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Original Article

Isolation and Identification of Phenol and Monochlorophenols-Degrading Bacteria: *Pseudomonas* and *Aeromonas* Species

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The phenol and monochlorophenols-degrading bacteria were isolated from the soil samples adjacent to textile, pharmaceuticals, industries and automobile workshops. Twenty seven isolates were recovered including twelve phenol, four 2-chlorophenol (2-CP) and eleven 4-chlorophenol (4-CP) degrading organisms. Among them five isolates were chosen as potential monochlorophenol-degrader; four of them were identified as species of *Pseudomonas* and one as *Aeromonas*. The biodegradability of phenol and monochlorophenol followed the order: phenol > 4-chlorophenol > 2-chlorophenol. *Pseudomonas* sp. AP2, *Pseudomonas* sp. AP3, *Pseudomonas* sp. AP4 and *Aeromonas* sp. PP3 degraded 400 and 600 ppm phenol within 48 and 72 h, and *Pseudomonas* sp. A4-CP2 degraded 600 and 800 ppm phenol in 48 and 72 h respectively. *Pseudomonas* sp. A4-CP2 also degraded 200 ppm 2-chlorophenol within 96 h and 200 and 350 ppm 4-chlorophenol within 48 and 75 h respectively. Bacterial isolates grew well in liquid culture containing 600 ppm phenol, while the growth was scanty in the medium containing 400 or 800 ppm phenol, except for *Pseudomonas* sp. A4CP2. The isolate *Pseudomonas* sp. A4CP2 also grew on 2-CP and 4-CP at concentrations of 200 and 350 ppm respectively. The correlation coefficient (at 0.01 level of significance) between bacterial growth and degradation capability of the isolates AP2, AP3, AP4, A4CP2 and PP3 were -0.835, -0.847**, -0.700**, -0.946** and -0.401 respectively.**

Keywords: Biodegradation, Phenol, 2-Chlorophenol, 4-Chlorophenol, *Pseudomonas*, *Aeromonas*

Introduction

Environmental pollution due to the release of natural phenolic compounds from agro-industrial operations has become widespread in the world. The structure of the long lived compounds are frequently found in many industrial effluents and residues like those produced in wine distillery, green olive embittering, wood debarking and coffee production[1]. Phenol is an aromatic compound derived from benzene, the simplest aromatic hydrocarbon, by adding a hydroxy group to a carbon to replace hydrogen. It is regulated by the Clean Water Effluent Guidelines for the following industrial point sources: electroplating, organic chemicals, asbestos, timber products processing, metal finishing, paving and roofing, paint formulating, ink formulating, electronic components[2]. 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[3] and has been assigned a reportable quantity (RQ) limit of 1,000 pounds[4]. It remains in the air, soil and water for much longer periods of time if a large amount of it is released at one time, or if it is constantly released to the environment.

Chlorophenols are a group of chemicals in which chlorines (between one and five) have been added to phenol. Anthropogenic introduction of monochlorophenols in the environment primarily results from industrial and agricultural usage of the compound[5]. The principal use of the monochlorophenols has been as intermediates for the production of higher chlorinated phenols[6]. 2-Chlorophenol (2-CP) has been detected in the leachate from municipal and industrial landfill. It is used as an intermediate for the synthesis of pesticides, medicines, phenolic resins, dyes, aroma compounds and other organic chemicals. 4-Chlorophenol (4-CP) is known to be mutagenic to animals; it produces reproductive effects, and in high concentrations, it causes methemoglobinaemia, liver and kidney damage, and skin and gastrointestinal irritation. Because of its toxicity, longevity and the possibility that it is a carcinogen, 4-chlorophenol is undesirable in the water supply. At concentration as low as 0.005 mg/dm^3, chlorophenolic compounds impart objectionable taste and odour to drinking water[7]. Besides, they are harmful to organisms and toxic to humans.

