INVESTIGATIONS OF MICROBIAL ALKALINE PHOSPHATASE PRODUCTION FROM FIELD SOILS OF NORTH GUJARAT REGION, INDIA.

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ABSTRACT

Biodiversity of Alkaline phosphatase producing microbes from field soil samples from GSFC farm, Bhilot, Patan District, North Gujarat region was found to be represented by 35 microbes, 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. 22 bacteria and 6 fungi produced alkaline phosphatase (ALP). The best four ALP producing bacteria were further characterized by morphological-biochemical tests. All the four bacteria produced several other hydrolytic enzymes. ALP production was significant in the alkaline pH range. ALP production was induced by organophosphorous insecticide Acephate in all the four isolates but maximum in Bacillus sp. SF15 which can mineralize xenobiotic environmental contaminants like organophosphate.

Keywords: *Alkaline phosphatase; Organophosphorous insecticide; Methyl Green dye; Phenolphthalein diphosphate*

1. INTRODUCTION:

Phosphorus, like carbon and nitrogen is an essential element in all living systems. It is required for the synthesis of nucleic acid molecules (DNA, RNA). It is a vital component of the biological energy molecule adenosine triphosphate (ATP). It is essential component of cell membranes as phospholipids. Under normal conditions, phosphorus is not an abundant component of the ecosphere. When it is present, it may not be available for assimilation by organisms due to its tendency to precipitate in the presence of metals such as calcium, magnesium and ferric ions at neutral to alkaline pH. Therefore, it often limits the growth of primary producers such as algae, other aquatic plants, cyanobacteria and photosynthetic bacteria.

Most phosphorus transformations mediated by microorganisms involve the mineralization of organic to inorganic phosphates or the conversion of insoluble, immobilized forms of tertiary phosphate into soluble mobile primary phosphates that are more readily used by organisms. One such transformation is mediated by phosphatase enzymes often microorganisms produced by bacteria, fungi, and some algae as exoenzymes (Valsami-Jones, 2002). Phosphatases are classified on the basis of their pH optima: Acid and Alkaline phosphatases. The role of acid phosphatase as a phosphate solubilizer in the field soils is reported more frequently than the alkaline phosphatases.

The 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

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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 the field soil samples from the North Gujarat region, India. Selected microorganisms were also evaluated for their contribution in the bioremediation of Acephate an oraganophosphorous insectiside.

2.0 METHODS & MATERIALS:

SOIL SAMPLES AND ANALYSIS:

The soil samples were collected in the monsoon season, August 2009 from GSFC farm sites of Patan district, North Gujarat region. Soil samples were serially diluted and checked for microbial counts (colony forming units per gram of soil = CFU/g) by standard plate count (SPC) technique. The soils were also tested for their pH, Electrical conductivity (E.C.), Phosphorous content, Potash and Organic Carbon.

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. Four bacterial cultures with intense green color (SF13, SF15, SF18 and SF21) 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, Capsule Staining and motility testing by Hanging drop technique. Biochemical characterization of the best isolates were done by using HiAssorted biochemical test kit and HiBacillus™ 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 4 best ALP producing isolates were 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 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 hrs.

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 ÏC 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 four 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.

3.0 RESULTS AND DISCUSSION:

SOIL ANALYSIS:

The general properties of the soils were: pH 7.82, E.C. 1.68 mili mho/cm, Phosphorous 11.49 Kg/hector, Organic Carbon 0.61% and Potash 228 Kg/hector. The microbial population in soil was found to be in the range of 4×10^7 to 6×10^7 CFU/g. The soil factors do not exhibit any inter-relationship nor do they correlate with population and microbial types.

