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Enzymatic Dehalogenation of Pentachlorophenol by *Pseudomonas fluorescens* of the Microbial Community from Tannery Effluent

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Abstract. Four different bacterial isolates obtained from a stable bacterial consortium were capable of utilizing pentachlorophenol (PCP) as sole carbon and energy source. The consortium was developed by continuous enrichment in the chemostat. The degradation of PCP by bacterial strain was preceded through an oxidative route as indicated by accumulation of tetrachloro-ρ-hydroquinone and dichlorohydroquinone as determined by high performance liquid chromatography (HPLC). Among the four isolates, *Pseudomonas fluorescens* exhibited maximum degradation capability and enzyme production. PCP-monooxygenase enzyme was extracted from culture extract and fractionated by DEAE-cellulose ion exchange chromatography. The molecular weight of the enzyme, purified from *Pseudomonas fluorescens*, determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration chromatography was found to be 24,000 Da.

The advances in organic synthesis have led the introduction of numerous new organic compounds in the environment, whose susceptibilities to biotreatment processes are unknown. Pentachlorophenol (PCP) is one such compound, widely used as a wood preservative, fungicide, insecticide, algicide, and found in effluents of pulp and paper mills and other commercial processes [6, 15]. PCP is highly toxic and recalcitrant compound capable of being biodegraded by only a limited number of bacteria [8, 18]. The recalcitrance of PCP to biodegradation by microbial population in soil and water reflects the difficulty and special constraints involving dehalogenation of phenolic compounds. However, the treatment of PCP-contaminated water with PCP-degrading microorganism is one avenue for addressing this problem [5]. Few PCP-degrading microorganisms have been isolated from the natural environment [1, 12, 16, 17]. Bacterial isolates obtained from nature have not been proved effective in the complete degradation of PCP and related intermediary metabolites at contaminated sites. Genetic analysis has been performed for degradation of PCP and there are limited reports of the role of genomic and plasmid DNA in degradation of PCP [19, 20]. Consequently a microbial consortium is usually required to provide all the metabolic capabilities for complete mineralization of PCP [3].

Based on the isolation of metabolites extracted from the culture media of these bacteria, various degradation pathways for PCP have been proposed [2, 9, 17]. All these pathways have in common that PCP is dechlorinated step by step to less chlorinated compounds and only after the removal of several chlorine atoms, the aromatic ring is cleaved. Therefore, these bacteria must contain enzymes, which are able to cleave carbon-chloride bond in PCP.

The bacterial enzyme PCP-4-monooxygenase from *Flavobacterium* sp. strain ATCC 39723 catalyzes the oxygenolytic removal of the first chlorine from PCP. PCP-4-monooxygenase is a FAD-binding, NADPH-requiring oxygenase with similar functional domains as other bacterial flavoprotein monooxygenase specific for phenolic substrate [4]. PCP-4-monooxygenase converts PCP to 2, 3, 5, 6-tetrachloro-ρ-hydroquinone (TeCH) in the presence of oxygen and NADPH which is further converted to 2,6-dichlorohydroquinone (DiCH) by tetrachloro-ρ-hydroquinone reductive dehalogenase enzyme [21, 23]. This enzyme not only catalyzes dehalogenation but also removes hydrogen, nitro, amino, and cyno

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groups from the benzene ring at the *para* position in relation to the hydroxyl of phenol [22].

Here we report on a PCP-degrading community, enriched by continuous process in the chemostat from contaminated sites. Identification of metabolites and substrate utilization tests were performed to obtain initial enzyme information on the pathway. Identification, purification, and characterization of PCP-4-monooxygenase that converts PCP to TeCH from *P. fluorescens* are also reported.

Materials and Methods

Sediment samples and culture conditions in chemostat. Sediment samples together with liquid effluent (1:10 wt/vol) were collected from three sites of the main channel of tanneries located at Jazmau, Kanpur, India. The microbial community capable of indicating utilization of PCP in MSM-PCP agar plates was enriched in a chemostat, and continuous enrichment was performed using a mineral salt medium and PCP (0.1 g/l) in 2-l glass vessels, as described earlier [14]. The growth of the bacterial community was determined by measuring A_{560}.

Isolation and identification of PCP-degrading bacteria. Bacterial community obtained from chemostat after continuous enrichment was spread over MSM-PCP agar plate and purified by repeated streaking and restreaking on nutrient agar plate [18]. The bacterial colonies appeared on nutrient agar plates were morphologically characterized and purified by repeated culturing. The morphologically distinct isolates were identified biochemically [10] and Biolog test method based on the utilization of 96 carbon sources. In this method, bacterial isolates grown at 24 h were removed from the petriplates and diluted with saline. The homogeneous mixture of bacterial cell was dispensed in 96 well microplates and incubated at 30°C. A_{590} was determined after 7 h and 24 h on microtitre plate reader. The isolates were identified using the microlog software.

