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Bioremediation of phosphate solubilising bacteria isolated from the nearby areas of Sundarban

Biswajit Saha*
Aparna Sen**

Abstract

Phosphate solubilizing bacteria (PSB) enhances phosphorous availability in soils through dissolving inorganic P pool. Microbial biodegradation is the use of bioremediation & biotransformation methods to harness the naturally occurring ability of microbial xenobiotic metabolism to degrade, transform or accumulate environmental pollutants, including hydrocarbons such as oil, polyaromatic hydrocarbon (PAH), metals etc. Biological processes play a major role in the removal of contaminants & take advantage of the catabolic versatility of microorganisms to degrade or convert such compounds. In this study we have isolated three prominent phosphate solubilising bacteria that also have different hydrocarbon degrading property. PSB were screened on Pikovskaya Agar media. The efficiency of different PSB isolates for phosphate solubilization was evaluated from the zone they formed on agar plates of PVK by solubilising the tri calcium phosphate of the medium. Hydrocarbons of different origin like petrol, diesel, kerosene etc were used in 1% concentration in Bushnell Hass media to determine their degradation capabilities. Different microbiological & biochemical test were performed to determine the optimum condition required for microbial growth. The results of this phosphate solubilization & hydrocarbon degradation are encouraging & need to be confirmed under field condition.

Keywords:

PSB;
Xenobiotic;
Polyaromatic hydrocarbon;
Pikovskaya Agar;
Bushnell Haas media.

Author correspondence:

Biswajit Saha*

Lecturer, Department of Microbiology, Bijoy Krishna Girls College, Howrah.
E-mail:- biswajit.saha1402@gmail.com

1. Introduction (12pt)

Oil spills are one of the most damaging forms of water pollution, and is one of the significant problem in the industrialized and developing world today (Song H G and Bartha R, 1990). Biodegradation is being used as a treatment option at many hydrocarbons contaminated sites (Braddock at al 1997), which exploits the ability of microorganisms to degrade and/or detoxify organic contaminants.

Petroleum (crude) oil is complex mixture of many thousands of compounds. Petroleum hydrocarbons are the major constituents of crude oil (50-98%) and alkenes represent 20-50% of oil, depending on the source of the oil. Petroleum

* Lecturer, Department of Microbiology, Bijoy Krishna Girls College, Howrah.

** Associate professor, Head, Department of Microbiology, Lady Brabourne College, Kolkata

hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae. However, bacteria play an imperative role in hydrocarbon degradation. Microorganisms are endowed with metabolism machinery to use petroleum products as a carbon and energy source. The extent of biodegradation of hydrocarbons in contaminated sites is dependent on several factors such as molecular composition of the hydrocarbons, the type of microbial population, and optimal environmental conditions to stimulate the bioavability of the contaminants to microorganisms (Huesemann, 1995). Some petroleum products have been found to exert carcinogenic and neurotoxic effect 2. Though several mechanical and chemical methods have been implemented for the degradation of these products, the rate of contamination is quite high and the cost involved is also high 3. In view of this situation bioremediation gives a better solution compared to the currently existing methods. It provides efficacy, safety on long term use, cost and simplicity of administration with promising opportunity for creating better environment (Arora et.al.1979).

Phosphate-solubilizing microorganisms play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Microorganisms are involved in a range of process that affect the transformation of soil Phosphorus (P) and thus are integral component of the soil 'P' cycle. Several mechanisms like lowering of soil pH by acid production, ion chelation, and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by PSMs. Phosphate-Solubilizing Bacteria (PSB) is slowly emerging as important organisms for the soil improvement. Aim of our present study is to isolate & identify the PSB from the soil from the nearby areas of Sundarban & to check their bioremidial properties against different environmental pollutants.

2. Research Method

Collection of soil samples: Soil samples were collected from neighboring places of riverside Matla, South 24 parganas. Samples were collected from the selected sites at a depth of 15cm from 6 different points within the area. The samples were then air-dried, powered and mixed well to represent a single sample. The sample was then taken for the study.

