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Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill

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Abstract

A pentachlorophenol (PCP) mineralizing bacterium was isolated from the secondary sludge of pulp and paper mill and identified as *Pseudomonas stutzeri* strain CL7. This isolate used PCP as its sole source of carbon and energy and was capable of degrading this compound as indicated by stoichiometric release of chloride and biomass formation. *P. stutzeri* (CL7) was able to mineralize a high concentration of PCP (600 mg/L) than any previously reported *Pseudomonad* with PCP as sole carbon source. As the concentration of PCP increased from 50 to 600 mg/L, the reduction in the cell growth was observed and the PCP degradation was more than 90% in all studied concentrations. This isolate was able to remove 66.8% of PCP from the secondary sludge of pulp and paper mill when supplemented with 100 mg/L of PCP and grown for two weeks. This study showed that the removal efficiency of PCP by CL7 was found to be very effective and can be used in PCP remediation of pulp paper mill waste in the environment.

Key words: pentachlorophenol; 16S rRNA; secondary sludge; bioremediation; pulp and paper mill

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Introduction

The manufacturing process of pulp and paper utilizes huge amount of lignocellulosic components of plants and chemicals and generates effluents consisting complicated mixtures of several hundred types of compounds. Chlorinated phenols are major environmental pollutants discharged from pulp and paper mill. Chlorophenols from the pulp and bleaching process are found both in free (hexane extractable) and bound (extractable with strong alkali) forms in dissolved organic matter and particles (Palm et al., 1995). Among the chlorophenols, pentachlorophenol (PCP) is expected to be recalcitrant to aerobic biodegradation due to its high chlorinated ring structure; generally aromatic compounds with higher amounts of chlorine are more recalcitrant to biodegradation (Anandrajah et al., 2000). The United States Environmental Protection Agency has registered PCP in the list of priority of pollutants and the safe permissible limits of PCP in water is 0.30 μg/L (US EPA, 1999). However pulp and paper mill effluent contains far above the permissible limit of PCP even after the treatment at industrial scale (Raj et al., 2005). In addition, this compound is very harmful to microorganisms because it destroys membrane function due to its ability to uncouple oxidative phosphorylation (Copley, 2000; Ito and Ohnishi, 1982). The accumulation of PCP through the food chain has been established and it is considered to be mutagenic or at least co-mutagenic to human, thus the PCP exposure in environment poses significant health hazards (Chandra et al., 2008). In spite of these properties, several microorganisms have been isolated which have the ability to degrade PCP.

A large variety of bacteria are known which can utilize chlorophenols as a carbon and energy sources under aerobic conditions. Lee et al. (1998) reported that *Pseudomonas* sp. (Bu34) was able to grow up to 4000 mg/L of PCP. Radehaus and Schimdt (1992) reported complete mineralization of PCP (160 mg/L) in a week by *Pseudomonas* sp. strain RA2. Shah and Thakur (2002) observed 72% PCP removal by *Pseudomonas fluorescence* (TE3) in 96 hours when grown with 100 mg/L PCP. Sharma and Thakur (2008) observed that *Pseudomonas aeruginosa* (PCP2) utilized 60% of PCP within 96 hours of incubation. Premlatha and Rajkumar (1994) recorded the complete degradation of PCP (800 mg/L) by *Pseudomonas aeruginosa* in 6 days with glucose as a co-substrate. Singh et al. (2007) reported that the bacterial isolate *Serratia marcescens* from pulp and paper mill waste was able to degrade 90.3% of PCP within 168 hr when grown with 300 mg/L of PCP. There are still many unknown bacteria that have tremendous degradation capacity for PCP present in nature and it is important to asses the potential of bacterial strain indigenous to sites contaminated with PCP. In the present study, PCP degrading bacterium *Pseudomonas stutzeri* was isolated from the secondary sludge of pulp and

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paper mill and characterized. The PCP degradation ability of this isolate with different concentrations of PCP and the removal of PCP from the secondary sludge of pulp and paper mill were also determined.

