A sustainable approach to soil bioremediation: Degradation of commercial phosphate fertilizer and pesticide by phosphatase producing soil bacteria from Sunderbans

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Sundarbans harbours diverse microbial communities, capable of thriving in extreme environmental conditions. Application of inorganic phosphate fertilizers and organophosphorus (OP) herbicide and pesticide deliberately to agricultural field, worsen the soil quality which may be minimized by indigenous soil-borne phosphate solubilizing microorganisms. This study investigated ability of phosphate solubilization by bacteria, isolated from Sundarban soils, their growth pattern and tolerance level of commercially available inorganic phosphate fertilizers, low grade rock phosphate (LGRP), high grade rock phosphate (HGRP), or OP sources, herbicides glyphosate and pesticide chlorpyrifos in combination with cypermethrin (5%, v/v), and will be compared with those of tricalcium phosphate (TCP) in Pikovskaya's (PVK) medium base. Altogether eight morphologically distinct bacterial strains showing phosphate solubilization index (PSI) 1.25 to 2.5, were tested. Among them, strain K24PM01, a gram-positive bacterium exhibited notable growth in presence of all tested phosphates, when used as sole source of phosphorus. Vigorous growth of organism was observed with chlorpyrifos-cypermethrin mixture (CCM) which is potentially toxic to environment. Furthermore, organism was assessed for phosphatase synthesis, in presence of CCM (0.01%-0.2%, v/v), considering TCP (5%, w/v) containing PVK medium as control. With increase in concentration of CCM 0.01to 0.5%, phosphatase synthesis declined sharply from 6.58 EU to 0.33EU. However, it showed reverse results for growth with concomitant fall of medium pH. Results demonstrated that strain K24PM01 may effectively metabolize CCM and utilize as sole source of phosphate.

Keywords : Bioremediation, Chlorpyrifos, organophosphorus, phosphate solubilizing bacteria

INTRODUCTION

The unlimited application of inorganic phosphate fertilizers and hazardous organophosphorus (OP) as herbicide and pesticides is highly questionable and poses a significant threat to environment including human civilization. Excessive use of those compounds has resulted in significant contamination of soil and ground water and subsequent pollution of food webs in terrestrial and aquatic ecosystem (Mali *et al*. 2023).Though rock phosphate (RP) is highly popular fertilizer in the agriculture world, immense application of rock phosphates as commercial fertilizers alters the soil physical status (Iseki *et al.* 2024) and its microbiome functions.

This problem can easily be overcome if RP is used along with soil-borne phosphate solubilizing microorganisms (de Amaral Leite *et al.* 2020) and additionally this can reduce mobilization of heavy metals to plants (Xu *et al.* 2019). Similarly, OP holds the global market as base chemical for herbicide and pesticide (Mali *et al.* 2022).These are esters of phosphoric acid with alkyl and aromatic substituents and organo-chlorine which are widely used as pesticides like chlorpyrifos, quinalphos, malathion, parathion and dimethoate (Mulla *et al,* 2020). The toxicity related to OP seem highly potent to reproductive capacity, endocrine, nervous system, cardiovascular system and respiratory system of mammals (Bose *et al.* 2021).

Bioremediation is the most authentic strategy to resolve this problem by degrading complex

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chemicals into simpler forms. A novel strategy for environmental clean-up by using microorganism to detoxify, degrade and eliminate toxic OP and immobilize phosphates from contaminated soil, providing a highly efficient, affordable and environment-friendly approach to remediate ecosystem (Bose *et al.* 2021). Bacterial strains such as *Bacillus, Enterobacter, Klebsiella, Pseudomonas* etc. have ability to degrade such chemical and its metabolites (Kumar *et al.* 2021) following variable catalytic activities (Dash and Osborne, 2020; Santillan *et al.* 2020).

The Sundarbans, mangrove forest delta soils are rich in biodiversity and microbial hotspot (Roy *et al.* 2018). Physiochemical condition of Sundarbans soil undergoes changes continuously due to flooding, salinity, temperature and lead to evolution of microorganism (Das *et al.* 2024). These indigenous microorganisms from Sundarbans soil play different roles in bioremediation. Mangrove mud soils are also major reservoirs for phosphate solubilizing bacteria (PSB) which are effective in phosphate solubilization *i.e.,* mobilization and mineralization of different RP and OP sources (Behera *et al.* 2014).

