Research Paper

Volume : 3 | Issue : 6 | June 2014 • ISSN No 2277 - 8179

Biodegradation of PCB118 by Pseudomonas mendocina strain CL-10.4.coated with superparamagnetic Fe3O4 nanoparticles

Microbiology

KEYWORDS : PCB 118; Pseudomonas mendocina strain CL-10.4.; superparamagnetic Fe3O4 nanoparticles

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ABSTRACT

PCB118 is a compound used in commercial PCB mixtures. A novel bacterial strain degrading PCB 118 was isolated from soil identified as Pseudomonas mendocina strain CL-10.4. by 16S rRNA sequence analysis. PCB-degrading activity was checked by GC-MS analysis and Enoic acids, Acetophenones, Acetic acids were degradation products. Pseudomonas mendocina cells were coated with magnetic Fe3O4 nanoparticles, immobilized by external application of magnetic field. Nanoparticles were monodispersed in aqueous solution with an average size of 9.56 nm, magnetization of about 15emu/g and near zero coercivity indicating that the Fe3O4 nanoparticles had superparamagnetic properties. Degradation of PCB 118 by free and coated cells of this bacterium was studied. The rate of degradation of PCB118 coated cells was at par to the free cells with advantage of magnetic separation and possible reuse for second batch of PCB degradation.

1. Introduction

Polychlorinated biphenyls (PCBs) are mixtures of chlorinated hydrocarbons that have been used extensively in various industries as dielectrics, as heat exchange fluids, as paint additives, in carbonless copy papers and in plastics. The toxicology of PCBs is affected by the number and position of the chlorine atoms. PCBs accumulate in the fat of animals, are lipophilic and they tend to become concentrated at higher levels of the food chain. They are carcinogens and can damage the adult reproductive system (Faroon et al., 2003).

Incineration, thermal desorption, chemical dehalogenation, solvent extraction, soil washing and solidification/stabilization are some of the established remedial technologies for their removal from the environment. (Weston, 2005). Most of these technologies are not cost effective; hence the industries generating PCB containing wastes are tempted to discard these residues without treatment into soil or water. Bioremediation using microorganisms (free or immobilized) can be used as a biofriendly method for removal of PCBs from the environment. The microbial degradation of PCBs has been extensively studied by scientists employing aerobic bacteria and other microorganisms such as white rot fungi. PCBs are also reductively dehalogenated by anaerobic microbial consortia. Biphenyl-utilizing bacteria can metabolize many PCB congeners to chlorobenzoates and further too, by biphenyl-catabolic enzymes (Furukawa, 2000).

Studying a single PCB congener is preferred over mixtures as it helps in understanding response of the organism towards a particular congener indicating its capability of utilizing higher or lower chlorinated congener and better elucidation of the degradation pathway. The current study focuses on degradation of PCB 118 (2, 3', 4, 4', 5- pentachlorobiphenyl) (Faroon et al., 2003) which is categorized as a higher chlorinated PCB congener. It has been listed as one of the priority congeners by National Oceanic and Atmospheric Administration Mussel Watch Program (NOAA, 1989) (McFarland and Clarke, 1989). Priority congeners are the ones with greatest environmental importance based on potential toxicity, frequency of occurrence in environmental samples, and relative abundance in animal tissue.

To our knowledge, this is the first study reported on efficient PCB 118 degradation using Pseudomonas mendocina strain CL-10.4 .The degradation was studied employing free cells and cells coated with superparamagnetic Fe_3O_4 nanoparticles. Coating the cells of PCB degrader with Fe_3O_4 nanoparticle facilitates easy separation of the PCB degrader by an externally applied magnetic field after the degradation process is over. The study of difference in the degradation capacity of the free and immobilized cells of Pseudomonas mendocina strain CL-10.4 is discussed in this paper which would aid in setting up bioreactors employing cells coated with superparamagnetic Fe_3O_4 nanoparticles, thus facilitating bioremediation of PCB118 contaminated effluent.

2. Materials and methods

2.1. Sample

Soil samples were collected and screened for PCB 118 degrading bacteria.