1 Materials and methods

1.1 Sample collection and isolation of PCP degrading bacterium

Secondary sludge samples of pulp and paper mill were collected from M/s Shree Gopal Unit (BILT) Yamunanagar, Haryana (India). The samples were taken in pre-sterilized 250 mL conical flasks and immediately preserved at 4°C. Bacteria were isolated by the serial dilution technique, and purified by repeated streaking on nutrient agar plates. Colonies appearing after incubation at 37°C for 48 hr were selected for further screening. PCP tolerant bacterial strains were isolated by the nutrient enrichment technique in mineral salt medium (MSM) (Sharma et al., 2009) supplemented with 50 mg/L pentachlorophenol as the sole carbon source for energy. The medium contained the following components (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 (mg/L): $FeSO_4{\cdot}7H_2O$, 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; EDTA, 2.5 and agar 1.0%. The pH was adjusted to 7.3 ± 0.2. PCP was added to the medium after autoclaving. The potent isolates were screened for the degradation studies and one of the efficient isolates was selected and designated as CL7 for further studies.

1.2 Morphological and biochemical characterization

The identification of PCP degrading bacterium (CL7) was conducted according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The CL7 isolate was subjected to microscopic examination for the shape and size. Gram stain, catalase, oxidase, citrate utilization, lysine, ornithine TDA, nitrate reduction, urease and starch hydrolysis tests were performed by standard methods. Antibiotic profiling were performed by using ICOSA universal -1 kit (Hi-Media Laboratories, Mumbai, India) having twenty different antibiotics of various concentrations according to the manufacturer's instructions. A total of 35 carbohydrate fermentation tests were performed according to the manufacturer's direction (Himedia Lab., Mumbai, India).

1.3 16S rRNA gene amplification and sequence analysis

Genomic DNA extraction, amplification of 16S rRNA gene and sequence analysis were performed as described in Krishna et al. (2008). The 16S rRNA gene sequence determined in this study was deposited in the GenBank of NCBI data library under the accession number EU784654.

1.4 Degradation of PCP by the isolated strain

The degradation studies were performed by inoculating 1% inoculum (10^6 cfu/mL) of CL7 in 250 mL Erlenmeyer flasks containing 50 mL of MSM supplemented with 100 mg/L PCP. The flasks were incubated at 37°C under shaking conditions (120 r/min) up to 168 hr. The growth of the bacterial cells was determined by measuring the optical density at 600 nm. The degradation of PCP in the culture filtrate was determined by high-performance liquid chromatography (HPLC) as described by Yang et al. (2006). Briefly, the cell suspension was centrifuged to separate the biomass at 8000 r/min for 5 min and the supernatant was passed through 0.22 μm filters. HPLC was performed with a PerkinElmer System (USA) equipped with a Merck Lichrospher 100 RP-18 (USA) end capped (5 μm) column at flow rate of 1 mL/min. The solvent system was methanol, water and glacial acetic acid in the volume ratio of 90:10:0.02. The UV detector absorbance wavelength was fixed at 280 nm. A standard PCP was run under the same conditions. The percent utilization was estimated by measuring the peak area of the PCP.

Chloride ion released in the aqueous media was determined at every 24 hr of interval up to 168 hr using 5 mL of culture filtrate. The level of chloride ion was measured with an Orion ion analyzer model 940 (NyCo Systems, USA) using calibrated selective chloride ion electrode. Chloride concentration was determined using a calibration curve plotted from the log of chloride molarity for a series of standard samples ranging from 10 to 1000 mg/L.

The effect of pH on the degradation of PCP was studied by growing the bacterial cells at different initial pH of 7.5, 8.5 and 9.5 in MSM supplemented with 100 mg/L of PCP as sole source of carbon. 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 three different temperatures 25, 30 and 37°C under shaking condition.

The effect of different concentrations of PCP on the growth of CL7 and degradation ability of this strain was also studied. The bacterial strain CL7 was inoculated (10^6 cfu/mL) to 250 mL Erlenmeyer flasks containing 50 mL of MSM supplemented with different concentrations (50, 100, 200, 400, 600 mg/L) of PCP. The flasks were incubated at 37°C under shaking conditions for 168 hr. The growth of the bacterial cells was determined by measuring the optical density at 600 nm and the degradation of PCP in the culture filtrate by HPLC.

1.5 Degradation of PCP in the secondary sludge

The PCP degradation ability of CL7 was analyzed in the secondary sludge by inoculating 5% of inoculum in 2.5 L conical flasks containing one liter of sludge supplemented with 100 mg/L of PCP. The flasks were incubated at 37°C under shaking condition for two weeks. PCP was extracted from the sludge by the method described by Chandra et al. (2008) with a little modification. The sonicated sludge sample was acidified by 1 mol/L HCl to pH 2.0; extracted three times with an equal volume of

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ethyl acetate by intermittent shaking for 30 min in standard separating funnel. The organic layer was dried with anhydrous sodium sulphate to absorb excess of water. Filtered samples were evaporated to dryness at 40°C, subsequently resuspended in 1 mL of methanol. Quantification of PCP present in the bacterial degraded sludge was determined by HPLC.