Wide variety of PSB were examined for production of intracellular and extracellular phosphatase enzyme which could degrade organophosphorus by breaking P-O and P-S linkage (Ambreen *et al.* 2020). It has been reported that bacteria like *Sphingomonas sp.* and *Brevundimonas sp.* have ability to degrade chlorpyrifos organophosphorus present in waste water (Santillan *et al.* 2020) and similar action was also shown by *Enterobacter aerogenes* CP2 and *Streptococcus pyogenes* CP11 with organophosphate hydrolase activity (Lourthuraj *et al.* 2022).

This study was designed to isolate PSB bacteria from Sundarban agricultural fields where enormous amount of RP and OP are being applied, to search for potent bacterial strain (s) which would be able to tolerate and utilize RP and OP both as sole sources of phosphorus. Attempts have also been made to determine the nature of organism and its biochemical activities with time in relation to phosphate degradation.

MATERIALS AND METHODS

Soil sample collection

Rhizospheric soil samples were collected from five different sites of Sundarban delta (*viz.* Canning, Dobanki, Frezerganj, Kumirmari, Gadkhali) using sterile glass container and were refrigerated within six hours. These soils were considered as sources of PSB.

Isolation of phosphate solubilizing bacteria

To isolate PSB, 1g of soil sample was suspended in 9 ml of saline water and was serially diluted up to 10^{-3} dilution followed by spreading (0.1ml from each dilution tube) on Pikovskaya's (PVK) agar medium supplemented with 5% (w/v) tricalcium phosphate (Sanchez-Gonzalez *et al.* 2022). The inoculated agar plates were incubated for 4-7 days at 37^0C. Based on discrete colony morphology bacterial colonies were selected as individual strain and all were individually sub cultured on PVK medium repeatedly for obtaining their pure culture and for confirming their phosphate solubilizing ability.

Determination of phosphate solubilization index (PSI)

Phosphate solubilization ability of the bacterial isolates was evaluated by spot inoculation the isolates on PVK with 5% (w/v) TCP and on PVK base with different phosphate sources (each at 0.5%, w/v) like low grade rock phosphate (LGRP, Commercial grade), high grade rock phosphate (HGRP, Sigma Aldrich, USA), and commercial organophosphorus sources, herbicide Glyphosate [(N-phosphonomethyl) glycine], pesticide Hamla (trade name) containing chlorpyrifos (50%,v/v) [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate)] and cypermethrin, a synthetic pyrethroid (5%, v/v).All plates were incubated for 3-5 days at 37^0C. Phosphate solubilization ability was indicated by clear halo zones formation at around bacterial colonies. Efficiency of a bacterial sample was calculated as phosphate solubilizing index (PSI) by using following formula (Aliyat *et al.* 2022): Phosphate Solubilizing Index (PSI) = (CD + HD) / CD, where CD= Colony Diameter (cm) and HD=

Halo Zone Diameter (cm). Potent PSB isolates were selected on the basis of PSI calculated in TCP as well as in other phosphate sources respond and the strain had been selected for further studies.

Determination of morphological and biochemical features of potent bacterial isolate

Colony morphology of isolate was obtained on PVK agar medium. Cell morphology, Gram character and other cellular features had been determined following standard microbiological methods and microscopic observation of the bacterial cells was done using crystal violet solution, Gram's Iodine and safranin according to standard microbiological protocol. Bacterial cells were observed under light microscope to determine gram character. Biochemical features of the organism were confirmed following the parameters described in Bergey's Manual of Determinative Bacteriology (9th Ed.).

Determination of growth of organism and pH

Bacterial growth on solid PVK agar had been measured by determination of colony diameter. The growth in broth medium was calculated as the cell number per unit volume determination using haemocytometer and microscopic observation. pH of the growth medium and buffer, used for enzyme assay were determined by pH paper (Fisher Scientific) and pH meter (LOBAL, DPH500, DIGITAL pH METER).