Degradability of PCP by the isolates. The bacterial isolates were grown in minimal salt medium containing PCP (100 mg/l) as the source of carbon and energy. The growth of the bacterial cells was determined by measuring optical density at 560 nm (A_{560}) and utilization of carbon source was measured by extraction and determination of PCP by spectrophotometer at 320 nm and HPLC [11, 14]. The biodegradation of PCP was determined by extraction of metabolites from the culture medium that contained MSM and PCP as sources of carbon and energy.

Metabolites were detected in the culture fluid after removing the bacterial cells by centrifugation at 7000 rpm for 10 min. The supernatant was initially extracted with dichloromethane for 10 min in a separating funnel. The process was repeated two times. The dichloromethane fraction was extracted twice with 0.5N NaOH (10 ml). The aqueous phase was combined, and the absorbance of the sample was measured at 320 nm in UV-Vis spectrophotometer.

The combined aqueous phase was acidified with HCl (6N) to a pH < 2. The acidified aqueous phase was extracted with dichloromethane three times. The organic phase was saved and dried over anhydrous sodium sulphate. The solvent was removed under vacuum by rotavapour. Dried samples were dissolved in 1 ml acetonitrile. A 20-μl sample was analyzed with Shimandzu, SP10 HPLC. HPLC was equipped with UV-Vis detector. Detector was set at wavelength of 254 nm. A reverse phase C18 column (size 250 × 3.9 mm), particle size 5 μm, was used for the study. The isocratic mobile phase was acetonitrile and sodium acetate 25 mM (pH 3.8), in the ratio of 70:30 vol/vol, and flow rate was 1 ml/min.

A PCP standard was run under the same conditions. The intermediary metabolites production was determined by HPLC as for the PCP. Standard, 2,6-dichloro-ρ-hydroquinone was run under the same conditions for comparison. Percent utilization was estimated by measuring the peak area of the metabolites.

Preparation of crude extract and enzyme assay. All the isolates were grown in MSM-PCP for a period of 12 h. Cultures were centrifuged at 10,000 rpm for 10 min. Precipitate was dissolved in phosphate buffer and dithithretiol was added. Cells were disrupted through sonicator at 20,000 Hz for 5 min, at 20-s intervals. The lysate was centrifuged at 8000 rpm for 10 min and specific enzyme activity was measured for 0, 6, and 24 h grown cultures. The standard reaction mixture contained 1.5 μmol of PCP, 10 μmol of EDTA, 1 μmol of NADPH, and 600 μl of enzyme extract in a total volume of 800 μl. The reaction was started by addition of PCP. The enzymatic activity was measured by decrease in PCP concentration in the mixture [13]. Based on the result of comparative enzyme activity of different strains, PCP-monooxygenase enzyme was purified from *Pseudomonas fluorescens.* Strain was inoculated in 5 l MSM containing PCP (100 ppm) as the sole carbon source, and placed on a rotatory shaker for 12 h at 270 rpm. Cells were disrupted, lysate were centrifuged, as described earlier, and supernatant was used for further experiment.

Fractionation and purification of enzyme. The cell extract was precipitated by ammonium sulphate. Ammonium sulphate was added slowly under continuous stirring to achieve about 80% saturation. The ammonium sulphate fraction was dialyzed against phosphate buffer in a dialysis bag of convenient length.

The enzyme preparation was fractionated by ion exchange chromatography. DEAE cellulose ion exchange chromatography column (size, 2.0 × 20 cm) was prepared by filling phosphate buffer (pH 7.5) to one-third from the bottom. A thick slurry of DEAE-cellulose was poured into the column by side to avoid entrapment of air and allowed to settle under gravity until half the column was packed with DEAE, and column was equilibrated with phosphate buffer (pH 7.5) until pH of the elutant buffer reached to 7.5. Desalted lyophilized protein was eluted from the column at a flow rate of 30 ml/h with a linear gradient of NaCl (0−0.5 M). The absorbance of fractions was measured at 280 nm. The eluant fractions that gave higher optical density were assayed for dehalogenation activity.

The purity and molecular weight of the enzyme was determined by gel filtration chromatography. The glass column, size 2 × 60 cm, was packed with Sephadex G-100. The column was washed extensively. Enzyme preparation of ion exchange column was loaded on the column. The column was washed with phosphate buffer (150 ml) at a flow rate of 20 ml/h. Absorbance of each fraction was measured at 280 nm, and fractions that gave higher absorbance were checked for enzyme activity as described earlier.

