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Phenol biodegradation by Pseudomonas putida CP1 and A(a)

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***Abstract*— Phenolic compounds are common pollutants in wastewater of Bangladesh. Biodegradation of phenol by Pseudomonas putida strains A(a) and CP1 was investigated and it was shown that A(a) and CP1 completely degraded phenol up to 1000 ppm and 800 ppm, respectively. In the presence of phenol as the sole carbon source, the removal rate was higher by A(a) than CP1, however, when 0.5% glucose was added additionally, the rate was higher by CP1. Growth yield of the strains was increased with increasing concentrations of phenol. In addition, pH change was negligible (< 0.10) without the presence of glucose, and this change was higher (0.50) when 0.5% (w/v) glucose was added in the medium. Although flocculation was detected when cells of A(a) and CP1 were grown in ≥ 800 ppm and ≥ 400 ppm phenol, respectively, this phenomenon was absent with additional presence of 0.5% (w/v) glucose in 500 ppm phenol for both of the strains.**

INTRODUCTION

Phenolic compounds are widely distributed in the effluents of many industrial processes, such as, oil refineries, petrochemical plants, coal conversion plants, and phenolic resin industries etc. Due to high water solubility of phenol, these compounds lead to widespread contamination of river, lake, estuarine, and other aquatic environments. Since the compounds are toxic to microorganisms, even a low concentration of phenolic compounds often collapse wastewater treatment plants by hampering their growth [1]. Among different approaches, use of microorganisms in treatment of phenolic compound polluted wastewater is beneficial especially because of their potentiality to almost complete degradation of phenol by production of nontoxic end products, and with generation of minimum secondary wastes [2]. Due to widespread distribution of phenol in the environment, some microorgamisms adapted to use the compound both as carbon and energy source. These microorganism use both aerobic and anaerobic pathway for phenol degradation and aerobic biodegradation has been studied since the beginning of the 1900s [3]. A number of microorganisms have been reported to degrade phenol at low concentration, including *Alcaligenes entrophus* [4], *Bacillus stearothemophilus* [5], *Pseudomonas* sp. [6-8], *Rhodoccus* sp. [9], and *Trichos-poron cutaneum* [10]. Degradation of phenolic compounds by *P. putida* CP1 and A(a) has been reported earlier [11-14]. Since phenol is a widely distributed environmental pollutant in Bangladesh due to its common presence in the effluents of many industrial processes, the aim of this study was to investigate the degradation of phenol by *P. putida* A(a) and *P. putida* CP1 when the compound is used as the sole carbon source with or without additional presence of glucose.

MATERIALS AND METHODS

Culture Medium

The ingredients of the minimal medium [14] were combined in distilled water and the pH was adjusted to 7.0 with 2M NaOH. The trace salts solution was prepared separately in distilled water and was stored in a dark bottle for 6-8 weeks. Phenol was added to the minimal medium after sterilization. The ingredients of the per liter minimal medium are as follows: (K_2HPO_4, 4.36 g; NaH_2PO_4, 3.45 g; NH_4Cl, 1.0 g; $MgSO_4.6H_2O$, 0.912 g; pH, 7.0). Trace salts solution was added at a concentration of 1 ml^{-1}. The composition of the per 100 ml trace salts solution was as follows: ($CaCl_2.2H_2O$, 4.77 g; $FeSO_4.7H_2O$, 0.37 g; $CoCl_2.6H_2O$, 0.37 g; $MnCl_2.4H_2O$, 0.10 g; $Na_2MoO_4.2H_2O$, 0.02 g). Minimal media together with phenol was used for biodegradation studies. Separately sterilized glucose solution was added to the sterilized minimal media to give the appropriate final concentration.

Microorganisms

The isolate *P. putida* CP1 was obtained from Dr. Favio Fava, University of Bologna, Italy and the isolate *P. putida* A(a) was obtained from Dr. Brid Quilty, Dublin City University, Ireland. The strains were maintained on phenol agar. The organism was kept at 4^0C for around 1 month and then sub-cultured.

