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Novel Organophosphate Pesticide Utilizing Alkaline Phosphatase Producing Polyextremophile *Bacillus Flexus* from Lake Ecosystem of North Gujarat, India

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ABSTRACT: The biodiversity of alkaline phosphatase (ALP) producing microbes from the lake ecosystem of the North Gujarat region was explored. Methyl Green dye and Phenolphthalein diphosphate supplemented MG-PDP medium screened ALP producing microbes as deep green stained cultures. The best ALP producing isolate was put through phenetic and genetic identification on the basis of morphology, biochemistry, physiology and 16S rRNA analysis. This best ALP producer proved to be catalase and oxidase positive, spore forming, noncapsulated, motile, vancomycin sensitive Gram-positive rod which gives optimum ALP production at pH 9.0, 35ºC and 0.5% NaCl concentration. Scanning Electron Micrographs revealed unique pili like bridge between bacterial cells and no flagella. N-BLAST search of the 16S rRNA sequence of the isolate (GenBank accession no JN415115) exhibited maximum similarity (98%) with *Bacillus flexus*. Organophosphorous Pesticide Acephate was utilized as sole Carbon source accompanied with the release of inorganic phosphate indicating its role in organophosphate pesticide bioremediation.

KEYWORDS: Alkaline phosphatase, Methyl Green dye, Phenolphthalein diphosphate, *Bacillus flexus*, Acephate.

I. INTRODUCTION

The lake ecosystem in Patan in North Gujarat adjacent to Little Rann of Kutch consists of highly salinity affected arid grasslands, lakes and marshes. The soils in these areas are likely to harbor extremophiles, reported sources of useful microbial enzymes. The secretion of extracellular proteases, amylases, chitinase and lipases have been reported from haloalkaliphilic bacteria from sea waters, saline soils /other habitats along the coastal Gujarat [1]. There are, however, no reports available for the ALPs from alkaliphiles or haloalkaliphiles or halotolerant alkaliphiles from Gujarat.

Phosphatases promote the degradation of complex phosphorous compounds and have an essential function in the nutrient dynamics of lakes in absence of external natural phosphorus input process therein [2]. The ALPs (EC 3.1.3.1) 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 [3].

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 [4]. 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. Increased phosphatase activities have been reported in lake water / soil samples with high concentrations of insecticides, herbicides and fungicides. Applications of ALPs in molecular biology have made them popular in scientific studies [5, 6].

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The present study was taken up to isolate, characterize, and identify the best ALP producing microbe from the lake ecosystem of North Gujarat region and to investigate possible bioremediation of toxic organophosphate pesticide Acephate through utilization by the best ALP producer.

II. MATERIALS & METHODS

2.1 Screening for ALP producing Microbes

Microbial flora present in the sediment soil samples from the lakes at Lodra, Bhilot, Santalpur, Radhanpur and Sami collected in August, 2009 was isolated. 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.

These isolates were further grown in PDP broth (composition: Glucose 1 g/l, Peptone 10 g/l, Yeast extract 5 g/l, NaCl 10 g/l and PDP 1 g/l) or in 'A' broth (composition same as PDP but 1 g/l organophosphate Pesticide Acephate as sole Carbon source in place of Glucose 1 g/l,). 100 µl cell free supernatant was added to 1000 µl of *p*-nitrophenyl phosphate disodium salt (*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. ALP activity was measured spectrophotometrically by determining the release of *p*-nitrophenol (*p*-NP) at 400 nm [7, 8, 9]. The amount of orthophosphate released from the organophosphate Pesticide was estimated by Ascorbic acid reduction method at 880 nm [10]. The isolate with best ALP activity and orthophosphate released was selected.

2.2 Morphological and biochemical characterization of best ALP producer Microbe FPB17

Characterization of the best ALP producing isolate was carried out on the basis of techniques described by Coppuccino, 1998 [11], including Gram's staining, Anthony's method of Capsule staining, Schaeffer-Fulton method of Spore staining and motility testing by Hanging drop technique. Gram's reaction of the isolate was also confirmed by Vancomycin sensitivity test using 30 µg sensitivity discs M/s Tulip Diagnostics (P) Ltd., Goa, India. Biochemical characterization of the isolate was conducted by using HiAssorted biochemical test kit and HiBacillusTM identification kit M/s HiMedia Laboratories, Mumbai, India and Staph Identification kit and Listeria Identification kit M/s Tulip Diagnostics (P) Ltd., Goa, India. This isolate was also checked for the production of other enzymes *viz.* Amylase, Protease, Lipase, DNAse, Lecithinase, Gelatinase and Oxidase on respective enzyme specific media by observing substrate clearance zones. The antibiotic sensitivity of the best strain was determined using antibiotic discs and Mueller Hinton Agar (MHA) of M/s HiMedia laboratory, Mumbai. The plate was checked for susceptibility after incubation at 37ºC for 24 h as per the chart provided with the antibiotic kits by HiMedia.

