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Original Article

Degradation of Monochlorophenols by *Pseudomonas putida* CP1 in the Presence of Growth Supplements

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Influence of readily degradable additional sources of carbon and nitrogen on the degradation of monochlorophenols by *Pseudomonas putida* CP1 was investigated. The organism grew on all three isomers of monochlorophenols when supplied as the sole source of carbon and energy. Low concentrations (0.01 to 0.5%, w/v) of yeast extract enhanced degradation of monochlorophenols. The order in terms of rate of removal of monochlorophenols was 4-chlorophenol > 2-chlorophenol > 3-chlorophenol, both in the presence and absence of the growth supplements. The rate of removal of monochlorophenols was highest when carbon:nitrogen (C:N) ratio maintained at optimum level (3:1) with monochlorophenols and growth substrates. The organism clumped when grown in the presence of monochlorophenols alone. The degree of clumping decreased with the addition of growth supplements.

Keywords: Biodegradation, Growth supplement, Monochlorophenol, *Pseudomonas putida* CP1

Introduction

Chlorophenols are a group of toxic chemicals have been in use as biocides to control bacteria, fungi, algae, mollusks, insects, slime, and other biota. The inadequate handling of chlorophenol-treated materials, accidental spills, and leaching from dumping sites have resulted in the serious contamination of soil and ground water[1]. Monochlorophenols can be formed during wastewater chlorination, and as a result of breakdown of pesticides and chlorinated aromatic compound, and since their solubility in water is relatively high, chlorophenols can easily migrate within different aqueous environments and contaminate ground waters[2].

In the natural environment toxic or inhibitory components will be found in mixtures with non-toxic or conventional wastes. When alternative carbon sources are presented to the microbial population, substrate interactions can occur[3]. Several investigators showed the facilitation of chlorophenol degradation by using conventional carbon sources such as glucose, sodium glutamate, and yeast extract[4-8]. Their findings suggested that the addition of some conventional carbon sources might aid in reducing the toxicity and growth inhibition of xenobiotics on cells, thereby increasing the transformation rates of xenobiotics.

The aims of this study were to study the influence of readily degradable additional sources of carbon and nitrogen on the degradation of monochlorophenols and the effect of carbon nitrogen (C:N) ratio on the biodegradation of monochlorophenols by *Pseudomonas putida* CP1 during mixed substrates study.

Materials and Methods

Source of chemicals and strain

2-Chlorophenol and 4-chlorophenol were obtained from Sigma Chemical Co (UK). 3-Chlorophenol was obtained from Aldrich Chemical Co (UK). D-Fructose and yeast extract were obtained from BDH Chemical Ltd. (UK) and Difco Laboratories Ltd (UK) respectively. The isolate *Pseudomonas putida* CP1 was obtained from Dr. Favio Fava, University of Bologna, Italy.

Pseudomonas minimal medium

The ingredients of the minimal medium[9] were combined in distilled water and the pH was adjusted to 7.0 with 2 *M* NaOH. The trace salts solution was prepared separately in distilled water. The monochlorophenols were added to the minimal medium after sterilization. The ingredients of the per litre minimal medium are as follows: K_2HPO_4, 4.36 g; NaH_2PO_4, 3.45 g; $(NH_4)_2SO_4$, 1.26 g; $MgSO_4.6H_2O$, 0.912 g; pH 7.0). Trace salts solution was added at a concentration of 1 ml/l. 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).

Maintenance of Pseudomonas putida CP1

The bacterium was maintained on chlorophenol agar together with different isomers of monochlorophenols. The organism was kept at 4°C for around 1 month and then subcultured.

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Culture conditions

Pseudomonas putida CP1 was grown overnight in nutrient broth, centrifuged at 5,000 rpm for 10 min and washed twice with 0.01 *M* sodium phosphate buffer. Five millilitre culture suspension was used to inoculate 95 ml sterile minimal medium[9] containing 2-chlorophenol, 3-chlorophenol and 4-chlorophenol in 250-ml conical flasks. Separately sterilized additional carbon and nitrogen sources were added to the sterilized minimal medium to give the appropriate final concentration, where added carbon and nitrogen effects were being studied. After inoculation, flasks were incubated in an orbital shaker at 150 rpm at 30°C. Samples were aseptically removed at regular intervals and analyzed for growth, pH, chlorophenol removal, chloride release, chemical oxygen demand (COD) and for reducing sugar where appropriate.

