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STUDY ON MICROBIAL ALKALINE PHOSPHATASE PRODUCTION FROM NORTH GUJARAT FIELD SOILS

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ABSTRACT

For the study of biodiversity of Alkaline phosphatase producing microbes, field soil samples were collected from GSFC farm, Bhilot, Patan District, North Gujarat region. 35 microbes were isolated by using different enrichment media, to study the biodiversity and to select the best isolate for alkaline phosphatase production. Primary characterization of the isolates were done by staining techniques and screening was done on Methyl Green dye and Phenolphthalein diphosphate supplemented MG-PDP / Modified Dermatophyte test agar (MDTA) media. Among the 35 microbes, 22 bacteria and 6 fungi were alkaline phosphatase producer. The best four bacterial alkaline phosphatase producers were further characterized by capsule and spore staining, biochemical tests and by determining the alkaline phosphatase production in the liquid medium. All four isolates also produced other biotechnologically important enzymes at alkaline pH. The isolates were able to degrade organophosphorous insecticide Acephate.

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 as hydrophilic phosphate groups, are in phospholipids, essential components of cell membranes

Under normal conditions, phosphorus is not an abundant component of the ecosphere. When it is present, it may not be in a form that is readily available for assimilation by organisms since its availability is restricted by its tendency to precipitate in the presence of metals such as calcium, magnesium and ferric ions at neutral to alkaline pH. As an essential element, therefore, it often limits the growth of primary producers such as algae, other aquatic plants, cyanobacteria and photosynthetic bacteria.

Microbial transformations of phosphorus also occur. They involve the mineralization of organic to inorganic phosphates (a process also referred to as "decomposition") or the conversion of insoluble, immobilized forms of tertiary phosphate into soluble, mobile primary phosphates that are more readily used by organisms. One such type of microbial transformation includes mineralization of organic to inorganic phosphate catalyzed by phosphatase enzymes, which are specifically involved in this conversion. Many microorganisms produce these enzymes but only bacteria, fungi, and some algae are able to secrete them outside of their cells. As exoenzymes, they participate in the dissolution and mineralization of organic phosphate compounds in the environment[1]. Without phosphatase enzymes, the presence of inorganic phosphorus would be limited. Phosphatases are further 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 was reported many times, as compare to alkaline posphatase. An interesting feature of microbial phosphatases, especially alkaline phosphatase, is that they are not released in the presence of excess dissolved phosphate[1].

The other applications of ALPs in diagnostics, immunology, clinical medicine and molecular biology has made them popular in scientific studies and commercial utility[2-10]. 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[11].

The purpose of the present study was to examine, under laboratory conditions, the ALP activities of the microorganisms in the field samples from north Gujarat region, India. Selected microorganisms were also evaluated for their contribution in the bioremediation of Acephate an oraganophosphorous insectiside.

MATERIALS AND METHODS

i) Soil Samples:

The soil samples were collected in the monsoon season, August 2009 from GSFC farm sites of Patan district, North Gujarat region. All soil samples were serially diluted and checked for colony forming units per ml (CFU/ml) by standard plate count (SPC) technique. They were also tested for their pH, E.C. and Phosphorous content at the soil testing laboratory.

ii) Enrichment and isolation of ALP producing microbes:

Thronton's medium[12] and Martin's Rose Bengal Chloramphenicol agar medium[13] were used as enrichment medium for isolation of microbes for bacteria and fungi respectively. From a mixed population of microbes isolated by spread plate technique, pure isolates of bacteria and fungi were obtained by repeated streak-plate method on Nutreint agar medium and Potato Dextrose agar medium respectively[13]. Then the pure isolates of bacteria were streaked on MG-PDP medium and fungi were on Modified Dermatophyte test agar medium to check the ALP production. The MG-PDP medium was supplemented with Methyl Green dye 50 mg/ml, Phenolphthalein diphosphate tetra sodium salt (PDP) 1 g/l, Peptone 10 g/l, Glucose 1 g/l, Yeast extract 5 g/l, Agar 20 g/l, pH variable (7.5, 8.0, 8.5, 9.0). The Modified Dermatophyte test agar medium was supplemented with Methyl Green dye 50 mg/ml, Phenolphthalein diphosphate tetra sodium salt, pH variable (7.5, 8.0, 8.5, 9.0) [13].

