# Comparative Study of the Phenol Biodegradation Potential of Free Cells and Immobilized *Pseudomonas Resinovorans*

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## ABSTRACT:

Microorganisms are capable of degrading xenobiotic compounds such as phenol, producing innocuous end products. In the present study a bacterium, *Pseudomonas resinovorans* was investigated for its ability to grow and degrade phenol as sole source of carbon and energy. Experiments were also carried out with established phenol degrading microbes to draw a comparison study of the biodegradation potential under same physiological conditions. The biodegradation assays were performed in liquid medium with phenol as single substrate, with initial concentration of phenol ranging from 100 to 1000 ppm. With higher initial phenol concentration the lag phase and degradation time has been found to increase. It is found that *Pseudomonas resinovorans* is able to degrade phenol up to 750ppm in 120 hrs as free cell and 1000ppm of phenol in 60 hrs when immobilized in the calcium alginate beads. The higher concentration of phenol is lethal to microbe. *Pseudomonas putida* has been found to be the most efficient phenol degrading one over the others with degradation time of 144hrs for initial phenol concentration of 1000 ppm while it is able to degrade 750 PPM of phenol in 132 hrs. *Pseudomonas resinovorans* shows better degradation potential than Pseudomonas putida & Pseudomonas aeruginosa until the initial phenol concentration of the contamination is 750 PPM. Increasing the initial substrate concentration from 750 PPM inhibits the growth of the microbe. Result from this study indicates that the microbe can be used for the treatment of the phenol contaminated industrial waste water.

**Keywords**: Biodegradation, xenobiotes, phenol, immobilized.

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## INTRODUCTION:

Phenols, sometimes called ***phenolics*** are the class of aromatic hydrocarbons with a hydroxyl group bonded directly to it. These are widely used as raw materials in petrochemical, petroleum refineries, chemical and pharmaceutical industries. The simplest of the class, phenol find its application in pulp and paper mills, coking operations, coal refining, in the production of phenolic resins, caprolactan and bisphenol A, slimicides, disinfectants, antiseptics and medicinal preparations such as ear and nose drops, mouthwashes and sore throat lozenges, tannery and foundries. Phenolic compounds are among the most frequently found xenobiotes in rivers, industrial effluents, and landfill runoff waters. These are toxic either by ingestion or by contact or inhalation even at low concentrations. Acute exposure of phenol causes central nervous system disorders, which leads to collapse and coma. Muscular convulsions with significant reduction in body temperature are also noted due to phenol toxicity, and this is known hypothermia. Renal damage and salivation may be induced by continuous exposure to phenol [1].

United State Environmental Protection Agency (USEPA) has set a water purification standard, in which, surface water must contains less than 1.0 μg/L phenol [2]. Owing to its toxic nature and its consequent health hazard, industrial effluents containing phenol require proper treatment prior to its discharge into the environments. Several physiochemical treatment technologies exist for the removal of phenol from the industrial effluents. However conventional methods such as solvent extraction, activated carbon adsorption, and chemical oxidation often suffer from serious drawbacks including high cost and formation of hazardous byproducts [3]. Hence the environmentalists focused on the development of technology which will emphasize on the degradation and detoxification of the pollutant without producing any unwanted secondary by-product. Thus biodegradation turned out to be the only alternative favorable for the degradation of the xenobiotes since it is an ecofriendly and cost effective method [2].

## OBJECTIVE:

Most of the phenol degrading bacteria are known to utilize the compound as the sole source of carbon. Biodegradation of phenol by bacteria and their isolation were extensively studied. But the study is mostly limited to the isolation of the microbe from the contaminated site and investigation of the degradation potential of the microbe till the concentration of the phenol

present as a contaminant in the particular site. The objective of the current study is exhaustive study of the phenol degradation potential in both free cell as well as immobilized form of bacterium ***Pseudomonas resinovorans*** (**NCIM: 2599**). Researchers till date have only reported that the microbe is able to degrade phenol. In the present study we have investigated the maximum phenol degrading capacity of the microbe and supported it with assaying the dehydrogenase activity of the microbe. We have also compared the degradation potential of the microbe with other known phenol degraders like *Pseudomonas putida* and *Pseudomonas aeruginosa*.

