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ORIGINAL PAPER

Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP-3 from effluent discharge site

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Abstract Three pentachlorophenol (PCP) degrading bacterial strains were isolated from sediment core of pulp and paper mill effluent discharge site. The strains were continuously enriched in mineral salts medium supplemented with PCP as sole source of carbon and energy. One of the acclimated strains with relatively high PCP degradation capability was selected and characterized in this study. Based on morphology, biochemical tests, 16S rDNA sequence analysis and phylogenetic characteristics, the strains showed greatest similarity with *Acinetobacter* spp. The strain was identified as *Acinetobacter* sp. ISTPCP-3. The physiological characteristics and optimum growth conditions of the bacterial strain were investigated. The results of optimum growth temperature revealed that it was a mesophile. The optimum growth temperature for the strain was 30°C. The preferential initial pH for the strain was ranging at 6.5–7.5, the optimum pH was 7. The bacterium was able to tolerate and degrade PCP up to a concentration of 200 mg/l. Increase in PCP concentration had a negative effect on biodegradation rate and PCP concentration above 250 mg/l was inhibitory to its growth. *Acinetobacter* sp. ISTPCP-3 was able to utilize PCP through an oxidative route with *ortho* ring-cleavage with the formation of 2,3,5,6-tetrachlorohydroquinone and 2-chloro-1,4-benzenediol, identified using gas chromatograph–mass spectrometric (GC–MS) analysis. The degradation pathway followed by isolated bacterium is different from previously characterized pathway.

Keywords *Acinetobacter* sp. ISTPCP-3 · Biodegradation · Characterization · Gas chromatograph–mass spectrometer · Pentachlorophenol

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Introduction

A large variety of chemicals are being synthesized and produced each year. Compounds are finally discharged into the environment during their manufacturing and use of these chemicals. Over last decades, chlorophenolic compounds have been used extensively as wide spectrum biocides in industry and agriculture. These are among the most persistent environmental pollutants because of their physico-chemical characteristics (Annachhatre and Gheewala 1996). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke

and Knackmuss 1988). Among chlorinated phenols; pentachlorophenol (PCP) and its sodium salt have been widely used as wood and leather preservative, owing to their toxic effect on bacteria, mould, fungi and algae (Kaoa et al. 2004). PCP is toxic to all life forms as it is an inhibitor of oxidative phosphorylation (Yang et al. 2006). Extensive exposure to PCP could cause cancer, acute pancreatitis, immunodeficiency and neurological disorders (Sai et al. 2001). The US Environmental Protection Agency (USEPA) has listed PCP as a priority contaminant (Bock et al. 1996). PCP may be washed into streams and lakes due to surface runoff or may infiltrate and contaminate groundwater. Its large amount finally gets deposited onto sediments thus persisting in the environment (Shiu et al. 1994). Moreover, PCP is recalcitrant to degradation because of its stable aromatic ring system and high chloride content, thus persisting in the environment (Saber and Crawford 1985).

The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Anaerobic biodegradation of PCP in aquatic, sludge and soil environment has been studied by various researchers (McAllister et al. 1996; Wang et al. 1998; Vallecillo et al. 1999; Tartakovsky et al. 2001; Thakur et al. 2001). Reductive dechlorination has been suggested as the primary PCP biodegradation mechanism. The aromatic ring is thus totally dechlorinated prior to ring cleavage (Wang et al. 1998; Tartakovsky et al. 1999, 2001). Aerobic degradation of PCP has also been studied extensively and several bacterial strains capable of degradation have been reported such as *Flavobacterium*, *Arthrobacter*, *Pseudomonas*, *Sphingomonas* and *Sphingobium* (Edgehill and Finn 1983; Crawford and Mohn 1985; Saber and Crawford 1985; Xun and Orser 1991; Orser et al. 1993; Edgehill 1994; Miethling and Karlson 1996; Chanama and Crawford 1997; Leung et al. 1999; Thakur et al. 2002; Yang et al. 2006; Dams et al. 2007). To the best of our knowledge, this is the first report of PCP degradation by *Acinetobacter* sp. and the degradation pathway reported here is different from previously characterized pathway.

