



## Original Article

# Degradation of Phenol by Mixed Culture of Locally Isolated *Pseudomonas* bacteria

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Textile, pharmaceuticals and automobile waste most often contain phenolic compounds. An attempt was made to degrade phenol using locally isolated bacteria. Moreover, as in nature pure culture is rarely found rather than mixed culture, therefore, attempt was also made to improve the degradation using mixed culture of *Pseudomonas* species. All isolates could completely degrade phenol up to 600 ppm. Isolate *Pseudomonas* FA degraded 800 ppm phenol completely in 72 hours, whereas the isolates *Pseudomonas* SA, TK and KA degraded only 39.33, 43.83 and 33.16% of 800 ppm phenol respectively in 96 hours. Complete removal time was also shorter for the isolate *Pseudomonas* FA as compare to the other isolates. Pattern of growth were similar for all of the isolates, but maximum growth was found in the isolate FA on 600 ppm phenol. Complete degradation time of phenol was increased with increasing of mixed culture with a removal rate of 25 ppm/h. This was higher than that of the pure culture. In mixed culture study, the growth of bacteria was also increased.

**Key Words:** Biodegradation, phenol, mix culture, *Pseudomonas*.

### Introduction

The production and uses of synthetic organic chemicals has increased enormously during the last few decades. Phenol is the simplest aromatic compound derived from benzene by adding a hydroxyl group to a carbon to replace hydrogen. It is found in nature in some foods, in human and animal wastes and decomposing organic material<sup>1</sup>. United States Environmental Protection Agency regulates phenol under the Clean Water Act (CWA) and the Clean Air Act (CAA) and has designated it as a hazardous substance and a hazardous air pollutant (HAP)<sup>2</sup>. Phenol is on the list of chemicals appearing in "Toxic Chemicals Subject to Section 313 of the Emergency Planning and Community Right-to-Know Act of 1986" and has been assigned a reportable quantity (RQ) limit of 1,000 pounds<sup>2</sup>. Phenol is also considered to be an extremely hazardous substance as it can remain in the air, soil and water for much longer periods of time if a large amount is released at a time, or if it is constantly released to the environment. It is moderately toxic and can be absorbed into the body by ingestion, inhalation and contact. It affects the central nervous system, and so symptoms of exposure can include muscle weakness and tremors, loss of coordination, paralysis, convulsions, coma and respiratory arrest<sup>3</sup>. Phenol is harmful for the non living environment also. Effects on the non-living

environment include damage to structures by acidic air pollutants, effects on ozone layer and the earth's heat balance and reduced visibility<sup>4</sup>.

Mixed culture means that several kinds of bacteria are mixed simultaneously<sup>5</sup>. A defined mix culture is a miniature of complex microorganisms in nature. To treat special waste, adaptation is generally needed. If microorganisms that can degrade these materials are added, the adaptation time might be reduced. In this study, pure culture of few locally isolated *Pseudomonas* sp. were carried out to see their ability to degrade various concentrations of phenol as *Pseudomonas* being a most widely prevalent microorganisms in the environment and it generally dominates the biological treatment system. Two known bacteria, *Pseudomonas putida* CP1, *Pseudomonas putida* A (a) were also used for the mixed culture study. Studying the interactions of different species in xenobiotics degradation is highly relevant for natural processes and also for bioremediation purposes under non sterile conditions<sup>6</sup>. The study therefore aims to isolate some potential bacteria that can degrade the toxic aromatic compounds like phenol, to study the rate of degradation of phenol supplied as a sole source of carbon at various concentrations by the bacterial isolates and finally, to evaluate the degradation capability of bacterial isolates by mix culture in order to determine their practical applicability.

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## Materials and Methods

### Microorganisms

*Pseudomonas putida* CPI and *Pseudomonas putida* A (a) was collected from the stock culture of Food Microbiology and Industrial Irradiation Division, Institute of Food and Radiation Biology, Bangladesh Atomic Energy Research Establishment, Savar, Dhaka. Identification of these two isolates were described elsewhere<sup>7</sup>. The remaining four locally isolated bacterial were *Pseudomonas* SA, TK, KA and FA. Identification of the local isolates was also described<sup>8</sup>.