## MATERIAL AND METHODS:

### *Microorganism and Inoculum Preparation*

The bacterium, *Pseudomonas resinovorans* (NCIM: 2599) was used throughout this study. This bacterium was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The bacterium from the stock culture was grown in nutrient broth for 24 h and the culture was used as a standard inoculum for all cultivation and degradation experiments.

### *Cultivation and Phenol degradation experiments*

The bacterium was grown in the minimal salt medium containing phenol as the sole source of carbon and energy. Liquid mineral salt medium (MSM) consisted of (g/L): $K_2HPO_4$, 0.4; $KH_2PO_4$, 0.2; NaCl, 1; $MgSO_4$, 0.1; $MnSO_4$, 0.01; $FeSO_4$. $H_2O$, 0.01; $Na_2MoO_4.2H_2O$, 0.01; $(NH_4)_2SO_4$, 0.4; was used in all cultivation and phenol degradation experiments [4]. The initial pH value of the medium is adjusted to 7.5 prior to autoclaving. Phenol was sterilized separately by filtration using 0.2 μm regenerated cellulose membrane filter and added to the sterilized medium after cooled down to room temperature**.** 10% (v/v) Inoculum was inoculated to initiate the cultivation of the microbe and degradation of phenol. The flask was incubated at 30$^{o}$C on a rotary shaker, agitated at 140 rpm.

### *Analytical Procedures*

To obtain the growth curve of the microbe, the optical density of the culture was measured at 600nm using a spectrophotometer. 1 ml of the culture was centrifuged and the pellet wash

washed twice before resuspending it in 1 ml of double distilled water and OD value of the cell concentration was taken.

Determination of the phenol concentration in the media was done using the 4-aminoantipyrine colorimetric test [5]. Samples were analyzed at 510nm and the results were correlated with the previously prepared calibration plot. All the readings were taken in triplicates and the average of them has been considered for the analysis.

To determine dehydrogenase activity, the isolates were grown at 30 °C on different concentrations of phenol in test tubes for 24 h. A 50-ml aliquot of an electron acceptor solution, triphenyl tetrazolium chloride (TTC) solution containing 0.25 g TTC in 100 ml tris buffer (l00mM, pH 7), was added to each well and the tubes were incubated at room temperature for 3 hrs for colour development. The O.D. was measured against a blank at 490nm [6].

***Degradation study as a function of phenol concentration:***

To determine the degradation capacity of the microbe, the initial phenol concentrations varied from 250–1000 mg/l while the cell density in each sample was constant ($OD_{600}$= 0.1). Time course sampling was carried out by centrifugation of the culture medium at 12,000 ×g for 10 min. The supernatant was collected in separate clean test tubes and the residual phenol content at each condition was determined using colorimetric biochemical assay as described above.

***Immobilization:***

The microbe was encapsulated in Ca-Alginate beads. Liquid cultures were centrifuged in a 50-ml plastic centrifuge tube (2,500 g) at room temperature for 10 min. and the supernatant was discarded. The pellet was resuspended with a previously autoclaved solution of sodium alginate to a final concentration of 4% (w/v) and 10% (v/v) bacterial biomass. The alginate-bacterial mixture was added drop wise with sterile syringe (20 ml) fitted with a wide bore needle (1 mm diameter) from a height of about 20 cm into an autoclaved solution of calcium chloride (3% (w/v), adjusted to pH 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4°C before being harvested by filtration [7]. After each cycle, the beads were filtered, washed and were used in the subsequent experiment.

## RESULTS & DISCUSSION:

### *Growth Curve and Phenol Removal by the Microbe:*

The growth curve as well as the degradation curve of the microbe to different concentration of phenol was prepared by measuring the OD at 600 and 510nm respectively. Fig 1 indicates that the microbe grows optimally till 500 PPM of phenol as the initial concentration of the substrate. But on increasing the concentration of the phenol to 750 PPM, the biomass concentration decreases. Hence the above graph implies that the microbe can resist the xenobiotes to a concentration as high as 0.7 g/L but above it the xenobiotes inhibit the growth. Similarly the rate of substrate degradation decreases with increase in intial phenol concentration which implies that the microbe finds it difficult to grow rapidly with increase in the concentration of the xenobiotes. The microbe is able to degrade up to 750 PPM of initial concentration of phenol in only 120 hrs.