In this study, a bacterium identified *Acinetobacter* sp. was isolated from acclimated bacterial consortium isolated from sediment core of pulp and paper mill effluent discharge site. It was able to grow and degrade relatively high concentrations of PCP. The strain was characterized for optimization of growth conditions such as pH and temperature. The metabolic profile was also studied in order to deduce degradation pathway. This highly potent bacterial strain could be applied for bioremediation of sites contaminated with PCP.

Materials and methods

Chemicals and solvents

The following chemicals were purchased from Sigma-Aldrich (USA): PCP (FW 266.30), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), anhydrous sodium sulphate (AR). Organic solvents (GC/MS grade) ethyl acetate, hexane and acetone were obtained from Merck, India. All other inorganic chemicals were of analytical grade obtained from Qualigens fine chemicals, GSK, India. Anhydrous sodium sulphate was heated at 130°C for 24 h prior to its use.

Growth medium

Mineral salts medium (MSM) was used in enrichment culture and degradation studies. The medium contained the following components at the specified concentrations (in mg/l): KH_2PO_4, 800; Na_2HPO_4, 800; $MgSO_4{\cdot}7H_2O$, 200; $CaCl_2{\cdot}2H_2O$, 10; NH_4Cl, 500; plus 1 ml of trace metal solution which includes $FeSO_4{\cdot}7H_2O$, 5; $ZnSO_4{\cdot}7H_2O$, 4; $MnSO_4{\cdot}4H_2O$, 0.2; $NiCl{\cdot}6H_2O$, 0.1; H_3BO_3, 0.15; $CoCl_2{\cdot}6H_2O$, 0.5; $ZnCl_2$, 0.25; and EDTA, 2.5. PCP was added to the medium after autoclaving. Solid MSM plates were prepared, if necessary, by adding 1.55 (w/v) bacteriological grade agar.

Enrichment and isolation of PCP degrading bacterium

Sediment sample was collected from the effluent discharge site of a pulp and paper mill at Nainital, Uttarakhand state, India located at foothills of Himalayas. The site was having a history of PCP contamination. The samples were taken into sterilized tube and preserved at 4°C. Sediment sample (2 g) was added to MSM (100 ml) with PCP (10 mg/l) as sole source of carbon and energy and incubated at 30°C for 5 days in a rotary shaker at 150 rpm. Enriched cultures, 5 ml, showing degradation of PCP were transferred to 100 ml fresh MSM with PCP

(50 mg/l). Six sub-cultures were performed before the isolation of effective strains. The final enriched cultures were spread on MSM agar plates. Three phenotypically different colonies were picked and purified by repeated streaking on the same medium. Of the three isolated bacterial strains, one pure isolate with highest PCP degradation efficiency was designated as ISTPCP-3 and selected for further study.

Biochemical and phenotypic characteristics

The identification of PCP degrading bacterium was done according to Bergey's Manual on Systematic Bacteriology (Holt et al. 1994). Cell morphology of the isolated strain was observed by scanning electron microscopy (JEOL JSM-6360LV, Japan). Samples were picked from agar culture onto a glass cover slip and fixed with buffer containing glutaraldehyde. Fixed samples were dehydrated by passing through series of ethanol solutions with concentration 30, 60, and 90% of ethanol for 10 min each. Finally, cover slips containing bacteria were kept in absolute ethanol for 24 h. Samples were vacuum dried followed by gold shadowing. The samples were observed under SEM with magnification at 15,000×.