ISOLATION AND CHARACTERIZATION OF MICROBES:

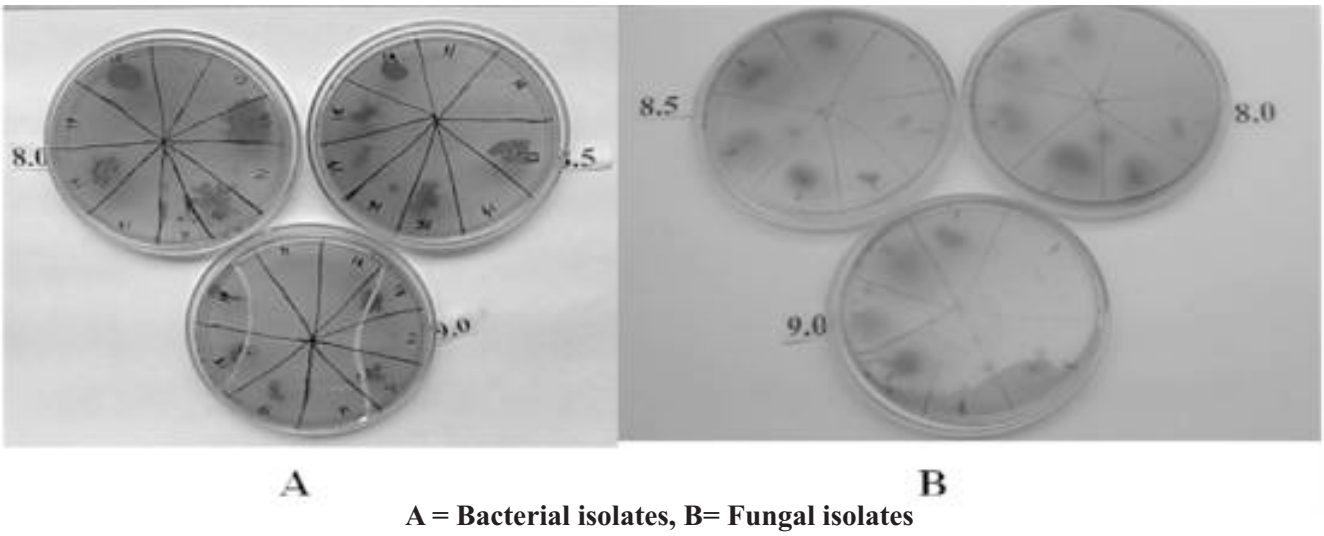
The microbes isolated and characterized from field soil samples on the basis of the colony and staining reactions are listed in Table 1.

Table 1: Biodiversity of microbes from field soil samples of Patan

Site of isolation	Microorganisms	No. of isolates	No. of isolates with ALP activity*
GSFC Field sites, Patan	**Bacteria**		
	Gram positive rods	18	16
	Gram positive cocci	7	4
	Gram positive filamentous rods	2	1
	Gram negative rods	1	1
	Fungi	7	6

* = green colored colony

The growth and degree of green staining of bacterial colonies grown on MG-PDP medium and fungal 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 & 3 and Fig.1.



A = Bacterial isolates, B= Fungal isolates

Fig. 1: Production of ALP by bacterial & fungal isolates at various pH levels as indicated by intensity of green color.

Table 2: Production of ALP by bacterial isolates (on MG-PDP medium) at various pH levels as indicated by intensity of green color.

Bacterial isolate no.	pH 7.5	pH 8.0	pH 8.5	pH 9.0
SF1	NG	NG	±	NG
SF2	NG	NG	-	++
SF3	NG	-	±	++
SF4	NG	-	-	++
SF5	NG	NG	NG	+
SF6	NG	NG	±	++
SF7	NG	-	++	+
SF8	NG	-	-	-
SF9	NG	++	++++	+++
SF10	NG	++++	++++	++++
SF11	NG	-	-	-
SF12	NG	-	-	-
SF13	NG	+++	++++	++++
SF14	NG	+	±	±
SF15	NG	+++	++++	++++
SF16	NG	+	++	
SF17	NG	-	+++	++++
SF18	-	+++	++++	++++
SF19	NG	-	-	+
SF20	NG	±	+	++
SF21	NG	+++	+++	++++
SF22	NG	-	-	-
SF23	-	+++	++++	+++
SF24	NG	-	NG	NG
SF25	NG	NG	±	±
SF26	NG	-	-	+
SF27	NG	-	-	NG
SF28	+	+++	++++	+++