Quantitative estimation of phosphatase enzyme

Phosphatase synthesized by the organism grown in broth culture was assayed. Culture supernatant was considered as the crude enzyme. Buffers (0.1M) - sodium citrate buffer (pH3.0-6.0), saline -citrate buffer (pH7.0) and glycine-NaOH buffer (8-9) were used as and when required for enzyme assay. 0.1ml of p-nitro phenyl phosphate (p-NPP), 10mM in required buffered system was used as substrate and 0.5ml of culture supernatant was used as enzyme source and the mixture was incubated at 37^0C for 30 mins. The reaction was stopped by adding 2ml of NaOH (1N). Optical density of the resultant solution was measured at 420 nm using UV-VIS spectrophotometer (UV-1900 Shimadzu, Japan). The amount of p-NP product was determined from a previously made standard curve of the same. One unit of enzyme activity was defined as the amount of enzyme required to produce 0.1µM of product per min.

Determination of pH and temperature optima of crude phosphatase

The optimum pH of crude enzyme was determined using buffer of different pH range (5.0-9.0). Variable temperature (20^0C -60^0C) was applied for incubation of different assay sets. Optical density of the resultant solution was measured at 420 nm using UV-VIS spectrophotometer (UV-1900 Shimadzu, Japan) and the amount of product calculated from standard curve of the same.

RESULTS AND DISCUSSION

Isolation of PSB and selection of potent strain

Altogether eight morphologically distinct bacterial strains were isolated from Sundarban soils and they showed visible efficiency of phosphate solubilization PVK agar supplemented with TCP (Table1). All of them were aseptically transferred for growth on PVK base agar plates containing LGRP, HGRP, OPs - glyphosate and CCM (each at 0.1% w/v) and TCP (5%, w/v) as sole source of phosphorus in individual set of medium. Medium with TCP was accounted as control for each case. RPs and OPs generally showed inhibitory effects for growth and hence, lower concentration of those was applied for initial study. Among the eight PSB, C24PM01 showed highest PSI with TCP and also with glyphosate; however, strain K24PM01 showed ability to utilize all five sources phosphorus with PSI ranging between 1.25±0.003 to 2.0±0.073 within 48h of incubation (Table 2) and was chosen as the subsequent experimental strain and CCM as the test chemical which is known to be highly toxic (Banaee *et al*. 2019) compared to others chemical used here.

Characterization of isolate K24PM01

PVK grown fresh colony and cells of strain K24PM01 was taken for determination of its

Table1: Growth and phosphate solubilization of PSB isolated from Sundarban soils

Site	Strain	Growth* on PVK	Phosphate Solubilisation[#]
Canning	C24PM01	++	+
Dobanki	D24PM01	+	+
	D24PM02	+	+
Frezerganj	F24PM01	+++	++
Gadkhali	G24PM01	+	+
	G24PM02	+	+
	G24PM03	+	+
Kumirmari	K24PM01	+++	++

+ = Low; ++ = Moderate; +++ = High
*Growth was recorded from colony diameter
[#]Solubilization of phosphate as per appearance of halo zone at around the colony

Table 2 : Phosphate solubilization index showed by Sundarban isolates in presence of TCP and other different phosphorus sources

| STRAIN	TCP	HGRP	LGRP	GLYPHOSATE	CCM
C24PM01	2.33±0.011	1.2±0.007	1.0±0.011	1.33±0.008	0
D24PM01	1.25±0.003	1.0±0.03	1.0±0.01	1.0±0.04	1.0±0.021
D24PM02	2.5±0.023	0	0	1.0±0.01	0
F24PM01	2.0±0.01	1.25±0.01	1.0±0.01	1.0±0.011	1.0±0.02
G24PM01	2.0±0.01	1.0±0.011	1.0±0.03	1.0±0.02	0
G24PM02	1.5±0.012	1.0±0.006	1.0±0.01	1.0±0.01	0
G24PM03	1.25±0.016	1.07±0.001	0	1.0±0.01	0
K24PM01	2.0±0.073	1.25±0.07	2±0.04	1.25±0.003	1.42±0.01

HGRP: High grade rock phosphate; LGRP: Low grade rock phosphate; Glyphosate: Commercial organophosphorus herbicide; CCM: Commercial organophosphorus pesticide mixture of Chlorpyrifos 50%(v/v) and Cypermethrin 5%(v/v)
TCP at 5%, w/v and the rest at 0.1%, w/v were used in PVK agar base medium. Plates were incubated for 48h at 37^{0}C.