16S rDNA sequence and phylogenetic analysis

Genomic DNA was isolated according to procedures described by Singh et al. (2003) with some minor modifications. 1.4 kb fragment of 16S rDNA was amplified using a Gene Amp 2400 PCR System (PE, USA). A 5 µl purified genomic DNA served as a template in the PCR reaction mixture with the following set of primers: upstream primer 27°F (GAGAGTTTGATCCTGGCTCAG), and downstream primer 1495R (CTACGGCTACCTTGTTACGA). Each 200 µl PCR micro-tube contained 5 µl purified extracted DNA; 2 µl of dNTP at 2.5 mM; 2.5 µl of 10× *Taq* DNA polymerase buffer; 11.3 µl of sterile MQ water and 2 µl of amplification primers (10 pmol each). The tubes were then subjected to the following thermal cycling programme: denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and chain extension at 72°C for 2 min with an additional extension time of 7 min on the final cycle, for a total of 30 cycles. The 16S rDNA sequence was compared against the available DNA sequence from type strains in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN sequence match tool. The sequences were aligned using multiple sequence alignment software CLUSTALW (v 1.81). A phylogenetic tree was then constructed using MEGA software (v 3.1) based on 16S rDNA sequences of closely related bacterial strains (Kumar et al. 2004).

Inoculum preparation for degradation studies

Unless otherwise stated, all aerobic batch cultivations were carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid culture. The strain ISIPCP3 was grown of MSM containing PCP (100 mg/l) as sole carbon source for 24 h. The cells were pelleted by centrifugation at 7,000*g* for 10 min, cells pellets were washed twice with fresh MSM. Cell density was monitored by spectrophotometer at 600 nm (OD_{600}) (Shimadzu UV2410, Japan). For all experiments 10^6 CFU/ml were used and samples were incubated at 30°C and shaking at 150 rpm in dark.

Degradation of PCP by the isolated strain

The degradation ability of the isolated strain ISTPCP-3 was analyzed in MSM containing PCP. Degradation efficiency was determined and estimated by loss of PCP from culture medium. In extraction of metabolites, the cell suspension (50 ml) was clarified by centrifugation at 7,000 rpm for 8 min. The cell free supernatant fractions were extracted thrice with an equal volume of ethyl acetate by shaking for 45 min. The organic layer was dried with anhydrous sodium sulphate, and the solvent was removed by gently blowing under a stream of N_2. The residue was derivatized in 300 µl of ethyl acetate with 100 µl of BSTFA and analyzed immediately on a GC–MS. The GC–MS analyses were performed in electron ionization (EI) mode (70 eV) with an Agilent 6,890 N gas chromatograph, equipped with 5973 MSD (Agilent Technologies, Palo Alto, CA, USA). A HP-5MS (Agilent, USA) capillary column (5% phenyl 95% methylpolysiloxane; 30 m length × 0.025 mm id × 0.25 µm film thickness) column was used at a temperature programme of 45°C for 1.5 min, increased to 100°C at 10°C/min, increased to 180°C at 4°C/min and finally increased to 300°C at 40°C/min and held at 290°C for 5 min. Helium was used as the carrier gas at a constant flow of 1.2 ml/min. The samples were analyzed in split mode (1:10) at an injection

temperature of 250°C, an EI source temperature of 230°C and a quadrupole analyzer temperature of 150°C, unit mass resolution, scan range m/z 35–500, with a scan cycle of three scans/s. The injected volume was 1 µl. Rothera test was performed with some modifications as described by Holding and Collee (1997) for detection of *ortho* or *meta* ring cleavage. Cell lysate (0.5 ml) was incubated with 2-chloro-1,4-benzenediol (0.5 mM) in 2 ml of tris buffer (0.02 M; pH 8) for 20 min, followed by addition of ammonium sulphate crystals (1 g), 1% sodium nitropruside solution (freshly prepared) and then ammonia solution (0.5 ml). Tubes were incubated for 1 h at 30°C in shaking condition. Appearance of a deep violet coloration indicates the *ortho* ring cleavage of the substrate.