### Chemicals and Media

Phenol used in this study was obtained from Sigma-Aldrich Co., UK. Bacteria capable of degrading phenol were cultured on minimal medium<sup>9</sup> comprised of  $K_2HPO_4$ - 4.36 g/l;  $Na_2HPO_4$ -3.45 g/l;  $NH_4Cl$ - 1.0 g/l;  $MgSO_4 \cdot 6H_2O$  - 0.912 g/l; trace salt solution- 1ml/l. The pH of the medium was adjusted to 7.0 with 2M NaOH. The trace salt solution contained 4.77 g of  $CaCl_2 \cdot 2H_2O$ ; 0.37 g of  $FeSO_4 \cdot 7H_2O$ ; 0.37 g of  $CoCl_2 \cdot 6H_2O$ ; 0.10 g of  $MnCl_2 \cdot 4H_2O$  and 0.02 g of  $NaMoO_4 \cdot 2H_2O$  in 100 ml water.

### Inocula

Cells from the *Pseudomonas* minimal medium were used to inoculate nutrient broth (15 ml). The harvested cells were centrifuged at 5000 rpm for 10 minutes and washed twice with 0.01M sodium phosphate buffer and final pellet resuspended in the same buffer. Bacterial suspension ( $\approx 10^7$ /ml) was used to inoculate 95 ml sterile minimal medium containing phenol in 250 ml conical flasks to give the appropriate final concentration of the added substrates. After inoculation, flasks were incubated in an orbital shaker at 120 rpm  $30^\circ C$ . Samples were aseptically removed at regular intervals and analyzed for growth, substrate removal and pH. Growth of the organisms

was monitored by measuring optical density at 660 nm. For mixed culture study, equal amounts of different inoculums were taken and the ultimate volume was fixed to 5 ml in 250 ml conical flasks to give the appropriate final concentration of the added inoculums. After inoculation, flasks were incubated in an orbital shaker at 120 rpm at  $30^\circ C$ . Rest of all the procedures were followed as above.

### Chemical Analyses

Samples were then centrifuged at 5000 rpm for 10 minutes; the supernatants were then analyzed for phenol. Phenol concentration was determined by using the 4-aminoantipyrine colorimetric method<sup>10</sup>.

## Results

Degradation of various concentrations (400 to 800 ppm) of phenol by the *Pseudomonas* isolates when grown as single culture; Table 1 illustrates removal rate, complete removal time and removal efficiency of the *Pseudomonas* isolates FA, SA, TK, and KA when grown on various concentrations (400 to 800ppm) of phenol as sole source of carbon and energy. All isolates could completely degrade phenol up to 600 ppm. Isolate *Pseudomonas* FA degraded 800 ppm phenol completely in 72 hours, but the isolates *Pseudomonas* SA, TK and KA degraded only 39.33, 43.83 and 33.16% of 800 ppm phenol respectively in 96 hours. Complete removal time was also shorter for the isolate *Pseudomonas* FA compares to the other isolates.

The growth of potential isolates (*Pseudomonas* SA, FA, TK and KA) when grown on various concentrations (400 to 800 ppm) is shown in Table 2. Maximum growth was found with the isolate FA on 600 ppm phenol. Patterns of growth were similar for all of the isolates. *Pseudomonas* FA also grew in the presence of 2- and 4-chlorophenol<sup>8</sup>.