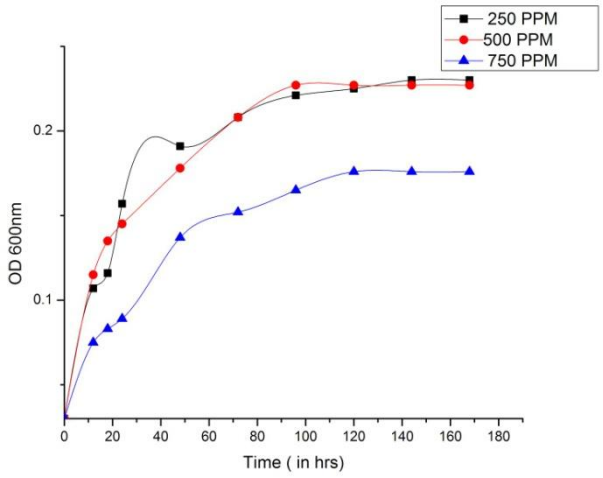


**Fig 1: Growth Curve of *Pseudomonas resinovorans* at different initial concentration of phenol.**

But increasing the initial concentration of phenol above it inhibits the growth. The growth pattern of the microbe suggests that the microbe not only can survive but also degrade efficiently at 750 PPM and hence this characteristics of the microbe makes it a potential candidate for bioremediation of phenol.

### *Respiration at Different Concentration of Phenol:*

The result of the microbe using TTC as indicator of dehydrogenase activity, indicated that the respiratory activity of the microbe is maximum at 0.6g/L of phenol in the medium. But the dehydrogenase activity of the microbe decreases all of a sudden with a phenol concentration

more than 0.7g/L of the substrate. This supports the result obtained from phenol degradation and growth curve mentioned above. Johnsen et al. (2002) [12] used the tetrazolium salt WST-1 as an indicator of microbial dehydrogenase activity in microtitre plate wells containing polycyclic aromatic hydrocarbons. Giti et al. (2005) [13] used the respiratory assay to measure the dehydrogenase activity of the isolates from the soil sample. With this method it is proved that at such high concentration of phenol (>0.6g/L) the microbes remain alive and and exhibited signs of respiration showing that at such concentration viable non-floculating microbes exist in the nature.