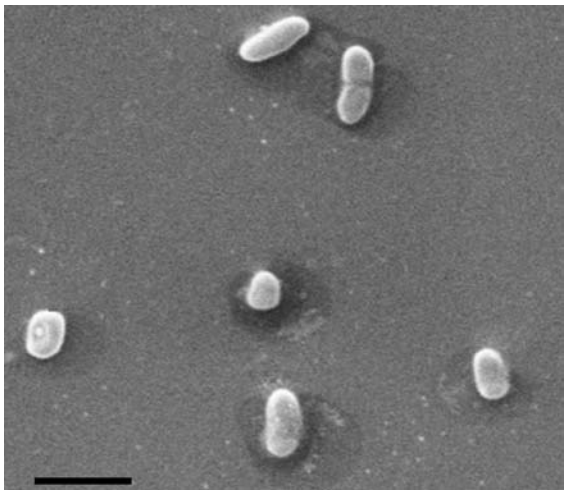

Fig. 1 Scanning electron micrograph of strain ISTPCP-3 at ×15,000 (*Bar* represents 1 µm)

Results

Isolation and identification of PCP degrading strain

The bacterial isolates from the sediment core of pulp and paper mill effluent discharge site were extracted and enriched with MSM in presence of PCP by continuous process. Three isolates were obtained that grew on MSM plates with PCP (100 mg/l) as sole source of carbon. All isolates were tested for their PCP degrading capacity. Out of three acclimated bacterial strain, ISTPCP-3 showed relatively high degradation capacity. The strain was able to degrade 100 ppm of PCP in 48 h. The strain was selected for further characterization. On MSM agar plate, the colonies were cream, non-spreading, smooth, wet, convex, and non transparent. The color of the colonies changed to brown in 72 h. The strain ISTPCP-3 was Gram negative, non-encapsulated and non-motile. Size and shape of the cells were determined by scanning electron micrograph (Fig. 1). Cells were coccobacilli, with 0.2–0.4 × 0.5–0.9 µm in size. The biochemical test showed negative for urease, oxidase, indole and catalase production. The strain was a non-fermenter.

16S rDNA sequence based phylogenetic analysis

Using universal primers, 1.4 kb of 16S rDNA sequence of strain ISTPCP-3 was amplified, sequenced and submitted to GenBank and accession number EF432791 was obtained. The BLAST searches of the obtained sequence showed high degree of sequence similarity with *Acinetobacter* spp. and were 99% similar with strain *Acinetobacter* sp. H104 (GenBank accession No. EF204271) and an uncultured *Acinetobacter* clone 1P-1-K14 (GenBank accession No. EU704861). A phylogenetic tree was constructed based on 16S rDNA sequence (Fig. 2) by Neighbor-joining method using MEGA software (v 3.1). The phylogenetic sequence analysis attested the results obtained from morphological and biochemical analysis. Therefore, the isolate was designated as *Acinetobacter* sp. ISTPCP-3.

The optimum growth conditions of strain ISTPCP-3

The growth of bacterial strain ISTPCP-3 and degradation of PCP were observed under culture conditions such as different temperature, initial pH, and initial PCP concentration. Figure 3 shows the degradation of PCP with different temperatures. The result reveals that *Acinetobacter* sp. ISTPCP-3 is a mesophilic bacterium. The bacterium was able to grow well in the temperature range of 25–35°C. The optimum growth temperature was 30°C by utilizing 50 ppm of carbon source. The degradation was weak as observed at 20 and 45°C. The effects of initial pH value on growth and degradation of PCP are shown in Fig. 4. The results indicate higher PCP degradation