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [7]. In this method acrylamide gel (10%) was used, and electrophoresis was performed. Gels were stained for protein with Coomassie Brilliant blue R-250.

Results and Discussion

Chemostat culture and PCP-degrading bacteria. Indigenous population, capable of degrading PCP, was enriched in a chemostat to expand the possibilities of bioremediation techniques [15]. A mixed stable community was obtained from a sediment core after continuous enrichment in MSM-PCP as sole source of carbon and

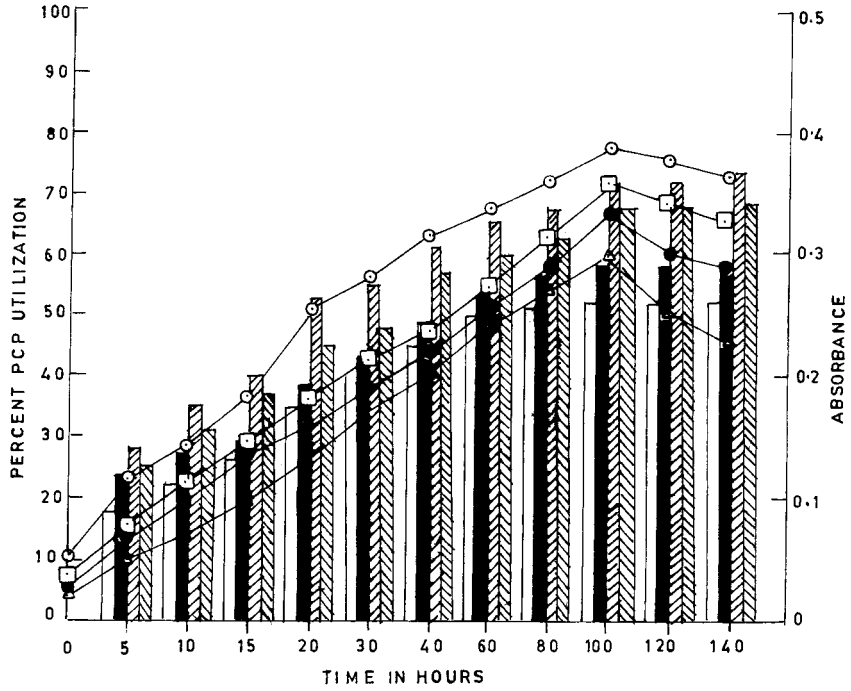

Fig. 1. Growth pattern of bacterial strains in minimal salt medium with PCP as sole source of carbon and energy in batch culture. Growth estimated by A_{560} indicated as TE_1, △; TE_2, ●; TE_3, ⊙; TE_4, ⊡. Utilization of PCP indicated as: TE_1, white bars; TE_2, black bars; TE_3, striped bars (running up left to right); TE_4, striped bars (running down, left to right).

energy in the chemostat for a period of eight months. After some initial fluctuations the culture turbidity increased, and stabilized on day 220 as described earlier [14]. The culture medium removed under aseptic condition on day 220 in tenfold dilution, and plated on nutrient agar. Four different isolates were obtained on nutrient agar plates. They were characterized morphologically, biochemically, and on the basis of utilization of carbon source by Biolog test method as *Serratia marcescens* (three isolates, TE_1, TE_2, and TE_4) and *Pseudomonas fluorescens* biotype A (one isolate, TE_3).

Utilization of PCP by bacterial strains. All four strains (TE_1, TE_2, TE_3, and TE_4) were subjected to shake-flask batch culture for the utilization of PCP (100 mg/l). Results of the study indicated significant increase in number of cells by bacterial strains at 120 h by measuring absorbance at 560 nm (A_{560}). The data of the experiment indicated higher increase in absorbance of TE_3 strains, followed by TE_4, TE_2, and TE_1. Utilization of PCP by four strains was determined by spectrophotometer and HPLC analysis after extraction of PCP in dichloromethane. It was observed that TE_3 utilized most of the PCP (72%), followed by TE_4 (61%), TE_2 (57%), and TE_1 (51%) respectively at 120 h (Fig. 1). The result of the study indicated significance of TE_3 in the consortia, and suggests that the strain may have a prevalent role in the degradation of PCP.