Cultural conditions

The organisms was grown overnight in nutrient broth, centrifuged at 5000 rpm for 10 minutes and washed twice with 0.01M sodium phosphate buffer. Five ml was used to inoculate 95 ml sterile minimal medium [15] containing phenol in 250 ml conical flasks. The concentrated stock solution of glucose was separately sterilized prior to their addition to the sterilized minimal medium to give the appropriate final concentration. After inoculation, flasks were incubated in an orbital shaker at 150 rpm at 30^0C. Samples were aseptically removed at regular intervals and analyzed for growth and pH. Samples were then centrifuged at 5000 rpm for 10 minutes, and the supernatants were analyzed for phenol removal and for glucose utilization where appropriate.

Measurement of growth of the organisms

Growth was monitored by using optical density measurement at 600 nm.

Chemical analysis

Penol assay: Phenol concentrations were determined by using the 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater [16].

Glucose estimation: The glucose concentrations were determined by the dinitrosalicylate (DNS) colorimetric method [17].

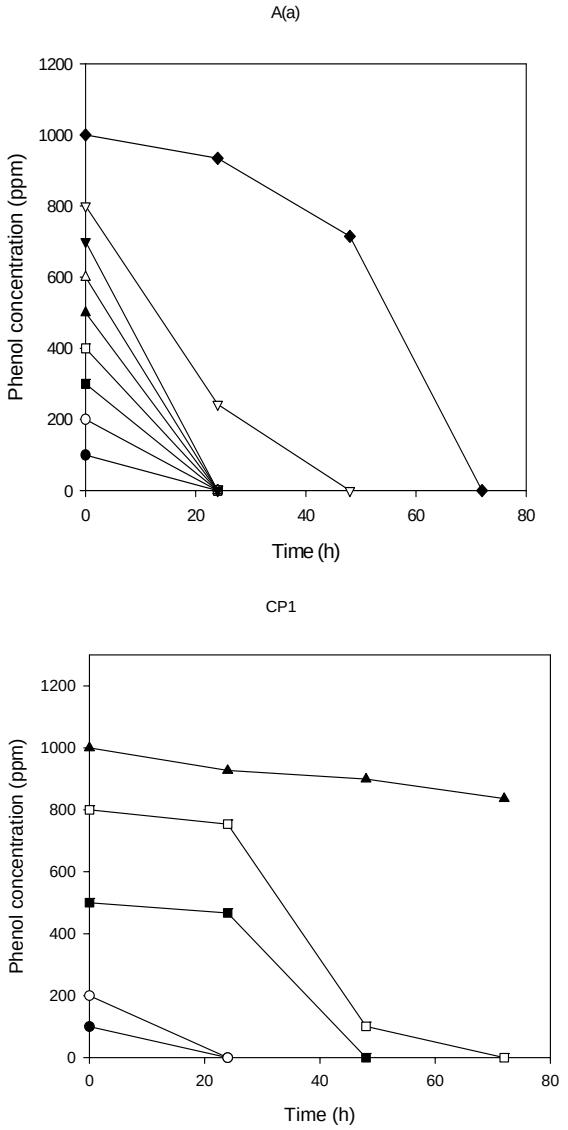

Fig. 1. Removal of various concentration of phenol by *P. putida* A(a) and *P. putida* CP1 when supplied as the sole source of carbon and energy

Data Analysis

The results presented were the mean of duplicate treatments and all experiments were repeated to confirm the data obtained. Standard errors were determined using Sigma Plot 2000. In all cases, the standard errors between runs were less than 5%.

RESULTS AND DISCUSSION

Phenol biodegradation by P. putida strains

P. putida A(a) was found to degrade completely upto 700 ppm phenol within 24 hours. The strain required 48 and 72 horu for complete degradation of the phenol concentration of 800 and 1000 ppm, respectively. However above 100 ppm phenol concentration substrate inhibition interfered the degradation.