Isolation & screening of phosphate solubilising activity:- The soil samples were processed on the same day. Samples of 1.0 g soil were suspended in 9.0 ml of phosphate buffer saline (pH 7.2) and serial dilutions (1:10) were spread on Pikovskaya's (PVK) agar containing TCP as the phosphate source. Plates were incubated at 30 °C for 7 days and colonies with a clear halo were marked positive for phosphates solubilization. The phosphate solubilizing potential of PSB in liquid medium was estimated by inoculating separated colonies in 100 ml PVK broth containing 0.5%, TCP, adjusted to 7.0 and incubating at 30 °C on rotary shaker (130 rev min^{-1}). 5.0 ml of the cultures were taken every 24 h for 7 days, centrifuged, and the content of soluble-P estimated by colorimetry (Jackson 1973). The formula for calculating solubilisation index is

$$\boldsymbol{SOLUBILIZATION\ INDEX} = \frac{\textbf{colony diameter} + \textbf{halozone diameter}}{\textbf{colony diameter}}$$

Screening of Oil Degrading Bacteria:- After second sub culturing oil degrading microorganisms were isolated in Bushnell Haas media, composition:-Magnesium sulphate 0.20gm/lt, Calcium chloride 0.020gm/lt, Monopotassium phosphate 1.0gm/lt, Dipotassium phosphate 1.0gm/lt, Ammonium nitrate 1.0gm/lt, Ferric chloride 0.05gm/lt, Final pH (at 25°C) 7.0±0.2.Screening of petroleum degrading isolate was carried out by growing the isolates in Bushnell- Hass (BH) broth overlaid with 1% v/v oil & incubated

for 7 days at 37degree C in a BOD incubator with 120 rpm shaker. Their ability to tolerate the oil content was accessed by measuring the turbidity using absorption spectrophotometer at 660nm.

Identification of the bacterial isolates:- Morphological and biochemical characteristics of the all isolated strain were studied either on nutrient agar or in nutrient broth as described earlier (Claus D *et al*., 1986). Gram reaction, motility, shape and color of colony and acid / gas production from carbohydrates and sugars fermentations were performed as recommended by (Ventosa *et al*. 1982). Biochemical tests catalase, urease activities, nitrate reduction, Indole production etc were tested as recommended by (Smibert and Krieg 1994). Based on the test results the preliminary identification of the isolated bacterial strain was done.

Molecular Identification of isolates:- Extraction of DNA was done from the best phosphate solubiliser after overnight growth in LB plates. The cell were suspended in 0.5 ml of distilled water, boiled for 10 min in a water bath and then centrifuged for 10 min at 13,000 rpm. The supernatant (total DNA) was placed in a new tube for PCR amplification. The primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (3′-ACGGCTACCTTGTTACGACTT-5′) were used for amplification of 16S rRNA gene (Weisburg et al. 1991). The total PCR reaction mixture was 50.0 µl comprising 200 µM dNTPs, 50 µM each primer,1X PCRbuffer,3 U *Taq* polymerase, and 100 ng genomic DNA. The thermocycling conditions involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min and final extension at 72 °C for 8 min. The 16S rRNA gene was purified by gel electrophoresis The sequencing of bacterial 16S rDNA amplicons was performed after purification, using Applied Biosystems 3500 Series Genetic analyzer system. The obtained 16S rDNA sequence of each isolate was used to determine the most closely related sequence of available sequences in Gene Bank database using the Blast server at NCBI.

Test for IAA production:- To determine the IAA produced by each isolate, a colorimetric technique was performed with Van Urk Salkowski reagent using the Salkowski's method (Ehmann, 1977). The isolates were grown in yeast malt dextrose broth (YMD broth) (Himedia, India) and incubated at 28 °C for 4 days. The broth was centrifuged after incubation. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$solution) and kept in the dark. Appearance of colour change with respect to blank indicates positive IAA production.

Preservation and Subculture of the Isolates:- The isolates were preserved in 50% (v/v) glycerol at -80°C. For the daily requirement of the culture, the isolates were streaked on nutrient agar slants and stored at 4°C. The sub-culturing was done every 15-20 days.