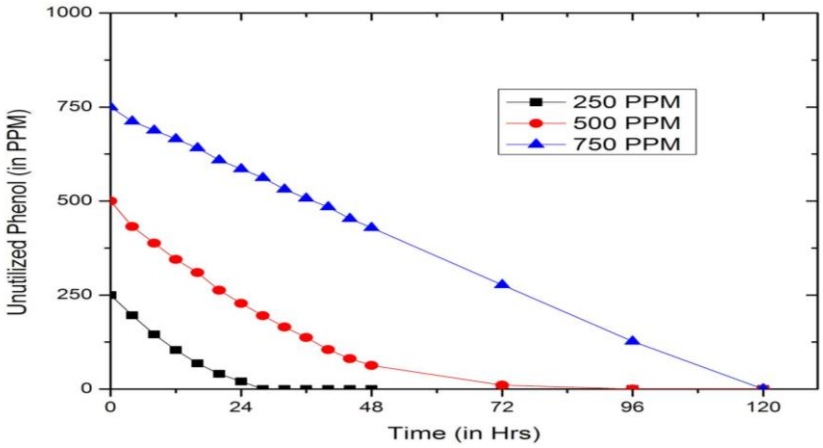


**Fig 2: Degradation Curve of *Pseudomonas resinovorans* at various initial concentration of phenol.**

### *Immobilization:*

The cells immobilized in the Ca-Alginate beads were not only able to tolerate high concentration of phenol but also are able to degrade it completely in a very short span of time (Fig 4). Where the microbe is able to degrade 750 ppm of phenol in 120 hrs in its free cell form, the microbe embedded in Ca-Alginate bead is able to degrade 1000 ppm of phenol completely in just 60 hrs. The microbe is able to degrade 500 ppm of phenol completely in 20 hrs while the free cell form of the microbe is able to degrade the same concentration in 96 hrs. When the microbe is embedded in Ca-Alginate beads, its exposure to the stress condition reduces. As a result, the microbe is able to degrade the xenobiotes faster and more easily as compared to its free cell state.

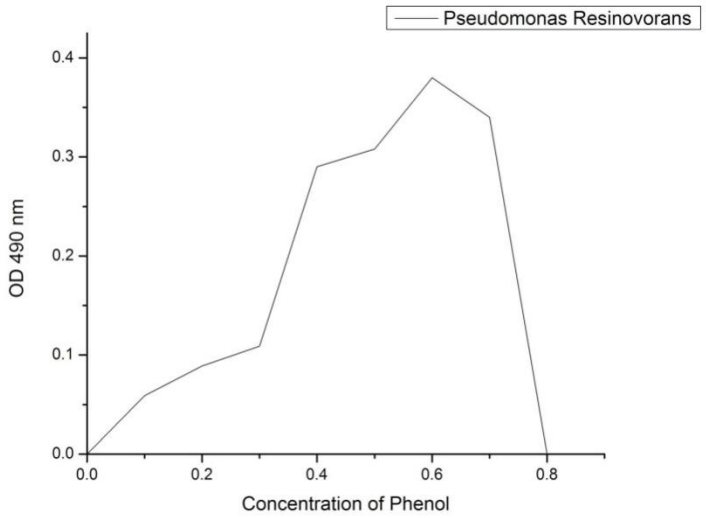


**Fig 3: Respiratory Assay Curve of *P.resinovorans* at phenol concentration ranging from 0.1g/L to 1 g/L.**

### *Comparision of Phenol degrading capacity with known microbes:*

*Pseudomonas putida* and *Pseudomonas aeruginosa* are the two microbes which are earlier been reported to degrade phenol in industrial waste water. P.putida is able to degrade up to 1000 ppm of phenol efficiently in 168 hrs while the other microbes shows inhibition at such high concentration of phenol. The microbes were reported to degrade up to 750 PPM of phenol efficiently and hence we compared their degradation potential till that concentration. Fig 5 is a comparative curve of the degradation potential of three microbes at 750 PPM of phenol. Since the maximum degradation potential of p.putida is 1000 PPM and of p.resinovorans & p.aeruginosa is 750 PPM, we compared the degradation potential of all the three microbes at 750 PPM. The above figure indicates that if the level of phenol contamination at any site is 750 PPM then this microbe is a best option for its biodegradation since p.resinovorans can degrade phenol better than p.putida when the initial phenol concentration is less than equal to 750 PPM. The microbe is able to degrade 750 PPM of phenol in just 120 hrs while p.aeruginosa is not able to degrade more than 50 % of the substrate and p.putida requires more than 144hrs for the degradation. Thus pseudomonas resinovorans is a better option for phenol contamination where the concentration of phenol is less than equal to 750 PPM.