2.3 ALP production & Organophosphorus pesticide utilization by best ALP producer FPB17

5% v/v of inoculum from actively grown parent culture (A_{600} = 0.8 - 1.2) of FPB17 was transferred to 50 ml of PDP Broth in 250 ml Erlenmeyer flasks and incubated at 35 °C, at 120 rpm for 24 h. Utilization of Organophosphorus Pesticide by the best ALP producer was studied by growing the isolate in A Broth, The ALP activity and release of phosphate were determined.

2.4 Molecular characterization of FPB17

DNA of FPB17 strain was isolated and quality evaluated on 1.2% Agarose Gel [12]. Fragment of 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR). The PCR amplicon was purified and forward and

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reverse DNA sequencing reaction of PCR amplicon carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the help of NCBI Genbank database.

2.5 Scanning electron microscopy (SEM) of FPB17

For three-dimensional SEM images of the bacterial cell surface structure, a thin smear of overnight culture of FPB17 was taken on a cover slip, air dried and fixed with 2 % Glutaraldehyde solution and washed and air dried. The cover slip was coated with a thin layer of gold (~ 50 or 100 microns), to get 6000x and 9000x magnification photographs of FPB17 in Scanning Electron Microscope Model number LEO *s*- 440*i*).

2.6 Optimization of incubation parameters for production of ALP by FPB17

The effect of pH (5–13), temperature (4–55°C) and NaCl concentrations (0-12%) in the Nutrient broth on the growth (A_{600} at 24h) and production of ALP by FPB17 was studied at 120 rpm. Cell free supernatant of the fermented broth centrifuged at 7000 g at 4°C for 15 min was assayed for ALP productivity at 24 h intervals. Enzyme thermostability was also checked. All the experiments were conducted in triplicate and average values are presented**.**

III. RESULTS

3.1 ALP producing Microbes from lake ecosystem of North Gujarat

The soil samples yielded 42 bacterial isolates and 15 fungal isolates. 34 of these bacterial isolates exhibited ALP production capability. None of bacterial isolates exhibited ALP at pH 7.5. As evident from Table 1, 12 higher ALP producing isolates appeared at pH 8.0, 4 higher ALP producing isolates at pH 8.5 and 8 higher ALP producing isolates at pH 9.0. Based on the ALP activity related green pigment, the bacterial isolates: FPB15, FPB17, FPB23 and FPB28 stood out as the highest ALP producers. The highest quantum of ALP was exhibited by FPB15 at pH 8.0, by FPB28 at pH 8.5 and by FPB17 and FPB23 at pH 9.0. Unlike the other 3 strains, FPB17 exhibited ALP only at pH 9.0. However, the only 2 fungal strains exhibiting ALP production did so at all the pH levels.

Table 1: Effect of pH on ALP production of by bacterial isolates (FPB) and fungal isolates (FPF) from lake ecosystem as indicated by intensity of green color of colonies.

Isolate nos.	pH 7.5	pH 8.0	pH 8.5	pH 9.0	Isolate nos.	pH 7.5	pH 8.0	pH 8.5	pH 9.0
FPB1	NG	+++	++	+	**FPB24**	NG	+++	+	-
FPB2	NG	+++	++	++	**FPB26**	NG	+++	-	-
FPB4	NG	+++	++	-	**FPB27**	NG	+++	±	±
FPB5	NG	+++	++	-	**FPB28**	NG	++	++++	+++
FPB13	NG	+	+++	+	**FPB29**	NG	+++	+	-
FPB15	NG	++++	+++	+	**FPB34**	NG	NG	++	+++
FPB17	NG	NG	±	++++	**FPB37**	NG	++	+++	+
FPB20	NG	+++	+	±	**FPB39**	NG	NG	+	+++
FPB21	NG	+++	-	-	**FPB40**	NG	NG	NG	+++
FPB22	NG	+++	-	-	**FPB41**	NG	NG	±	+++
FPB23	NG	NG	++	++++	**FPB42**	NG	NG	NG	+++
FPF1	+++	+++	++++	++	**FPF12**	++	++	+++	++++

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

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3.2 Morphological and biochemical characterization of best ALP producer

The morphological and biochemical characteristics of FPB17 presented in Table 2, assign this highest ALP producer strain as nonpathogenic vancomycin sensitive Gram +ve, sporulating, noncapsulated motile *Bacillus*. In addition to the ALP producing capability, *B. flexus* FPB17 exhibited amylase, protease, lipase, catalase, oxidase and gelatinase producing capability also.

