ve	+ve	+ve
10	Raffinose Utilization	+ve	+ve	-ve	+ve	+ve	+ve
11	Trehalose Utilization	+ve	+ve	+ve	+ve	+ve	+ve
12	Maltose Utilization	+ve	+ve	+ve	+ve	+ve	+ve
13	Methyl Red Test	+ve	+ve	-ve	+ve	-ve	+ve
14	Voges Proskauer	-ve	-ve	+ve	-ve	+ve	-ve
15	Nitrate Reduction	-ve	-ve	+ve	-ve	-ve	+ve
16	Catalase Detection	+ve	+ve	+ve	+ve	+ve	+ve
17	Esculin Hydrolysis	-ve	+ve	+ve	+ve	+ve	+ve
18	ONPG Utilization	+ve	+ve	+ve	+ve	+ve	+ve
19	Urease Detection	-ve	-ve	+ve	-ve	-ve	-ve
20	Arginine Utilization	-ve	-ve	+ve	-ve	+ve	-ve
21	Alkaline phosphatase	+ve	+ve	+ve	+ve	+ve	+ve

+ve = substrate utilized / reaction positive, -ve = substrate not utilized / reaction negative

Table 6: Enzyme spectrum of the best bacterial ALP producers from Patan Lake Ecosystem.

Bacterial isolate	Enzyme Production						
	Amylase	Protease	Lipase	DNAse	Gelatinase	Oxidase	Lecithinase
FPB17	+ve	+ve	+ve	-ve	+ve	+ve	-ve
FPB23	+ve	+ve	+ve	-ve	+ve	+ve	+ve
FPB28	+ve	+ve	+ve	-ve	+ve	+ve	+ve
FPB34	+ve	+ve	+ve	-ve	+ve	+ve	+ve
FPB37	+ve	+ve	+ve	-ve	+ve	+ve	+ve
FPB39	+ve	+ve	+ve	-ve	+ve	+ve	-ve

Selection of best ALP producer:

The 6 best ALP producer bacterial isolates were analyzed for production of ALP in the production medium containing PDP or Acephate as sole C source. The results are depicted in Fig. 3. Very minor differences in the ALP levels of different six isolates were observed in the PDP containing medium. However, in the medium with organophosphorus insecticide Acephate, isolate FPB17 exhibited 1.36 fold increased ALP activity, whereas the other five isolates had slightly decreased ALP production. Addition of high concentrations of insecticides, herbicides and fungicides increased phosphatase activities in lake water samples incubated at laboratory conditions as reported by Lo´pez *et al.* (2006). It can be seen that utilization of Acephate as sole C source induced the production of ALP in the isolate FPB17. This may lead to utilization of *Bacillus* sp. FPB17 to detoxify and mineralize a xenobiotic environmental contaminant like organophosphates and future use as a pollution controlling agent.

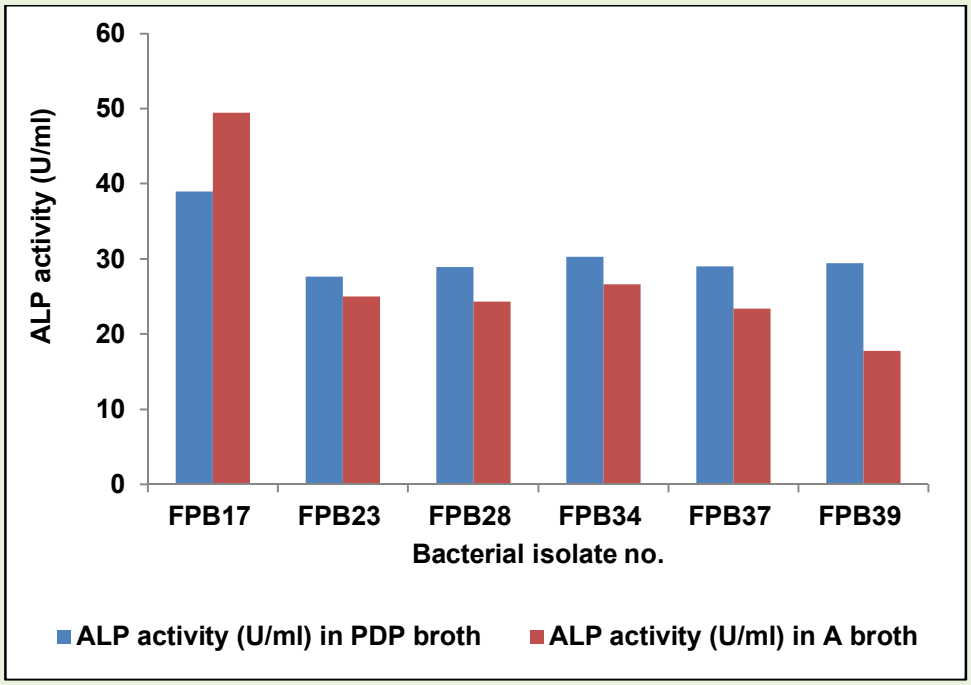


Fig. 3: Determination of ALP activity (U/ml) in liquid medium.

CONCLUSIONS:

The Alkaline Phosphatases are the most important group of enzymes in the aquatic environment. The investigations on the Alkaline Phosphatase production by microbes from Lake ecosystem shows: [1] The bacteria are more promising in ALP production in the lake ecosystem than the fungal isolates. [2] *Bacillus* is a dominant genus in sediment soil samples of the lake ecosystem in Patan. [3] Most of the facultative alkalophiles produce ALP when the pH is above 7.5. [4] Incorporation of PDP as the substrate and Methyl Green as an indicator dye is useful in the detection of ALP production, ALP producing colonies get stained with deep green color. [5] Morphological and biochemical characterization of high ALP producers confirm them as *Bacillus* and [6] Organophosphate insecticide Acephate induced the production of ALP in the isolate FPB17 which proved its utility in the utilization of the insecticide and the future use as a pollution controlling agent.

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