Measurement of growth of Pseudomonas putida CP1

Growth of *Pseudomonas putida* CP1 was monitored by using a number of methods including the measurements of turbidity, dry weight and bacterial number by the pour plate method. *P. putida* CP1 flocs were disrupted by sonication at 50 W for 30 sec prior to measurement of cell numbers and the deflocculation was observed microscopically.

Chemical analyses

Monochlorophenols 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[10]. The fructose concentrations were determined by the dinitrosalicylate (DNS) colorimetric method[11]. Chloride release was analyzed with an Orion bench top pH/ISE meter (Model 920A). The chemical oxygen demand (COD) was determined using a modification of the method outlined in Standard Methods for the Examination of Water and Wastewater[10].

Results and Discussion

The removal of 200 mg/l monochlorophenols by *Pseudomonas putida* CP1 in the presence of various concentrations (0.01-1.00%, w/v) of yeast extract was investigated. Chemical oxygen demand (COD) removal, chlorophenol removal, changes in pH and chloride release is shown in Table 1. There was a lag in the removal of chlorophenol in all cases and the lag in the removal of chlorophenol decreased with increasing concentrations of yeast extract. The change in pH was very slight in the controls and this was also the case in the treatments containing less than 0.2% (w/v) yeast extract. Significant increase in the pH of the growth medium was observed with 1% (w/v) yeast extract that was attributed to the utilization of amino acids, peptides and proteins and the subsequent production of nitrogenous metabolites and the rate of removal of chlorophenols decreased with the increase in pH. When yeast extract was present the chlorophenols were completely removed and there was residual COD, which increased with increasing concentrations of yeast extract.

The rate of removal of chlorophenol increased with increasing concentrations of yeast extract up to a value of 0.5% (w/v). At 1% (w/v) yeast extract there was a decrease in the rate of chlorophenol removal. In the absence of yeast extract, there was a stoichiometric release of chloride. However, in the presence of yeast extract, the release was not always stoichiometric (Table 1). As yeast extract contains complex sources of nutrients the chloride produced following degradation may have been bound by some of the constituents of the yeast extract. Other investigators showed

Table 1. *Lag periods, C:N ratio, changes in chloride and pH, and the rates of chlorophenol and COD removal by Pseudomonas putida CP1 when grown on 200 mg/l monochlorophenols in the presence of various concentrations of yeast extract (YE)*

