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Characterization of pentachlorophenol degrading *Bacillus* strains from secondary pulp-and-paper-industry sludge

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ABSTRACT

Pentachlorophenol (PCP) degrading *Bacillus* strains CL3, CL5, and CL11 were isolated from the secondary sludge of a pulp paper mill and characterized. These isolates were identified as *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus thuringensis* based on their 16S rRNA sequence analysis. These isolates were able to grow and utilize PCP as a carbon and energy source. HPLC analysis and stoichiometric release of chloride in the medium confirmed the degradation ability of these isolates. The removal efficiency of PCP by these bacterial isolates was highly significant and they were able to degrade more than 90% of PCP when grown at high concentration of PCP (600 mg l^{-1}). Consortia of these isolates removed 77% of PCP from the sludge. The removal efficiency of PCP by the bacterial isolate used in this study was found to be more efficient than what has been reported with other *Bacillus* species. These results suggest that the bacterial isolates are very effective PCP degraders and can be used in remediation of PCP-contaminated sites.

1. Introduction

Chlorophenolic compounds are common environmental contaminants, mainly due to their use as wide-spectrum biocides in industry and agriculture, their formation during pulp bleaching, and direct industrial waste discharge (Vallecillo et al. 1999). The most common sources of chlorinated phenols in the environment include production of chlorine from the bleaching of pulp, combustion of organic matter, partial transformation of phenoxy pesticides such as 2,4-dichlorophenoxyacetic acid and 2,4,6-trichlorophenoxyacetic acid, treatment of wood against fungi and insects, and preservation of rawhides in leather tanning industries (Shukla et al. 2001). The toxicity of these compounds tends to increase with their degree of chlorination (Fetzner and Lingens 1994). Among chlorinated phenols, pentachlorophenol (PCP) has been widely used as a wood and leather preservative owing to its toxicity toward bacteria, mould, algae, and fungi (Kaoa et al. 2004). Pentachlorophenol is toxic to all forms of life since it is an inhibitor of oxidative phosphorylation (Shen et al. 2005). Moreover, it is recalcitrant to degradation because of its stable aromatic ring and high chloride content, thus persisting in the environment (Copley 2000). This compound is listed among the priority pollutants of the U.S. Environmental Protection Agency (EPA 1987).

Chemical and physical-based clean-up methods have some disadvantages, including their high costs and the possibility of causing secondary pollution. The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Several bacterial strains capable of degrading PCP under aerobic conditions have been reported, principally members of the genus *Pseudomonas* sp., *Sphingomonas chlorophenolica*, *Rhodococcus chlorophenolicum*, *Flavobacterium* sp., *Arthrobacter* sp., and *Corynebacterium* sp. (Field and Sierra-Alvarez 2008, and references therein). Very few PCP-degrading *Bacillus* have been reported (Chandra et al. 2006). In this investigation, we have isolated and characterized three *Bacillus* strains that are capable of degrading high concentrations of PCP.

2. Material and methods

2.1. Isolation and enrichment

Pulp and paper mill secondary sludge samples were collected from the M/s Shree Gopal Unit (BILT) Yamunanagar, Haryana, India. The samples were collected into pre-sterilized 250-ml conical flasks and immediately transferred at 4 °C. The potential PCP-degrading bacterial strains were isolated by the serial dilution technique, and purified by repeated streaking on nutrient agar

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plates. Colonies appearing after incubation at 37 °C for 48 h were selected for further screening. Selection of PCP-tolerant bacterial strains was done in mineral salt medium (MSM; Dams et al. 2007) supplemented with 50 mg l^{-1} (0.19 mM) PCP as the sole carbon source. The composition of MSM (in mg l^{-1}) was KH_2PO_4, 800; Na_2HPO_4, 800; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 10; NH_4Cl, 500; and 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; $CoCl2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; and EDTA, 2.5. PCP was added to the medium after autoclaving. The pH was adjusted to 7.3 ± 0.2 prior to autoclaving. Three potent isolates were selected for the degradation studies. These strains were designated as CL3, CL5, and CL11.

2.2. Morphological and biochemical characterization

The biochemical characterization of the three isolates was done in accordance with *Bergey's Manual of Systematic Bacteriology* (Holt et al. 1994). Each pure culture was subjected to microscopic examination for morphological analysis. Capsule stain was determined with 20% copper sulfate. Catalase activity was determined based on formation of bubbles in the presence of 3% H_2O_2 solution. Oxidase was performed on paper discs using tetramethyl-*p*-phenylenediamine. Nitrate reductase was detected on nitrate agar plates and DNase on DNase agar medium with methyl green as an indicator. Starch hydrolysis was demonstrated from clearing zones formed around the colonies grown on starch containing agar. Motility, indole formation, and urease activities were determined by using an MIU-media kit (Hi-Media Laboratories, India). Antibiotics profiling was performed with an ICOSA universal-1 kit (Hi-Media Laboratories, India).