3. Results and Analysis

Soil and water contamination with hydrocarbons caused extensive damage of the local system, this contamination are crisis to plants and animals. An efficient way of remediation the oil-contaminated sites could be employment of special microorganisms, such as bacteria, microalgae, and fungi [14] Bacteria are the most important microbes in this process because they break the dead materials into organic matter and nutrients [15].Petroleum hydrocarbon is composed of carbon and hydrogen and many microorganisms have the ability to utilize the hydrocarbon as a sole source of carbon energy and these isolates are widely present in soil. In this present study a total of 13

isolates were initially isolated from which 3 bacterial isolates named BS1, PS2, G were further analysed as they were potent phosphate solubilising bacteria. The solubilising index of the isolates were between 2.8- 3.5.

Fig1:- Phosphate Solubilization by BS1

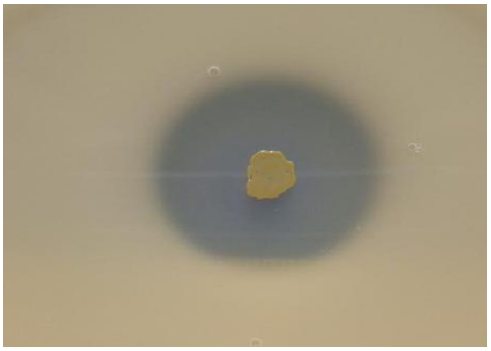
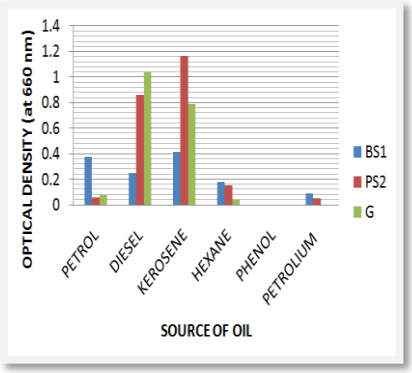


Fig2:- Tricalcium phosphate Solubilisation in Burks media.

Oil degradation potential were tested in Bushnell haas media where appearance of growth in 1% concentration of six different pollutants such as Petrol, Diesel, Kerosin, hexane, phenol & petroleum were observed after 7 days of incubation. The corresponding optical densities were taken which indicates that all of these organisms can utilise petrol, diesel, kerosin, hexane & petroluem but not phenol at 1% concentration. BS1 & PS2 utilises kerosin most where G utilises diesel most.



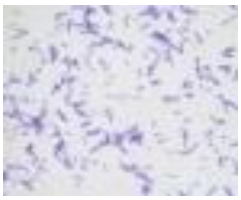
Degradation potential of isolates

Fig:-3

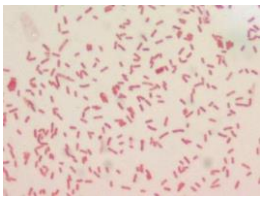
Fig:-4

Fig:-3- Turbidity in BH media contains kerosin with respect to control for culture PS2.
Fig:-4- Turbidity in BH media contains Diesel with respect to control for culture G.

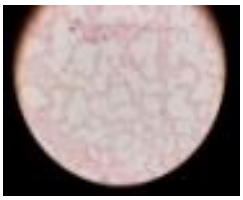
Identification of the bacterial isolates:- Bacterial identification were done by using different techniques like staning and biochemical charecterisations. BS1 is Gram positive; other two bacteria are Gram Negative. The charecteristics of colonies are given in table no.1.



BS1



PS2



G

Fig.5:- Gram staining of BS1, PS2, G

COLONY CHARECTERISTICS:-
Table no.1

Characteristics	BS1	PS2	G
SIZE	Medium	Medium	Medium
SHAPE	Circular	Circular	Circular
COLOUR	White	Green	Green
TEXTURE	Smooth	Smooth	Smooth
OPACITY	Opaque	Opaque	Opaque
CONSISTENCY	soft	soft	soft

IMViC for BS1
Fig:-6

IMViC for PS2
Fig:-7

IMViC for G
Fig:-8

Fig:-9 –Motility test test

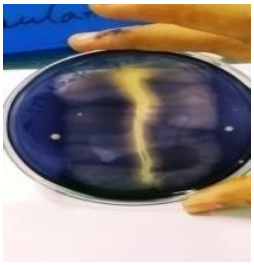


Fig:-10-Amylase production By PS2

Fig:-11-IAA production

Table no.2

TEST	BS1	PS2	G
Indole	-	-	-
Methyl red	-	-	+
Voges Proskauer	+	+	+
Citrate	-	+	+
Motility	+	+	+

