BIODEGRADATION OF 2-CHLOROPHENOL BY BACILLUS SUBTILIS ISOLATED FROM INDUSTRIAL SLUDGE

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INTRODUCTION
Chlorophenols are organic chemicals in which one or more hydrogen atoms of phenol (1-hydroxybenzene) are replaced by one or more atoms of chlorine. Chlorinated aromatics, pesticides and herbicides are environmental contaminants of great health concern (ATSDR, 1999). Among the nineteen different chlorophenols, 2-CP has been listed as a priority pollutant (EPA-US, 2002). EPA recommends that drinking water contain no more than 0.04mg/L of 2-chlorophenol for a life time exposure for an adult, and 0.05 mg/L for a 1-day, 10-day, or longer exposure for a child (ToxFAQs, 1999).

Chlorophenols belong to the group of toxic and persistent to microbial attack xenobiotics. Nevertheless, due to the adaptation microorganisms acquire the ability to use chlorophenols as the sole source of carbon and energy. Microbial dechlorination is a common metabolic pathway for remediation of chlorophenol pollutants. Under anaerobic conditions, bacteria can dechlorinate polychlorophenols to less-chlorinated phenols (Takeuchi et al., 2000). Bacteria with a potential to degrade chlorinated benzoic acid and other aromatic compounds have been isolated. Recently, some anaerobic bacteria capable of aryl-dechlorination have been obtained from methanogenic enrichments (Bae et al., 2002) in the form of pure cultures.

The present study describes the pathway for the utilization of halogenated phenol by bacteria B. subtilis with the emphasis on the main reactions and intermediates formed enzymes responsible for these reactions.

MATERIALS AND METHODS
Bacterial isolation and characterization
The microorganism degrading 2-CP was isolated from effluents of paper and wood preserving industry effluents by enrichment culture technique. The bacteria was purified by adopting standard microbiological procedures and characterized according to the Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994) and on the basis of rDNA
phylogeny analysis.

Media and growth conditions
The bacterium was cultured in a liquid mineral salt medium (MSM) comprising of (g/L) of NaHCO$_3$ (2.0), NH$_4$Cl (2.0), NaCl (2.0), MgSO$_4$ (0.4), K$_2$HPO$_4$ (0.4), NaNO$_3$ (2.0), Yeast extract (0.02%) and peptone (0.04%). 2-CP (0.2% w/v) was incorporated as the growth substrate after autoclaving the medium. The bacterial growth was monitored at 660 nm with a spectrophotometer at regular intervals by withdrawing a known volume of the medium aseptically. Bacterium was maintained as liquid cultures by transfer to fresh medium weekly.

rDNA gene and phylogeny analysis
PCR was performed on the extracted genomic DNA of the environmental isolate (Royal life sciences, Hyderabad). The PCR product obtained was purified and analyzed by the Sequence analyzer. The phylogeny was analyzed by conducting BLAST analysis for the consensus sequences of reverse and forward rDNA sequences. The similar sequences obtained were aligned employing clustal W program (Thompson et al., 1994). The alignment output file generated a phylogenetic tree using Phylodraw tool.

Bacterial degradation of 2-Chlorophenol
The degradation rate of 2-CP was determined by culturing the bacterium in screw cap serum bottle containing 100mL MSM supplemented with 2-CP (0.2% w/v). The decrease in concentration of the 2-CP was determined at regular time intervals as per the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). Alternatively the degradation rate of 2-CP was also monitored at 285nm at regular intervals.

Extraction and characterization of metabolites
Metabolites that accumulated in the spent medium during the growth of bacteria on 2-CP as substrate were isolated by solvent extraction. The spent medium after acidification with dilute HCl (0.1M) was extracted with diethyl ether (1:3 v/v) three times. Later the resulting extract was dried over anhydrous sodium sulphate and evaporated to dryness. The residue obtained was dissolved in methanol and characterized by TLC and GC-MS analysis.

Preparation of cell free extract
The bacterium was harvested in its log phase (25 days) of growth by centrifugation at 5000 rpm for 15 min. The cells were repeatedly washed with 50mM sodium phosphate buffer of pH-7.0. After final suspension of the cells in 0.1M KH$_2$PO$_4$ buffer, (pH-7.8) containing 1mM ascorbic acid, 10% acetone, 10% glycerol and 100μM ferrous sulphate, they were subjected to sonication (Vibracell, Sonics and Materials, CT, USA, Model VC 130) for a total of 3 minutes at 4ºC. After sonication, the cell debris and unbroken cells were separated by centrifugation at 10,000 rpm for 20 minutes. The resulting supernatant was used as the crude source of enzymes. The protein in the enzyme preparation was estimated according to Folin-Ciocalteu method (Lowry et al., 1951).

Chlorophenol dehalogenase (CD) assay
The CD activity was measured in a reaction mixture (5 mL) containing: 25mM phosphate buffer (pH 7.2), 1 mM chlorophenol (substrate) and enzyme (0.04mg). Before initiation of the reaction, the reaction mixture was equilibrated at 30ºC in a water bath for 10 min. The reaction was then initiated by the addition of enzyme, after which the free halide was as described earlier. Enzyme activity unit is defined as the amount of enzyme that catalyses the formation of 1μmol halide ion/minute (Jing et al., 2008).

Chlorophenol-NADPH-oxido-reductase (CNOR) assay
The CNOR activity was measured in a reaction mixture (5 mL) containing 25 mm phosphate buffer (pH 7.2), 0.17mM NADPH, 0.04mg of enzyme; the reaction was initiated by adding 0.08mM chlorophenol. Enzyme activity was monitored by noting the decrease in absorbance at 340nm due to the substrate dependent oxidation of NADPH at 25°C. One enzyme unit is defined as the amount of enzyme which in presence of chlorophenol causes the oxidation of 1μmol NADPH per minute.

