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Isolation, Characterization and Growth Study of *Pseudomonas sp.* from Plastic Contaminated Soil

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Abstract: ***An aerobic microorganism with the ability to utilize phenol as a carbon and energy source was isolated from a site contaminated with plastic wastes. The isolate was identified as a Pseudomonas sp. based on morphological, physiological, and biochemical tests. The isolated strain showed optimal growth at 25 ℃ and pH of 7. The phenol utilization studies with the Pseudomonas sp. showed that the complete assimilation occurred in 24 hours. The microorganism metabolized phenol up to 53mM concentrations. The bacterial strain was immobilized in alginate beads and its phenol degradation efficiency was observed to increase many folds.***

Keywords: Aerobic Microorganism, Phenol utilization, *Pseudomonas sp.*, Plastic Waste Contamination, Alginate Bead Immobilization

1. Introduction

The phenolics comprise characteristic pollutants in wastewater and effluents discharged from petrochemical, textile, tannery, and coal gasification units [1]. Since these compounds are toxic even at low levels, they pose a threat to the biosphere and especially to aquatic life. Biodegradation of phenolics by certain anaerobic, aerobic bacteria and fungi has been reported [2]- [12]. Diverse microorganisms, including many species of bacteria and fungi, have evolved the metabolic capacities to degrade hydrocarbons. Bacteria are often the dominant hydrocarbon degraders in aquatic systems. Microbial cell remediation efficiency for xenobiotic pollutants remains a major challenge to microbial and process engineers. Hence, some strategies have been proposed to overcome the issue. The use of microbial immobilization technique is one of them. Nevertheless, the application of free microbial cells for wastewater treatment in activated sludge processes creates issues such as solid waste disposal. Immobilized microorganisms have proved to be effective in addressing phenol-containing wastewater with little sludge yield and have been receiving increasing attention [13]-[15].

Immobilized cell technology has been widely applied in various research and industrial applications. The purpose of the present investigation has been to study the biodegradation of phenol using free and immobilized *Pseudomonas sp.*

2. Materials and Methods

The soil collected from the plastic contamination site was used to isolate microorganisms adopting the selective enrichment culture technique. The bacterial strain was grown on mineral salt medium (MSM) containing (g l)1) K2HPO4, 1.6;48 KH2PO4, 0.2; (NH4)2SO4, 1.0; Mg SO4 Æ 7H2O, 0.2; NaCl, 0.1; CaCl2 Æ 2H2O, 0.02; FeSO4. H2O, 0.01; Na2MoO4 Æ 2H2O, 0.05; MnSO4 Æ H2O, 0.05; Na2WO4 Æ 2 H2O, 0.05; supplemented with phenol up to 5 mM as the sole source of carbon and energy [16]. In the metabolic studies, growth substrate (2–5 mM) was incorporated into the sterilized MSM aseptically. The flasks were inoculated with 5% (v/v) of inoculums and incubated on a rotary shaker (110 rpm) at 25±2 ℃. Un-inoculated flasks were incubated in parallel as controls. The purified bacterium was identified and characterized based on the morphological, physiological, andbiochemical features as described in Bergey's Manual of Systematic Bacteriology [17].

2.1 Measurement of Cell Growth

The growth curve of the organism was followed by a measurement of the optical density at 600 nm. The viablecounts were established by the pour plate technique. The growth response of the organism on other substrates was assessed by harvesting phenol-metabolizing cells by centrifugation at 5000 rpm at 5℃ for 10 min. The cell pellet obtained was resuspended in the sterile MSM containing different substrates (5 mM each). All operations were performed under aseptic conditions. All the results represent data from at least three independent and repetitive experiments.

2.2 Immobilization of microorganisms

The phenol-degrading bacteria were harvested after 12 hours of growth from 1 liter of culture medium. The cell pellet obtained by centrifugation at 5000 rpm for 10 min at 4 ℃, obtained was and used for immobilization in alginate and

agar matrices. The alginate entrapment of cells was performed according to Bettemann and Rehm [18]. Alginate (4% w/v) was solubilized in boiling water and autoclaved at 121 ℃ for 15 min. A 10 ml bacterial cell suspension was added to 100 ml of sterilized alginate solution and mixed by stirring on a magnetic stirrer. This alginate-cell mixture was extruded drop by drop into a cold, sterile 0.1 M calcium chloride solution through a burette. The gel beads formed were left in the solution for one hour before being filtered off. The beads were washed with distilled water and used for experiments. Agar entrapment of cells was carried out in a sterile 4% (w/v) agar saline solution [19]-[20]. Briefly, a 12-hour bacterial cell suspension (10 g wet weight) was mixed with 10 ml of saline and extruded into a sterile agar-saline solution to obtain cell-entrapped beads. These agar beads were washed successively with distilled water and saline for further applications. A 25 g each, of the alginate and agar immobilized beads containing 2.1 $\times 10^{11}$cfu g^{-1} and 2.3 $\times 10^{11}$cfu g^{-1} beads, respectively, were added to 250 ml conical flask containing 100 ml of mineral salt medium to which 17 mM phenol was supplemented. The fermentation was carried out at 25 ℃ on a rotary shaker at 150 rpm for the desired incubation period. Samples of the culture broths were taken each day for residual phenol analysis. Control flasks were incubated in parallel under the same conditions to ascertain the evaporation losses of phenol.