**Table 1.** Degradation Capabilities of phenol by *Pseudomonas* isolates SA, TK, FA and KA

Isolates no.	Concentration								
	400 ppm			600ppm			800ppm		
	Degradation rate (ppm/h)	Degradation Time(h)	Degradation efficiency (%)	Degradation rate (ppm/h)	Degradation Time	Degradation efficiency (%)	Degradation rate (ppm/h)	Degradation Time(h)	Degradation efficiency (%)
FA	16.67	24h	100	18.75	48h	100	21.37	72h	100
SA	16.67	24h	100	19.87	72h	100	5.58	96h	39.33
TK	16.67	24h	100	10.58	72h	100	6.5	96h	43.83
KA	16.67	24h	100	7.08	72h	100	3.2	96h	33.16

**Table 2.** Growth rate (OD at 660 nm) of the isolates (*Pseudomonas* SA, FA, TK and KA) grown on different phenol concentrations

Isolates	Growth (OD at 660 nm)		
	400 ppm	600 ppm	800 ppm
<i>Pseudomonas</i> SA	0.316	0.644	0.2695
<i>Pseudomonas</i> TK	0.352	0.507	0.3825
<i>Pseudomonas</i> KA	0.26	0.514	0.173
<i>Pseudomonas</i> FA	0.38	0.42	0.55

**Degradation of 600 ppm of phenol by the mixed cultures of *Pseudomonas* isolates:** Since all these bacteria degraded 600 ppm phenol, this concentration was selected for mix culture study. Different combination of isolates was used for mixed culture study. Figure 1 and Table 3 describe the comparison between mixed and pure cultures in terms of the removal rate, complete degradation time and growth.

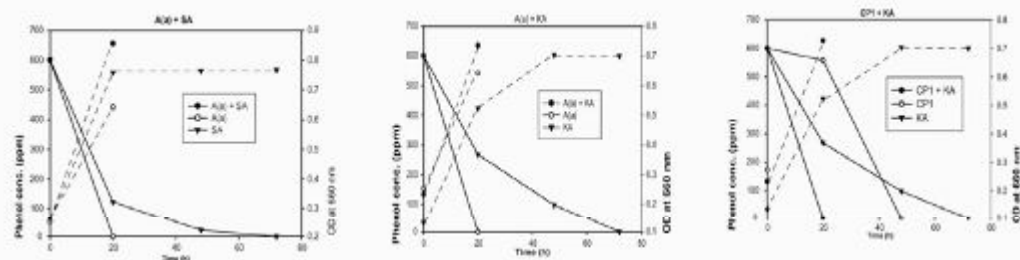
From mixed cultures of *Pseudomonas putida* A (a) and *Pseudomonas* sp. SA, it was found that *P. putida* A (a) degraded 600 ppm phenol in 24 hour and *Pseudomonas* sp. SA degraded the same concentration in 72 hour when they were cultured individually. The mixed culture of this two *Pseudomonas* sp. degraded the same concentration in 20 hour (Figure 1). The OD of mix culture ranged from 0.25- 0.856 whereas the OD of *Pseudomonas*. sp. SA and *P. putida* A (a) was 0.2525- 0.6420 and 0.26-0.772, respectively.

From mixed cultures of *Pseudomonas putida* A (a) and *Pseudomonas* sp. KA, it was observed that *P. putida* A (a) and

*Pseudomonas* sp. KA degraded 600 ppm phenol in 20 hour in a mixed culture, 72 hour and 24 hour were needed to complete the degradation by *Pseudomonas* sp. KA and *P. putida* A (a) respectively (Figure 1). The growth of the mix culture ranged from 0.2330-0.7350 while the growth ranged from 0.2525 to 0.6420 for *P. putida* A (a) and 0.1320 to 0.70 for *Pseudomonas* sp. KA.

From mixed cultures of *Pseudomonas putida* CPI and *Pseudomonas* sp. KA, it was observed that *Pseudomonas putida* CPI and *Pseudomonas* sp. KA degraded 600 ppm phenol in 20 hour while individually they completed degradation in 48 hour and 72 hour respectively (Figure 1). The growth under mixed culture (i.e., OD at 660 nm) was ranged between 0.23-0.727 during different periods was higher than that of single culture.

The removal rate of phenol was 25 ppm/h in mixed culture of all combinations and was high than the pure culture of the isolates.