2.2. Chemicals

PCB118 was obtained from Sigma Aldrich Co., Germany. n-Hexane, acetone, ammonium hydroxide, oleic acid, FeCl2, FeCl3, biphenyl were of analytical grade and obtained from Merck & Co. All other chemicals were commercially available.

2.3. Enrichment, isolation and identification

1gm Paddy field soil sample was suspended in 10ml of sterile phosphate buffered saline and 5ml of supernatant was inoculated in 250ml Erlenmeyer flasks containing 95ml of Mineral salts medium (MSM) [(NH4)2SO4 (0.1 g); Na2HPO4.7H2O (0.82 g); KH2PO4(0.27 g;) MgSO4.7H2O (0.01 g); Ca (NO3)2 (0.05 g); and FeSO4.7H2O (0.025 g)] with 1000 ppm of biphenyl (Bokvajova et al., 1994).

The isolates showing growth in biphenyl were then cultivated in Luria Bertani medium for 24hrs at 37°C. Cells were harvested, washed with saline. The selected isolate was inoculated (0.2 ml, 0.1 O.D530nm) in 10 ml of assay medium MSM supplemented by 100 ppm of PCB 118 and incubated for 7 days at 28°C under shaker condition. Thereafter flasks were treated in a water bath (80-100°C) for 10 minute to terminate the growth of the isolate.

Metabolites produced after biodegradation of PCB118 were extracted with 2 ml hexane incubated at 20°C under shaker conditions for 1h and subjected to GC-MS analysis. The promising isolate selected for present study was based on GC-MS analysis of degradation products which were non-toxic and low molecular weight compounds. Identification of the isolate was done on the basis of morphological, cultural and biochemical tests and the strain was confirmed as Pseudomonas mendocina strain CL-10.4 by 16S rRNA analysis carried out at SciGenom Labs Pvt Ltd, Kerala, India.. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

2.4. Synthesis and modification of Fe_3O_4 magnetic nanoparticles

Fe_3O_4 nanoparticles were synthesized by co-precipitation method and modified by addition of ammonium oleate. 23.5g

$FeCl_3.6H_2O$ and 8.6g $FeCl_2.4H_2O$ dissolved in 600 ml deionized water with mechanical stirring at 800 rpm and 85°C and then quickly added 30 ml of 7.1M NH_4OH. To the resulting suspension, 16 ml of oleic acid was added drop wise over a period of 30 min. After 45 min the magnetic precipitate was separated by magnetic decantation and washed several times with acetone. Magnetic precipitate was modified with about 4 ml of 7.1M NH_4OH to form the hydrophilic Fe_3O_4 magnetic nanoparticles, which in an aqueous solution were monodispersed.

2.5. Coating of microbial cells by Fe_3O_4 nanoparticles

Fifteen millilitres of a magnetic suspension was mixed with 100 ml of a cell suspension. The microbial cells were coated by adsorbing the Fe_3O_4 nanoparticles. The coated cells could be concentrated on the side of the vessel containing the suspension and separated from the suspension medium by decantation (Shan et al., 2005).

2.6. Biodegradation assay of PCB118

The assay medium was MSM supplemented by 100ppm of PCB 118 (Bokvajova ml of et al., 1994). Free cells (0.2ml/ 0.1O.D530nm) and magnetic Fe_3O_4 coated cells Pseudomonas (0.2ml) were inoculated in two separate flasks and incubated for 10 days at 28^0C under shaker condition. Aliquots were taken from each of the flasks with an interval of 3, 6 and 10 days. The end products obtained were analyzed by GC-MS analysis.

2.7. Analytical methods

2.7.1. Characterization of nanoparticles

X-Ray diffraction of Fe3O4 nanoparticles was carried out using Analytical Expert Pro Magnetic Particle Diffractor with copper anode, wavelength 15040 A0 , Power 10kV, 30mA detector and accelerator RTMS with diffracted beam monochromator. A glass slide was loaded with silicon grid and the amorphous powder of the nanoparticles was sprinkled on and loaded into the instrument for analysis.

