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Removal of Phenol in Batch Culture by *Pseudomonas putida* AP11, AP9, AP6 and AP7 Isolated from the Aromatic Hydrocarbon Contaminated Soils

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Abstract

Phenol widely used in industries, are of growing concern owing to their toxicity and wide distribution in industrial wastes. The aims of the study were to characterize some of the locally isolated bacteria and to develop suitable methods for the degradation of phenol using them. Locally isolated AP11, AP9, AP6 and AP7 were identified as *Pseudomonas putida* using the classical methods. *Pseudomonas putida* AP11 and AP9 were able to remove 600 ppm phenol completely, but *Pseudomonas putida* AP6 and AP7 were able to remove 400 ppm phenol completely. The maximum degradation rates for freely suspended culture of *Pseudomonas putida* AP11, AP9, AP6, AP7 were 10.83, 10.42, 8.33, and 8.33 (ppm/h) respectively. The isolates AP11, AP9, AP6 and AP7 can be used to wastewater containing phenol in effluent treatment systems.

Keywords: Aromatic hydrocarbon; Carbon source; Contaminated soil; Phenol; *Pseudomonas.*

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1. Introduction

Phenols are toxic to human beings and affect several biochemical functions [1]. Phenol is a listed priority pollutant by the U.S. Environmental Protection Agency [2] and is considered to be a toxic compound. The toxicity of phenol has been widely documented and their disastrous effect toward human and environment is a great concern [3-5]. The greatest potential source of exposure to phenol is in the occupational setting, where phenol is used in manufacturing processes. People are also exposed via consumer products, such as medicines and lotions, and some foods and tobacco smoke. Phenol has been found in drinking water [2]. Phenol is currently removed by different methods such as precipitation/coagulation, osmosis, ion-exchange, ultrafiltration, electrodialysis, electro-chemical degradation, floatation, etc., which are costly and inefficient. These current

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treatments often produce other toxic end products, requiring further processing steps [6-8]. On the other hand, biodegradation has been studied as an alternative approach due to the low costs associated with this option, as well as the possibility of complete mineralization of the xenobiotic [9]. Phenol biodegradation has been studied detail using pure and mixed cultures of suspended bacteria [10-11]. Phenol and other phenolic compounds are common constituents of many industrial effluents and suitable microorganism based process need to be developed for the effective degradation of phenol and then the effluents could be disposed safely [12].

The aim of this study was to identify and characterize the potential phenol degrading bacteria, isolated from the contaminated sites and their ability to degrade various concentrations of phenol when supplied as the sole carbon source was investigated.

2. Materials and Methods

2.1. *Test organisms and identification*

Isolates were obtained from Microbiology and Industrial Irradiation Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka, Bangladesh. The organisms were previously isolated from soils of aromatic compound contaminated sites. The isolates were identified using cultural, morphological characteristics and biochemical tests according to methods described in Bergey's Manual of Systematic Bacteriology [13]. The bacteria were maintained on phenol agar medium and stored at 4^0C for around 1 month and then sub-cultured.

2.2. *Pseudomonas minimal medium*

The ingredients of minimal medium [14] were dissolved in distilled water and the pH was adjusted to 7.0 with 2 M NaOH. The composition of minimal medium per litre were as follow: ($KHPO_4$, 4.36 g; NaH_2PO_4, 3.45 g; $MgSO_4$, 0.912 g; NH_4Cl, 1.0 g; pH, 7). Trace salt solution was added at a concentration of 1 ml per litre. The composition of trace salt solution per 100 ml was as follows: $CaCI_2.2H_20$, 4.77 g; $FeSO_4.7H_20$, 0.37 g; $CoCl_2.6H_20$, 0.37 g; $MnCl_2$, 0.19 g; $NaMoO_4.2H_20$, 0.02 g.

2.3. *Phenol agar*

Bacteriological agar at a concentration of 1.5% (w/v) was added to the minimal medium. Following sterilization by autoclaving, the medium was allowed to cool. Immediately prior to pouring phenol was added to the medium to give the appropriate concentrations. Phenol broth was used for biodegradation studies, the composition of which was exactly similar to the phenol agar except that no agar was added to it.

2.4. *Cultural conditions for biodegradation studies*

Isolates were grown in nutrient broth for 24 hours, centrifuged at 5000 rpm for 10 minutes and washed twice with potassium phosphate buffer. Five ml of bacterial suspension

(10^7cells/ml) was used to inoculate 95 ml sterile minimal medium [14] containing phenol in 250 ml conical flasks. After inoculation, flasks were incubated in an orbital shaker at 120 rpm at 37°C. Control flasks were run in parallel. Samples were aseptically removed at regular intervals and analyzed for growth and substrate removal. The study period for phenol was 0-96 h.

2.5. *Measurement of the growth of bacterial cells*

Growth of the bacterial cells was monitored turbidimetrically by measuring the optical density (OD) at 660 nm using a UV visible spectrometer.