Table 3 : Morphological and biochemical characterization of bacterial isolate K24PM01
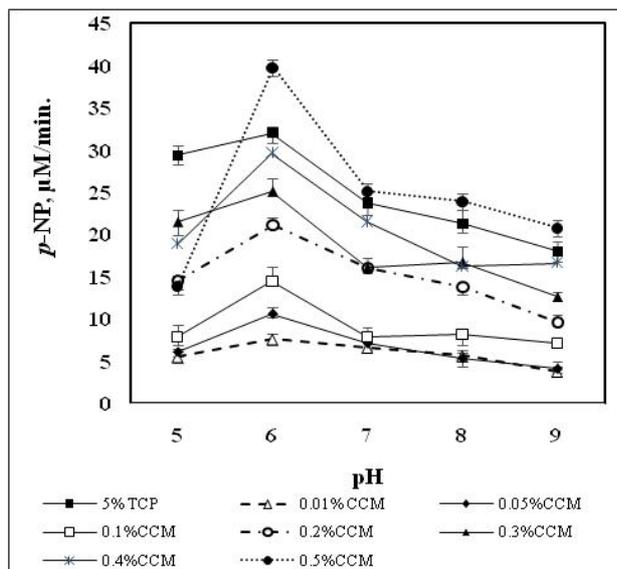
Features	Observation after 48h of growth
Colony morphology	Undulated, white, rough, circular, flat, Matte
Gram nature	Gram positive
Pigment synthesis	Nil
Cell shape & Cell arrangement	Coccus, Single and in chains
Motility	Nil
Sporulation	Nil
Salt tolerance	NaCl, 1-4 % (w/v)
Catalase	Positive
*Sugar utilization profile:	
Dextrose, sucrose, cellobiose, cellulose	Positive
Xylose, Maltose, Lactose, Fructose, Starch	Negative

