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Pentachlorophenol Remediation by *Enterobacter* sp. SG1 Isolated from Industrial Dump Site

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Abstract: Chlorophenols contamination is serious concern to the environment due toxicity to all forms of life. Among all the chlorophenols, pentachlorophenol (PCP) is more detrimental to the environment. Pentachlorophenol used as pesticide, herbicide, antifungal agent and wood preservative which causes environmental pollution. In the present research a PCP degrading bacterium was isolated and characterized from industrial dump site. 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, ring cleavage activity and biomass formation. Based on morphological, biochemical and 16S rRNA gene sequence analysis this strain was identified as *Enterobacter* sp. SG1. Gas Chromatography (GC) analysis revealed that this strain was able to degrade PCP up to a concentration of 2 mM. This study showed that the removal efficiency of PCP by SG1 was found to be very effective and can be used in degradation of PCP contaminated site or waste in the environment.

Key words: Pentachlorophenol, *Enterobacter* sp., 16srRNA gene sequence, GC-analysis, bioremediation, industrial dump site

INTRODUCTION

The chlorophenolic compounds are major environmental contaminants giving global concern mainly due to use of these compounds as wide spectrum biocides in industry and agriculture. Over recent decades, significant quantities of industrial, agricultural and domestic chemicals have been released into the environment (Sharma and Thakur, 2008). PCP and its sodium salt have toxic effect on bacteria, mould, fungi and algae (Kao *et al*., 2005). Humans are exposed to PCP through inhalation, absorption through the skin and consumption of contaminated food and water (Nnodu and Whalen, 2008). PCP was registered for public use by the United States Environmental Protection Agency (USEPA) until 1984. The safe permissible limit of PCP in water is 0.30 μg L^{-1} but many industries throughout the world releasing sludge and effluents is far above than mentioned concentration (EPA, 1999).

Several methods like physical, electrophysical chemical methods have been attempted for the removal of this chlorophenols. All these method converting it chemically to even more hazardous by-products and additional treatment is frequently needed to destroy hazardous material (Benitez *et al*., 1999). An alternative to these methods focuses on biological methods of hazardous waste treatment usually defined as bioremediation. Bioremediation offers the possibility of detoxifying or degrading contaminants to harmless by-products. Bioremediation is also often the least expensive method of cleanup. Aerobic degradation of PCP has been studied extensively and several bacterial isolates such *Kocuria* sp. CL2 (Karn *et al*., 2011) *Bacillus* sp. (Karn *et al*., 2010) *Novosphingobium lentum* (Dams *et al*., 2007) *Pseudomonas* sp. (Kao *et al*., 2005) and *Sphingomonas chlorophenolica* (Yang *et al*., 2006) were found to utilize PCP as a sole source of carbon and energy. Earlier *Enterobacter* sp. was reported for the degradation of pentachlorophenol (100 mg L^{-1}) and some other hydrocarbon at very less concentration. In this study, a PCP degrading bacterium was isolated and characterized from industrial dump site and tested for their ability to degrade PCP under *in vitro* conditions.

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MATERIALS AND METHODS

Reagent sources: PCP was purchased in 98% purity from Aldrich Chemical Company Inc. USA. Stock solution of 10 mM PCP was prepared in methanol (purchased from SD Fine Chemicals, Mumbai, India) and water (1:1).

Sampling, isolation and enrichment of PCP degrading bacteria: Sludge samples were collected from industrial dumpsites of distilleries industries at Noida (Near Delhi), India in sterile reagent bottles and stored at 4°C. After one day Screening of PCP degrading bacteria was done using nutrient enrichment technique in mineral salt medium (MSM) described (Karn *et al*., 2011) containing PCP as sole source of carbon and energy. Colonies appeared after 48 h were selected. MSM was used as growth medium (pH 7.3) supplemented with PCP. PCP was added to the medium after autoclaving, PCP was sterilized by syringe filter through 0.2 μm membrane). Bacteriological agar at 1.5% (w/v) was added for MSM plate preparation.