Treatment	Lag -cp (h)	C:N ratio	△ Cl^- (mg/l)	△ pH	Chlorophenol removal rate		COD removal rate	
					(mg/l/h)	(mg/l/h/mg)	(mgO_2/l/h)	(mgO_2/l/h/mg)
4-cp only	6	0.427	62.05 ± 3.50	-0.1	4.64 ± 0.02	0.0082 ± 0.0000	20.35 ± 1.48	0.052 ± 0.004
+ 0.01% (w/v) YE	6	0.479	75.30 ± 4.00	-0.1	4.82 ± 0.02	0.0101 ± 0.0000	23.36 ± 1.46	0.067 ± 0.004
+ 0.05% (w/v) YE	6	0.648	90.00 ± 4.00	-0.08	4.80 ± 0.11	0.0105 ± 0.0002	30.61 ± 0.01	0.082 ± 0.000
+ 0.1% (w/v) YE	6	0.804	82.05 ± 0.15	-0.03	7.71 ± 0.10	0.0088 ± 0.0002	47.27 ± 0.09	0.122 ± 0.000
+ 0.2% (w/v) YE	3	1.013	76.10 ± 4.10	0.05	10.70 ± 0.26	0.0125 ± 0.0003	55.39 ± 1.46	0.162 ± 0.004
+ 0.5% (w/v) YE	3	1.291	80.00 ± 3.00	0.29	10.48 ± 0.04	0.0104 ± 0.0004	76.50 ± 4.50	0.279 ± 0.015
+ 1% (w/v) YE	3	1.459	43.50 ± 0.50	0.98	4.76 ± 0.02	0.0061 ± 0.0000	129.58 ± 5.5	0.483 ± 0.016
3-cp only	22	0.427	59.50 ± 0.50	-0.1	1.03 ± 0.01	0.0021 ± 0.0000	10.01± 0.50	0.024 ± 0.001
+ 0.01% (w/v) YE	22	0.479	72.50 ± 0.50	-0.09	1.59 ± 0.15	0.0025 ± 0.0002	12.69 ± 0.68	0.043 ± 0.002
+ 0.05% (w/v) YE	22	0.648	83.15 ± 3.75	-0.08	2.68 ± 0.03	0.0028 ± 0.0000	14.86 ± 0.00	0.053 ± 0.000
+ 0.1% (w/v) YE	6	0.804	86.40 ± 5.00	-0.03	3.01 ± 0.05	0.0031 ± 0.0000	19.13 ± 0.70	0.076 ± 0.003
+ 0.2% (w/v) YE	6	1.013	117.5 ± 0.35	0.08	4.60 ± 0.00	00044 ± 0.0000	30.78 ± 0.37	0.114 ± 0.001
+ 0.5% (w/v) YE	6	1.291	108.5 ± 0.50	0.41	4.57 ± 0.01	0.0043 ± 0.0000	47.96 ± 0.09	0.220 ± 0.000
+ 1% (w/v) YE	22	1.459	127.5 ± 5.50	1.33	1.76 ± 0.02	0.0017 ± 0.0000	82.13 ± 1.67	0.424 ± 0.007
2-cp only	9	0.427	59.3 ± 0.00	-0.1	2.11 ± 0.010	0.0035 ± 0.0000	10.25 ± 0.33	0.021 ± 0.000
+ 0.01% (w/v) YE	9	0.479	72.05 ± 0.35	-0.09	2.22 ± 0.015	0.0036 ± 0.0000	12.59 ± 0.68	0.026 ± 0.001
+ 0.05% (w/v) YE	6	0.648	80.05 ± 4.24	-0.06	2.98 ± 0.025	0.0038 ± 0.0000	18.18 ± 2.18	0.058 ± 0.006
+ 0.1% (w/v) YE	6	0.804	81.3 ± 1.70	-0.03	3.94 ± 0.07	0.0037 ± 0.0001	29.09 ± 2.91	0.082 ± 0.008
+ 0.2% (w/v) YE	6	1.013	102.5 ± 3.50	0.06	4.44 ± 0.100	0.0041 ± 0.0000	45.08 ± 1.45	0.134 ± 0.000
+ 0.5% (w/v) YE	6	1.291	111.5 ± 5.50	0.32	4.83 ± 0.03	0.0041 ± 0.0000	56.18 ± 1.14	0.214 ± 0.003
+ 1% (w/v) YE	6	1.459	46.00 ± 0.50	1.03	2.99 ± 0.15	0.0021 ± 0.0001	101.5 ± 7.25	0.448 ± 0.025

± = Standard error; -cp = Chlorophenol; C:N = Carbon:nitrogen; △ Cl^- = Change in chloride; COD = Chemical oxygen demand.

that yeast extract and possible constituents of yeast extract enhance the survival of xenobiotic degrading organisms[2,6]. The results of their work also indicated that the complex mixture, yeast extract enhances the growth and dechlorination activity of the organisms. Loh and Tan[12] reported an improvement of phenol degradation rates when yeast extract was supplemented at concentrations from 0.2 g/l to about 2 g/l, but deterioration with further increases to 4 g/l. The organism flocculated when grown in the presence of chlorophenol as the sole source of carbon. When yeast extract was introduced to the medium, the degree of flocculation decreased with increasing concentrations of yeast extract.

An attempt was made to observe the effect of a combination of yeast extract and fructose on the degradation of monochlorophenols by *P. putida* CP1 using an optimal carbon nitrogen ratio[13]. A combination of yeast extract (0.1%, w/v) and fructose (0.2%, w/v) with a C:N ratio 3:1 was chosen. Substrate removal was measured in terms of COD, reducing sugars and chlorophenol removal. The pH and OD of the culture were also measured. The carbon to nitrogen ratio (C:N) for each system is listed in Table 2. There was little change in pH in any of the systems. The rate of removal was greater in the presence of fructose than yeast extract but was greatest in the presence of both substrates. The order in terms of rate of removal found to be 4-chlorophenol > 2-chlorophenol > 3-chlorophenol both in the presence and absence of additional carbon sources. The rate of removal of fructose increased in the presence of yeast extract. Clumping was reduced in the presence of both fructose and yeast extract when added individually to the medium. When both substrates were added to the growth medium the clumping was reduced further.

The degree of growth and the rate of growth increased with increasing COD concentrations in the case of 3-chlorophenol. Whereas for both 4-chlorophenol and 2-chlorophenol the maximum growth was found in the presence of both yeast extract and fructose, followed by yeast extract, which was followed by fructose (Table 2). Yu and Ward[5] reported the use of various combinations of carbon and nitrogen sources including glucose and yeast extract supplementation for the degradation of pentachlorophenol by mixed bacterial cultures and noticed significant levels of enhancement. They reported maximum pentachlorophenol degradation with the medium supplemented with glucose and peptone.