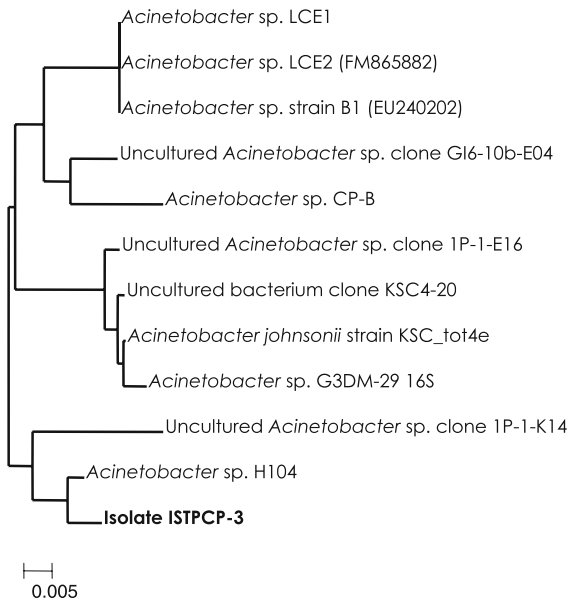

Fig. 2 Phylogenetic tree based on 16S rDNA gene sequence of ISTPCP-3 strain and related species

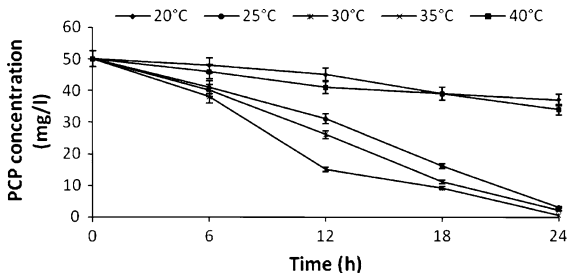

Fig. 3 Effect of temperature changes on degradation of PCP by *Acinetobacter* sp. ISTPCP-3

between pH 6.5 and 7.5. The optimum value for maximum degradation was at pH 7.0. Not much degradation was observed at extreme pH values of 5.0 and 9.0. The growth of bacterial strain was measured at absorbance 600 nm. Figure 5 shows the removal of different concentrations of PCP by isolated strain ISTPCP-3. The bacterial strain was able to completely degrade PCP at all concentrations at and lower than 100 mg/l. When the initial PCP concentrations were 50 and 100 mg/l, the strain was able to completely degrade PCP within 24 and 48 h, respectively. At PCP concentration of 200 mg/l, degradation was incomplete with curve showing an extended lag phase. The possible explanation for

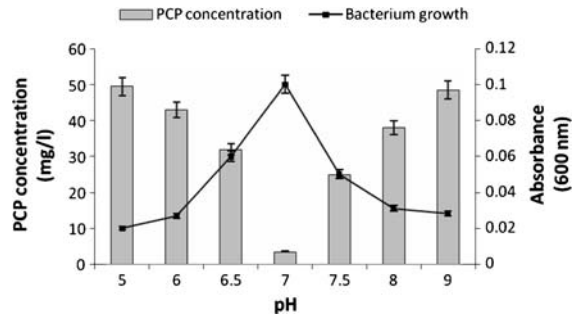

Fig. 4 Effect of initial pH changes on degradation of PCP by *Acinetobacter* sp. ISTPCP-3

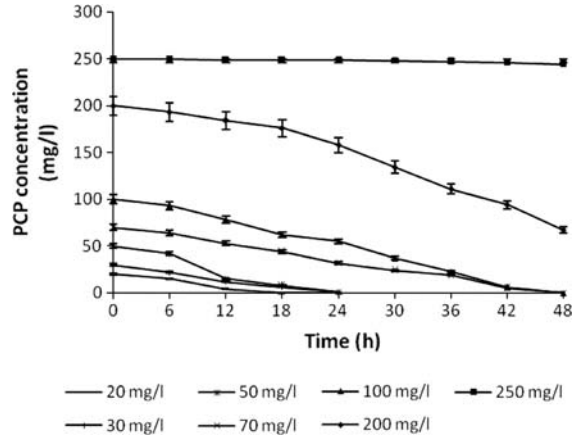

Fig. 5 The removal of different concentrations of PCP by *Acinetobacter* sp. ISTPCP-3

observed incomplete degradation of PCP by bacterial strain at 200 mg/l could be due to decreased activity of the degrading enzymes at lower pH, as the pH of the medium decreased significantly at that concentration (data not shown). No significant degradation of PCP was observed by bacterial strain, when the PCP concentration was 250 mg/l. All samples were taken in triplicates and the line in graphs represents average value with bars represents standard error.