Fresh bacterial culture grown on PVK agar plate was taken for all test.
Sugars individually was used at 1% w/v level as sole source of carbon in PVK medium.
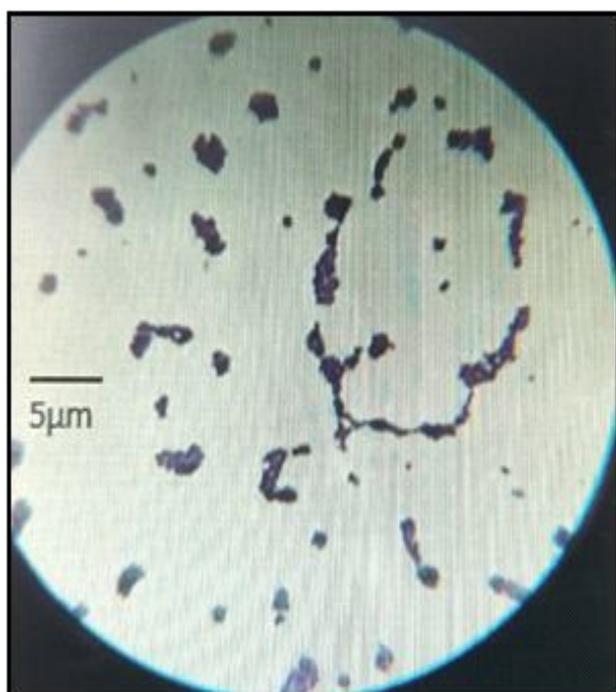
Fig. 1: Bacterial strain K24PM01 after Gram staining
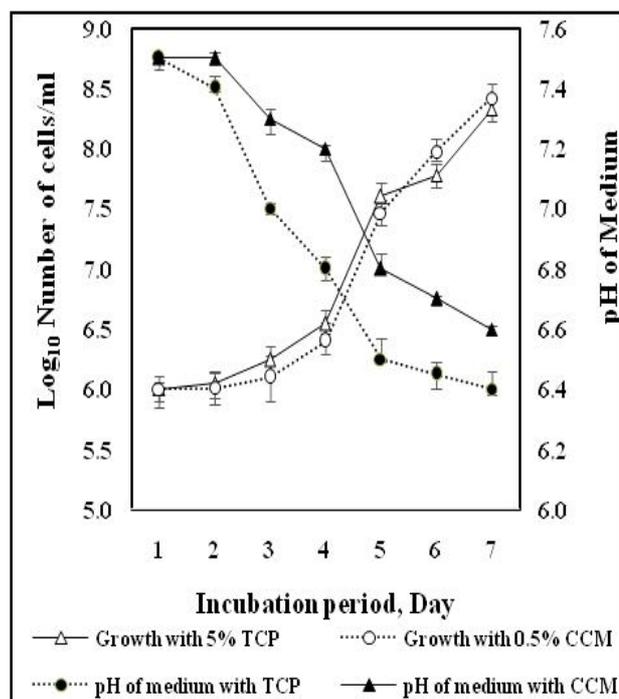
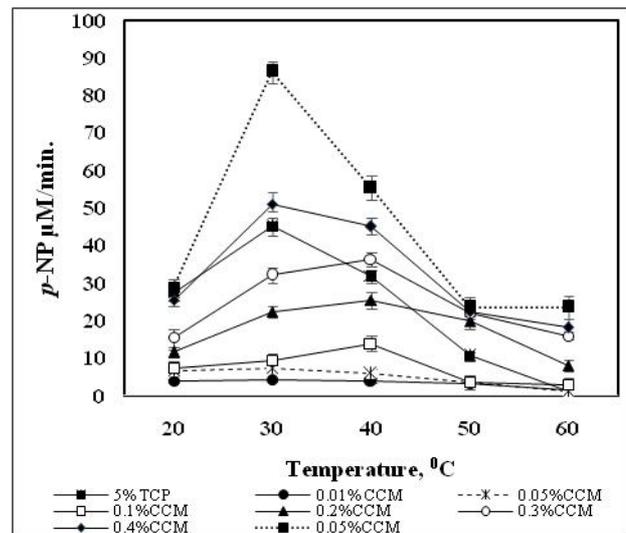**Fig.2**: Time course of growth and changes in pH by K24PM01 with TCP, 5% (w/v) and CCM,0.5%(v/v) in PVK base medium
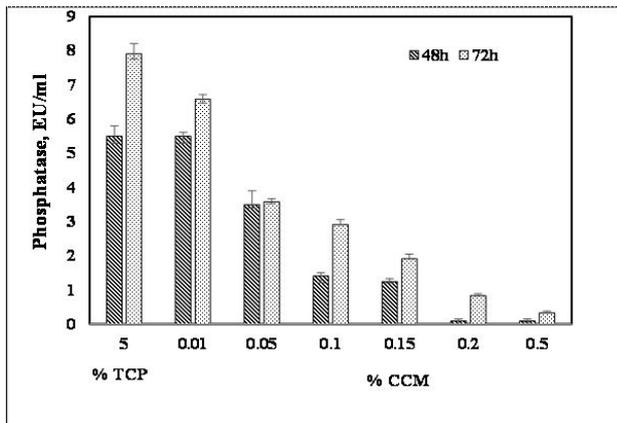
Fig.3. Synthesis of phosphatase by strain K24PM01in presence of TCP, 5% (w/v) and CCM, 0.01- 0.5%(v/v) in PVK base medium.One unit of enzyme activity is defined as the amount of enzyme required to produce 0.1µM of product per min

Fig. 5: Determination of temperature optima of phosphatase in terms of *p*-NP formation. Source of enzyme was culture filtrate of strain K24PM01 grown with TCP and variable conc. of CCM.