Morphological and biochemical characterization: The identification of PCP degrading bacterium was carried out according to Bergey's Manual on Systematic Bacteriology by Holt *et al*. (1994). Catalase activity was determined by detective bubble formation with 3% H_2O_2 solution. Oxidase was determined by using paper disc with tetramethyl-p-phenylenediamine. Nitrate reduction by using nitrate agar plate; DNAase test by DNAase agar medium with methyl green as an indicator. Starch hydrolysis was determined on starch hydrolyzing agar by detecting cleared zones formed around the colonies. Antibiotic profiling was also done by using Hexa disc G plus 1 and minus 9 (Hi-Media Laboratories, Mumbai, India) having twelve different antibiotics of various concentrations according to the manufacturer's instructions.

Amplification of 16S rRNA gene and sequence analyses: Genomic DNA extraction, amplification of 16S rRNA gene and sequence analysis was performed as described by Krishna *et al*. (2008). The sequences were compared against the available DNA sequences in GeneBank (http://www.ncbi.nlm.nih.gov/) using the BLASTN tool. The 16S rRNA gene sequence determined in this study was deposited in the GenBank of NCBI data library under the accession number JX448403.

Screening, Inoculum preparation and Growth analysis: Screening of PCP degrading bacterial strain was done by growing the bacterial isolates in MSM supplemented with 0.4 mM of PCP and placed on shaking incubator at 120 rpm at 37°C for 24 h. Growth of the culture was observed by UV-VIS spectrophotometer at wavelength 600 nm (Shimadzu, UVmini-1240 Tokyo, Japan). Culture was used as inoculum when absorbance was reached approximately 0.5. Growth of the bacterial cells was observed in MSM containing different concentration of PCP (0.4, 0.8, 1.5 and 2 mM) by taking absorbance at 600 nm on spectrophotometer from 0 to 168 h at an interval of 24 h.

Chloride release assay: The chloride ions released during degradation of PCP in MSM were measured by titration method. To 1.0 mL of culture filtrate added to the titration dish, there was added 2 to 3 drops of phenolphthalein indicator. If a pink color appeared, it was titrated with N/50 sulfuric acid until the color changed from pink that of the original sample. Next was added 10 to 15 drops of potassium chromate indicator to give the filtrate a bright yellow color. Silver nitrate (0.028 N) was added from a pipette drop wise, stirring continuously with a stirring rod, until the sample just turned from yellow to an orange-red. A calculation was done by part per million (ppm) = mL of silver nitrate used x 1000 (OFI Testing Equipment, Huston, USA).

Bromothymol blue test and determination of ring cleavage: Presumptive dissimilation tests were performed in agar plates containing MSM plus 20 ppm of PCP and 20 mg L^{-1} of bromothymol blue as an indicator. This test is based on the acidification of the medium due to release of HCl, when dechlorination takes place. Overnight grown cells were taken to determine ring cleavage activity; 10 mL cell suspension was centrifuged at 7000 rpm for 10 min to separate the biomass. The supernatant and biomass were collected for extracellular and intracellular activity respectively. To check intracellular activity bacterial cells were lysed by adding 2 mg mL^{-1} of lysozyme in TES buffer (1 mL) in pellet portion and kept for 1 h for proper lysis of bacterial cell. The mixture was treated with toluene (1 mL) and 0.01 M catechol (1 mL). The development of the yellow color was observed for meta cleavage. In absence of color, the mixture was shaken for 1 hour at 170 rpm for formation of b-ketoadipic acid (Rothera reaction) which indicated the presence of ortho fission Kharoune *et al*. (2002). In this procedure, the above mixture was acidified with 2 mL HCl (10.2 M). One milliliter $NaNO_3$ (1%) was added to the above followed by concentrated ammonia 15 mL and 1 mL ferrous sulfate solution (10%). In the supernatant, the same process was followed for meta and ortho cleavage. The development of a reddish brown color indicated a typical Rothera reaction and the presence of ortho cleavage. MSM containing PCP with no bacterial culture was used as a control for the ring cleavage assay.

