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Short Communication

Optimisation of pH, Temperature and Carbon Nitrogen Ratio for the Degradation of *m*-Chlorophenol by *Pseudomonas putida* CP1

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Aromatic pollutants like *m*-chlorophenol is toxic to the environment and chlorophenol containing a *meta*-chlorine are more persistent under aerobic conditions than compounds lacking a chlorine substituent in positions *meta* to hydroxyl group. Therefore, it should be removed effectively from the environment. In order to increase the degradative activity, the optimum conditions for *m*-chlorophenol degradation by *Pseudomonas putida* CP1, some physicochemical conditions like pH, temperature and carbon nitrogen ratio for the growth and degradation of most persistent monochlorophenol, *m*-chlorophenol by the organism was optimised. The pH optimum for *m*-chlorophenol degradation by the bacterium was between pH 6.5 and 7.0 and the temperature optimum was 30°C for removal activity. Carbon : nitrogen (C:N) ratio of 3:1 was found best for effective removal of chemical oxygen demand (COD) and *m*-chlorophenol by the bacterium.

Keywords: *m*-Chlorophenol degradation, *Pseudomonas putida* CP1, Chemical oxygen demand (COD)

Halogenated aromatics, particularly chlorinated aromatics, are produced in vast quantities due to their numerous applications such as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers, and intermediates for chemical synthesis. Because of their toxicity, bio-concentration, and persistence, the ubiquitous distribution of the halogenated compounds in the biosphere has caused public concern over the possible effects on the quality of life[1]. Though some chlorinated aromatic compounds are biodegradable, they are often recalcitrant and natural purification of contaminated sites seems slow because microorganisms able to degrade the contaminant are missing or that the environmental conditions e.g. temperature, redox potential, pH, and concentration of contaminant are such that degradation is not promoted. Environmental pH is the most important factor affecting chlorophenol adsorption and mobility. Soil organic content is another important factor affecting chlorophenol mobility[2].

Chlorophenols are much more environmentally stable than the parent unsubstituted phenol. As the number of chlorine substituents increases the rate of aerobic decomposition decreases[3]. Compounds containing a *meta*-chlorine are more persistent under aerobic conditions than compounds lacking a chlorine substituent in positions *meta* to hydroxyl group[4-5]. Persistence of chlorophenols in the environment depend on the presence of microbial populations and environmental parameters such as pH, temperature, aeration rate, available nutrients, the absence or presence of inhibitory co-pollutants, and the absence or presence of substances changing the electron flow in the system. Chlorinated phenols may be removed from a water body via volatilisation, photo-degradation, adsorption onto suspended or bottom sediments, and microbial degradation[6]. The aim of the present study was to optimise some physicochemical conditions such as pH, temperature and carbon nitrogen ratio for the growth and degradation of most persistent mono-chlorophenol, *m*-chlorophenol by *Pseudomonas putida* CP1.

P. putida CP1 was obtained from Dr. Favio Fava, University of Bologna, Italy. It was maintained on agar medium containing *m*-chlorophenol (Aldrich Chemical Co, UK) at 4°C for and subcutured monthly. Inoculum was prepared by growing the bacterium overnight in nutrient broth, harvesting cells by centrifuged at 5,000 rpm for 10 min, washing twice with 0.01 *M* sodium phosphate buffer and re-suspending in distilled water. Culture suspension (5 ml) was used to inoculate 95 ml sterile minimal medium[7] containing *m*-chlorophenol in 250-ml conical flasks. The minimal medium with an initial pH 7.0 contained 4.36 g/l K_2HPO_4, 3.45 g/l NaH_2PO_4, 1.26 g/l $(NH_4)_2SO_4$, 0.912 g/l $MgSO_4.7H_2O$ and 1 ml/l trace solution. Trace salts solution contained (per 100 ml) 4.77 g $CaCl_2.2H_2O$, 0.37 g $FeSO_4.7H_2O$, 0.37 g $CoCl_2.6H_2O$, 0.10 g $MnCl_2.4H_2O$ and 0.02 g $Na_2MoO_4.2H_2O$. After inoculation, flasks were incubated in an orbital shaker at 150 rpm at 30°C. Samples were aseptically removed at regular intervals and analysed for growth, pH, chlorophenol removal, COD and for reducing sugar where appropriate.