In contrast, CP1 strain showed lower efficiency in phenol degradation and it could degrade only 100 to 200 ppm phenol within 24 hours, and the strain degraded 500 and 800 ppm phenol within 48 and 72 hours, respectively. The growth of CP1 was inhibited with 1000 ppm phenol. Since both of the strains degraded upto 500 ppm phenol within short period of time, this concentration was selected for further study.

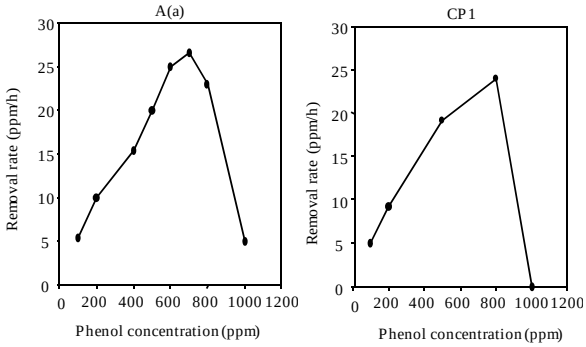

Fig. 2. Rates of phenol removal by *P. putida* A(a) and *P. putida* CP1 when grown on various concentrations (100 to 1000 ppm) of phenol as the sole source of carbon.

Fig. 2 shows the phenol removal rate by *P. putida* A(a) and CP1. The removal rate was increased with the increasing concentration of phenol upto 800 ppm and with all tested phenol concentration removal rate was higher for *P. putida* A(a) than *P. putida* CP1. Due to substrate inhibition removal rate was decreased above 800 ppm for both strains and complete inhibition was found with 1000 ppm for CP1, although A(a) still had a slower removal rate at this concentration.

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 [6, 18-19]. In the present investigation, both of the *P. putida* strains, CP1 and A(a) were found to be capable of phenol degradation, where A(a) was found to be more efficient in phenol degradation than CP1.

Biodegradation of phenol by P. putida A(a) and CP1 in the presence of glucose

When *P. putida* A(a) and CP1 were grown in the presence of 500 ppm phenol supplemented with 0.5% (w/v) glucose at 30^0C in shaking condition without pH control, both of the substrates were removed concurrently from the medium. The growth yield of the strains increased with the reduction of the substrate concentrations from the media. Phenol was completely removed within 48 h by both *P. putida* A(a) and CP1 (Fig 3). However, glucose suppressed phenol degradation in case of *P. putida* A(a). When *P. putida* A(a) was grown in only 500 ppm phenol (Fig. 1) complete degradation was found within 24 h but with presence of 0.5% (w/v) glucose it took higher time (48 h) for complete removal. On the other hand, glucose had no effect on the removal of phenol in case of *P. putida* CP1. In both cases 500 ppm phenol was removed completely by *P. putida* CP1 within 48 h. Suppression of the degradation of phenolic compounds in the presence of more easily degradable carbon sources was also well documented by a number of investigators [15,20-23].