iii) Some characterization of the isolates:

Colony characterization of the bacterial isolates were carried out from Nutrient agar medium and fungi from Potato Dextrose agar medium. Morphological characterization of the isolates were carried out on the basis of Gram's Staining. Selection of the bacterial isolates producing ALP were done from the MG-PDP medium and fungi from MDTA medium. Selected isolates were further characterized by Schaeffer-Fulton method of Spore staining, Anthony's method of 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 Pvt. Ltd., Mumbai; Staph Identification kit and Listeria Identification kit of Tulip Diagnostics (P) Ltd., Goa, India.

iv) Enzymatic characterization of the isolates:

The best isolates were checked for the production of different other enzymes *viz* Amylase, Protease, Lipase, DNAse, Lecithinase, Gelatinase and Oxidase than ALP in the different solid mediums Starch agar, Casein agar, Tributyrin agar, DNAse test agar, Egg yolk agar, Gelatin agar and Nutrient agar respectively.

v) Production of ALP in liquid medium:

The pure cultures of the best isolates were maintained on nutrient agar slants. Inoculum preparation was carried out in nutrient broth containing 1% peptone, 1% meat extract, 0.5% NaCl with initial pH 9.0. Inoculum was developed by transferring single colony from the grown culture in 25 ml N. broth in 100 ml Erlenmeyer flask, incubated on an orbital shaker at 35 °C and 120 rpm for 6 h and was used 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 broth containing PDP 1 g/l, Glucose 1 g/l, Peptone 10 g/l, Yeast extract 5 g/l and NaCl 10 g/l in 250 ml Erlenmeyer flask and incubated at 35 °C, 120 rpm for 24 h.

vi) 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[14-16]. 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.

vii) Role of ALP in Organophosphorus insecticide degradation:

Isolates were grown 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

i) 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 x 10^7 to 6 x 10^7 CFU/g. The soil factors do not exhibit any inter-relationship nor do they correlate with population and microbial types.

ii) 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

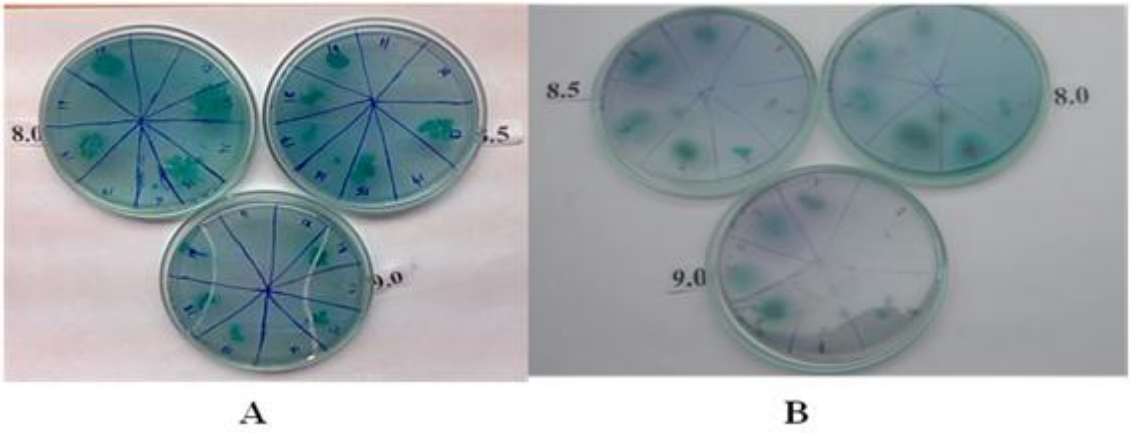
Site of isolation	Microorganisms	No. of isolates	No. of isolates with ALP activity*
GSFC Field sites, Patan	**Bacteria**		
	Gram positive rods	18	16
	Gram positive cocci	7	4
	Gram positive filamentous rods	2	1
	Gram negative rods	1	1
	Fungi	7	6