Degradation products and pathway for PCP

The degradation products of PCP by bacterial strain ISTPCP-3 were extracted and identified by GC–MS. The GC chromatograms are shown in Fig. 6. The metabolite peaks were identified using documented data from National Institute of Standards and Technology (NIST) library database. The results of the

study indicated degradation of 100 mg/l PCP. In control sample (0 h) only PCP peak (RT = 17.2 min) was observed. Two new peaks of metabolism were observed at 48 h, the peaks were identified as 2,3,5,6-tetrachlorohydroquinone (RT = 18.5 min) and 2-chloro-1,4-benzenediol (RT = 9.8 min). At 48 h about 95% of PCP had been degraded indicating transformation of PCP into dechlorinated products. The selected mass-to-charge fragmentation pattern and relative intensity of various peaks of metabolism is shown in Table 1. The identification of 2,3,5,6-tetrachlorohydroquinone and 2-chloro-1,4-benzenediol suggests that these products were produced due to dechlorination of parent PCP compound. A positive Rothera test, indicative of deep violet color formation confirmed the ring cleavage through *ortho* pathway. Heat-killed cells were taken as control; it did not show any products or metabolites from PCP. Formation of low molecular weight compounds from PCP degradation was previously reported by various researchers (Crawford and Mohn 1985; Xun et al. 1992; Ohtsubo et al. 1999; Cai and Xun 2002).

Discussion

The PCP biodegradation pathway has been studied extensively using soil and aquatic bacteria such as *Sphingobium chlorophenolicum*. These bacteria have evolved pathways to degrade PCP and use the ring-