The data were analyzed by analysis of variance, and the means were compared with Tukey's test at $p < 0.05$. Three replicates were maintained for each treatment

2 Results and discussion

2.1 Characterization of CL7 isolate

The biochemical characterization of CL7 isolate is presented in Table 1. CL7 is Gram negative, motile, aerobic, oxidase- and urease positive and able to hydrolyze starch. Susceptible to different antibiotics such as calithromycin, co-trimoxazole, netilin, cefaclor and ampicillin. Acid is produced from fructose, sodium gluconate, glycerol, salicin, ONPG, esculin, citrate, and malonate. The bacterium was further identified by 16S rRNA sequence analysis. The nucleotide BLAST and RDP-II analyses showed that CL7 belong to the phylum proteobacteria and family *Pseudomonadaceae*. Phylogenetic analysis revealed that CL7 have very close similarity with *Pseudomonas stutzeri* and hence identified as *P. stutzeri* (Fig. 1).

2.2 Degradation of PCP by CL7

P. stutzen CL7 was able to grow and utilize PCP as an energy source. The growth of bacterial strain was significantly increased up to 72 hr and decreased thereafter and attained stationary phase up to 168 hr. It was observed that CL7 utilized more than 60% of PCP within 24 hr and above 90% at 72 hr. The PCP was completely mineralized after 120 hr of incubation (Fig. 2). The bacterial growth curve with correspondence to liberation of chloride ion as shown in Fig. 3, revealed that the strain CL7 achieved good growth with simultaneous liberation of chloride ion. The initial concentration of chloride ion was 200 mg/L but during the course of bacterial treatment the liberation of inorganic chloride ion in culture medium increased up to 478 mg/L at the 168 hr treatment. The growth of CL7 and degradation of PCP were observed under culture conditions such as different temperatures and initial pH values. The results revealed that CL7 was able to grow well at 37°C and degrade 93.5% of PCP at this temperature compared to 25 and 30°C (Fig. 4a). The effects of initial pH value on growth and degradation of PCP are shown in Fig. 4b. These results indicated a higher PCP degradation (96.5% and 94.5%) between pH 7.5 and 8.5. The growth

Table 1 Cultural and biochemical characteristics of *P. stutzeri* (CL7) isolated from the sludge of pulp and paper mill

Characteristics	Observation
Form	Rods
Gram stain	–
Motility	+
Catalase test	–
Oxidase test	+
Nitrate reduction test	–
Urease test	+
Starch hydrolysis	+
Antibiotic sensitivity	Calaritromycin; co-trimoxazole; netillin; cefaclor; ampicillin/sublactam
Fructose, sodium gluconate glycerol, salicin, ONPG, esculin, citrate, malonate	+

+: positive; – : negative.

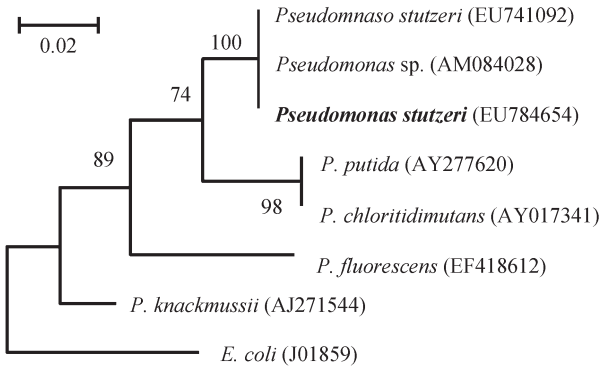

Fig. 1 Neighbor-joining tree based on 16S rRNA sequence CL7 of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1500 replicates.