Table 3: Production of ALP by Fungal isolates (on modified Dermatophyte test agar medium) at various pH levels as indicated by intensity of green color

Fungal isolate no.	pH 7.5	pH 8.0	pH 8.5	pH 9.0
Sfu1	++	++	+++	++
Sfu2	NG	NG	NG	NG
Sfu3	+	++	+	+
Sfu4	+	++++	++	NG
Sfu5	+	+++	++	++
Sfu6	+	+	+	++
Sfu7	+	++	++	+++

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: SF13, SF15, SF18 and SF21 and fungal isolate Sfu4 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). 9 out of the 28 bacterial isolates exhibited significant ALP activity at some or the other pH level, whereas only 4 of the 7 fungi exhibited good ALP activity.

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 Biochemical and morphological characteristics of the 4 good ALP producers are presented in Tables 4 & 5. Most of these isolates are aerobic, sporulating, motile Gram positive bacilli (Table 5). 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: Biochemical characteristics of the best bacterial ALP producers.

S. No.	Biochemical test	Bacteria			
		SF13	SF15	SF18	SF21
1	Glucose Utilization	+ve	+ve	+ve	+ve
2	Xylose Utilization	-ve	-ve	-ve	-ve
3	Lactose Utilization	-ve	-ve	-ve	-ve
4	Mannitol Utilization	+ve	+ve	+ve	+ve
5	Rhamnose Utilization	-ve	-ve	-ve	-ve
6	Alpha-Methyl-D-Mannoside Utilization	-ve	-ve	-ve	-ve
7	Ribose Utilization	-ve	-ve	-ve	+ve
8	Arabinose Utilization	-ve	-ve	-ve	+ve
9	Sucrose Utilization	+ve	+ve	+ve	ND
10	Raffinose Utilization	-ve	-ve	-ve	-ve
11	Trehalose Utilization	+ve	+ve	+ve	+ve
12	Maltose Utilization	+ve	+ve	-ve	+ve
13	Methyl Red	-ve	-ve	-ve	-ve
14	Voges Proskauer	+ve	+ve	+ve	+ve
15	Nitrate Reduction	-ve	-ve	+ve	-ve
16	Catalase Detection	+ve	+ve	+ve	+ve
17	Esculin Hydrolysis	+ve	+ve	+ve	+ve
18	ONPG Utilization	+ve	+ve	+ve	+ve
19	Urease Detection	-ve	-ve	-ve	+ve
20	Arginine Utilization	-ve	+ve	-ve	+ve
21	Alkaline phosphatase Detection	+ve	+ve	+ve	+ve

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

Table 5: 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
SF13	+ve	Thin, large rods	Noncapsulated	Sporulating	Motile
SF15	+ve	Thick, large rods	Noncapsulated	Sporulating	Motile
SF18	+ve	Thin, large rods	Noncapsulated	Sporulating	Motile
SF21	+ve	Thin, large rods	Capsulated	Sporulating	Motile

Table 6: Enzyme spectrum of the best bacterial ALP producers.

Bacterial isolate no.	Amylase	Protease	Lipase	DNAse	Gelatinase	Oxidase	Lecithinase
SF13	+ve	+ve	+ve	-ve	+ve	+ve	-ve
SF15	+ve	+ve	+ve	-ve	+ve	+ve	+ve
SF18	+ve	+ve	+ve	-ve	+ve	+ve	-ve
SF21	+ve	+ve	+ve	-ve	+ve	+ve	-ve

Selection of best ALP producer:

The 4 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 four isolates were observed in the PDP containing medium. However, in the medium with organophosphorus insecticide Acephate, isolate SF15 exhibited 1.28 fold increased ALP activity, which was higher than the ALP activity of the other three isolates in the same medium.