Fig.4: Determination of pH optima of phosphatase in terms of *p*-NP formation. Source of enzyme was culture filtrate of strain K24PM01 grown with TCP and variable conc. of CCM

features (Table 3). This Gram-positive short rod to cocci (Fig.1) appeared as single cell and also in chain. It was an opaque non-sporulating, non-motile halotolerant bacterium which showed NaCl tolerance level up to 4% (w/v). Biochemical tests showed that K24PM01 strain was a catalase positive strain. Dextrose, sucrose, and cellobiose could be utilized by the organism for good growth and phosphate solubilization; however, cellulose itself showed good growth but poor PSI (data not shown). Fructose, lactose, maltose, xylose and starch were not suitable for its growth. Based on limited characterization, the strain has been

tentatively recorded as a species of *Arthrobacter*. Soil *Arthrobacter* shows ability of cellulose degradation (Das *et al.* 2022) and phosphate ester degradation too (Jiang *et al.* 2022). Having mangrove vegetation, Sundarban mud soil is rich in organic matter (Hasan *et al.* 2025) and must contain cellulose or its derivative like cellobiose which could be a good source of carbon substrate for PSB strain K24PM01 and its phosphatase enzyme synthesis.

Time course of growth of potent isolate in presence of TCP and CCM

The strain K24PM01 showed a very slow growth in presence of inorganic phosphorus TCP (5%, w/v) and low concentration of organophosphorus CCM (0.5%, v/v). Because of the high inhibitory effect to microorganisms, CCM was used at 1/10th concentration of TCP. In each case, observed growth in terms of $\log_{10}$ number of cells/ml run almost parallelly. Exponential phase of growth initiated after 3rd day which continued even after 7th day of incubation. pH of the growth medium declined sharply in both cases, concomitant with early log (3rd day) to mid log phase (5th day) of growth and the values were pH 6.4 and pH 6.6 in media with TCP and CCM, respectively (Fig.2). Lowering of pH might be due to acid secretion by the organism which is a common phenomenon showed by most PSB, although chlorpyrifos degrades slowly in acidic

soil compare to that of in alkaline and neutral soil (Singh *et al.* 2023).

Determination of phosphatase enzyme synthesis by K24PM01

Strain when grown in broth medium with TCP and CCM of variable concentration (0.01% - 0.5%, v/v) individually, enhanced concentration of CCM showed gross inhibitory effect on organism's phosphatase enzyme synthesis (Fig.3). The lowest conc. of CCM (0.01%, v/v) was able to support phosphatase synthesis which was almost comparable with that of 5% TCP and it might happen when CCM components were degraded and used up as source of phosphates even in acidic growth medium (Fig. 2) rather, more than 55% suppression of enzymatic degradation and utilization of CCM had been noticed with 10 times increment in its concentration (Fig.3).

Determination of pH and temperature Optima of crude phosphatase synthesized by K24PM01

To determine pH optima and temperature optima of the crude phosphatase culture filtrate of strain K24PM01 was taken. Filtrate of 5% TCP containing medium was considered as the control and the same of 0.01% - 0.5%CCM containing PVK broth medium were the test sets for these experiments. The crude enzyme activity in terms of formation of product *p*-NP (µM/min) showed peak at pH 6.0 in each case including the control set and then decline slowly in individual buffered system (Fig.4). This activity further showed much enhanced values with increase in concentration of CCM. Maximum activity was recorded with 0.5% CCM (39.75µM/min) which was more than 24% greater than the control set with 5% TCP. The temperature optima of this crude acid phosphatase synthesized by strain K24PM01 showed its maxima at 30^0C when bacteria grown in 5% TCP and higher conc. of CCM (0.4-0.5%). However, filtrate from lower conc. of CCM (0.01-0.03%) containing medium showed shifting their peak to 40^0C and then went down with minimum activity at 60^0C (Fig. 5).

CONCLUSION

This study demonstrated that: (i) the Sundarban halotolerant PSB strain K24PM01 could utilize TCP, inorganic rock phosphates and commercial organophosphates like glyphosate and chlorpyrifos-cypermethrin combination (CCM) as their sole sources of phosphate. (ii) The organism resulted in comparable growth while using 5% TCP and 0.5% CCM and (iii) showed probable utilization and degradation of phosphates by means of acid phosphatase synthesis or might be due to the presence of mild dose of exogenous organic acids as indicated by lowering of pH in growth medium. Bioremediation of organophosphates present in agricultural runoff in Sundarban biosphere reserve by this indigenous PSB strain could be one of the low-cost major steps to control OP pollution. Following molecular identification of the potent strain, field application will be suggested.

ACKNOWLEDGEMENT

DECLARATION

Conflict of interest**.** Authors declare no conflicts of interest.

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