Table 2: Morphological and Biochemical characteristics of the FPB17 isolate

Colony morphology		**Cell morphology**			
Form	Mycelioid	Shape	Thick and large rods	Arrangements	in chains
Elevation	Low convex	Motility	Motile	Gram stain	Positive
Color	Creamy white	Spore formation	Terminal spores	Capsule	Absent

Biochemical Tests					
ALP production	+	Catalase production	+	Oxidase production	+
Urease production	-	Starch Hydrolysis	+	Esculin Hydrolysis	-
Casein & Gelatin Hydrolysis	+	Tributyrene Hydrolysis	+	Deoxyribo nucleic acid Hydrolysis	-
Phospholipid Hydrolysis	-	Arginine Utilization	-	ONPG Utilization	+
Methyl Red	+	Voges Proskauer	-	Nitrate Reduction	-
Utilization as sole carbon source					
Mannitol	+	Lactose	-	Maltose	+
Sucrose	+	Alpha-Methyl-D-Mannoside	-	Arabinose	-
Raffinose	+	Trehalose	+	Glucose	+
Rhamnose	-	Ribose	+	Xylose	-
Antibiotic Sensitivity					
Amoxycillin 10 µg	R	Augmentin 30 µg	R	Cephotaxime 30 µg	S
Ceftriaxome 30 µg	S	Cefuroxime 30 µg	S	Cefoperazone 75 µg	S
Ceftazidime 30 µg	R	Erythromycin 15 µg	I	Lincomycin 2 µg	R
Ciprofloxacine 5 µg	S	Ofloxacin 5 µg	S	Pefloxacin 5 µg	S
Lomefloxin 30 µg	S	Tobramycin 10 µg	S	Amikacin 30 µg	S
Gentamycin 10 µg	S	Netilmicin 30 µg	S	Vancomycin 30 µg	S

+ = substrate utilized / reaction positive, - = substrate not utilized / reaction negative,
S = Sensitive, R = Resistant, I = Intermediate Resistant

3.3 ALP production and phosphate status in submerged fermentation containing PDP or Acephate as sole C source

As indicated in Table 3 the production of ALP by FPB17 in the production medium containing PDP was 38.94 U/ml, while in the production medium with Acephate as sole C source the production was 49.47 U/ml (1.36 fold increased ALP activity). Phosphate, initially not indicated in the medium, showed its presence at 24 h growth, mainly because of utilization of the P containing substrates PDP and Acephate. The phosphate increased with the growth of FPB17 being highest at 48 h, particularly in A broth as compared to PDP broth.

Table 3: Release of Phosphate during PDP and Acephate substrate utilization by isolate FPB17.

Age (h)	**ALP Activity (U/ml)**		**PO_4^{-3} (mg/ml)**	
	PDP broth	**A broth**	**PDP broth**	**A broth**
0	0.000	0.000	0.000	0.000

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6	0.000	0.000	0.000	0.001
12	0.000	0.000	0.000	0.006
24	13.19	20.14	0.010	0.032
48	38.94	49.47	0.016	0.068
72	31.94	43.06	0.012	0.037

3.4 Molecular characterization and Bioinformatic analysis of *Bacillus* sp. FPB17

By using BACTERIA_8F_S4406_016_A02.ab1 forward primer and BACTERIA_1492R_S4406_014_B02.ab1 reverse primer, 1428 base pair (bp) long consensus sequence of 16S rRNA of *Bacillus* sp. FPB17 was obtained. It has been indicated as novel sequence and has been submitted to NCBI GenBank with accession number as JN415115.

16S rRNA sequences of 10 different *Bacillus* species taken from NCBI database were compared with the 16S rRNA sequence of *Bacillus* sp. FPB17 using Neighbour-Joining method of Saitou and Nei, 1987 [13]. Comparison study using BLAST showed that16S rRNA sequence of *Bacillus* sp. FPB17 had highest homology (98%) with *Bacillus flexus* NBRC 15715. This strain therefore is designated as *Bacillus flexus* FPB17

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3.5 Scanning Electron Microscopy (SEM) of the *Bacillus* flexus FPB17

SEM of the *Bacillus* flexus FPB17 strain indicated medium sized rods with spores (Fig. 1), unique pili like bridge between bacterial cells, matching to the characteristic stacking pattern of growth in certain groups of bacteria [14], but no flagellum has been seen.