2.7.2. Magnetization studies on Fe3O4 nanoparticles using Quantum Design Superconducting quantum interference (SQUID) magnetometer

The Fe3O4 nanoparticle powder sample was weighed and wrapped in paper and packed in gelatin capsules and properly centered in the straw. The magnetization measurements at room temperature were performed between -2T to 2T (1Tesla = 10 kOe) to obtain the M-H curves.

2.7.3. STEM (Scanning Transmission electron microscopy)

Scanning Transmission electron microscopy was done using FEI Tecnai F20ST TEM/STEM instrument. Fe_3O_4 nanoparticles were dispensed in methanol, and a drop of this suspension was put on a copper grid for STEM imaging.

2.7.4. Analysis of PCB degradation brought about by free cells and magnetic Fe_3O_4 coated cells by GC-MS analysis

The incubation of PCB-degrading cultures (free and coated) cells was stopped by heating the flasks at 80-100°C for 10min and resulting metabolites were extracted with 2 ml hexane at 20°C on a shaker for 1h. The hexane layer obtained was used for GC-MS analysis. 10µl of hexane layer was diluted with hexane to a final volume of 1 ml (Bokvajova et al., 1994). Samples were analysed on a Hewlett -Packard column Model: GCD – HP1800A Mass range: 10 - 425 amu integrated gas chromatograph - electron ionization detector operated through a data system. HP1 column was 30m long with internal diameter 0.25mm. Temperature of injector in the column was 100°C increased 100/ min and reached up to 250°C with 3min holding time. Temperature of detector increased 300 °C /min and reached up to 280°C where holding time was 8min. The carrier gas was helium and flow rate in column was 1ml/min.

2. Results and Discussion

2.1. Screening and identification of the PCB118 degrading organisms

The enrichment approach employed in this work was based on growth stimulation of biphenyl degrading bacteria. Screening of PCB118 degrading organism from water and soil samples collected at different locations, 4 isolates namely (A, B, C, D) were chosen as potential PCB degrading cultures since they:

1. Showed highest growth biphenyl containing media
2. Established stable population densities within 1 month of enrichment.
3. Based on GC-MS analysis of degradation products which were non toxic and low molecular weight compounds

The promising isolate B was selected for present study based on degradation products .The cultural, morphological and biochemical tests identified this promising isolate as Pseudomonas mendocina strain CL-10.4 and the strain was confirmed by 16S rRNA gene sequence analysis. The nucleotide sequence analysis of the sequence was done at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/ BLAST) and corresponding sequences were downloaded. Isolated pure bacterial species was designated as Pseudomonas mendocina strain CL-10.4 with NCBI accession number HQ113219.1.

Pseudomonas mendocina strain CL-10.4 was employed to carry out further degradation studies because this microorganism has extradiol dioxygenases which are related to enzyme families of 2, 3 and 3, 4 di-oxygenases (Robertson et al.,2007). These enzymes are known to be key enzymes in PCB degradation.

2.2. Characterization of Fe_3O_4 nanoparticles

The analytical methods used for nanoparticles characterization were Scanning transmission electron microscopy (STEM), X-ray diffraction (Shan et al., 2005; Niederdraenk et al., 2006) and magnetization studies were done on the magnetic nanoparticles (MNP) using Quantum Design SQUID magnetometer (Shan et al., 2005, Hoa et al., 2009). Microbial cells were then coated with magnetic nanoparticles by adsorption. The nanoparticles were strongly adsorbed on the cell surfaces. It was possible to concentrate the dispersed coated cells by application of a magnetic field. Pseudomonas mendocina strain CL-10.4 were coated with magnetic Fe_3O_4 nanoparticles and then immobilized by external application of a magnetic field, surfaces were hydrophilic.

2.2.1. X-ray diffraction

X- ray diffraction was used to determine adequate size of the nanoparticles. The data obtained after X-ray diffraction was combined computationally with complementary chemical information to produce and refine a model of the arrangement of atoms within the crystal. Diffractogram of the nanoparticles synthesized having average size of 9.56nm.

The positions of diffraction angle also coincided with the standard Crystallographic diffraction data corresponding to Fe_3O_4.