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Growth of *P. putida* CP1 was monitored by turbidity measurement. For the determination of pH dependency on *m*-chlorophenol removal by the bacterium, the pH of the minimal medium was adjusted to various pH values (5.0-8.0), and then *m*-chlorophenol (200 ppm) was added and they were incubated at 30ºC and 150 rpm in an orbital shaker for desired period. To optimise the cultivation temperature, the bacterium was grown in the minimal medium with an initial pH 6.5 at different temperatures (20-37°C) under shaking (150 rpm in an orbital shaker. After incubation cells were separated from culture medium by centrifugation. Clear supernatant was used for the assay of the *m*-chlorophenol. pH and biomass level were also determined at different intervals. *m*-Chlorophenol concentrations were determined by using the 4-aminoantipyrene colorimetric method essentially as described in APHA[8]. Fructose concentration was determined by the dinitrosalicylate (DNS) method[9]. Chemical oxygen demand (COD) was determined using a modification of the method essentially as described in APHA[8].

The initial pH of the culture media has a profound effect on the growth and removal of *m*-chlorophenol by *P. putida* CP1 (Figure 1). The maximum degradation of *m*-chlorophenol observed at pH between 6.5 and 7.0, which corresponded to complete removal of 200 ppm *m*-chlorophenol within 190 h. The organism could not grow or remove *m*-chlorophenol at pH 5.0 or above pH 7.0. At pH 6.0, only about 50% chlorophenol degradation was observed in 48 h. Khan *et al*.[10] reported an optimum degradation of 4-aminophenol by *Pseudomonas* species ST-4 at pH 8.0.

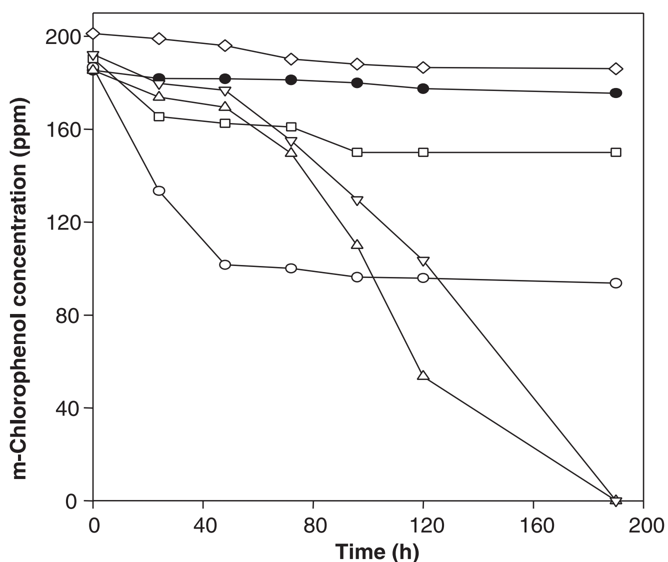

Figure 1. R*emoval of m-chlorophenol Pseudomonas putida CP1 at different pH containing minimal medium.*
● *= pH 5;* □ *= pH 5.5;* ○ *= pH 6;* ∆ *= pH 6.5;* ∇ *= 7;* ◇ *= pH 7.5*

Effect of cultivation temperature on *m*-chlorophenol removal by *P. putida* CP1 is shown in Figure 2. The bacterium showed the temperature optimum for removal *m*-chlorophenol at 30°C. It was unable to completely remove *m*-chlorophenol at a concentration of 200 ppm at temperatures above and below 30°C. The 4-aminophenol degradation by *Pseudomonas* species ST-4 had been reported at 30°C by Khan *et al*.[10].

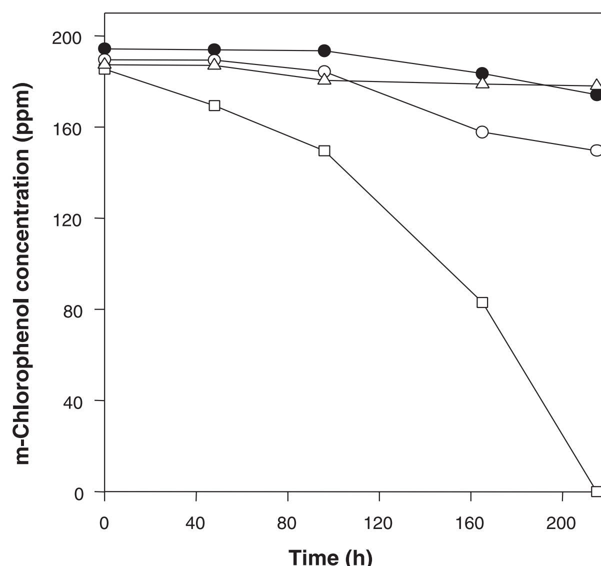

Figure 2. *Removal of m-chlorophenol by Pseudomonas putida CP1 at different temperature at pH 6.5 containing minimal medium*
● *= 20°C;* ○ *= 25°C;* □ *= 30°C;* ∆ *= 37°C*