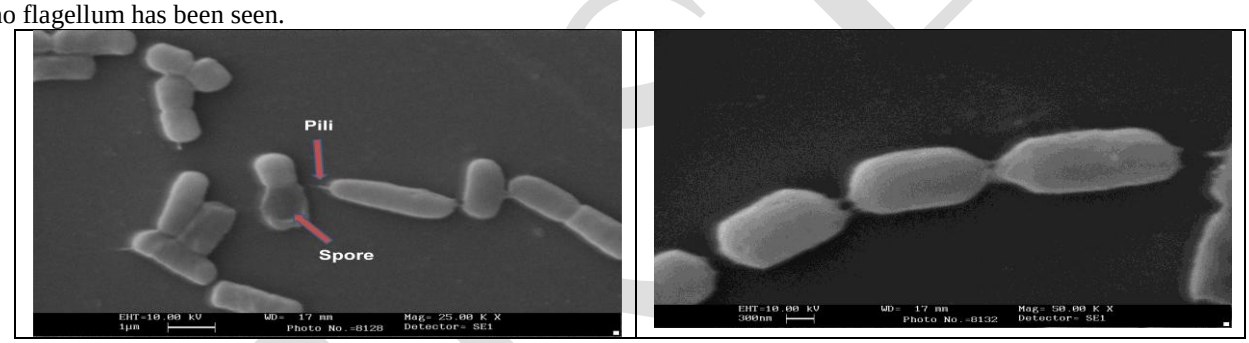

Fig. 1 Scanning electron microscopic view of *Bacillus flexus* FPB17

3.6 Effect of environmental factors on ALP of *B. flexus* FPB17

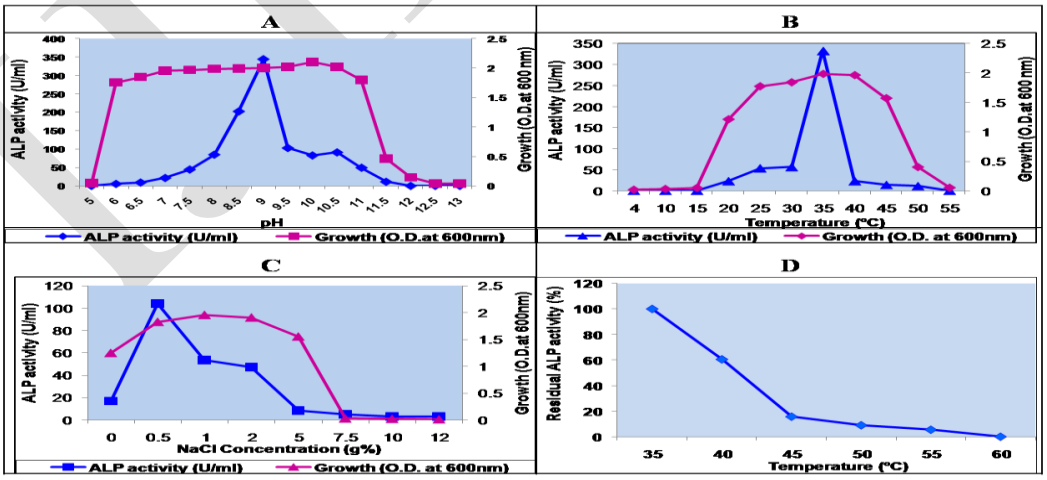

Fig. 2 Impact of pH (A), Temperature (B) & NaCl concentration (C) on the yields of ALP production by *Bacillus flexus* FPB17 and Thermostability of ALP of *Bacillus flexus* FPB17 (D)

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While the growth of this strain was optimal at pH 10.0, there was almost no impact of pH between 7.0 and 11.0 on the growth, but the ALP production was optimal at pH 9.0 and sharply declined below and above the pH optima (Fig. 2(A)). The ALP enzyme production was maximum at 35ºC and was very sensitive to temperature deviations, getting to nil at 55ºC or below 15ºC (Fig. 2(B)). The maximum enzyme activity was obtained in the presence of 0.5 g% NaCl concentration. Further increase in NaCl, resulted in the decreased production (Fig. 2(C)). The growth of the isolate dropped drastically after 5% NaCl level indicating FPB17 as halotolerant rather than a halophile. The enzyme exhibited stability at temperatures below 35ºC (Fig. 2(D)).