2.3 Gram staining

This technique involves making a thin, homogenous smear of the organism on a slide, airdrying it, adding crystal violet stain, Gram's iodine, ethanol, and safranin, air drying, and observing under a 100X lens. Initially, all the cells take up crystal violet but only Gram-positive organisms retain the primary stain due to the presence of multiple layers of peptidoglycan. Dye is washed off from the cell walls of Gram-negative organisms, upon the addition of decolorising agent. Gram-negative organisms take up the counterstain safranin.

2.4 The catalase test:

It is used to find out if the given bacteria contain the catalase enzyme. By generating water and oxygen, the catalase enzyme aids in the detoxification of hydrogen peroxide. The creation of bubbles, a sign of oxygen gas, is seen as a sign of a positive test result.

2.5 Oxidase test:

This test determines whether an organism possesses cytochrome c oxidase enzyme. A positive test indicates a purple color on the oxidase disc. It is used to differentiate between species of *Pseudomonas*, *Neisseria*, *Moraxella* etc...

2.6 Carbohydrate fermentation test:

This test is performed to check whether the microorganism can utilize certain carbohydrates like Lactose, Sucrose, Glucose, etc. The results of the test are indicated by the change in color from blue to yellow of the test sample which indicates that the organism produces acid and is acid positive. The formation of a gas bubble in Durham's tube indicates it is gas-positive.

2.7 IMVIC test

The IMVIC test comprises of Indole, Methyl red, Voges Proskauer (VP), and Citrate. The indole test determines whether the given bacteria can break down the important amino acid tryptophan. The development of a red color ring on the broth's topmost layer indicates a successful test. A methyl red test is performed to check the fermentation. A methyl red indicator is used for the test. The positive results are indicated by methyl red, a pH indicator that turns red in acidic conditions below pH 4.5. In negative test conditions, the test sample remains yellow color beyond the pH range of 5. In the VP test, results are indicated as positive if the test sample turns brownish-red to pink color. If the results are negative then the test solution turns yellow. The citrate test is performed to determine the ability of the bacteria to use sodium citrate as the only source of carbon and inorganic ammonium hydrogen phosphate as a source of nitrogen. This test is possible only if if the organisms are capable of fermenting citrate. The positive test result appears blue and negative results remain green.

2.8 Cetrimide Agar test

Cetrimide agar is a solid selective medium used for the isolation and identification of *Pseudomonas sp.* from a source. Cetrimide inhibits bacterial growth except Pseudomonas and enhances fluorescein and pyocyanin pigment production.

2.9 Motility test

Motility is the ability of the organism to move by itself using special fibrils like flagella that produce a gliding form of motility. It has been recognised as an important taxonomic tool for the biological characterization of microorganisms, motility by bacterium is mostly demonstrated in a semi-solid agar medium which is a combination of different parameters that help in motility. The inoculum is spread on this medium and observed for motility under the microscope. The motility test helps us to differentiate between genera and species of bacteria. There are a variety of ways to determine the motility of a bacterium; biochemical tests as well as microscopic analysis. The *Pseudomonas sp.* visualized under 45x microscopic view were motile and swarming.

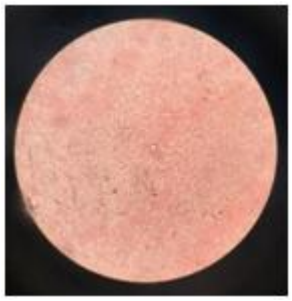

Figure 1: Gram staining; Gram-negative rods

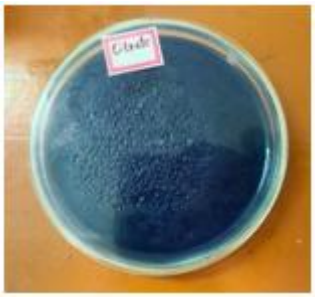

Figure 2: Citrate test

3. Result and Discussion

Microorganisms can adapt to a variety of environmental conditions. The present work's preliminary objective was to isolate a microorganism that could grow on phenol. A microorganism was obtained that could metabolize phenol up to 53 mM, by adopting the enrichment culture technique.