2.2.2. Magnetization studies of Fe_3O_4 nanoparticles

Figure 1. Shows the magnetization measurement of the ferrite nanoparticles performed at room temperature on a Superconducting quantum interference device (SQUID) magnetometer. The magnetization measurements at room temperature were performed between -2T to 2T (1Tesla = 10 kOe) to obtain the M-H curves. The M-H plots of Fe_3O_4 nanoparticles show magnetization of about 15emu/g and near zero coercivity. This was indicative of superparamagnetic behaviour that is characteristic of the Fe_3O_4 nanoparticles.

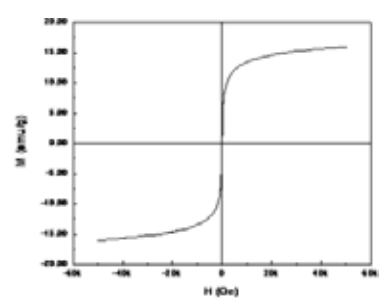

Fig.1. Magnetization measurement of Fe3O4 nanoparticles

Volume : 3 | Issue : 6 | June 2014 • ISSN No 2277 - 8179

2.2.3. STEM (Scanning Transmission electron microscopy)
The Fe_3O_4 nanoparticles in suspension did not settle. Using Scanning transmission electron microscopy (STEM) single Fe3O4 nanoparticle grain size was found to be 3.5nm.

2.2.4. STEM imaging of free and coated cells
To confirm the coating by adsorption, STEM imaging of coated cells and uncoated cells (free cells) of Pseudomonas mendocina strain CL-10.4 was done (Figure 2 and 3). Fe_3O_4 nanoparticles had a narrow size distribution in aqueous solution. It was observed that the nanoparticles had strongly adsorbed on the surface of the cells. Cell immobilization by adsorption is currently gaining considerable importance because of a major advantage, namely, reducing or eliminating the mass transfer problems associated with the common entrapment methods (Meikap, 1995; Shan et al., 2005).

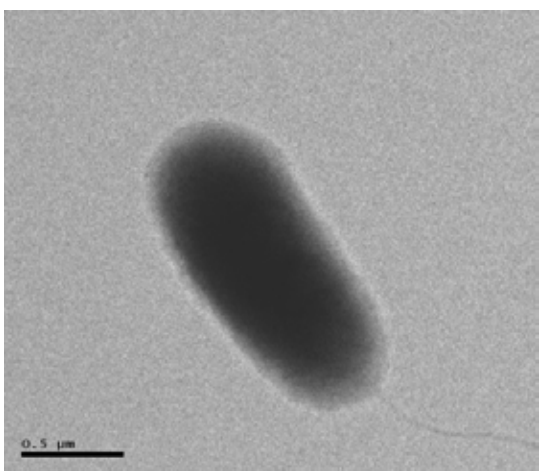

Fig.2. STEM image of free cells.

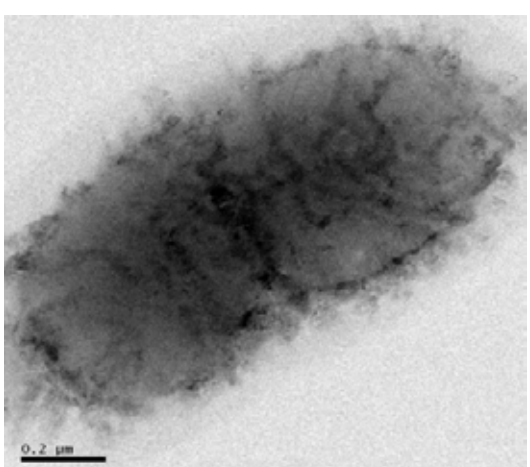

Fig. 3. STEM image of cells coated with Fe_3O_4 nanoparticles.

2.3. Immobilization of coated cells
The cells coated with superparamagnetic Fe_3O_4 particles could be immobilized by external application of magnetic field. The magnetic field can be applied by using a magnet. The nanoparticles were strongly adsorbed on the cell surfaces because of their high specific surface area and high surface energy. Thus it was possible to concentrate the dispersed coated cells by application of a magnetic field. When dispersed, the coated cells experienced minimal mass transfer problems. It can also overcome drawbacks such as limitations in biomass loading and in the loss of cells from the carrier associated with conventional immobilization by adsorption.