Table no.3

ENZYME PRODUCTION	BS1	PS2	G
Amylase	+	+	-
Catalase	-	+	-
Nitrate reductase	-	+	-
Urease	-	-	-
Phenylalanine deaminase	-	-	-

All of the isolates were indole negative & VP positive. G shows positive in MR, VP & citrate test where PS2 shows positive in VP& Citrate only. BS1 is positive to VP only (Table no.2) . All three strains are motile (Table no.2& Fig no.9). Several enzymatic tests were conducted (Table no.3) BS1& PS2 shows positive in amylase production in starch agar plate. Only the organism PS2 is able to produce catalase enzyme as they hydrolyses 3% hydrogen per oxide .all of them are urease & phenylalanine deaminase negative (table no.3). IAA production detection was taken positive after addition of salkowsky reagent and incubation. All three organisms' shows pink coloration indication that they are capable of producing Indole acetic acid (Fig. no.11)

Molecular identification: After performing 16s rRNA isolation by PCR and Sangers method of DNA sequencing it has been observed that the most promising phosphate solubilising strain in terms of solubilisation index (BS1) is *Bacillus cereus*. The phyllogenetic tree has been constructed.

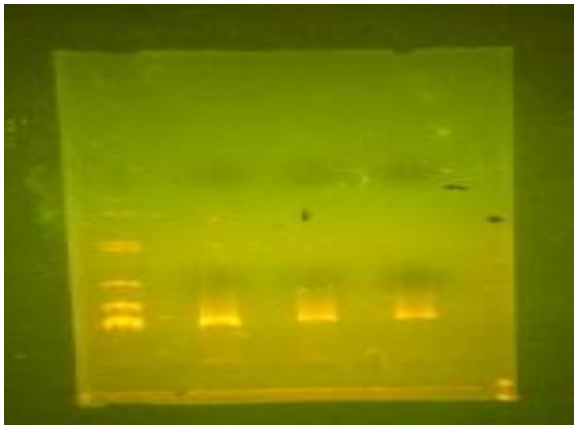


Fig no:-12-Genomic DNA Isolation Left-Marker-BS1-PS2-G-Right

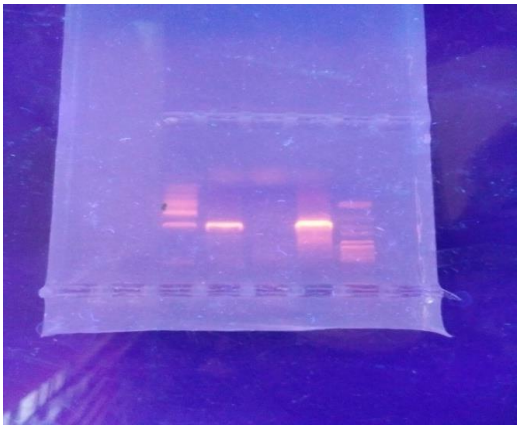


Fig.no-13- PCR amplified 16srRNA Left-Marker-BS1-PS2-G-Marker-Right

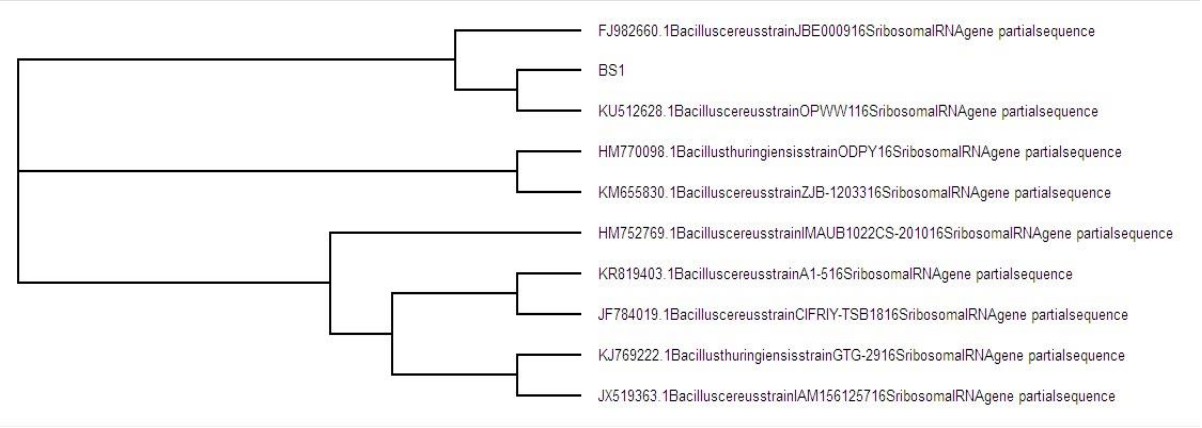


Fig.No.14-Phyllogenetic tree for BS1

4. Conclusion:-

A total of 13 bacterial strains were initially isolated from the site, from which three most promising phosphate solubilising organisms were chosen for further analysis. Phosphate solubilisation parameter shows that BS1 is most potent phosphate solubiliser in terms of solubilisation of insoluble tricalcium phosphate in both burks media & Pikovskaya agar media. All strains can more or less utilize different complex organic compounds like petrol, diesel, kerosin etc when used 1 % concentrations, so they have some bioremidial properties. Although the percentage of bioremediation need to be study by gravitometric analysis & Gas chromatography. All three strains are capable of producing some industrially important enzymes like amylase, phosphatases, catalases etc.