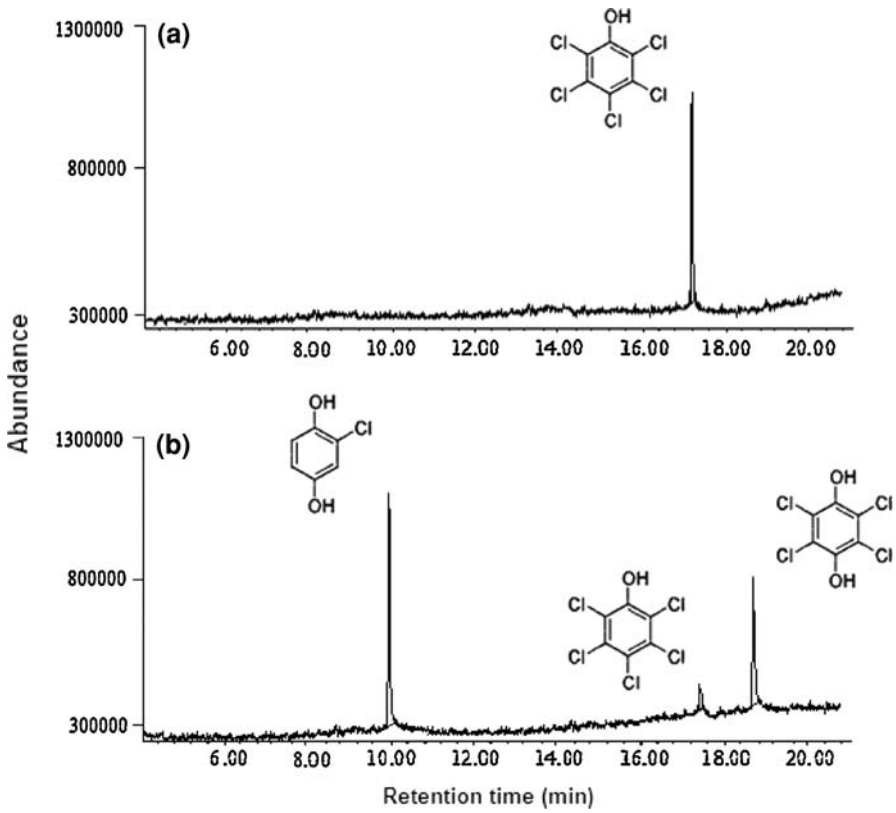

Fig. 6 Gas chromatograms of extracts obtained from the liquid culture; (**a**) 0 h and (**b**) after 48 h of incubation with strain ISTPCP-3

Table 1 Retention time, molecular weight, and mass spectra fragmentation of trimethylsilyl derivative compounds

Compound	RT (min)	MW	Selected fragments m/z (relative intensity)
2-Chloro-1,4-benzenediol	9.8	288	257 (100) 147 (52) 95 (18)
Pentachlorophenol	17.2	336	323 (72) 325 (24) 93 (100)
2,3,5,6-Tetrachlorohydroquinone	18.5	390	392 (100) 340 (42) 267 (30)

cleavage products of PCP as their source of carbon and energy. So far, two types of pathways for aerobic degradation of PCP have been described; one is through formation of chloro-catechols and other is through formation of subsequent hydroquinone. In pathway via chloro-catechols, the subsequent chlorophenols formed are further metabolized via *ortho* or modified-*ortho* ring cleavage pathways (Thakur et al. 2002). In the hydroquinone pathway, subsequent dechlorination leads to formation of hydroquinone, which is subsequently cleaved by *ortho* ring cleavage enzyme (Chanama and Crawford 1997; Hu et al. 2006).

According to earlier reports of PCP degradation pathway in bacteria (Crawford and Mohn 1985; Xun et al. 1992; Ohtsubo et al. 1999; Cai and Xun 2002), contains five catalytic enzymes, which are responsible for its mineralization. The bacterial enzyme PCP-4-monooxygenase catalyzes the oxygenolytic removal of the first chlorine from PCP to tetrachlorohydroquinone using nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate. However, recent reports showed the enzyme converted PCP to tetrachlorobenzoquinone rather than tetrachlorohydroquinone (Chen and Yang 2008). In our studies, we did not get tetrachlorobenzoquinone, as product of metabolism from PCP. Tetrachlorohydroquinone is further converted trichlorohydroquinone by reductive dechlorination and subsequently to dichlorohydroquinone was formed by reductive dehalogenase enzyme. Dichlorohydroquinone acts as a precursor for ring-cleavage enzyme, which converts it to chloromaleylacetate, an open ring structure.

We report here; novel degradation pathway followed by isolated acclimated bacterium *Acinetobacter* sp. ISTPCP-3. In case of pathway followed by the isolated strain, PCP is converted to 2,3,5,6-tetrachlorohydroquinone, which is further converted to 2-chloro-1, 4-benzenediol, which acts as a ring cleavage precursor instead of dichlorohydroquinone. Work related to characterization of enzymes involved at each step of the pathway is being carried out in this laboratory.

In summary, an indigenous bacterial population capable of PCP degradation was enriched. Initially, three bacterial strains capable of degrading PCP were isolated. One potent bacterial strain with relatively high PCP degradation capability was isolated and characterized further. The strain was identified as *Acinetobacter* sp. ISTPCP-3. The ability of the strain for degrading PCP, as sole carbon source, was investigated under different physiological conditions. The strain was a mesophile and showed maximum degradation at neutral pH. The strain could degrade PCP (100 mg/l) completely within 72 h. The PCP degradation pathway followed by the bacterial strain was different from previously characterized pathways. These results highlight the potential of this bacterium to be used in for bioremediation of sites contaminated with PCP.

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