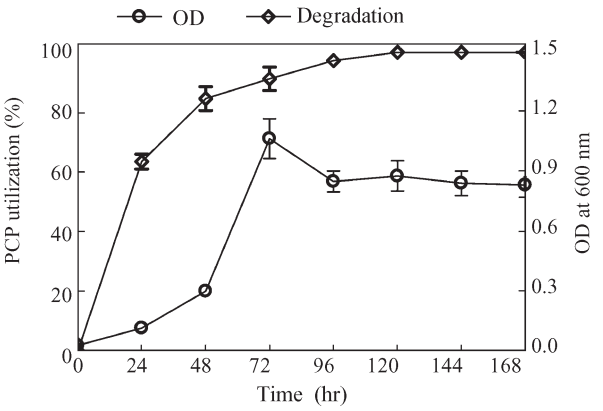

Fig. 2 Growth curve of *P. stutzeri* CL7 in mineral salt media containing 100 mg/mL of PCP as a sole carbon or energy source.

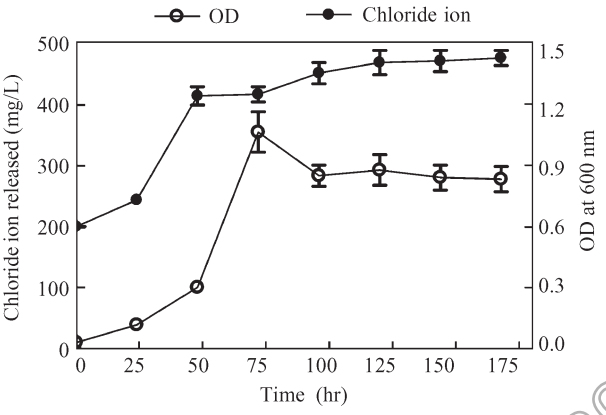

Fig. 3 Release of chloride ion in the medium at different time intervals due to the degradation of PCP by *P. stutzeri* CL7.

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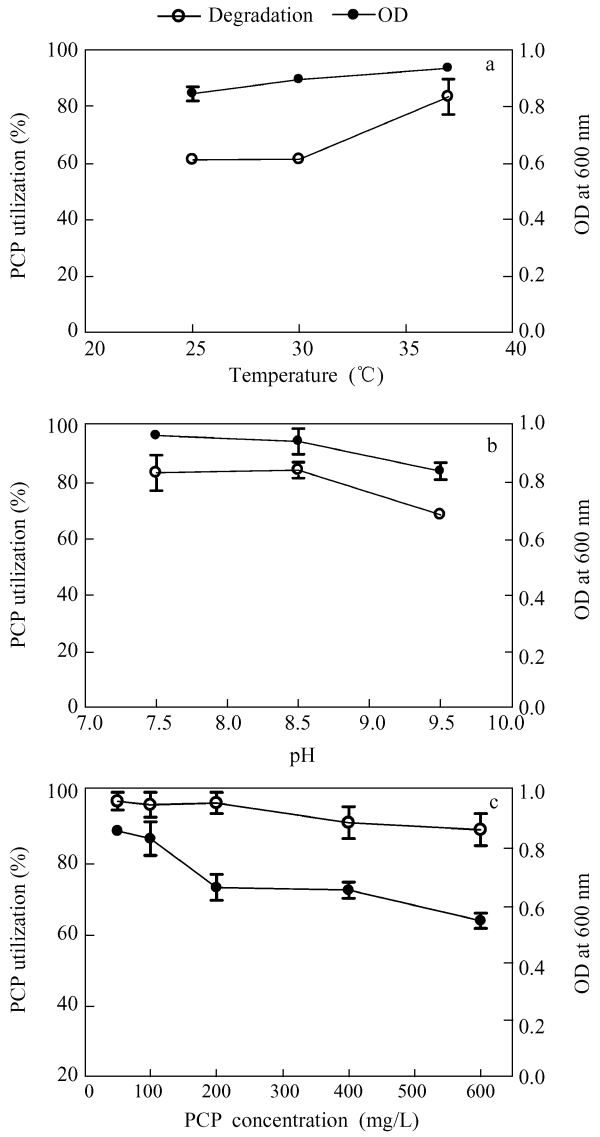

Fig. 4 Effect of temperature (a) and pH (b) and different PCP concentrations (c) on the growth and degradation of PCP by *P. stutzeri* CL7.

of the bacterial strain and the degradation of PCP were reduced at pH 9.5. The growth of CL7 was significantly reduced as the concentration of PCP in the mineral salt medium increased. It was able to degrade 89% PCP at all concentrations. More than 95% degradation of PCP was recorded up to 200 mg/L of PCP and decreased at 400 and 600 mg/L (Fig. 4c).