*** = green colored colony**

iii) Screening of ALP producers by evaluation of green colony stain:

Screening of the phosphatase activity of bacterial colonies growing on plates generally involves the use of an artificial enzyme substrate which, upon cleavage, yields a colored product. One such substrate is the chromophore *p*-NPP[17]. Plates to be assayed are flooded with a solution containing Tris-HCl buffer (pH 8.5), $MgCl_2$, and *p*-NPP. Those colonies displaying the wild type ALP activity stain an intense yellow, while colonies of putative mutants for ALP activity are white[18]. The main disadvantage of methods such as this is that they require the addition of reagent solutions to the surface of the plate after the colonies have appeared. This can introduce contaminants to the plate, or promote cross-contamination of isolated colonies. Another method

uses a precipitating fluorescent probe 2-(5'-chloro-2'-phosphoryloxyphenyl)-4-[3H]-quinazolinone (CPQP) to screen phosphatase producing colonies. Upon enzymatic cleavage, this molecule yields the stable, highly fluorescent precipitate 2-(5'-chloro-2'-hydroxyphenyl)-4-[3H]-quinazolinone (CPQ)[19]. To test ALP activity in broth one method involves indolyl phosphate as a substrate and enzyme production is detected by a blue color resulting from conversion of indolyl phosphate to indigo[20]. The method described in this paper uses PDP as an artificial enzyme substrate and Methyl Green as an indicator dye for detection of ALP production. Colonies producing ALP stained with deep green color, whereas the other colonies remained colorless (Fig 1) [7, 21, 22]. This method has several benefits over the other methods described above. Less chance of contamination as compared to *p*-NPP containing medium, preparation of medium is easy as compared to CPQP containing medium and as an additional benefit it also distinguishes between activity of excreted and cell associated phosphatase.



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.

The products of the phosphatase reaction form, with the stains, high pigmented insoluble complexes that precipitate at the site where the reaction has occurred. Since, in most bacteria, phosphatases are membrane bound, in the presence of phosphatase substrates and stains, intensely stained precipitates are formed on his bacterial component. Such precipitates make the cells become stained and cause the intense pigmentation of the phosphatase positive colonies[23].

The degree of green staining of colonies grown on MG-PDP media and MDTA medium was scored as follow: NG not grown; - grown but no stain; ± weak stain; +, ++, +++, ++++ progressive intensity of staining[22].

Bacteria are an inevitable source of phosphatases for recycling organic phosphorus compounds in freshwater ecosystems [24]. (Table 2)

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 color

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
SF-1	NG	NG	±	NG	SF-15	NG	+++	++++	++++
SF-2	NG	NG	-	++	SF-16	NG	+	++	
SF-3	NG	-	±	++	SF-17	NG	-	+++	++++
SF-4	NG	-	-	++	SF-18	-	+++	++++	++++
SF-5	NG	NG	NG	+	SF-19	NG	-	-	+
SF-6	NG	NG	±	++	SF-20	NG	±	+	++
SF-7	NG	-	++	+	SF-21	NG	+++	+++	++++
SF-8	NG	-	-	-	SF-22	NG	-	-	-
SF-9	NG	++	++++	+++	SF-23	-	+++	++++	+++
SF-10	NG	++++	++++	++++	SF-24	NG	-	NG	NG
SF-11	NG	-	-	-	SF-25	NG	NG	±	±
SF-12	NG	-	-	-	SF-26	NG	-	-	+
SF-13	NG	+++	++++	++++	SF-27	NG	-	-	NG
SF-14	NG	+	±	±	SF-28	+	+++	++++	+++
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**
Sfu-1	++	++	+++	++	Sfu-5	+	+++	++	++
Sfu-2	NG	NG	NG	NG	Sfu-6	+	+	+	++
Sfu-3	+	++	+	+	Sfu-7	+	++	++	+++
Sfu-4	+	++++	++	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

Characterization of the good ALP producers:

The morphological and biochemical characteristics of the 4 good ALP producers are presented in Tables 3 & 4. 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 5). *Bacillus* spp. have been found to be the dominant phosphatase producing bacteria [24].