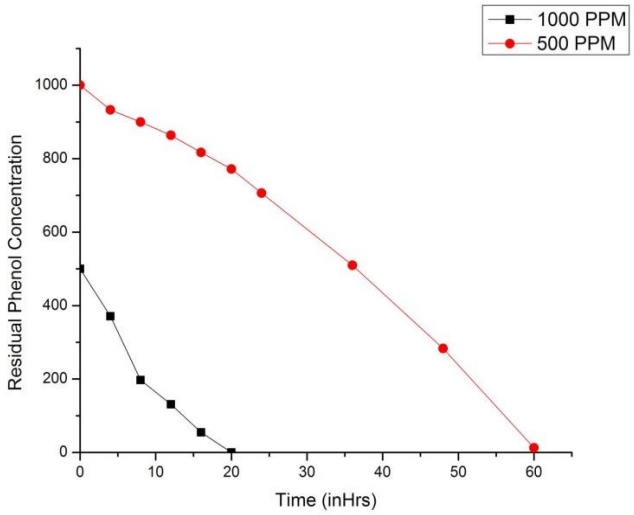


**Fig 4: Phenol Degradation Curve by P.resinovorans immobilized on Ca-alginate beads.**

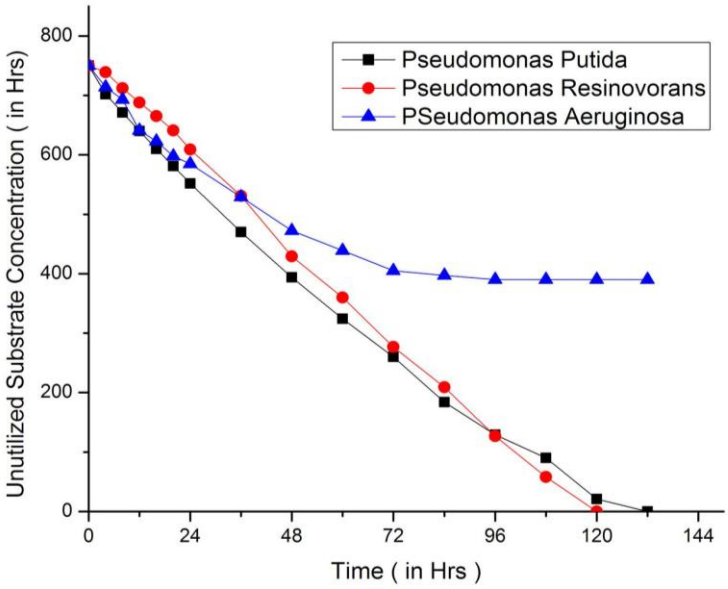


**Fig 5: Comparative Study of the degradation potential of *Pseudomonas putida*, *Pseudomonas aeruginosa* & *Pseudomonas Resinovorans* at 750PPM of initial phenol concentration.**

### *Free cell vs Immobilized cells:*

Above we have discussed and we found that the cells immobilized in Ca-Alginate beads have a better degradation potential than the freely suspended cells of *p.resinovorans*. Fig 6 is a comparative study of the phenol degrading potential of both. While the freely suspended cells

cannot tolerate a phenol concentration of more than 800 PPM, the immobilized cells are able to degrade up to 1000 PPM of phenol.

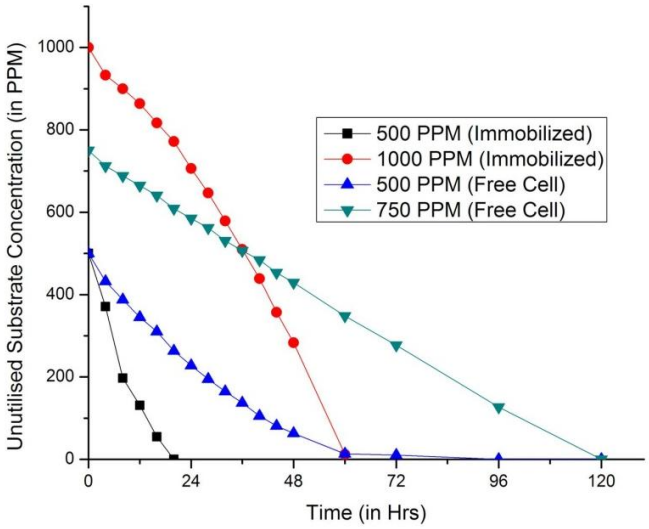


**Fig 6: Comparision study of the phenol degrading capacity of free cell form with the immobilized ones of *p.resinovorans* at different concentration of phenol.**

When compared with the setup where initial concentration of phenol is 500 PM, the freely suspended cells were able to degrade substrate in 72 hrs while the immobilized cells were able to degrade the same in less than 24 hrs. Hence we can conclude that the immobilized microbe degrades the same concentration of phenol in less than 1/3$^{rd}$ the time taken by the freely suspended cells.