The bacterial strain CL7 was capable of completely mineralizing PCP when grown in 100 mg/L of PCP utilizing it as sole source of carbon. Some previous reports also showed the degradation of PCP by other *Pseudomonas* species (Radehaus and Schimdt, 1992; Shah and Thakur, 2002; Sharma and Thakur, 2008). The release of chloride ion in the medium increased with increase in time. The liberation of chloride ion from the medium can be considered as the result of mineralization of chlorinated compounds by the bacterium thereby release of chloride in the medium. Available data of earlier studies indicated that chlorinated phenols were mineralized to chlorine free end products (Homada et al., 1987; Kennes et al., 1996; Radehaus and Schimdt, 1992; Mohn and Kennedy, 1992). The maximum PCP degradation was observed at pH between 7.5 and 8.5 in this study. Wolski et al. (2005) reported the degradability of PCP at pH values from 6.3 to 8.0, with maximum rate of PCP degradation at pH 6.3 by *Pseudomonas* species. The optimum temperature for the growth and degradation of PCP was observed at 37°C in this study. Sharma et al. (2009) reported the temperature range of 25–35°C for PCP degradation by *Acinetobacter* species. The growth and degradation of PCP decreased in the medium as the concentration of PCP increased from 50–600 mg/L. The possible explanation for reduction in degradation of PCP by the bacterial strain CL7 might be due to decreased activity of the degrading enzymes at lower pH, as the pH of the medium decreased significantly at higher concentration (data not shown). The present study results showed that CL7 was able to utilize PCP as a sole source of carbon.

2.3 Degradation of PCP in sludge

The bacterial strain has been shown to degrade PCP in mineral salt medium at higher concentrations in this study. Therefore, this strain was used for the treatment of pup and paper mill sludge. The initial PCP concentration of the sludge was about 0.029 mg/L, and therefore, 100 mg/L of PCP was amended to the sludge to study the efficacy of this strain in degradation of PCP. The survival of this strain was monitored by determining the growth in the form of colony forming units (cfu). The growth of the bacterial strain increased from the initial cfu of 2.38×10^4 to 10.78×10^6 after two weeks. HPLC analysis shown in Fig. 5 revealed that this strain is capable of mineralizing PCP from the sludge. It is able to remove up to 66.8% PCP from the sludge.

Many biological treatment methods are utilized to clean up soils contaminated with chlorinated phenols. One approach towards *in-situ* remediation is the addition of microbial cells or enzyme to enhance the bioremediation process of contaminated site. Although *in-situ* remediation of PCP studies was reported previously, they mainly deal with compost, manure and soil (Miethling and Karlson, 1996; Laine and Jorgensen, 1997). Very few reports are

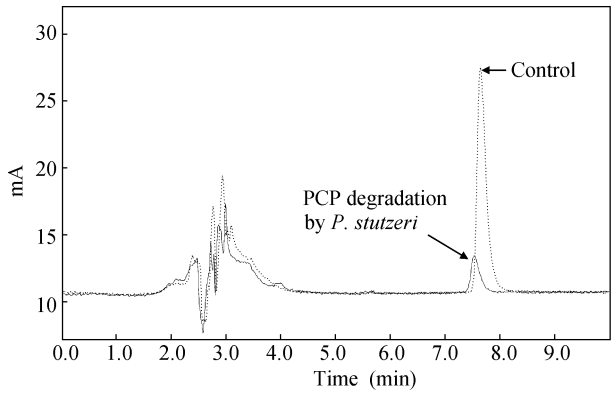

Fig. 5 HPLC chromatogram of PCP degradation in the sludge by *P. stutzeri* CL7 compared with control.

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available with removal of PCP from the sludge. In the present study, CL7 mineralized 66.8% PCP from the sludge where the PCP concentration was more than 100 mg/L. Chandra et al. (2008) also reported the removal of PCP from the effluent of pulp and paper by 85% and 90% by *Bacillus cereus* and *Serratia marcescens* respectively where the PCP concentration was 50.3 mg/L. These results suggested that CL7 is more efficient in degradation of PCP from pulp and paper mill sludge.

3 Conclusions

In conclusion, the results obtained by this study indicated that the bacterial strain CL7 is able to mineralize high concentrations of PCP. This strain also degraded 66.8% of PCP from the sludge within two weeks of treatment. These results highlight the potential of this bacterium to be used in bioremediation of high strength PCP contaminated pulp and paper mill sludge.

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