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ORIGINAL PAPER

Degradation of pentachlorophenol by *Kocuria* sp. CL2 isolated from secondary sludge of pulp and paper mill

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Abstract A pentachlorophenol (PCP) degrading bacterium was isolated and characterized from sludge of pulp and paper mill. 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. Based on morphology, biochemical tests, and 16S rRNA gene sequence analysis this strain was identified as *Kocuria* sp. CL2. High Performance Liquid Chromatography (HPLC) analysis revealed that this strain was able to degrade PCP up to a concentration of 600 mg/l. This is first time we are reporting the degradation of PCP by the *Kocuria* species. This isolate was also able to remove 58.64% of PCP from the sludge within two weeks. This study showed that the removal efficiency of PCP by CL2 was found to be very effective and can be used in degradation of PCP containing pulp paper mill waste in the environment.

Keywords *Kocuria* sp. CL2 · Sludge · Biodegradation · Characterization · Chloride ion · Pentachlorophenol

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Introduction

The chlorophenolic compounds are common environmental contaminants originating mainly from the use of the compounds as wide spectrum biocides in industry, agriculture, and their formation during pulp bleaching and some direct industrial waste discharge (Vallecillo et al. 1999). These are most persistent environmental pollutants because of their physicochemical 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) has widely been used as wood and leather preservative, owing to their toxic effect on bacteria, mould, fungi and algae (Kaoa et al. 2005). PCP is toxic to all life forms as it inhibits the oxidative phosphorylation (Yang et al. 2006) and 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). 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. Aerobic degradation of PCP has been studied extensively and several bacterial isolates such as *Mycobacterium chlorophenolicum* (Wittmann

et al. 1998), *Rhodococcus chlorophenolicum* (Apajalahti et al. 1986), *Flavobacterium* (Gonzalez and Hu 1991), *Novosphingobium lentum* (Dams et al. 2007) *Pseudomonas* species (Kaoa et al. 2005) and *Sphingomonas chlorophenolica* (Yang et al. 2006) were found to utilize PCP as a sole source of carbon and energy. 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 isolates indigenous to sites contaminated with PCP. In this study, a PCP degrading bacterium was isolated and characterized from sludge of pulp and paper industry and tested for its ability to degrade PCP under in vitro and in situ conditions.

Material and methods

Isolation and enrichment of PCP degrading bacterium

Sludge sample was collected from the effluent treatment plant site of a pulp and paper mill at M/s Shree Gopal Unit-Yamunanagar, Haryana, India. The samples were taken into sterilized conical flask and 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 h were selected for further screening. Screening of PCP tolerant bacterial strains were done by the nutrient enrichment technique in mineral salt medium (MSM) (Dams et al. 2007) supplemented with 50 mg/l (0.185 mM) of pentachlorophenol as a sole source of carbon for energy. 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. The pH of the medium was adjusted to 7.5. PCP was added to the medium after autoclaving. All reagents used were of analytical grade. Synthetic PCP was purchased from Sigma Aldrich chemicals (USA). All solutions were prepared in sterile Milli-Q water (Millipore direct Q3, Bangalore, India). Pentachlorophenol was dissolved in small amounts of methanol and Mili1-Q water was added to make the required volume where the methanol concentration did not exceed more than 0.2%.

One bacterial isolate with highest PCP degradation efficiency was selected and designated as CL2 for further study. This isolate was deposited at Microbial Culture Collection Centre (MTCC), Institute of Microbial Technology, Chandigarh, India.