Degradation of PCP by *Pseudomonas fluorescens.* The metabolites released during degradation of PCP was extracted and analyzed by HPLC. The culture medium was extracted twice with dichloromethane (neutral extract). The extracted solution was acidified to pH 2.0 and extracted twice with dichloromethane (acidic extract). As can be seen in Fig. 2, PCP was degraded at 6 h, and two new peaks appeared which were detected as tetrachloro-ρ-hydroquinone (TeCH) and 2,6-dichloro-ρ-hydroquinone (DiCH) in the neutral extract. It was observed that the peak area appeared at RT 4.538 reduced at 48 h, and the area of RT 4.296 has increased. The result of the study indicated conversion of PCP into TeCH which further converted to 2,6-DiCH. The identification of intermediary metabolites is a significant step in complete mineralization of PCP. Extraction methods are critical steps and selection of time points for sampling is another step, which must be optimized for degradation of PCP. Two methods are adopted for extraction of PCP and its intermediary metabolites for the complete mineralization of PCP.

PCP-monooxygenase enzyme fractionation and purification. The enzyme preparation obtained from four bacterial strains (TE_1, TE_2, TE_3, and TE_4) indicated that strain TE_3 produced maximum monooxygenase in 12 h (0.25 U/mg specific activities) as compared to other strains (Table 1). The culture filtrate of TE_3 was subjected to isolation of the PCP-monooxygenase enzyme. The first step of enzyme purification facilitated with ammonium sulphate resulted in a 1.4-fold increase in

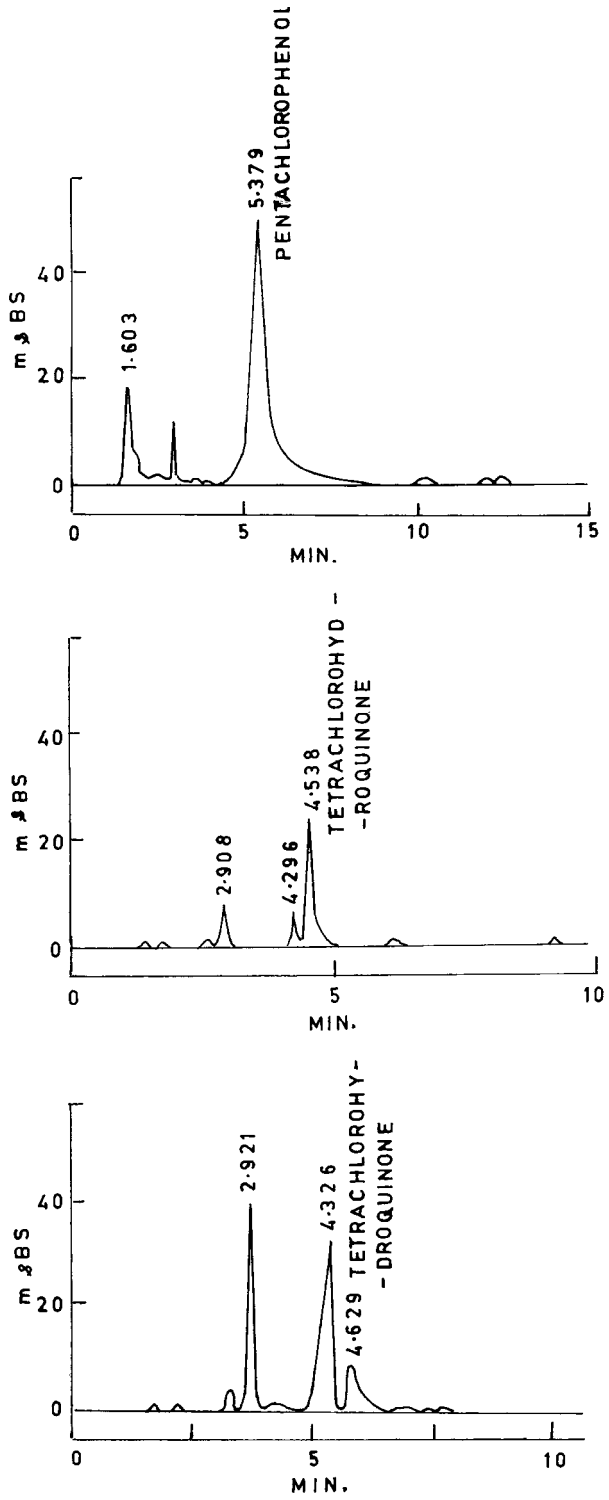

Fig. 2. HPLC profile of PCP degradation and metabolite formed by strain TE_3. RT, PCP 5.379; RT, TeCH 4.538, and RT, DiCH 4.538.