Utilizing microorganisms to degrade toxic organo-pollutants is an efficient, economical approach that has been successful in laboratory studies. Biodegradation can occur under both aerobic and anaerobic conditions. Biodegradation is unique in that the end result is often the complete conversion of the organic

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substance to inorganic products (*e.g.*, carbon dioxide and water). Certain bacteria can degrade certain aromatic compounds. The most important hydrocarbon-degrading bacteria in waste treatment systems are basically *Pseudomonas*, *Aerobacter*, *Aeromonas*, *Alcaligens*, *Bacillus*, *Mycobacterium*, *Comamonas*, *Cryptococcus*, *Crynebacterium*, *Rhizobiaceae* and *Flavobacterium* and others[8]. As the awareness over the environmental pollution increases, interest has been focused from the fact that many aromatic hydrocarbons are classified as environmental pollutants.

The aims and objectives of the present investigation were to isolate some potential bacteria that could degrade the toxic aromatic compounds like phenol, 2-chlorophenol and 4-chlorophenol and to determine the rate of degradation when they supplied as a sole source of carbon at various concentrations.

Materials and Methods

Sources and collection of samples

Soil samples were collected from three locations of Savar, Dhaka adjacent to the textile and pharmaceuticals industries, and automobile workshops, which were expected to contain phenolic contaminants in the soil. A total of 12 samples (4 samples from each site) were collected under 3 inches depth from the surface soil.

Chemicals and media

The phenolic compounds used in this study were phenol, 2-chlorophenol (2-CP) and 4-chlorophenol (4-CP) obtained from Sigma-Aldrich Co, UK. Total bacterial count was determined by plating on nutrient agar and incubated at 30°C for 24-72 h. Bacteria capable of degrading aromatic substrates (*e.g.*, phenol, 2-chlorophenol and 4-chlorophenol) were isolated on minimal medium[9] composed of K_2HPO_4, 4.36 g/l; Na_2HPO_4, 3.45 g/l; NH_4Cl, 1.0 g/l; $MgSO_4.6H_2O$, 0.912 g/l; and trace salt solution, 1 ml/l. The pH of the medium was adjusted to 7.0 with 2M NaOH. The trace salt solution contained $CaCl_2.2H_2O$, 4.77 g; $FeSO_4.7H_2O$, 0.37 g; $CoCl_2.6H_2O$, 0.37 g; $MnCl_2.4H_2O$, 0.10 g; and $NaMoO_4.2H_2O$ (in 100 ml water), 0.02 g.

Isolation of phenol-degrading bacteria

Nutrient agar and monochlorophenol/phenol agar plates were used for the isolation purpose. Morphologically dissimilar colonies were picked up from the plates and each individual colony was streaked on agar plate for pure culture. Isolates were marked according to their sources (T for textile industry P for pharmaceuticals industry and A for automobile workshop). The selected colonies were marked on the basis of their cultural characteristics such as shape, size, form, texture, opacity, pigment formation and were then purified through repeated streak plating on nutrient agar media and their cultural characteristics were recorded[10].

Inoculum

Cells from the *Pseudomonas* minimal medium were used to inoculate nutrient broth (15 ml). Cultures were incubated overnight at 30ºC and agitated at 120 rpm. The cells were harvested, centrifuged at 5,000 rpm for 10 min and washed twice with 0.01 *M* sodium phosphate buffer and final pellet resuspended in the same buffer. Bacterial suspension (~10^7/ml) was used to inoculate 95 ml sterile minimal medium containing phenol, 2-chlorophenol, or 4-chlorophenol in 250-ml conical flasks to give the appropriate final concentration. After inoculation, flasks were incubated (at 30ºC) in an orbital shaker at 120 rpm[9].