It was found in our previous studies[11-12] that fructose and yeast extract had positive influence on *m*-chlorophenol degradation by *P. putida* CP1. In this study, the influence of various combinations of fructose and yeast extract on degradation of *m*-chlorophenol by *P. putida* CP1 was studied. It was observed that more COD was removed in the presence of yeast extract plus fructose compare to either yeast extract or fructose alone (Figure 3). Complete removal of *m*-chlorophenol was achieved within 22 h in presence yeast extract plus fructose. The *m*-chlorophenol removal rate was 1.03 mg/l/h in the absence of fructose and yeast extract (Table 1). On the other hand, the rate was 7.467, 7.529, 3.01, 4.60, 9.31 mg/l/h in the presence of 0.2% fructose, 0.5% fructose, 0.1% yeast extract, 0.2% yeast extract, 0.1% yeast extract plus 0.2% fructose and 0.2 yeast extract plus 0.5% fructose respectively. Conventional carbon sources may have some other effects, as they may provide the reducing power for the degradation of recalcitrant organic compounds[13] or in some cases act as inducing agents for biodegradative enzymes[14]. Carbon : nitrogen ratio (3:1) was found best for the complete removal of COD by *P. putida* CP1, which correlated the optimum C:N for bacteria[15]. It could be concluded from the present study that *P. putida* CP1 could effectively remove COD and degrade *m*-chlorophenol in the presence of both carbon (fructose) and nitrogen (yeast extract) sources.

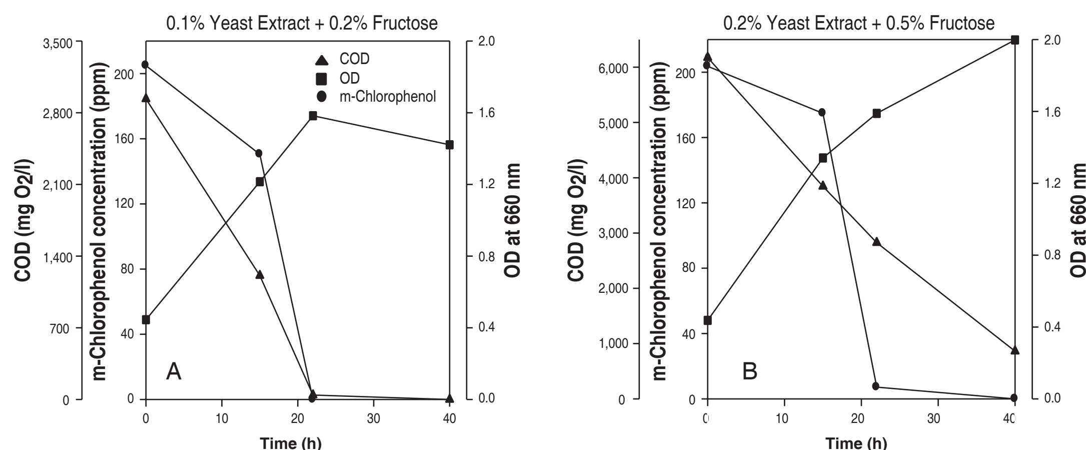

Figure 3. *Removal of m-chlorophenol (200 ppm) by Pseudomonas putida CP1 in the presence of (A) 0.1% yeast extract plus 0.2% fructose and (B) 0.2% yeast extract plus 0.5% fructose. COD = Chemical oxygen demand; OD = Optical density*

Table 1. *Influence of carbon : nitrogen (C:N) ratio on rate of degradation of m-chlorophenol (200 ppm) by Pseudomonas putida CP1.*

Treatment	C:N ratio	Δ pH	Lag-cp (h)	Chlorophenol removal rate (mg/l/h)	Fructose removal rate (mg/l/h)
cp alone	0.427	- 0.1	21	1.03	-
cp + 0.2% F	3.48	- 0.24	15	7.47	44.45
cp + 0.5% F	8.06	- 0.55	15	7.53	126.16
cp + 0.1% YE	0.804	- 0.02	6	3.01	-
cp + 0.2% YE	1.013	0.08	6	4.6	-
cp + 0.1% YE + 0.2% F	2.96	- 0.16	-	9.31	87.68
cp + 0.2% YE + 0.5% F	5.17	- 0.38	-	8.93	119.2

Lag-cp = Lag period for *m*-chlorophenol degradation; cp = *m*-chlorophenol; F = fructose; YE = yeast extract

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