**Figure 1.** The degradation of 600ppm phenol by *Pseudomonas putida* A (a), *Pseudomonas* sp. KA , *Pseudomonas* CPI, *Pseudomonas* sp. SA and the mixed cultures of the isolates.

**Table 3.** Comparison of degradation rate and time of 600 ppm phenol by mixed culture or pure culture of the isolates

Isolates No.	Mixed culture		1st isolate		2nd isolate	
	Complete Degradation time (h)	Removal rate (ppm/h)	Complete Degradation on time (h)	Removal rate (ppm/h)	Complete Degradation time (h)	Removal rate (ppm/h)
<i>P. putida</i> A (a) + <i>P. sp.</i> SA	20	25	24	23.30	72	19.87
<i>P. putida</i> A (a) + <i>P. sp.</i> KA	20	25	24	23.30	72	13.83
<i>P. putida</i> CPI + <i>P. sp.</i> KA	24	25	48	23.25	72	13.83

## Discussion

The ability of the bacteria particularly those of genus *Pseudomonas* to utilize aromatic hydrocarbons has been widely documented<sup>5, 11</sup>. Four isolates (*Pseudomonas* spp. SA, TK, and KA) were isolated which completely degraded 600 ppm of phenol within 72 hour but varying in their removal rate. The concentration above 600 ppm of phenol was degraded by *Pseudomonas* sp. FA. Among the bacteria tested, *Pseudomonas putida* CP1 was able to degrade 600, 800 ppm of phenol, 200 ppm of 2-chlorophenol and 300 ppm of 4-chlorophenol in 48, 72, 100 and 72 hours respectively, and *Pseudomonas putida* A (a) degraded 600 ppm phenol in 24h and 800 ppm phenol in 48 hours<sup>12-13</sup>.

The removal rates of the potential isolates were higher for phenol and they are almost similar for 400 ppm. But in case of 600 ppm of phenol the removal rate was varied by the bacterial isolates. The removal rate was lower in every case when the phenol concentration was 600 ppm except *Pseudomonas* sp. SA. The rate was 16.67 ppm/h for 400 ppm and increased to 19.87 ppm/h for 600 ppm. The degradation rate of *Pseudomonas putida* CP1 was 23.25 ppm/h at 600 ppm and increased to 24.58 ppm/h when the phenol concentration was 800 ppm and 23 ppm/h removal rate was recorded for 800 ppm phenol whereas it was 25 ppm/h at 600 ppm in case of *Pseudomonas putida* A (a)<sup>12</sup>.

The difference of structure and toxicity among phenolic compounds requires that various bacteria have specific qualities to degrade each compound and a mixed culture may be applied<sup>5</sup>. Degradation of mono-chlorophenol and phenol by mixed microbial community were reported<sup>7, 14, 15</sup>. Although four isolates capable of degrading phenol of various concentrations, most of the cases, the degradation rate of a mixed culture was determined as being significantly higher than that of any of the individual isolates. Individual organisms may metabolize only a limited range of substrates; therefore it is likely that assemblages of different bacterial species with broader enzymatic capabilities have a greater capacity to degrade complex phenolic compounds<sup>16</sup>. For this reason, mixed culture was carried out with the individual degrading isolates.

The removal rates of *Pseudomonas* sp. SA and KA were 19.87, 13.83 ppm/h, respectively and the removal rate of *P. putida* was 23.30 ppm/h while the phenol concentration was 600 ppm. When SA and KA mixed with *P. putida* A (a), the removal rate was increased 25 ppm/h in both cases. The degradation time was also decreased rather than the pure culture indicating synergistic interaction between them. When organisms were mixed with each other, the degradation time was decreased and the removal rate was increased. This observation was also supported by the higher bacterial growth and possibly both of the isolates contributed to degradation.

## Conclusion

*Pseudomonas* sp. SA, *Pseudomonas* sp. TK and *Pseudomonas* sp. KA were able to degrade 600 ppm and *Pseudomonas* sp. FA was able to degrade phenol up to 800 ppm of phenol. In mixed culture study, the growth of bacteria was increased and degradation rate was also increased.

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