The isolated strains have the capability to produce growth hormones (IAA) that could help to the plants to enhance their root and shoot growth. This shows the potential of these bacteria for use in crop production. It is known that rhizosphere microorganisms mediate many soil processes, such as decomposition, nutrient mineralization and nitrogen fixation. Other researchers have reported that bacteria in rice fields have the potential to produce IAA and are able to fix Nitrogen . In the current study, it was found that the isolated strains were able to solubilize phosphate in Burks and Pikovskaya media, with P solubilizing activity indicated by clear halo zone around colonies. The ability to enhance the growth of different plants need to be study and different parameters like antibiotic sensitivity test, heavy metal tolerance test plus effect of ultraviolet rays on the gowth of these microorganisms need to be study. The potential of these PSB should be exploited further for the sustainable management of acid sulfate soils, especially for rice production. For instance, they can be used as bio-fertilizers to improve the fertility of acid sulfate soils.

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References

1. De Freitas JR, Banerjee MR, Germida JJ (1997) Phosphate solubilizing rhizobacteria enhance the growth and yield but no phosphorus uptake of canola (*Brassica napus* L.). Biol Fertil Soils **24:** 358–364.

2. Ohtake H, Wu H, Imazu K, Ambe Y, Kato J, Kuroda A. Bacterial phosphonate degradation, phosphite oxidation and polyphosphate accumulation. A Res Conserv and Recycling 1996;18:125–34.
3. McGrath JW, Wisdom GB, McMullan G, Lrakin MJ, Quinn JP. The purification and properties of phosphonoacetate hydrolase, a novel carbon-phosphorus bond-cleaving enzyme from *Pseudomonas fluorescens* 23F. Eur J Biochem 1995;234:225–30
4. Illmer P, Schinner F. Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil Biol Biochem 1992;24:389–95.
5. Rodríguez H, Goire I, Rodríguez M. Caracterización de cepas de *Pseudomonas solubilizadoras de fósforo.* Rev ICIDCA 1996;30:47–54.
6. Arora D, Gaur C. Microbial solubilization of different inorganic phosphates. Indian J Exp Biol 1979;17:1258–61.
7. Thaller MC, Berlutti F, Schippa S, Iori P, Passariello C, Rossolini GM. Heterogeneous patterns of acid phosphatases containing low-molecular-mass Polipeptides in members of the family *Enterobacteriaceae.*Int J Syst Bacteriol 1995b;4:255–61.
8. Gügi B, Orange N, Hellio F, Burini JF, Guillou C, Leriche F, Guespin-Michel JF. Effect of growth temperature on several exported enzyme activities in the psychrotropic bacterium *Pseudomonas fluorescens.* J Bacteriol 1991;173:3814–20.
9. Richardson AE, Hadobas PA. Soil isolates of *Pseudomonas spp. that utilize inositol phosphates. Can J Microbiol*1997;43:509–16.