Biochemical and phenotypic characteristics

The identification of PCP degrading bacterium was carried out according to Bergey's Manual on Systematic Bacteriology (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. Motility, Indole and Urease activities were determined by using MIU-media kit (Hi-Media Laboratories, India) and antibiotic profiling was also done by using ICOSA universal-1 kit (Hi-Media Laboratories, India) having twenty different antibiotics of various concentrations according to the manufacturer's instructions. The antibiotics and their concentrations are: Norfloxacin (10 μg), Gentamicin (10 μg), Chloramphenicol (30 μg), Cefuroxime (30 μg), Ciprofloxacin (5 μg), Cefaperazone (75 μg), Ceftazidime (30 μg), Roxithromycin (30 μg), Calaritromycin (15 μg), Co-Trimoxazole (25 μg), Netillin (30 μg), Cefaclor (30 μg), Cephotaxime (30 μg), Cephadroxil (30 μg), Azithromycin (15 μg), Ampicillin/Cloxacillin (10/10 μg), Penicillin (10 units), Amikacin (30 μg), Sparfloxacin (5 μg) and Ampicillin/sublactam (10/10 μg).

16S rRNA sequence and phylogenetic analysis

Genomic DNA was extracted from overnight grown cultures by a simple lysis protocol, as described in Kapley et al. (2001). One μl of purified genomic DNA served as template. 16S rRNA gene was amplified using GeneAmp 2700 PCR systems (Applied Biosystems, USA) with the following set of primers: forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′ and

reverse primer: 5′-ACGGGCGGTGTGTTC-3′ as described in Weisburg et al. (1991). Reaction mixture for the PCR contained 1× 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 sterile MQ water. The PCR was performed with an initial denaturation at 92°C for 2 min followed by 36 cycles of 92°C for 1 min, 48°C for 30 s and 72°C for 2 min and a final extension of 72°C for 6 min. The amplification product was gel purified using QIAgel extraction kit (Qiagen, USA), and ligated into the pGEM-T easy vector as per manufacturer's instructions (Promega Inc., USA). Ligated plasmid was transformed into *E. coli* DH5α cells by calcium chloride treatment and heat shock method. After screening of the positive clones, the partial sequence was generated by chain termination method using an Applied Biosystems automated DNA sequencer (DNA sequencing facility, Delhi University, India). The sequence was compared against the available DNA sequences from type strains in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN sequence match tool. The sequences were aligned using MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html) program and the alignment was manually corrected and phylogenetic tree was constructed using the MEGA 4.0 (Tamura et al. 2007) software. The sequence was deposited in GenBank database under the accession number EU784649.

Degradation of PCP by isolated strain

The degradation studies were performed by inoculating 1% inoculum (10^6 CFU/ml) of the bacterial strain CL2 in 250 ml Erlenmeyer flasks containing 50 ml of MSM supplemented with 100 mg/l (0.37 mM) of PCP. The flasks were incubated at 37°C under shaking conditions (120 rpm) up to 168 h. Growth of the bacterial cells was determined by measuring the optical density at 600 nm and the PCP degradation was analysed by HPLC. The cell suspension was centrifuged (5 min, 8000 rpm) and supernatant was filtered through 0.22 μm filters. HPLC was carried out with purified samples on Perkin Elmer Series (200) system. Samples (10 μl) were injected and separated on reverse phase (Licrosphere® 100 RP-18 endcapped column, 250 mm × 4.6 mm i.d.) in an isocratic mode using aqueous methanol 90% (v/v) at a flow rate of 1 ml/min. The eluates were monitored at 280 nm with online diode array detector (series 200). PCP was quantified on the basis of standard curve prepared by taking known quantities of PCP (Sigma–Aldrich, USA). Chloride ion released in the aqueous medium was determined at every 24 h of interval up to 168 h using 5 ml of culture filtrate. The level of chloride ion was then measured with an Orion ion analyzer model 940 using calibrated selective chloride ion electrode. The electrode was calibrated by adding known concentrations of sodium chloride standards to the MSM used in the experiments.