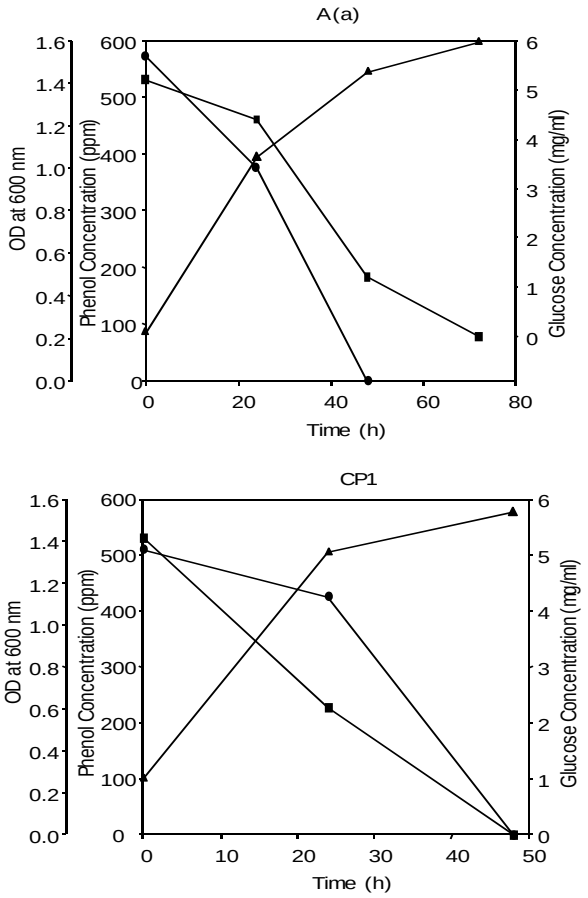

Fig. 3. Removal of 500 ppm phenol by *P. putida* A(a) and *P. putida* CP1 in the presence of 0.5% (w/v) glucose. Symbols: ▲, OD at 660 nm; ●, phenol concentration; ■, glucose concentration.

Swindoll *et al.* [20] also reported similar repressive effects on the mineralization of *p*-nitrophenol, phenol and toluene by mixed cultures. O'Sullivan [21] reported an inhibitory effect on the removal of phenol and mono-chlorophenols by a mixed microbial population in the presence of glucose. This study reported a suppression of phenol removal in the presence of glucose. The presence of glucose exerted repressive effects on phenol removal by *P. pictorum* [21]. Reduction of phenol removal rates in the presence of various concentrations of glucose was also reported for heterologous populations [24]

Growth of Pseudomonas putida strains in Pseudomonas minimal media

Table 1 shows the growth of both *P. putida* A(a) and CP1 in different phenol concentration with or without glucose at different time intervals. The data indicates the increase of cell growth with the increase of incubation time, which is proportional to substrate utilization. *P. putida* A(a) started to flocculate at ≥ 800 ppm phenol whereas *P. putida* CP1 started to flocculate at ≥400 ppm. Presence of glucose enhanced the bacterial growth for both strains and there was no flocculation in the presence of glucose.

The phenomenon of flocculation hampered growth measurement by noting optical density. A negligible change in pH in the medium was recorded with the complete removal of phenol. This change ranged from 0.07-0.10. Change in pH was significant in the presence of 0.5% (w/v) glucose, maximum 0.5 unit was found for CP1, this drop of pH may be due to the formation of acidic products like citric acid, pyruvic acid and acetic acid during glucose metabolism [25].

Table 1. Growth of *P. putida* strains on minimal mdium in presence of various concentrations of phenol with of withoutr glucose at 30ºC

Phenol conc.(ppm)	Hours.	OD at 600 nm *P. putida* A(a)	*P.*
100	0	0.282	0.236
	24	0.388	0.448
	48	[a] -	-
	72	-	-
200	0	0.279	0.285
	24	0.562	0.682
	48	-	-
	72	-	-
300	0	0.247	-
	24	0.723	-
	48	-	-
	72	-	-
400	0	0.254	0.296
	24	0.823	[b] F
	48	-	-
	72	-	-
500	0	0.345	0.256
	24	0.853	F
	48	-	F
	72	-	-
600	0	0.2525	0.273
	24	0.921	F
	48	-	F
	72	-	F
700	0	0.202	-
	24	0.93	-
	48	-	-
	72	-	-
800	0	0.265	0.271
	24	F	F
	48	F	F
	72	F	F
900	0	0.219	-
	24	F	-
	48	F	-
	72	F	-
1000	0	0.205	0.204
	24	F	F
	48	F	F
	72	F	F
500 ppm + glucose (0.5%)	0	0.227	0.264
	24	1.049	1.345
	48	1.425	1.536
	72	1.592	-

[a] -, not detected; [b] F, Flocculation.

Thus, *P. putida* A(a) and *P. putida* CP1 degraded phenol when supplied as the sole source of carbon and was able to degrade completely up to 1000 ppm and 800 ppm phenol respectively. Addition of 0.5% (w/v) glucose suppressed phenol removal by *P. putida* A(a) but the addition of 0.5% glucose had no effect on the degradation of phenol by *P. putida* CP1.

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