Degradation of PCP: The degradation ability of the isolated strain was analyzed in 250 mL of Erlenmeyer flask containing 50 mL of MSM supplemented with 0.4, 0.8,

1.5 and 2.0 mM of PCP separately. Next 5% inoculum was added to each flask and incubated on a shaker at 37°C and 120 rpm/min. Degradation efficiency was determined by consumption of PCP from the culture medium. In extraction of PCP, the cell suspension was clarified by centrifugation at 8,000 rpm for 5 min. The cell free supernatant fractions were extracted three times with an equal volume of n-hexane by shaking vigorously for 15 min in a standard separating funnel. The organic layer was dried with anhydrous sodium sulfate, the residue was finally dissolved in 50 μL mixture of n-hexane: ethyl acetate (10:1) and analyzed immediately on a GC. The GC analyses were performed in electron capture detection mode with a gas chromatograph Nucon GC-5765 (Centurion Scientific, New Delhi, India) capillary column DB-5 (30 m length 0.025 mm i.d. 0.25 μm film thickness) was used at a temperature programme of 50°C (2 min), then raised to 10°C min^{-1} to 280°C, where it was held for 10 min. Helium was used as the carrier gas at a constant flow of 1.2 mL min^{-1}. The samples were analyzed in split less mode at an injection temperature of 250°C and detector temperature 280°C, The injected volume was 0.1 μL.

Statistical analysis: Data were statistically analyzed by analysis of variance (ANOVA) and the mean differences were compared by Tukey-Kramer Multiple Comparison Test at $p<0.05$. All experiments were performed in three replicates and analyses were performed using GraphPad Prism (v 4.03) software. (CA, USA).

RESULTS

Isolation and screening: The isolation of bacteria was done by enrichment technique. The PCP degrading bacterial strain were isolated by the serial dilution method and purified by repeated streaking on MSM plates. Growth was observed after incubation at 37°C for 48 h. Colonies appear were selected for further screening. A total of seven bacteria were isolated on basis of their morphology from sludge sample and were represented as SG1 to SG7. These isolates were subcultured and transferred to MSM agar plates. These isolates were transferred in MSM broth supplemented with 0.4 mM PCP. Isolates which were able to grow in this concentration were selected for their ability to grow at higher concentrations of PCP. Only SG1 strain was selected for further degradation studies because other strains were not able to grow.

Morphological and biochemical and molecular characterization: The bacterial colonies grown on this media were further screened and the bacterial strain SG1 was selected based on the growth and utilization of PCP as sole source of carbon and energy. Isolate SG1 has gram negative rod shaped. The results of the biochemical characterization of the SG1 strain are presented in (Table 1). This isolate was sensitive to six different the antibiotics used in this study with varied concentration mentioned in (Table 2) and showed sensitive to three antibiotics Gentamicin (10), Chloramphenicol (30) and Co-Trimoxazole (25 mcg/disc). Partial sequence data of the isolate was analyzed by BLAST search. The BLAST analysis of this sequence showed 99% of sequence similarity with *Enterobacter* sp.

Table 1: Morphological and biochemical characteristics of SG1

Test	Results
Shape	Rod
Gram staining	-
Catalase test	+
Capsule staining	-
Oxidase test	-
DNase test	-
Nitrate reduction test	+
Starch hydrolysis	-

+: Positive, -: Negative

Table 2: Antibiotic sensitivity of SG1

Antibiotics (mcg/disc)	Sensitivity (S)/Resistant (R)
Gentamicin (10)	S
Nalidixic acid (30)	R
Chloramphenicol (30)	S
Co-Trimoxazole (25)	S
Clindamycin (2)	R
Cephalothin (30)	R
Gentamicin (10)	R
Erythromycin (15)	R
Penicillin G (10)	R