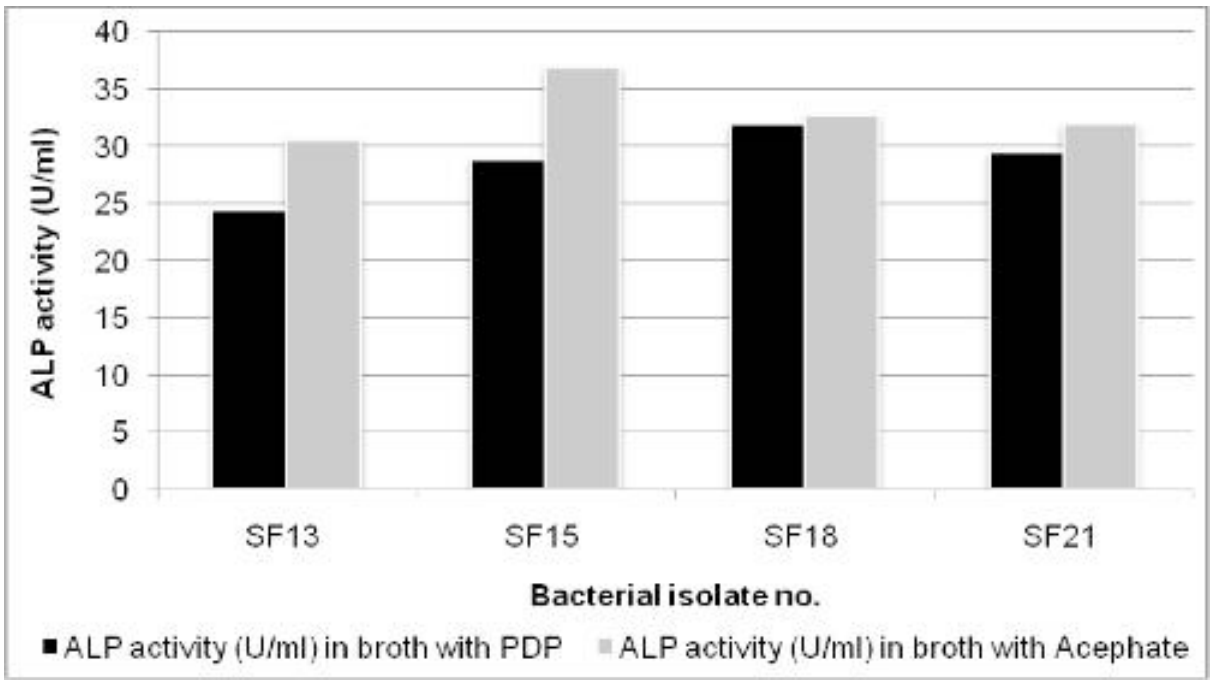


Fig. 3: Determination of ALP activity (U/ml) in medium with PDP / Acephate.

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 all the four isolates but maximum in isolate SF15. It is possible to utilize Bacillus sp. SF15 to detoxify and mineralize xenobiotic environmental contaminants like organophosphates as a pollution controlling agent.

4. CONCLUSIONS:

The studies on the Alkaline Phosphatase production by microbes from North Gujarat region show that: [1] The bacterial types were higher than the fungi in the field soil samples and also they were more promising in ALP production than the

fungal isolates. [2] Bacillus is a dominant ALP producing genus in field soil samples of Patan district. [3] Most of the facultative alkaliphiles produce ALP at pH above 7.5. [4] Incorporation of PDP as the substrate and Methyl Green as indicator dye provides useful detection method for ALP, ALP producing microbes get stained deep green. [5] Morphological and biochemical characterization of high ALP producers confirm them as Bacillus and [6] The production of ALP in SF15 was induced by Organophosphate insecticide Acephate and this isolate can utilize the insecticide and be used as a pollution controlling agent.

ACKNOWLEDGEMENTS:

The authors are thankful to the Management of Kadi Sarva Vishwavishvidyalaya (KSV), Gandhinagar, for facilitating this research. Sincere thanks are also due to Dr. M. C. Sharma, Professor & Head, Department of Biotechnology, Kadi Sarva Vishwavishvidyalaya, Gandhinagar for encouragement, guidance and suggestions for improvement in the manuscript.

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