Degradation study

Samples were aseptically removed at regular intervals and analyzed for growth, substrate removal and pH. Growth of the organisms was monitored by using optical density measurement at 660 nm. The study period for phenol and 4-chlorophenol was 0-72 h and 96 h for 2-chorophenol. Samples were then centrifuged at 5,000 rpm for 10 min. The supernatants were analyzed for phenol/ monochlorophenol. Phenol/monochlorophenol concentration was determined by using 4-amino antipyrene method based on the procedure using standard method[11].

Identification

Isolated bacteria were identified according to methods described in Bergey's Manual of Systematic Bacteriology[12].

Statistical analysis

Statistical analysis [Pearson correlation (2-tailed)] was performed for determining correlation between the bacterial growth (OD) and the phenol concentration (ppm) by using the Statistical Package for Social Science (SPSS).

Results

Twenty-seven bacterial isolates were recovered on the basis of their degrading capacity of phenol, 2-chlorphenol (2-CP) and 4-chlorophenol (4-CP) and were selected for further study in liquid culture. Among them, 3 isolates were capable of growing on phenol, 4-chlorphenol, or 2-chlorphenol when the substrate was supplied as the sole carbon source. Most of the colonies (44.4%) were isolated from phenol and 4-chlorphenol, while only four colonies were isolated from the medium containing 2-chlorophenol (14.8%).

Twelve isolates showed their ability to degrade phenol; among them four isolates, identified as *Pseudomonas* sp. AP2, *Pseudomonas* sp. AP3, *Pseudomonas* sp. AP4 and *Aeromonas* sp. PP3, degraded 600 ppm of phenol within 72 h with varying rates of phenol removal (Table 1). The growth of the organisms was well in 600 ppm of phenol. Another isolate, *Pseudomonas* sp. A4CP2 degraded 4-chlorophenol, 2-chlorophenol and phenol of various concentrations, which was selected as most potential isolate. All these five isolates exhibited good growth in liquid culture in the presence of phenol and 4-chlorphenol.

Table 1. *Phenolic compound removal rate, removal time and removal efficiency of five bacterial isolates*

Substrate	Concentration	Isolate	Removal rate (ppm/h)	Removal time (h)	Removal efficiency (%)
Phenol	400 ppm	AP2	16.67	24	100
		AP3	16.67	24	100
		AP4	16.67	24	100
		PP3	16.67	24	100
	600 ppm	AP2	19.87	72	100
		AP3	10.58	72	100
		AP4	7.08	72	100
		PP3	16.02	72	100
4-Chlorophenol	200 ppm	A4CP2	7.20	48	100
	350 ppm	A4CP2	4.29	75	100

The average removal rate of five isolates was varied from 9.125 to 22.13 ppm/h. The highest removal rate was recorded for *Pseudomonas* sp. A4CP2, while *Pseudomonas* sp. AP4 exhibited lower removal rate for various concentration of phenol. All isolates exhibited a lower removal rate when the substrate was 800 ppm of phenol.

Growth of the organisms, except *Pseudomonas* sp. A4CP2, was better when phenol concentration was 600 ppm compared to the growth in 400 or 800 ppm phenol concentration (Figure 1). *Pseudomonas* sp. A4CP2 grew only on 2-chlorophenol and 4-chlorophenol at a concentration of 200 ppm and 350 ppm respectively (Figure 2). Bacterial aggregation was observed with *Pseudomonas* sp. A4CP2 when 4-chlorophenol concentration was 350 ppm. Aggregation was also formed when phenol concentration was 800 ppm. *Pseudomonas* sp. A4CP2 could degrade 200 ppm of 2-chlorophenol within 96 h, and 600 and 800 ppm phenol within 48 h and 72 h respectively. No potential bacteria could be isolated from the culture of 2-chlorophenol.