The effect of different concentrations of PCP on the growth of CL2 and degradation ability of this strain was also studied. The bacterial strain CL2 was inoculated to 250 ml Erlenmeyer flasks containing 50 ml of MSM supplemented with different concentrations (50, 100, 200, 400, 600 mg/l or 0.185, 0.37, 0.75, 1.5 and 2.2 mM) of PCP. The flasks were incubated at 37°C under shaking conditions for 168 h. The growth and the degradation of PCP in the culture filtrate were determined as mentioned previously.

Degradation studies of PCP in sludge

The degradation ability of the isolated strain CL2 was analyzed in the sludge by inoculating 5% of inoculum in 2.5 l conical flasks containing 1 l of sludge supplemented with 100 mg/l (0.37 mM) of PCP. The flasks were incubated at 37°C under shaking condition for 2 weeks. PCP was extracted from the sludge by the method described in Chandra et al. (2006). The sludge sample was acidified by 1 N HCl to pH 2.0; extracted thrice with an equal volume of 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 under vacuum at 40°C, subsequently resuspended in 1 ml of methanol. Quantification of PCP present in the bacterial degraded sludge sample was determined by HPLC. The growth of CL2 was determined in both PCP amended and unamended sludge.

Statistical analysis

Data were statistically analysed by analysis of variance (ANOVA) and the mean differences were compared by Tukey–Kramer Multiple Comparison

Test at $p < 0.05$. Three replicates were maintained for each treatment. All the analyses were performed using GraphPad Prism (v 4.03) software.

Results

Isolation and identification of PCP degrading isolate

Totally 36 bacteria were isolated from the sludge and screened them on PCP containing media. The bacterial colonies grown on this media were further screened and the bacterial strain CL2 was selected based on the growth and utilization of PCP as sole source of carbon and energy. The colonies were morphologically vermilion in color, non-spreading, smooth, wet, and non transparent. The results of the biochemical characterization of the CL2 strain are presented in Table 1. The shape of the bacteria are cocci, motile and positive for Gram reaction, oxidase and nitrate reduction tests and negative for capsule, catalase, DNAase, indole, urease and starch hydrolysis. This isolate was sensitive to Calaritromycin; Cotrimoxazole; Netillin; Cefaclor; Ampicillin/sublactam. Partial sequence data of the isolate was analysed by BLAST search. The BLAST analysis of this sequence showed 99% of sequence similarity with *Kocuria polaris*. A phylogenetic tree was constructed based on 16S rRNA gene sequence by maximum parsimony using MEGA software (v 4.0). The phylogenetic analysis revealed that CL2 was grouped with *Kocuria polaris* and hence designated it as *Kocuria* sp. CL2 (Fig. 1).

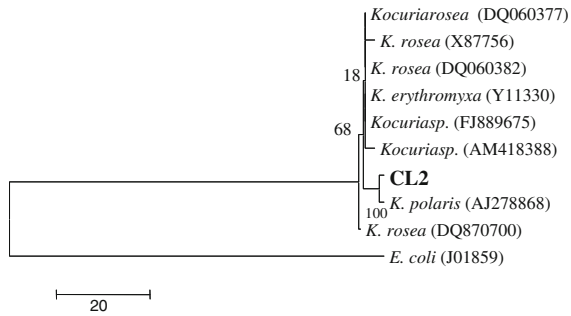

Fig. 1 Maximum parsimony tree based on 16S rRNA sequence of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates

Degradation studies

The bacterial strain CL2 was able to grow and utilize PCP as an energy source. The growth of bacterial strain was increased up to 120 h and then attained stationary phase. The CL2 utilized 55 % of PCP within 24 h and 95% after 96 h when 100 mg/l (0.37 mM) of PCP was used (Fig. 2). During the course of bacterial treatment PCP was mineralized and the liberation of inorganic chloride ion in culture medium was observed (Fig. 3). The chloride ion concentration increased with increase in PCP degradation. The amount of PCP utilized and the release of chloride ion (mentioned in parenthesis) for different

Table 1 Biochemical characteristic features of the aerobic bacterial isolate CL2 of the sludge and comparison with other *Kocuria* species