Total 9 antibiotic were tested

Degradation of PCP: Strain SG1 was able to grow and utilize PCP as an energy source. At concentration 0.4 mM of PCP, the growth of SG1 increased significantly up to 120 h (Fig. 1). Initially the growth was slower in the MSM but the growth increased in the medium after 24 h of incubation and the maximum growth was achieved at 72 and 96 h of the incubation period. At concentration 0.8 mM and 1.5 mM of PCP, SG1 has showed same growth pattern at 96 h after that there was decrease in growth at 1.5 mM. Further growth of SG1 increased up to 120 h and reached to stationary phase thereafter. At 2 mM maximum growth was observed on 96 and 120 h of incubation period. At initial stages SG1 showed similar growth pattern up to 48 h further there was decrease in the growth and remains stationary (Fig. 1). When strain SG1 were grown on MSM agar plates supplemented with 0.1 mM of PCP, formation of yellow color zone was observed in the surrounding bacterial growth (Fig. 2). Bromothymol blue is a pH indicator which gives green

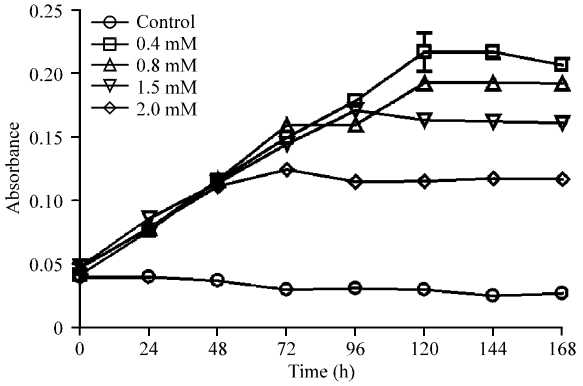

Fig. 1: Growth profiles of SG1 in MSM containing 0.4, 0.8, 1.5 and 2.0 mM of PCP as a sole carbon and energy source, error bars represent the standard deviation

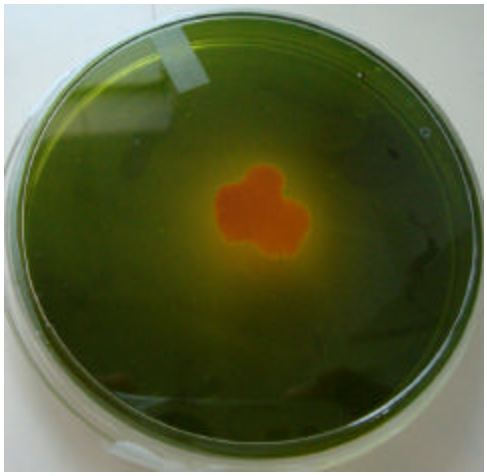

Fig. 2: Bromothymol blue test: yellow coloured zone were produced by strain SG1 on MSM medium containing PCP and bromothymol blue as indicator

color at pH 7.3 and turns to yellow as pH decreases. Here pH decreased is due to formation of HCl due to release of chloride in the medium. The ring fission of PCP was also determined. Yellow color not appeared after mixing catechol in cell suspension which indicated the absence of meta cleavage by visual inspection. However, a positive Rothera test was observed after 144 h. The exact enzymatic mechanism is not known for ring cleavage enzymatic activity at this stage, further study is needed. Chloride release assay was also observed during degradation studies by SG1 strain, released inorganic chloride ion was measured it was found that chloride ion concentration increased in the medium with increase of time. At concentration 0.4 mM of PCP, initial chloride ion