2.3. Amplification of 16S rRNA genes and sequence analyses

Genomic DNA was extracted according to Kapley et al. (2001) from overnight grown cultures. 16S rRNA genes were amplified from 1 μl purified genomic DNA using a GeneAMP 2700 PCR (Applied Biosystems, USA) with the following set of primers: forward: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse: 5′-ACGGGCG GTGTGTTC-3′ as described by Weisburg et al. (1991). Reaction mixture for the PCR contained 1X PCR buffer, 200 μM of dNTPs, 1.5 mM $MgCl_2$, 0.1 μM of each primer, and 2.5 units of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 μl. The PCR was performed with an initial denaturation at 92 °C for 2 min followed by 36 temperature cycles as follows: 92 °C for 1 min, 48 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 6 min. The amplicons were purified with the QIA gel extraction kit (Qiagen, USA), and ligated into the pGEM-T easy vector as per the manufacturer's instructions (Promega Inc., USA). Ligated plasmids were transformed into *Escherichia coli* DH5α cells. Recombinant plasmids were sequenced using an Applied Biosystems automated DNA sequencer (DNA Sequencing facility, Delhi University, India). The sequences were compared against the available DNA sequences in GeneBank (http://www.ncbi.nlm.nih.gov/) using the BLASTN tool. The sequences were aligned using the MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html) program and the alignment was manually corrected and a phylogenetic tree was constructed using the MEGA 4.0 (Tamura et al. 2007) software.

2.4. Degradation studies

The degradation studies were performed by inoculating strains CL3, CL5, and CL11 at 1% inoculum (10^6 CFU ml^{-1}) in 250-ml Erlenmeyer flasks containing 50 ml of MSM supplemented with 100 mg l^{-1} (0.37 mM) of PCP. The flasks were incubated at 37 °C at 120 rpm for up to 168 h. Growth was determined by measuring the OD_{600nm}, and PCP depletion was quantified by HPLC by sampling 1 ml of culture at 24-h intervals. Chloride ion concentrations in cultures were measured at 24-h intervals up to 168 h using a chloride ion-specific electrode (Orion ion analyzer model 940) that was standardized against a wide range of known chloride standards in MSM.

HPLC was carried out on a Perkin Elmer Series 200 system. The cell suspension was centrifuged (8000 rpm, 5 min) and the supernatant was filtered through a 0.22-μm filter. Ten μl samples were injected on a reverse phase Lichrosphere^R 100 RP-18 endcapped column (250 mm × 4.6 mm i.d.). The column was eluted in an isocratic mode using aqueous methanol (90%, v/v) at a flow rate of 1 ml min^{-1}. PCP was monitored at 280 nm with an online diode array detector (series 200). PCP was quantified from standard curve prepared by injecting known quantities of PCP (Sigma Aldrich, USA) on the same column.

The effect of PCP concentration on the growth of strains CL3, CL5, and CL11 and on their PCP-degrading ability were also examined. The isolates were inoculated to 250-ml Erlenmeyer flasks containing 50 ml of MSM supplemented with different concentrations (50, 100, 200, 400, and 600 mg l^{-1}, corresponding to 0.19 mM, 0.37 mM, 0.75 mM, 1.5 mM, and 2.2 mM) of PCP separately. The flasks were incubated at 37 °C with shaking for 168 h. The growth of the bacterial cells was measured at OD_{600nm} and the degradation of PCP in the culture by HPLC as described above.

The effect of pH on the degradation of PCP was studied by growing each strain at an initial pH of 7.5, 8.5, or 9.5 in MSM supplemented with 100 mg l^{-1} of PCP as the sole carbon source. The pH of the medium was adjusted with NaOH or HCl. The influence of temperature on PCP degradation was also determined by incubating the samples at 25 °C, 30 °C, or 37 °C under shaking.

2.5. Degradation studies of PCP in sludge

The degradation ability of a consortium comprised of strains CL3, CL5, and CL11 was analyzed in 2.5-l conical flasks containing 1 liter of sludge supplemented with 100 mg l^{-1} of PCP and inoculated with 5% of inoculum. The flasks were incubated at 37 °C under shaking for two weeks. PCP was extracted from the sludge according to Chandra et al. (2006). The sludge was sonicated and acidified to pH 2.0 with 1 N HCl. It was then extracted three times with an equal volume of ethyl acetate by intermittent shaking for 30 min in separating funnels. The organic layer was dried over anhydrous sodium sulphate. Filtered samples were evaporated under vacuum at 40 °C, subsequently resuspended in 1 ml of methanol, and injected on an HPLC to quantify the remaining PCP.

2.6. Statistical analysis

Data were subjected to analysis of variance using ANOVA software and the averages were compared by the Tukey–Kramer Multiple Comparison Test at $p < 0.05$. Three replicates were prepared for each treatment. All the analyses were performed using GraphPad Prism (v 4.03) software.

3. Results

3.1. Isolation, characterization, and identification

Three bacterial isolates, CL3, CL5, and CL11, were selected based on their ability to grow on PCP-amended medium. The biochemical characterization results are presented in Table 1.

The 16S rRNA gene sequences determined in this study have been deposited in GenBank of NCBI database under the accession numbers EU784650, EU784652, and EU784658 for CL3, CL5, and CL11, respectively. A BLAST search revealed that CL3 is closely