Table 3: Morphological characterization of the best bacterial ALP producers.

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 4: Biochemical characterization 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

Table 5: Enzymatic characterization 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 best ALP producer SF15 was selected on the basis of its highest activity in the PDP containing broth and Acephate containing broth as compare to the other isolates. The results obtained showed that there were very minor differences of the ALP activities of different four isolates in the PDP containing broth. The presence of oraganophosphorous insecticide Acephate induced the production of ALP in the selected four isolates (Fig. 2). The ALP activity increased 1.28 fold in isolate SF15 which proved its utility in the degradation of the insecticide and the future use as a pollution controlling agent. Further development of this work is required.

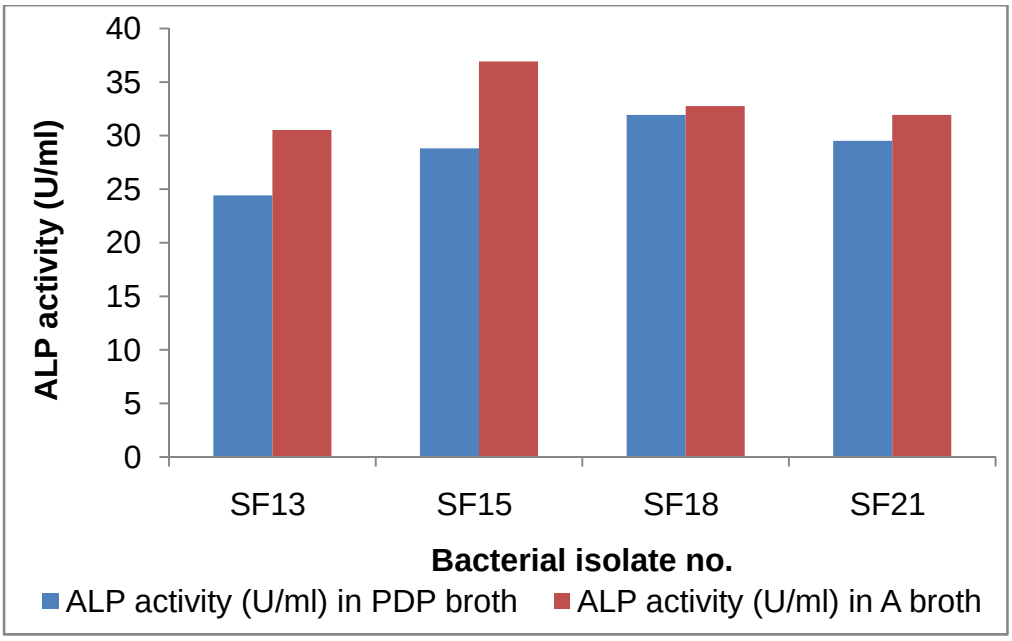


Fig. 2. Determination of ALP activity (U/ml) in liquid medium.

CONCLUSIONS

The studies on the ALP production by microbes from North Gujarat region shows: [1] The bacterial population was higher than the fungus in the field soil samples and also they were more promising in ALP production than the fungal isolates. [2] *Bacillus* genera were found to be dominant in field soil samples of Patan district. [3] Most of the isolates were facultative alkalophiles and produce ALP in the medium when the pH was above 7.5. [4] Organophosphate insecticide Acephate induced the production of ALP in the isolate SF15 which proved its utility in the biodegradation of the insecticide and the future use as a pollution controlling agent.

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