The degradation of monochlorophenols enhanced in the presence of low concentrations (0.01% to 0.05%, w/v) of yeast extract. The rate of removal was highest when the carbon: nitrogen ratio maintained at 3:1 in medium containing monochlorophenols and growth supplements. The organism flocculated when grown on monochlorophenols alone and the degree of flocculation decreased with the addition of growth supplements.

Table 2. *Growth rate, C:N ratio, changes in pH and rates of removal of monochlorophenols, COD and fructose during growth of P. putida CP1 on 200 mg/l monochlorophenols in the presence and absence of 0.1% (w/v) yeast extract (YE) and 0.2% (w/v) fructose (F)*

Treatment	Total COD	Growth rate, μ	C:N	△pH	Monochlorophenol removal rate		COD removal rate		Fructose removal rate	
	(mgO_2/l/h)	(per h)	ratio		(mg/l/h)	(mg/l/h/mg)	(mgO_2/l/h)	(mgO_2/l/h/mg)	(mg/l/h)	(mg/l/h/mg)
4-cp only	934.23	0.011 ± 0.0001	0.427	-0.11	4.64 ± 0.02	0.0082 ± 0.0000	20.35 ± 1.48	0.053 ± 0.0037	-	-
+ 0.1% (w/v) YE	1530.03	0.071 ± 0.0060	0.804	-0.03	7.71 ± 0.10	0.0088 ± 0.0001	47.27 ± 0.09	0.123 ± 0.0002	-	-
+ 0.2% (w/v) F	2722.15	0.025 ± 0.0001	3.48	-0.28	11.62 ± 0.10	0.0230 ± 0.0002	66.62 ± 2.02	0.170 ± 0.0051	67.00 ± 0.59	0.125 ± 0.0014
+ 0.1% (w/v) YE + 0.2% (w/v) F	3583.89	0.082 ± 0.0015	2.960	-0.20	11.68 ± 0.06	0.0114 ± 0.0000	102.05 ± 4.09	0.223 ± 0.0087	100.37 ± 0.65	0.144 ± 0.0012
3-cp only	863.30	0.012 ± 0.0000	0.427	-0.09	1.03 ± 0.01	0.0021 ± 0.0000	10.01 ± 0.05	0.024 ± 0.0001	-	-
+ 0.1% (w/v) YE	1484.89	0.016 ± 0.0001	0.804	-0.01	3.01 ± 0.05	0.0031 ± 0.0000	19.13 ± 0.70	0.076 ± 0.0028	-	-
+ 0.2% (w/v) F	2658.9	0.022 ± 0.0004	3.480	-0.25	5.07 ± 0.01	0.0074 ± 0.0000	63.54 ± 1.27	0.202 ± 0.0039	49.97 ± 0.59	0.073 ± 0.0003
+ 0.1% (w/v) YE + 0.2% (w/v) F	3021.58	0.026 ± 0.0002	2.960	-0.14	9.31 ± 0.30	0.0147 ± 0.0004	70.18 ± 2.96	0.220 ± 0.0094	87.68 ± 0.65	0.153 ± 0.0010
2-cp only	963.27	0.014 ± 0.0001	0.427	-0.10	2.11 ± 0.01	0.0035 ± 0.0000	10.25 ± 0.33	0.021± 0.0006	-	-
+ 0.1% (w/v) YE	1534.69	0.023 ± 0.0002	0.804	-0.02	3.94 ± 0.07	0.0037 ± 0.0000	29.09 ± 2.91	0.082 ± 0.0084	-	-
+ 0.2% (w/v) F	2791.84	0.020 ± 0.0001	3.480	-0.34	4.89 ± 0.15	0.0131 ± 0.0003	65.32 ± 0.10	0.162 ± 0.0002	77.62 ± 0.15	0.007 ± 0.0001
+ 0.1% (w/v) YE + 0.2% (w/v) F	3510.2	0.025 ± 0.0006	2.96	-0.29	11.34 ± 0.10	0.0138 ± 0.0001	79.58 ± 0.56	0.224 ± 0.0015	108.75 ± 0.65	0.117 ± 0.0007

± = Standard error; -cp = Chlorophenol; C:N = Carbon:nitrogen; COD = Chemical oxygen demand.

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