## CONCLUSION:

The result shows that *Pseudomonas resinovorans* is a potential biodegradation agent as compared to the others phenol degrading microbes till the initial concentration of the phenol as contaminant is 750 PPM. But on increasing the concentration of the xenobiote, the growth of the microbe gets inhibited. But on immobilizing the microbe in Ca-Alginate matrix the phenol does not have any negative effect on the growth of the microbe and hence the microbe is able to tolerate up to 1000 ppm of initial concentration of phenol. The usage of bead is an advantage over the freely suspended ones since they can be used repeatedly making it cost effective technique as we can use the beads repeatedly for biodegradation.

**REFERENCE:**


1. Nair, C.I., Jayachandran, K. and Shashidhar, S., Biodegradation of phenol, *Afr. J. Biotechnol.,* **7**(25), 4951-4958, **(2008)**.
2. Chung, T.P., Tseng, H.Y. and Juang, R.S., Mass transfer effect and intermediate detection for phenol degradation in immobilized *Pseudomonas putida* systems. *Process Biochemistry*, 38, 1497-1507, (2003).
3. Klein, J.A. and Lee, D.D. Biological treatment of aqueous wastes from usual conversion processes, *Biotechnol. Bioeng*., 8, 379–390 (1978).
4. Bai, J., Wen, J.P., Li H.M. and Jiang, Y., Kinetic modeling of growth and biodegradation of phenol and m-cresol using *Alcaligenes faecalis. Process Biochemistry,* 42: 510-517, (2007).
5. APHA, *Standard Method for the Examination of Water and Wastewater*. 2nd Edition. Method 5530, 540-544, (1998)
6. Alef, K., Nanniper, P., 1995. *Methods in Applied Soil Microbiology and Biochemistry*, 228–231.
7. Abd-El-Haleem, Beshay U, Abdelhamid A, Moawad H, Zaki S; Effects of mixed nitrogen sources on biodegradation of phenol by immobilized Acinetobacter sp. Strain W-17 ; *African Journal of Biotechnology*, 2 (1), 8–12, (2003).
8. Sa´ 1, C.S.A., Boaventura, R.A.R., Biodegradation of phenol by *pseudomonas putida* DSM 548 in a trickling bed reactor. *Biochemical Engineering Journal* 9, 211–213 (2001).
9. Neujahr, H.Y., Gaal, A., Phenol hydroxylase from yeast, *European Journal of Biochemistry* 58, 351–357 (1973).
10. Bai, J., Wen, J.P., Li H.M. and Jiang, Y., Kinetic modeling of growth and biodegradation of phenol and m-cresol using *Alcaligenes faecalis. Process Biochemistry,* 42: 510-517, (2007).
11. Kar S, Swaminathan T and Baradarajan A.; Biodegradation of phenol and cresol isomer mixtures by Arthrobacter; *World Journal of Microbiology & Biotechnology* 13, 659- 663
12. Johnsen, A., Bendixen, K., Karlson, U., Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates by using the respiration indicator WST-1. *Applied and Environmental Microbiology*, 68, 2683–2689 (2002).

13. Giti E, Mehdi H, Nasser G, Development of a microtitre plate method for determination of phenol utilization, biofilm formation and respiratory activity by environmental bacterial isolates; *International Biodeterioration & Biodegradation*, 56, 231–235, (2005)
14. Liu Y.J., Zhang A.N., Wang X.C., Biodegradation of phenol by using free and immobilized cells of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03, *Biochemical Engineering Journal*, 44, 187–192, (2009)
15. Yang Chu-Fang, Lee Chi-Mei, Enrichment, isolation, and characterization of phenol-degrading *Pseudomonas resinovorans* strain P-1 and *Brevibacillus sp.* strain P-6, *International Biodeterioration & Biodegradation*, 59, 206–210, (2007)
16. Nagamani A, Soligalla R and Lowry M, Isolation and characterization of phenol degrading *Xanthobacter flavus, African Journal of Biotechnology*, 8 (20), 5449-5453, (2009)

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