2.6. *Chemical Analyses*

Concentrations of phenol were determined by colorimetric method using 4-aminoantipyrene based on the procedure detailed in standard methods for the examination of water and wastewater [15].

3. Results and Discussion

3.1. *Identification and characterization of bacterial isolates*

Cultural and morphological characteristics of the isolates are shown in Table 1. The cell characteristics were noted following cultivation on nutrient agar at 30°C. All the isolates were gram negative, motile, non-spore forming and rod shaped. None of them showed acid fast staining. A variety of biochemical tests were performed on all the strains to

Table 1. Colony characteristics and microscopic observation of the bacteria isolated from aromatic hydrocarbon contaminated soils.

Colony character/ microscopic observation	Isolates			
	AP11	AP9	AP6	AP7
Size	Small	Moderate	Moderate	Small
Color	Colorless	Buff	Buff	Colorless
Colony shape	Circular	Circular	Circular	Circular
Elevation	Convex	Convex	Convex	Convex
Opacity	Translucent	Opaque	Opaque	Translucent
Cell shape	Rod	Rod	Rod	Rod
Gram reaction	-	-	-	-
Spore staining	-	-	-	-
Motility				
Acid fast staining	-	-	-	-

+ = positive reaction, - = negative reaction

enable their identification and the results are shown in Table 2. In the biochemical test, all of the isolates were found as oxidase and catalase positive. Production of H_2S and urease were not observed for any of the isolates. In Voges proskauer and methyl red test, different response was observed.

Table 2. Biochemical tests of four bacterial isolates.

Isolates	Biochemical Tests									
	Oxidase test	Catalase test	Indole production	Voges proskauer	Methyl red	Tween hydrolysis	Starch hydrolysis	H_2S	Urease test	Citrate
AP11	+	+	-	+	-	-	-	-	-	-
AP9	+	+	-	-	+	-	-	-	-	-
AP6	+	+	-	-	+	-	-	-	-	-
AP7	+	+	-	+	-	-	-	-	-	-

+ = positive reaction, - = negative reaction

A wide range of sugar utilization was also observed by all of the isolates (Table 3). Isolates AP11 and AP9 were able to grow on most of the sugars except lactose. The AP6 grew in the presence of four sugars out of ten while the AP7 utilized six sugars.

Table 3. Carbohydrate utilization by four isolates.

Carbohydrate	Isolates			
	AP11	AP9	AP6	AP7
Fructose	+	+	-	-
Arabinose	+	+	-	-
Glucose	+	+	+	+
Galactose	+	+	+	+
Maltose	+	+	-	-
Sorbital	+	+	+	+
Manitol	+	+	-	+
Sucrose	+	+	-	+
Xylose	+	+	+	+
Lactose	-	-	-	-

+ =Growth, - = No growth

Comparing the cultural, morphological and biochemical characteristics of isolates with the properties listed in the Bargey,s Manual for systematic Bacteriology [13], it was found that all of the isolates belong to *Pseudomonas putida*. *Pseudomonas putida* are regarded as one of the most common species of phenol degrading bacteria isolated from contaminated sites [16-19]. The ability of this species particularly to utilize aromatic hydrocarbons has been widely documented [20-21]. It was also able to use a wide

diversity of carbon energy substrates and this together with their ability to compete effectively with other bacteria, is undoubtedly responsible for their dominance [22]. *Pseudomonas putida* EKII, *Pseudomonas cepacia* G4, *Pseudomonas putida* Q5, *Pseudomonas putida* MTCC were investigated as phenol degrader as well as all of them were non pathogenic [16, 23-26].

3.2. *Growth of pseudomonas putida isolates and their degradation capacity in liquid culture media containing phenol in different concentrations*

All the organisms were grown in the liquid culture containing 100 ml minimal medium with various concentrations of phenol (400-800 ppm). The results of phenol degradation and their corresponding removal rates and removal efficiency are presented in Fig. 1 and Table 4.