Test	CL2	*K. rosea*[a]	*K. varians*[a]
Form	Coccus	Coccus	Coccus
Colony color (Nutrient Agar)	Vermilion	Orange/red	Yellow
Cell grouping	Individuals	Individuals	Individuals
Gram stain	+	+	+
Capsule stain	−	NA	NA
Motility	+	+	NA
Catalase test	−	+	NA
DNase test	−	NA	NA
Oxidase test	+	−	−
Nitrate reduction test	+	+	+
Indole test	−	−	NA
Urease test	−	−	+
Starch hydrolysis	−	+	−

+, Positive; −, Negative and NA, no data available

[a] Data obtained from Reddy et al. (2003)

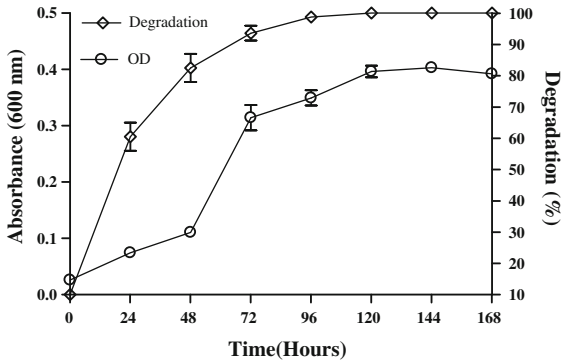

Fig. 2 Growth curve and degradation of *Kocuria* sp. CL2 in mineral salt media containing 100 mg/l (0.37 mM) of PCP as a sole carbon or energy source

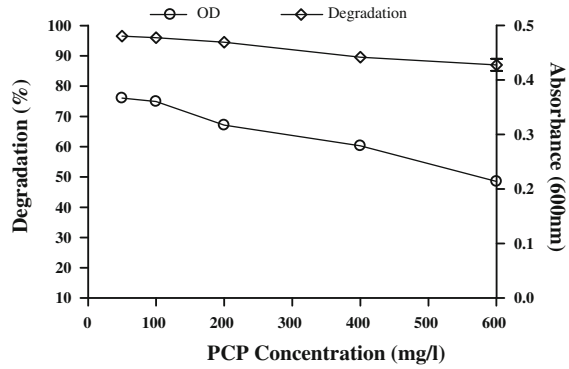

Fig. 4 Effect of different concentrations of PCP on the growth and utilization of PCP by *Kocuria* sp. CL2 grown for 168 h

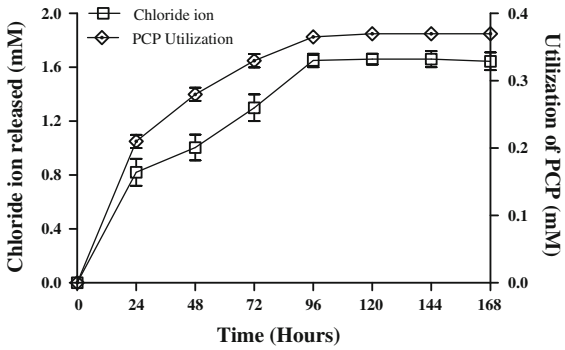

Fig. 3 Release of chloride ion in the medium at different time intervals due to degradation of PCP (100 mg/l or 0.37 mM) by *Kocuria* sp. CL2