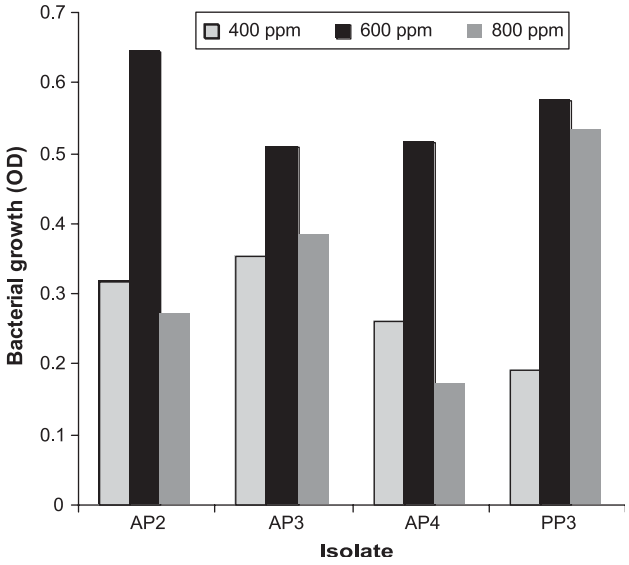

Figure 1. *Average bacterial growth (optical density, OD) of four potential isolates, namely Pseudomonas sp. AP2, Pseudomonas sp. AP3, Pseudomonas sp. AP4 and Aeromonas sp. PP3, on phenol of various concentrations (400-800 ppm).*

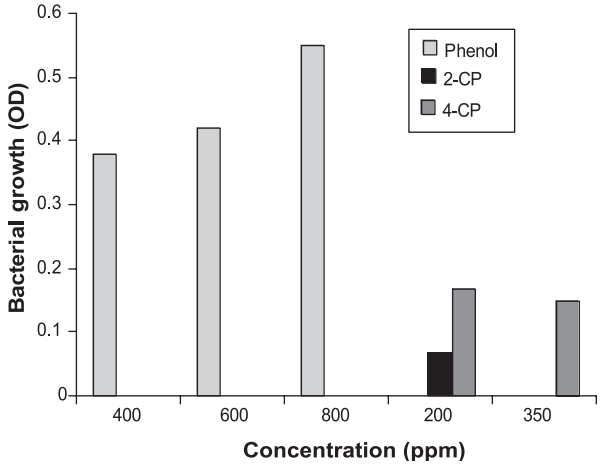

Figure 2. *Average bacterial growth (optical density, OD) of Pseudomonas sp. A4CP2 on phenol (400-800 ppm), 2-chlorophenol (200 ppm) and 4-chlorophenol (200-350 ppm).*

The coefficient of correlation is a measure of the relationship between two variables. From the Pearson's correlation it was revealed that bacterial growth (optical density, OD) and phenol degradation (concentration in ppm) were strong negatively correlated that was statistically significant at the 0.01 level (2-tailed), *i.e.*, when the bacterial growth was increased, the concentration of phenol was decreased. In every case, strong negative relationship found, except for *Aeromonas* sp. PP3 (r = -0.401). For *Pseudomonas* spp. AP2, A4CP2, AP3 and AP4, the r values were -0.835**, -0.847**, -0.700** and -0.946** respectively (Table 2).

Table 2. *Pearson's correlation matrix for bacterial growth and concentration of phenol of potential isolates*

Phenol	Parameter	Bacterial growth (Optical density, OD)				
(ppm)		AP2	A4CP2	AP3	AP4	PP3
AP2	Pearson correlation	-0.835**				
	Significant (2-tailed)	0.003				
	N	10				
A4CP2	Pearson correlation		-0.847**			
	Significant (2-tailed)		0.001			
	N		10			
AP3	Pearson correlation			-0.700**		
	Significant (2-tailed)			0.024		
	N			10		
AP4	Pearson correlation				-0.946**	
	Significant (2-tailed)				0.000	
	N				10	
PP3	Pearson correlation					-0.401**
	Significant (2-tailed)					0.251
	N					10

**Correlation is significant at the 0.01 level (2-tailed). AP2 = *Pseudomonas* sp. AP2; A4CP2 = *Pseudomonas* sp. A4CP2; AP3 = *Pseudomonas* sp. AP3; AP4 = *Pseudomonas* sp. AP4; PP3 = *Aeromonas* sp. PP3.