IV. DISCUSSION

A few bacterial and fungal isolates from lake ecosystem of North Gujarat exhibited high ALP production capability, particularly at pH above 8.5 on PDP medium with methyl green indicator dye, the high extra cellular ALP producers appearing as dark green colonies. This medium and dye has been earlier utilized by Satta et al., 1979 [15]; Riccio et al., 1997 [16] and Nilgiriwala et al., 2008 [17]. The other techniques for selecting phosphatase producers are Yellow colonies through *p*-NPP [18], indigo blue colonies through indolyl phosphate [19] and precipitating fluorescence using 2-(5'-chloro-2'-phosphoryloxyphenyl)-4-[3H]-quinazolinone [20]. The PDP plus methyl green method is advantageous as it avoids contamination and distinguishes between excreted and cellular phosphatase.

The biochemical and enzymatic characteristics of the bacterial isolate indicated that this being catalase and oxidase positive, belong to genus *Bacillus*. The isolate also exhibited important hydrolytic enzymes including amylase, protease, lipase etc. in addition to ALP which also confirms that it belongs to genus *Bacillus*. The highest ALP producer FPB17 strain proved to be vancomycin sensitive, Gram +ve, sporulating, noncapsulated motile *Bacillus*. Arthi et al., 2003 [21] used Vancomycin sensitivity test to confirm the bacteria as *Bacillus*. *Bacillus* sp. has been found to be the dominant phosphatase producing bacterium [22]. ALP has been reported from several *Bacillus* species including *Bacillus cereus* [23], *Bacillus intermedius* S3-19 [24], *Bacillus licheniformis* MC14 [25], *Bacillus megatarium* [26], *Bacillus stereothermophilus* [27], *Bacillus subtilis* [28, 29, 30], *Bacillus* sp. P9 [31] and *Bacillus* sp. [32].

1.36 fold increased ALP activity was exhibited by isolate FPB17 in medium with organophosphorus Pesticide Acephate as sole C source. This increased yield of ALP in medium with organophosphorus Pesticide Acephate is assignable to the phenomenon of "Induction" by this xenobiotic substrate, very commonly observed with enzymes and several other metabolites. Lo´pez et al., 2006 [33] had worked out that addition of high concentrations of Pesticides, herbicides and fungicides increased phosphatase activities in lake water samples incubated at laboratory conditions. Utilization of Acephate by FPB17 indicates the possible use of ALP as pollution controlling agent to detoxify and mineralize environmental contaminants like xenobiotic organophosphates. According to Barik and Purushothaman, 1999 [22], bacterial phosphatases have an important role in the recycling organic phosphorus compounds in freshwater ecosystems. 16S rRNA sequencing analysis and bioinformatic analysis confirms that the strain FPB17 exhibits 98% homology to *Bacillus flexus* NBRC 15715 and therefore can be designated as *Bacillus flexus*. This gene sequence is novel and has been submitted to GenBank and was assigned accession number JN415115.

The Scanning Electron Micrographs of this strain revealed unique pili like bridge between bacterial cells, matching to the characteristic stacking pattern of growth in certain groups of bacteria [14]. No flagellum was seen, but the organism was motile may be through the type 4 pili which are responsible for twitching type of motility.

FPB17 exhibited maximum production of ALP at pH 9.0, 35ºC incubation temperature and 0.5% NaCl. The enzyme activity declined sharply above 35º C and is thermolabile. ALP production under alkaliphilic pH range have been reported in *E. coli* (pH 8.3) by Danielle and Raymond, 1984 [34]. In case of fungi similar results have been reported by Dahot et al., 1986 [35] in *Penicillium expansum* (pH 9.5) and by Morales et al., 2000 [36] in *Neurospora crassa* (pH 9.5-10.5). Temperatures in the range of 37°C to 40°C have been reported as optimum for ALP production in *E. coli* by Danielle and Raymond, 1984 [34] and in *Penicillium expansum* by Dahot et al., 1986 [35]. *Bacillus flexus* is reported to be generally halotolerant upto 12% NaCl concentration [37], but this FPB17 strain is an exception as the growth drops drastically after 5% NaCl level. Thermolabile ALPs have been reported [38] to be better suited for applications in molecular biology

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V. CONCLUSIONS

A *Bacillus* isolate FPB17 from the soils of banks of lake ecosystem of North Gujarat region at Bhilot (Patan) was found to be high ALP producer on PDP medium with methyl green indicator dye appearing as dark green colonies. Results of 16S rRNA technique confirm that the strain FPB17 as *Bacillus flexus*. The 16S rRNA consensus sequence was found to be novel and was submitted to NCBI GenBank as accession number JN415115. *Bacillus flexus* FPB17 is mesophilic and facultative halotolerant alkaliphile. It utilized organophosphate pesticide Acephate as a carbon source & its presence induced the activity of ALP enzyme. It exhibited maximum induction (1.36 fold higher ALP activity). The utilization of the Pesticide opens the possible future use as a bioremediation agent for xenobiotic organophosphorus pesticides.

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