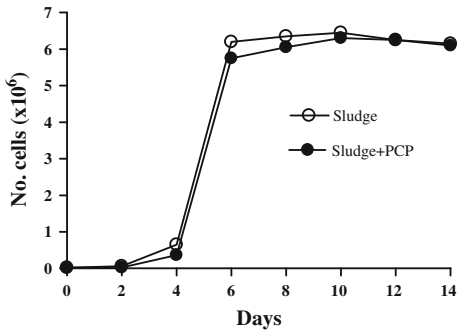

Fig. 5 Growth pattern of CL2 (CFU/ml) grown in the sludge supplemented with or without PCP

time intervals; 24, 48, 72, 96, 120, 144 and 168 h were 0.20 (0.82), 0.29 (1.10), 0.34 (1.30), 0.37 (1.6), 0.37 (1.6), 0.37 (1.6), and 0.37 (1.6) mM respectively. The liberation of chloride ion in the culture filtrate can be considered as the result of mineralization of PCP. The growth of CL2 was significantly reduced as the concentration of PCP in the mineral salt medium increased. Up to 200 mg/l, 95% removal of PCP was observed, and slightly decreased at 400 and 600 mg/l (Fig. 4).

Degradation of PCP in sludge

The removal of PCP from the sludge was tested by inoculating the bacterial strain CL2 into sludge. The initial PCP concentration in the sludge used for this study was about 0.029 mg/l, and therefore 100 mg/l of PCP was added 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 with time both in sludge supplemented with PCP and sludge alone. The growth of CL2 was higher in sludge alone compared to sludge amended with PCP (Fig. 5). HPLC analysis shown in Fig. 6 revealed that this strain is capable of mineralizing PCP from the sludge. It is able to remove up to 58.64% PCP from the sludge. These results suggested that the isolated bacterial strain CL2 have potential to remove PCP in the sludge.

Discussion

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

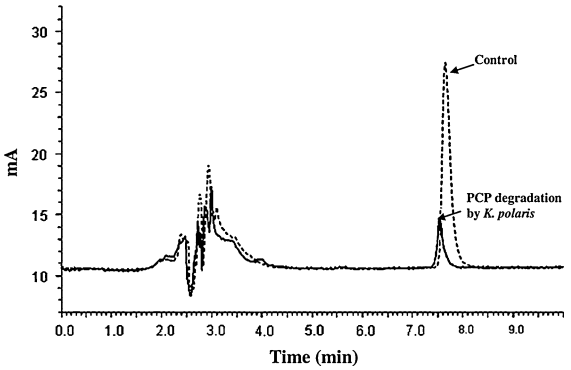

Fig. 6 HPLC chromatogram showing the PCP degradation in sludge by *Kocuria* sp. CL2

inorganic chloride and the concomitant production of biomass linked to chlorophenol utilization (Radehaus and Schmidt 1992). We are reporting for the first time that *Kocuria* sp. CL2 able to metabolize pentachlorophenol as carbon and energy source. This strain is capable of tolerating up to 600 mg/l of PCP and also degraded 90% of PCP in the culture medium.

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 CL2 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 (Homada et al. 1987; Radehaus and Schmidt 1992; Mohn and Kennedy 1992).

In the present study the bacterial strain CL2 was able to grow and remove the PCP more than 87% in 600 mg/l indicating the efficiency *Kocuria* sp. CL2 to remove PCP at higher concentrations. Apart from PCP, this strain also capable of degrading 2,4,5-Trichlorophenol and 2,3,4,6-Tetrachlorophenol (unpublished data). Numerous studies have evaluated the biodegradability of PCP in soil and compost to clean up the chlorinated phenols. Though 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 available with removal of PCP from the sludge. In the present study, CL2 mineralized 58.64% PCP from the sludge where the PCP concentration was more than 100 mg/l.

In conclusion, the results obtained by this study indicated that the bacterial strain CL2 is able to mineralize high concentrations of PCP. This strain also degraded 58.64% of PCP from the sludge within 2 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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References

Annachhatre AP, Gheewala SH (1996) Biodegradation of chlorinated phenolic compounds. Biotechnol Adv 14:35–56

Apajalahti J, Karpanoja P, Salkinoja-Salonen MS (1986) *Rhodococcus chlorophenolicus* new-species a chlorophenol mineralizing actinomycete. Int J Syst Bacteriol 36:246–251