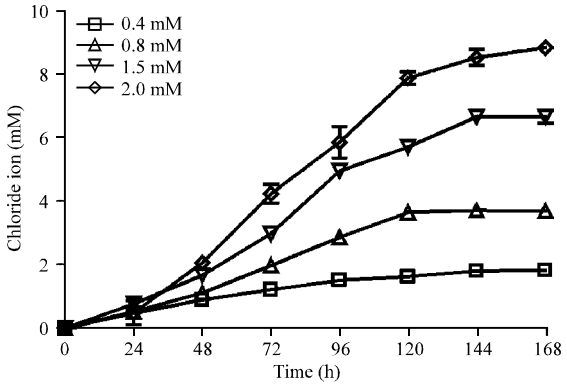

Fig. 3: Chloride ion released in MSM supplemented 0.4, 0.8, 1.5 and 2.0 mM of PCP by the bacterial isolates during course of incubation

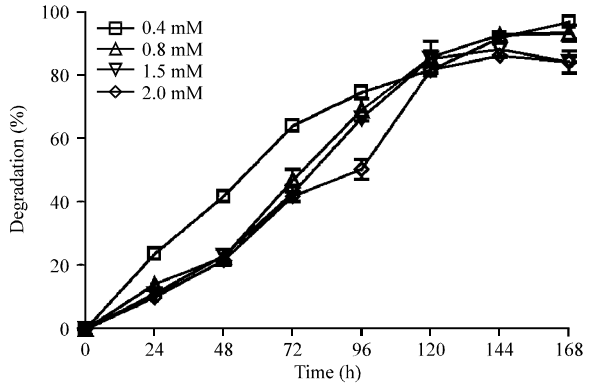

Fig. 4: Degradation of PCP at different concentration 0.4, 0.8, 1.5 and 2.0 mM of PCP and at different time intervals

was about 0.5 mM at the 24 h and reached up to 1.8 mM at 144 h of incubation period. At 0.8 mM initial releases was less but at 120 h reached to 3.7 mM at 1.5 mM and 2 mM maximum chloride released was 6.5 and 8.6 mM respectively (Fig. 3). Chloride release into MSM was linked with the utilization of PCP as carbon and energy source by the bacterial isolates Further PCP degradation was confirmed by GC-analysis shown in Fig. 4. *Enterobacter* sp. was able to degrade PCP up to 85% at 2 mM concentration.

DISCUSSION

Characterization of PCP degrading bacteria from industrial sludge: Microbial diversity is the most extraordinary reservoir of life in biosphere that can be exploited for innovative applications useful to mankind. These include the use of microorganisms for production

of novel compounds and biodegradation of xenobiotic pollutants. Microorganisms are natural recyclers, have the ability to transform and/or degrade xenobiotics. So microbial diversity offers an immense field of environment-friendly options for mineralization of contaminants. Many scientists have been exploring the microbial diversity, because of their varied effects on environment, particularly of contaminated areas in search for organisms that can degrade a wide range of pollutants. Microorganisms with specific degradation capabilities may be isolated by adding the compound of interest to enrich those cells from environmental samples. Isolates can then be obtained on culture medium containing the compound as a carbon source. In this manner one may select for those microorganisms that are able to metabolize or at least survive in the presence of pollutant compounds. Present study showed degradation of PCP by bacterial isolates from industrial dump site. In this study total seven bacteria were isolated from distillery dumpsite sludge using enrichment method. Only SG1 bacterial strain with relatively high degradation ability were selected and discussed in this work. The bacterial strain was identified morphologically and biochemically as *Enterobacter* sp. The strain were also identified by method based on 16s rRNA gene sequence analysis. BlastN analysis of 16s rRNA gene sequence confirmed as *Enterobacter* sp.