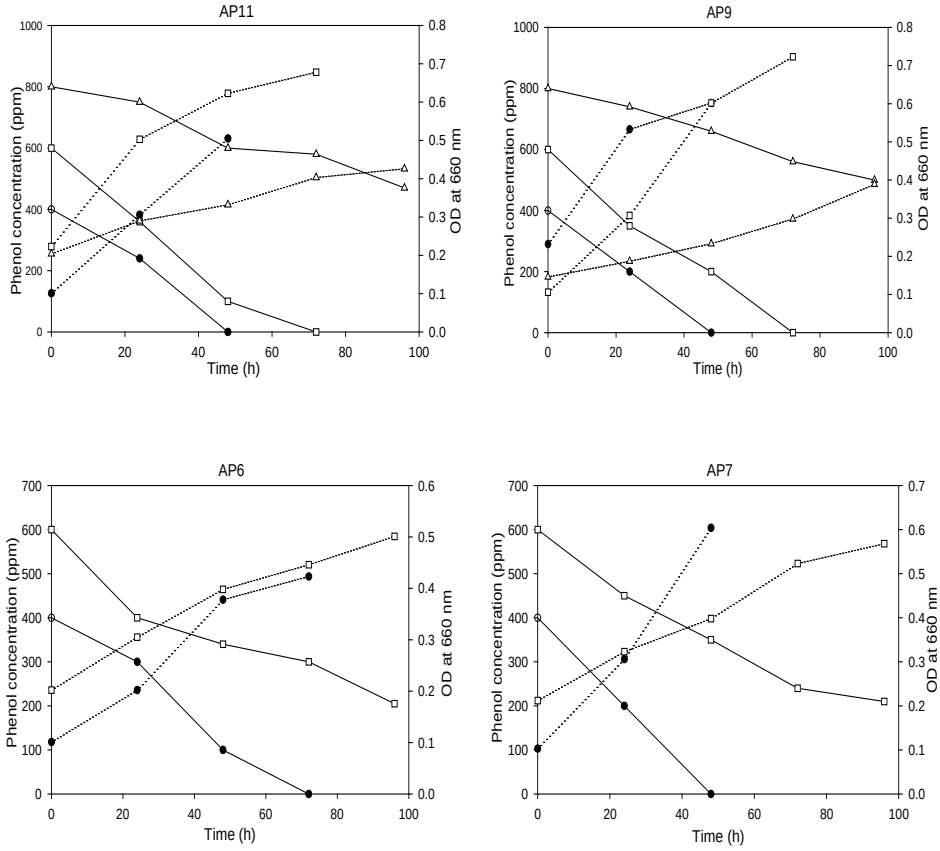

Fig. 1. The removal of various concentrations of (400 - 800 ppm) phenol by *Pseudomonas* species when supplied as the sole sources of carbon and energy (solid line represents degradation and corresponding broken line represents growth).

AP11 was found to be the most effective bacteria comparing with the others isolates. Complete removal of 400 and 600 ppm of phenol was observed by the organism. Removal efficiency was higher with the progressing growth. Highest bacterial growth (OD 0.507 at 660 nm) was observed at 72 h. However, complete removal of 800 ppm was not observed. It was possible to remove about 41.25% within the time 96 h and the bacterial growth was increasing slowly with removal of phenol (Table 4). Almost similar patterns of growth, phenol degradation, removal rate and removal efficiency were observed for the isolate AP9.

Pseudomonas putida AP6 degraded 400 ppm of phenol completely within 72 h. Complete degradation of 600 ppm phenol was not achieved within 96 h. It was possible to degrade only 66%. The bacterial growth was highest at 72 h for 400 ppm phenol. The increasing growth was not in similar fashion at 600 ppm compared to 400 ppm. Maximum growth (OD 0.370) was found at 96 h with 600 ppm phenol. Maximum removal rate was 8.33 (Table 4). The isolates AP7 also showed almost similar patterns of growth, phenol degradation, removal rate and removal efficiency as observed for AP6.

Table 4. Maximum concentration of phenol degradation, degradation time, and growth and removal efficiency of different isolates of *Pseudomonas putida* AP11, AP9, AP7 and AP6.

Isolate	Phenol conc. (ppm)	Max. deg. (ppm)	Time (h)	Growth (OD at 660 nm)	Deg. rate at 24 h (ppm/h)	Removal efficiency (%)
AP11	400	400	48	0.304	10.83	100
	600	600	72	0.507	10.00	100
	800	330	96	0.331	2.08	41.25
AP9	400	400	48	0.456	8.33	100
	600	600	72	0.435	10.42	100
	800	300	96	0.251	2.50	37.5
AP6	400	400	72	0.276	4.17	100
	600	395	96	0.370	8.33	65.83
AP7	400	400	48	0.338	8.33	100
	600	390	96	0.403	6.25	65

It was reported that *Pseudomonas putida* CP1 is capable to remove 600-800 ppm of phenol, *Pseudomonas putida* A(a) also degraded 600 ppm phenol within 24 h and 800 ppm phenol within 48 h [18]. The bacterial growth varied with time and concentration of phenol. The *Pseudomonas* sp. A4CP2, exhibited the highest growth (OD = 0.55) growing on 800 ppm phenol [19]. A number of bacteria have been evaluated for their usefulness in controlling phenol, a hazardous pollutant, which is produced in oil refineries, petrochemical plants, pharmaceutical industries etc. [27-29].The isolates AP11, AP9, AP6 and AP7 were capable to degrade phenol at a concentration very much similar to other

reported strains, therefore, the isolates can be used to wastewater containing phenol in effluent treatment systems.

4. Conclusions

Four isolates, namely AP11, AP9, AP6 and AP7 obtained from aromatic hydrocarbon contaminated soils were identified as *Pseudomonas putida*. Complete degradation of 600 ppm phenol was found for isolates AP11 and AP9, but complete degradation of 400 ppm phenol was found for isolates AP6 and AP7. The maximum degradation rates for suspended culture of *Pseudomonas putida* AP11, AP9, AP6, AP7 were 10.83, 10.42, 8.33, and 8.33 (ppm/h), respectively.

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