Bock C, Kroppenstedt RM, Schmidt U, Diekmann H (1996) Degradation of prochloraz and 2,4,6-trichlorophenol by environmental bacterial strains. Appl Microbiol Biotechnol 45:257–262

Chandra R, Ghosh A, Jain RK, Singh S (2006) Isolation and characterization of two potential pentachlorophenol degrading aerobic bacteria from pulp paper effluent sludge. J Gen Appl Microbiol 52:125–130

Dams RI, Paton GI, Killham K (2007) Rhizomediation of pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. Chemosphere 68:864–870

Gonzalez JF, Hu WS (1991) Effect of glutamate on the degradation of pentachlorophenol by *Flavobacterium* sp. Appl Microbiol Biotechnol 35:100–104

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore, pp 626–640

Homada MF, Haddad AI, Abd-El-Bary MF (1987) Treatment of phenolic wastes in an aerated submerged fixed-film (ASFF) bioreactor. J Biotechnol 5:279–292

Kaoa CM, Liu JK, Chen YL, Chai CT, Chen SC (2005) Factors affecting the biodegradation of PCP by *Pseudomonas mendocina* NSYSU chloride release. J Hazard Mater 124:68–73

Kapley A, Lampel K, Purohit HJ (2001) Rapid detection of *Salmonella* in water samples by multiplex PCR. Water Environ Res 73:461–465

Laine MM, Jorgensen KS (1997) Effective and safe composting of chlorophenol-contaminated soil in pilot scale. Environ Sci Technol 31:371–378

Miethling R, Karlson U (1996) Accelerated mineralization of pentachlorophenol in soil upon inoculation with *Mycobacterium chlorophenolicum* PCP1 and *Sphingomonas chlorophenolica* RA2. Appl Environ Microbiol 62:4361–4366

Mohn WW, Kennedy KJ (1992) Reductive dehalogenation of chlorophenol by *Desulfomonite tiedjei* DCB-1. Appl Environ Microbiol 58:1367–1370

Radehaus PM, Schmidt SK (1992) Characterization of a novel *Pseudomonas* sp. that mineralizes high-concentrations of pentachlorophenol. Appl Environ Microbiol 58:2879–2885

Reddy SNG, Prakash SSJ, Prabahar V, Matsumoto GI, Stackebrandt E, Shivaji S (2003) *Kocuria polaris* sp. nov., an orange-pigmented psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. Int J Syst Evol Microbiol 53:183–187

Reineke W, Knackmuss HJ (1988) Microbial degradation of haloaromatics. Annu Rev Microbiol 42:263–287

Saber DL, Crawford RL (1985) Isolation and characterization of *Flavobacterium* strain that degrade pentachlorophenol. Appl Environ Microbiol 50:1512–1518

Sai K, Kang KS, Hirose A, Hasegawa R, Trosko JE, Inoue T (2001) Inhibition of apoptosis by pentachlorophenol by v-myc-transfected rat liver epithelial cells: relation to down-regulation of gap junctional intercellular communication. Cancer Lett 173:163–174

Solyanikova IP, Golovleva LA (2004) Bacterial degradation of chlorophenols: pathways, biochemica, and genetic aspects. J Environ Sci Health B 39:333–351

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Vallecillo A, Garcia-Encina PA, Pena M (1999) Anaerobic biodegradability and toxicity of chlorophenols. Water Sci Technol 40:161–168

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Wittmann C, Zeng AP, Deckwer WD (1998) Physiological characterization and cultivation strategies of the pentachlorophenol degrading bacteria *Sphingomonas chlorophenolica* RA2 and *Mycobacterium chlorophenolicum* PCP-1. J Ind Microbiol Biotechnol 21:315–321

Yang CF, Lee CM, Wang CC (2006) Isolation and physiological characterization of the pentachlorophenol degrading bacterium *Sphingomonas chlorophenolica*. Chemosphere 62:709–714