Effect of 2-CP on bacterial growth
The inhibitory effect of 2-CP on bacterial growth was measured. 100 mL MSM containing serum bottles were supplemented with different concentrations of 2-CP (10-60mM), after ten days of incubation the bacterial growth was measured at 660nm with a spectrophotometer.

RESULTS

Bacterial characterization
The isolated bacterium was Gram positive, short rod, catalase positive, oxidase positive and motile. On subjecting the isolated bacterium for rDNA phylogenetic analysis (Table 1 and 2, Fig. 1) the bacterium isolated was found closely related to Bacillus subtilis. The blast similarity searches for various ribosomal RNA gene accessions from Bacillus subtilis represented close match (E = 0). The pairwise evolutionary distance derived from ClustalW established that the isolated bacterium is Bacillus subtilis (Table 2).

Biodegradation of 2-Chlorophenol by Bacillus subtilis
The bacterial growth increased with incubation time and the concentration of 2-CP decreased subsequently. This instance is an indication of the bacterial degradation (Fig. 2) of 2-CP.
The bacterium depleted 97% of the supplemented 2-CP in 40 days with stoichiometric release of chloride (Fig. 3). Further the cell free extracts from B. subtilis showed DH enzyme activity (specific activity = 0.114 μmol/min/mg) and CDOR (specific activity = 0.26 μmol/min/mg). These enzymes dehalogenated 2-CP to phenol and covert it to catechol a terminal intermediate required for ortho-cleavage. The bacterium was also shown to be resistant to 40mM 2-CP, which is rather a toxic dose for other microorganisms (Fig. 4).

Characterization of metabolites

Metabolites that accumulated in the spent medium during the growth of B. subtilis on 2-CP were isolated and characterized. The thin layer chromatography (TLC) analysis demonstrated accumulation of a metabolite with an Rf value of 0.72 that corresponded to phenol on co-chromatography. This result established that phenol was one of the intermediary metabolite during the degradation of 2-CP. It was also confirmed by GC-Mass spectra (Fig. 5). Hence B. subtilis initiated the degradation of 2-CP by reductive dechlorination to phenol. This compound may further follow an ortho-cleavage pathway under aerobic conditions (Fig. 6).

Table 1: Blast Similarity Searches for r-DNA obtained from bacterium (BCBUCCI)

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<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
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<td>Bacillus subtilis gene for 16S rRNA, partial sequence</td>
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Table 2: Pairwise evolutionary distance for rDNA gene (label-1) from 2CP degrading bacterium compared with database entries (Label-2)

<table>
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<th>Label-1</th>
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because they could be active under anoxic conditions (Kushalatha et al., 2010). In the current work we have concentrated on understanding the metabolic pathway of 2-CP in *B. subtilis*. However the higher concentrations (50mM) of 2-CP in the medium were found to inhibit growth (Fig. 4).

The blast searches for rDNA genes and pairwise evolutionary distance calculation of isolated bacterium established conclusively that the bacterium metabolizing 2-CP was *Bacillus subtilis*. There was a long-held belief that the gram-positive soil bacterium *Bacillus subtilis* is a strict aerobe. But recent studies have shown that *B. subtilis* will grow anaerobically, either by using nitrate or nitrite as a terminal electron acceptor, or by fermentation (Michiko and Zuber, 1998). (added extra information) Metabolic pathway of 2-CP in *B. subtilis* proceeded with an initial reductive dechlorination reaction. This is a usual pathway under anaerobic conditions but reductive dechlorination also occurs under aerobic conditions (Fetzner and Lingens, 1994). On the basis of metabolite extraction, characterization and enzyme analysis *B. subtilis* degrades 2-CP through the tentative pathway (Fig. 6).

Anaerobic dehalogenation of 2-CP, a common intermediate of polychlorophenol degradation, by mixed cultures was reported (Themel et al., 1996). Based on TLC and GC-MS profile, in *B. subtilis* the 2-CP is reductively dechlorinated to phenol. Further phenol may be degraded via ortho cleavage pathway involving catechol or may follow benzoate pathway (Zhang and Wiegel, 1992; Van Schie and Young, 2000). Catabolic plasmids are widespread in nature, and an increase in their frequency within a community has often been observed in pollutant-stressed environments. However the absence of any plasmids (data not shown) in *B. subtilis* during degradation of 2-CP suggest that catabolic pathway may be chromosomally encoded (Sayler et al., 1990). In addition *B. subtilis* could utilize nitrite as electron acceptor but it did not use sulphate, sulphite, thiosulphate, and nitrate.

The observation that bacteria can use chlorinated phenols may have important implications for the natural attenuation and bioremediation of contaminated aquifers. *B. subtilis* is capable of metabolizing 2-CP under hypoxic conditions by switching over to different metabolic mode. This metabolic versatility has raised interest in the research community and makes this bacterium suitable for potential use in biotechnological applications.

### DISCUSSION

One particular concern of release of 2-CP in to the environment is contamination of drinking water resources. Chlorophenols are not reduced significantly during conventional drinking water treatment processes. Bacteria have received attention because they could be active under anoxic conditions (Kushalatha et al., 2010). In the current work we have concentrated on understanding the metabolic pathway of 2-CP in *B. subtilis*. However the higher concentrations (50mM) of 2-CP in the medium were found to inhibit growth (Fig. 4).

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### REFERENCES


ToxFAQs for Chlorophenols: www.atsdr.cdc.gov/tfacts107.html