Degradation of PCP: There is extensive evidence that chlorophenols are mineralized by bacteria that utilize chlorophenolic compounds as a carbon and energy source. The evidence is based on the stoichiometric release of inorganic chloride and the concomitant production of biomass linked to chlorophenol utilization (Radehaus and Schmidt, 1992). This strain is capable of tolerating up to 2 mM of PCP and also degraded 85% of PCP in the culture medium. An increasing concentration has negative effect on growth and degradation. Previously Gautam *et al.* (2000) also reported that at 500 mg L^{-1} of PCP has negative effect on the bacterial consortium degrading PCP. Earlier Nandish (2005) observed *Enterobacter* sp. was able to degrade 70% of PCP at 100 mg L^{-1}. Saber and Crawford (1985) isolated *Flavobacterium* by enrichment method from PCP contaminated soils which were able to mineralize 100 to 200 ppm PCP as a sole source of carbon and energy and liberated chlorine as chloride into the medium. Radehaus and Schmidt (1992) reported complete mineralization of PCP (160 mg L^{-1}) in a week by *Pseudomonas* sp. strain RA2. Singh *et al.* (2005) reported that the bacterial isolate *Serratia marcescens* from pulp and paper mill waste was able to degrade 90.3% of PCP within 168 h when grown with 300 mg L^{-1} of PCP. Shah and Thakur (2002) observed 72% PCP removal by *Pseudomonas fluorescence* (TE3) in 96 h when grown with 100 mg L^{-1} PCP. Sharma and Thakur (2008) observed that *Pseudomonas aeruginosa* (PCP2) utilized 60% of PCP within 96 h of incubation. Premlatha and Rajkumar (1994) recorded the complete degradation of PCP (800 mg L^{-1}) by *Pseudomonas aeruginosa* in 6 days with glucose as a co-substrate. By observing the previous report present isolate *Enterobacter* sp. SG1 removal efficiency is quite comparable and showed potential to the other isolates.

The lower chlorinated phenols are attacked by monooxigenase yielding chlorocatechol as the first intermediates (Chlorocatechol pathway) which are subjected to ring cleavage prior to dechlorination. On the other hand polychlorinated phenols (3 to 5 chlorine) are converted to chlorohydroxyquinones as the initial intermediate (hydroxyquinone pathway). Subsequent reaction progressively removes chlorine from the ring prior to ring cleavage (Solyanikova and Golovleva, 2004). The results of this study clearly show that SG1 strain is able to utilize PCP as chloride ion concentration increased significantly with increase of PCP degradation. 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 are mineralized to chlorine free end products (Radehaus and Schmidt, 1992; Mohn and Kennedy, 1992). In the present study the bacterial strain SG1 was able to grow and remove the PCP more than 85% in 2 mM indicating the efficiency *Enterobacter* sp. SG1 able to remove PCP at higher concentrations. Apart from PCP, this strain also capable of degrading 2,4,-Dchlorophenol (unpublished data).

The critical step in the degradation of chlorinated compounds is the cleavage of the carbon-chlorine bond (Mohn and Kennedy, 1992). Two different catabolic pathways appear to have evolved for chlorophenols degradation. The first pathway involves dehalogenation after ring cleavage, common for mono, di and some trichlorophenols. The ortho ring cleavage of 3,5-dichlorocatechol (DCC) may be the rate-limiting step in the dehalogenation of PCP after ring cleavage. It has been postulated by Ditzelmuller *et al.* (1998) that the ring cleavage of halocatechols is the crucial reaction step in haloaromatic degradation because of the inefficient ortho transformation of halocatechols by ordinary (non-chloroaromatic) ring-cleavage enzymes (Clement *et al.*, 1995). PCP may be biodegraded through a similar pathway (Ettala *et al.*, 1992). In this study, ring fission of PCP was determined. Yellow color not appeared

after mixing catechol in cell suspension which indicated the absence of meta cleavage. However a positive rothera test was observed after 144 h. The exact mechanism for ring cleavage is not clear. These tests confirmed degradation of PCP by strain SG1. Further the degradation was also confirmed by the GC analysis. The study has, thus come out with an efficient, stable bacterial strain capable of degrading PCP. However, scale up studies need to be tested for bioremediation of PCP at industrial scale and also need to know the genes responsible for biodegradation of these compounds and their expression.

CONCLUSION

Enterobacter sp. SG1 is efficient aerobic bacterial strain having high degradation capability for PCP up to 2 mM concentration. Therefore this strain can be used for the removal of PCP containing soil and water in the environments.

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