activity. Precipitated protein was desalted by dialysis, resulting in a 4.3-fold purification and 88.6% yield of monooxygenase enzyme. On fractionation of crude culture filtrate on ion exchange column, three major protein peaks, with 0.25 M, 0.5 M, and 0.55 M sodium chloride, appeared. Peaks I (F-I) and III (F-III) showed no enzyme activity. However, enzyme present in one of the fraction (F-II), released by 0.5 M NaCl, exhibited significant enzymatic activity and utilization of PCP. This fraction resulted in an 8.7-fold purification and 73.1% enzyme activity. Positive fraction (F-II), obtained after ion exchange chromatography, was further purified through gel filtration chromatography. One major protein peak with enzyme activity was observed. The molecular weight of the enzyme was estimated by gel filtration chromatography using BSA, ovalbumin, chymotrypsin, and ribonuclease. The Kav value estimated for the active purified protein was 0.687, corresponding to molecular weight 24000 Da. This fraction resulted in a 13.4-fold purification with 68.2% enzyme activity (Table 2).

SDS-PAGE of purified PCP-monooxygenase revealed a single protein of 23,000 Da (Fig. 3). Similarly Xun and Orser purified a PCP-induced periplasmic protein of 30 kDa [20]. Xun et al. detected a major band, with molecular weight of 66 kDa, and a minor band of 132 kDa on the native gradient PAGE, indicating that it exists mainly as a monomer but also can associate as dimer under native electrophoresis conditions [21]. The data presented here suggest that the microbial community enriched using PCP as sole source of carbon and energy was able to degrade PCP, which may be useful for bioremediation of PCP remaining in the environment even after traditional bioremediation treatment methods have been applied. Further studies are necessary on the application of this community for bioremediation of PCP-polluted water and soil.

Conclusion

In this study, samples were collected from three sites of tannery effluent from Kanpur, India, and PCP was extracted and estimated by HPLC. An indigenous microbial community capable of degrading PCP was enriched for a period of eight months in a chemostat. The microbes' rapid utilization of PCP may be due to their prior enrichment in PCP as a source of carbon and energy. The members of the consortium were identified as *Pseudomonas* sp. (one strain TE_3) and *Serratia marscens* (three isolates TE_1, TE_2, TE_4). To elucidate the pathway for the degradation of PCP by these microbes, the growth of all strains in PCP was studied. Results obtained for degradation of PCP through HPLC analysis of the dichloromethane extractable products show formation of tetrachloro-ρ-hydroquinone and 2,6-dichlorohydroquinone in

Table 1. Identification of members of the bacterial consortium and PCP-monooxygenase enzyme activity

Isolate No. Biolog test method result	Specific enzyme activity (U/mg)			
	0 h	6 h	12 h	24 h
TE_1—*Serratia marcescens*	0	0.00	0.15	0.1
TE_2—*Serratia marcescens*	0	0.02	0.18	0.1
TE_3—*Pseudomonas fluorescens*	0	0.09	0.25	0.22
TE_4—*Serratia marcescens*	0	0.06	0.21	0.14

Table 2. Fractionation and purification of PCP-monooxygenase enzyme of *Pseudomonas fluorescens*

Purification	Total vol (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (v/n)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude culture cell extract	20	21.6	432	6.048	120.96	0.28	1.0	100
Ammonium sulfate	20	15.1	301.3	5.906	118.12	0.392	1.4	97.6
Dialysis	20	3.9	79.3	4.758	95.16	1.20	4.3	88.6
DEAE cellulose	15	1.62	24.3	3.87	60.4	2.48	8.7	73.1
Sephadex G-100	15	1.0	15.03	1.95	56.38	3.752	13.4	68.2

the extracts. The culture extract was subjected to estimation of PCP-degrading monooxygenase enzyme, and fractionated and purified. Cellulose ion exchange chromatography of one of the fractions of DEAE indicated enzymatic activity; gel filtration chromotography and SDS-PAGE showed the molecular weight to be 24 kDa. Further characterization of PCP-4-monooxygenase and molecular cloning of the gene encoding the enzyme is in progress.

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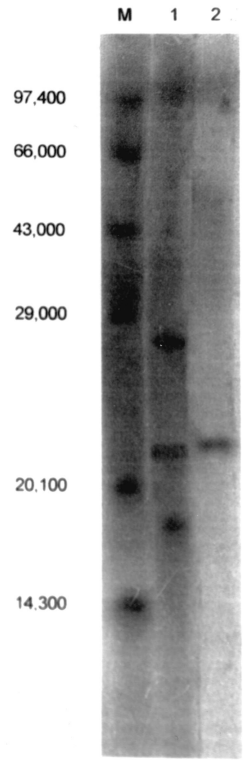

Fig. 3. SDS-PAGE profile of partially purified enzyme. Lane 1, molecular marker; Lane 2, crude extract; Lane 3, partially purified enzyme.

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