2.4 Analysis of PCB degradation by free cells and magnetic Fe_3O_4 coated cells by GC-MS analysis
The degradation capabilities congener-specific degradation of PCB 118 of coated cells and free cells of Pseudomonas mendocina were studied over a period of 10 days. GC-MS analysis showed that the standard PCB 118 peak was absent in both the samples suggesting its degradation by the organisms. The key metabolites (dihydrodiols, acetophenones and acetic acid) were detected for PCB degradation carried out by both types of cells.

Analysis of degradation of PCBs brought about by coated cells revealed that acetophenones (3-Dodecanone), dihydrodiols1 (3-Propanediol, 2-ethyl-2-(hydroxymethyl) and acetic acid were major products. Presence of Hydrochloric acid indicated dechlorination of the PCB 118.

Efficiency of degradation of PCB-118 brought about by free cells:
At the end of 3 days of incubation, GCMS analysis of metabolites of PCB118 degradation by Pseudomonas mendocina strain CL-10.4 showed formation of acetic acid and acetophenones which indicated that the degradation of the substrate had begun. The presence of acetophenones (7-Tridecanone) confirmed dioxygenases attack on PCBs. On the 6^{th} day, formation of diols proved that the enzymes had attacked the PCB 118 ring. Thus presence of dihydrodiols (Nonadecanediol, 1, 2-,) confirmed the dioxygenase attack on the chlorinated biphenyl. The increase in concentration of acetic acid (peak area 79.27%) proves more substrate breakdown. On the 10th day miniscule amounts of acetic acid (peak area 1.17%) was detected. Thus it can be assumed that acetic acid is utilised via TCA cycle for energy production and completion of aerobic degradation of PCB to CO_2 and water.

Efficiency of degradation of PCB-118 brought about by Fe_3O_4 magnetic nanoparticles coated cells:
At the end of 3 days of incubation, GCMS analysis of metabolites of PCB118 degradation by coated cells Pseudomonas mendocina strain CL-10.4 showed formation of moderate amount of acetic acid (peak area 19.07%) indicating that the degradation of PCB had started. After 6 days of incubation the concentration of acetic acid (peak area 1.79%) was not high but formation of dihydrodiols and acetophenones confirm that the substrate was attacked by dioxygenases. At the end of 10 days the formation of acetic acid (peak area 1.67) was not in higher concentration and can be presumed to be utilized via TCA cycle. The presence of Hydrochloric acid confirms that the products formed were dechlorinated.

The results suggest that the degradation probably proceeds from dihydrodiols through various other intermediates and together with acetophenones via catechol pathway. Acetic acid formation suggests complete degradation of PCB.

Formation of lesser amount of acetic acid by coated cells could be due to faster utilization of acetic acid for energy generation. Adsorption with nanoparticles did not affect degradation potential of the cells. Cells were viable and capable of carrying out degradation. This would indicate that $Fe3O_4$ nanoparticles were not toxic to the cells nor did they alter any characteristics of the cells on attachment. The cells could also be immobilized by external application of magnetic field facilitating their easy separation and possible reuse.

Conclusion
From the above study it can be concluded that Pseudomonas mendocina strain CL-10.4 was able to degrade PCB118 aerobically by biphenyl degrading pathway. $Fe3O_4$ nanoparticles that were synthesized using co–precipitation method adsorbed on the cells of Pseudomonas mendocina strain CL-10.4 and immobilized by external application of magnetic field did not affect the biodegradation capacity of the immobilized cells and also revealed the rate of degradation to be efficient as compared to free cells. The coated cells had an added advantage of magnetic separation from the medium and possible reuse for a second batch of PCB degradation.

PCB 118 is one of higher chlorinated congeners of PCBs and considered as one of the most toxic and persistent one. Overall findings suggest need to exploit this strain for bioremediation of PCB118 and other congeners (from hexa to deca chlorinated PCBs) contaminated effluents.

ACKNOWLEDGEMENT

We are grateful to University Grants Commission for extending financial assistance in the form of minor research project [47-13-14/ 10 (WRO)]

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