Discussion

Phenolic compounds are used for various purposes in many areas, for example chlorinated phenolic compounds are specifically utilized as preservatives that have been found to affect aquatic life, causing ecological imbalance[13]. Phenol, 2-chlorophenol and 4-chlorophenol degrading potential bacteria described in this study was isolated from the soils samples, collected from three locations of Savar, adjacent to the textile and pharmaceuticals

industries and automobile workshop and were identified as species of *Pseudomonas* and *Aeromonas* sp.

Among these five potentials, only one isolate *Pseudomonas* A4CP2 degraded monochlorophenols and the other four degraded phenol. The differences in the degradation between the monochlorophenols seemed to be due to the position of Cl^- substituent. Menke and Rehm[14] described the theoretical order of degradability of chlorophenols by considering the reaction-decelerating effect of Cl^- ion and the reaction accelerating effect of –OH group position. The order of mineralization was reported as: phenol > 4-chlorophenol > 2-chlorophenol suggesting that the substrate specificity of phenol hydroxylase was related to the position of the hydroxyl group in the chlorophenol molecule as described in the study and that of others in the laboratory[15]. Lu and Tsai[16] and Fakhruddin[17] also reported the same order of microbial degradation of phenolic compounds by a culture medium. It has reported that *Pseudomonas putida* CP1 is capable to remove 600-800 ppm of phenol, 200 ppm of 2-chlorophenol and 300 ppm of 4-chlorophenol within 48-72 h, 100 h and 72 h respectively[17]. *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 highest growth (OD = 0.55) was recorded in case of *Pseudomonas* sp. A4CP2 growing on 800 ppm phenol. Clump was formed by *Pseudomonas* sp. A4CP2 when grown on 800 ppm of phenol, 300 ppm of 2-chlorophenol and 350 ppm of 4-chlorophenol. *Pseudomonas putida* CP1 also aggregated and formed clumps in the presence of 2-chlorophenol and high concentrations (>200 ppm) of 4-chlorophenol[17]. Aggregation of microbial cell was a tactic for survival during starvation. Aggregation or flocculation was also a protective mechanism for the cell against toxicity and microbial metabolic activity could change after the formation of floc[15].

The ability of the bacteria particularly those of genus *Pseudomonas* to utilize aromatic hydrocarbons has been widely documented[19-20]. In case of *Pseudomonas* sp. A4CP2, the growth (OD) was highest on phenol rather than on 2-chlorophenol and 4- chlorophenol. The bacterial growth was inhibited due to substrate's toxicity. The average growth in 2-chlorophenol was 0.0652 at 200 ppm level, whereas the highest was 0.55 when the phenol concentration was 800 ppm (Figure 2). The removal rate was also varied depending on the substrate (Table 1).

In conclusion, the organisms isolated from phenolic waste contaminated sites were capable to degrade phenolic substances like phenol, 2-chlorophenol, or 4-chlorophenol of various concentrations. Among the isolates *Pseudomonas* sp. A4CP2 was able to degrade up to 800 ppm of phenol, 200 ppm of 2-chlorophenol and 350 ppm of 4-chlorophenol, while the other isolates, namely, *Pseudomonas* sp. AP2, *Pseudomonas* sp. AP3, *Pseudomonas* sp. AP4 and *Aeromonas* sp. PP3 could degrade only phenol of 400-600 ppm concentrations with maximum at a concentration of 600 ppm. The bacterial growth was also increased with time during the degradation of phenol and monochlorophenols. The correlation between bacterial growth and phenol concentration was strongly negative. The removal rate and bacterial growth decreased when isolates were grown on 4-chlorophenol than that on phenol.

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