The classification of bacteria was carried out entirely based on phenotypic characteristics, such as cell wall type, morphology, motility, and nutritional requirements. The isolated phenol metabolizing strain was thus subjected to various morphological, physiological, and biochemical tests.

Gram staining

It was observed that the bacteria seen under the microscope after Gram staining was reddish-pink and its shape was identified as small rods. As per observation, the bacteria identified are Gram-negative small rods.

Motility test

The small rods identified in the Gram staining were used for identifying the motility of the microbes, and it was seen under a 45x microscopic view. Where the flagellar movement was seen in the microbes.

Table 1: Results for biochemical tests

BIOCHEMICAL TEST							
Test / S. No.	INDOLE	METHYL RED	CITRATE	VOGES PROSKEUR	CETRIMIDE AGAR	CATALASE	OXIDASE
1	-	+	-	-	-	+	-
2	-	-	+	-	+	+	+

Figure 4: Indole test

Figure 5: Voges Proskauer test

Figure 6: Methyl Red test

Table 2: Results of Fermentation tests

S. No.	Carbohydrate Fixation Test									
	Glucose		Lactose		Sucrose		Mannitol		Maltose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
1	+	-	+	-	+	-	-	-	-	+
2	-	-	-	-	-	-	+	+	+	-

'+'- Positive result, '-' - Negative result

The results analyzed from Tables. 1 and 2 of the biochemical tests as well as the carbohydrate fermentation test suggest that *Pseudomonas sp.* may be present on the second plate. Based on the findings for positive in motility test as well as the negative results for Indole, Methyl Red, and Voges Proskauer and the positive results for Citrate, Cetrimide, Catalase, and Oxidase. The organisms on plate 2 in the carbohydrate fermentation test (acid and gas) produced positive results for Mannitol and Maltose (acid) and negative results for Glucose, Lactose, and Sucrose.Given the foregoing collection of results, it is reasonable to believe that the organisms on plate 2 might be any species in the genus Pseudomonas, which is one of the key indicators of the same

3. Growth Study and Degradation of Phenol

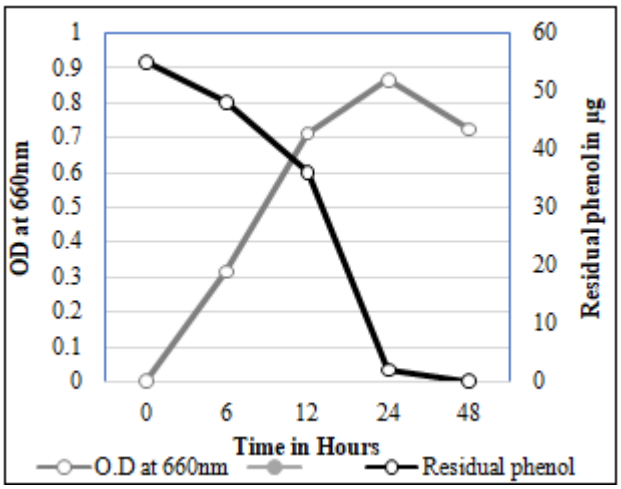

Figure 7: Growth response of *Pseudomonas sp.* and Degradation of Phenol

Studies on the degradation of phenol by *Pseudomonas sp.* indicated that with 53mM phenol utilization in 24 hours (Figure 7), the organism tolerated phenol concentration up to 55mM but at higher concentrations there was a decline in the growth due to substrate inhibition.

The degradation of phenol by free agar immobilized *Pseudomonas sp.* was also compared. The data obtained suggest that the rate of degradation of phenol was unaffected on immobilization and these cells could be used continuously for a week without loss of their biodegradative property.

This study demonstrates that the bacterial strain, *Pseudomonas sp.* isolated from the plastic-contaminated site can metabolize phenol in free as well as immobilized fermentation cultures. The study suggests that the bacteria already present at a polluted site can often adapt to degrade the plastic contaminants if sufficient time is given.

The use of biological systems for bioremediation is more cost-effective than traditional cleaning techniques. Most of the studies so far carried out depend on the use of cell-free systems. However, cell immobilization can be a better alternative for biotreatment. In the present investigation, the bacterial strain on immobilization in an agar matrix proved useful for developing biotechnology for treating phenol.

3. Conclusion

The microorganism isolated from plastic-contaminated sites used phenol as the sole source of carbon and energy. This was identified and characterized as *Pseudomonas sp.* The organism showed optimal growth at 25°C and a pH of 7, and the substrate concentration of up to 55mM of phenol was well tolerated by the organism. The study has also revealed that *Pseudomonas sp.* can efficiently degrade phenol at higher concentrations following immobilization, since the bacterium is capable of degrading phenol there exists a possibility for its use in the development of microbial technology for the bioremediation of plastics.

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Author Profile

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