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# Degradation of 2,4-dichlorophenol by *Bacillus* sp. isolated from an aeration pond in the Baikalsk pulp and paper mill (Russia)

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**Abstract**

A pure culture of 2,4-dichlorophenol (2,4-DCP)-degrading bacteria was isolated from a natural enrichment that had been adapted to chlorophenols in the aeration pond of the Baikalsk pulp and paper mill (Russia). The bacteria were identified by 16S rDNA intergenic region analysis, using PCR with universal primers. Comparative analysis of the 16S rDNA sequence (1545 bp) in the GenBank database revealed that these bacteria are related to *Bacillus cereus* GN1. Degradation of 2,4-DCP was studied using this culture in liquid medium under aerobic conditions, at initial concentrations of 20–560 μM 2,4-DCP. The 2,4-DCP degradation rates by *B. cereus* GN1 could be determined at concentrations up to 400 μM. However, higher concentrations of 2,4-DCP (560 μM) were inhibitory to cell growth.


*Keywords:* Baikalsk pulp and paper mill; 2,4-dichlorophenol; Degradation; *Bacillus*

## 1. Introduction

Toxic chlorophenols are ubiquitous organic pollutants in aquatic ecosystems and have both anthropogenic and natural sources. Anthropogenic sources are of industrial, agricultural, and domestic origin (Environmental Handbook, 1995; ATSDR, 1999; Davi and Gnudi, 1999). Chlorophenols of natural origin are formed as a result of chlorination of natural organic matter (Naturally Produced Organohalogens, 1995; Czaplicka, 2004), degradation of natural chlorinated fulvic acids (Flodin et al., 1997) and activities of some species of soil fungi, lichens, and insects (Dembitskiy and Tolstikov, 2003). In the Lake Baikal ecosystem (Russia, World Heritage Site since 1996), chlorophenols are formed when reactive chlorine is used for pulp bleaching in the Baikal pulp and paper mill (BPPM), located directly on the southern shore of the lake. The purified wastewater from BPPM is discharged through the aeration pond directly into the lake. Chlorophenols have been included in the list of "particularly hazardous compounds" for the Lake Baikal ecosystem. Total chlorophenol concentration in the purified wastewater ranges from 1.0 to 10.0 $\mu g\,mL^{-1}$ (Beim et al., 1997). The total concentration of 2-chlorophenol, 4-chlorophenol, and 2,4-dichlorophenol (2,4-DCP) in the aeration pond and in Lake Baikal is 5.76 and 1.86 $\mu g\,mL^{-1}$, respectively; in addition, high levels of chlorophenols were detected in the Selenga river (major tributary of Lake Baikal) (Batoev et al., 2005). Despite the fact that microbial degradation of chlorophenols has been investigated for many years, there is still considerable interest in the metabolic capacity of bacteria able to degrade chlorophenols within indigenous microbial consortia in various ecosystems (Annachhatre and Gheewala, 1996; Solyanikova and Golovleva, 2004). The indigenous bacteria adapted to chlorophenols in the aeration pond were proposed for application to the biological wastewater treatment in the BPPM. Most investigations are concerned with studying pathways and enzymes involved in biodegradation of chlorophenols,

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using culture collection strains, or with using engineered aerobic–anaerobic systems to improve biodegradability. The aim of the present work was to investigate 2,4-DCP-degrading bacteria newly isolated from an aeration pond of BPPM, where 2,4-DCP is a major pollutant (Batoev et al., 2005).

## 2. Materials and methods

### 2.1. Organism and culture conditions

A pure culture of 2,4-DCP-degrading bacteria was isolated from a natural enrichment culture that had been adapted to chlorophenols in the aeration pond of BPPM, using mineral medium (MM: g $L^{-1}$: $K_2HPO_4$-0.65; $KH_2PO_4$-0.19; $NaNO_3$-0.5; $MgSO_4 \cdot 7H_2O$-0.1; $FeSO_4 \cdot 7H_2O$-0.005; $(NH_4)_2SO_4$-0.5 (Loh and Wang, 1998)), supplemented with 2,4-DCP as a growth substrate. Enrichments were incubated in nutrient broth with shaking (143 rpm) at 28 °C for 24 h in sterile Erlenmeyer flasks (500 mL). Organisms were subsequently grown on nutrient agar plates to obtain single colonies. A pure culture of 2,4-DCP-degrading bacteria was isolated by cycles of replating on MM/2,4-DCP agar plates with 1% vitamin solution. For degradation studies, one colony was inoculated into each sterile shake-bottle containing 10 mL MM, 20–560 μM 2,4-DCP and 0.5% yeast extract and incubated under aerobic conditions on the shaker (143 rpm) at 28 °C.

### 2.2. Chemical analysis

2,4-DCP was determined by reverse phase high performance liquid chromatography (HPLC) with UV detection at 285 nm (Beckman, System Gold), using a C-18 RP-column (125 × 3 mm, 5 μm) and a mobile phase (60% methanol and 2.5% acetic acid in $H_2O$) with a flow rate of 0.5 mL $min^{-1}$. Samples were centrifuged (10 min, 13000*g*) prior to injection of the supernatant and were analyzed in triplicate.

### 2.3. PCR conditions

Genomic DNA from the bacteria was isolated from one-day liquid pure culture by the standard phenol-chloroform procedure. The PCR was performed in 30 μL reaction mixture containing dNTPs, 1 μM of each primer, 10 × buffer solution, $MgCl_2$, DMSO, 0.5 U of *Taq*-DNA Polymerase, template DNA, and sterile water. Amplification was carried out in a DNA thermocycler Biometra T-gradient. The initial denaturation at 95 °C for 5 min was followed by 25 cycles of denaturation at 94 °C for 0.5 min, annealing at 65 °C for 0.5 min, and extension at 72 °C for 1 min. The PCR products were loaded onto 1% agarose gel, and DNA fragments were separated at field strength of 4.4 V $cm^{-1}$ for 1.5 h, then stained with ethidium bromide and photographed.

### 2.4. Cloning and sequencing of amplified 16S rDNA

PCR products were cloned into vector pCR®II-TOPO and transformed in competent cells of *Escherichia coli* using the TOPO TA Cloning® Kit, according to the manufacturer's protocol (Invitrogene). Transformed *E. coli* (positive clones containing an insert of approx. 1.5 kb) were selected on solid media containing kanamycin by blue/white screening, and their 16S rDNA was amplified by PCR as described above and analyzed by agarose gel-electrophoresis. Using an Applied Biosystems 373 DNA automatic sequencer, 16S rDNA sequencing was carried out. The Thermo Sequenase Primer Cycle Sequencing Kit was used with universal primers M13univCS (5′-AGG GTT TTC CCA GTC ACG ACG TT-3′), M13revCS (5′-GAG CGG ATA ACA ATT TCA CAC AGG-3′) and one internal primer DiHo-1100-r (5′-GGG TTG CGC TCG TTG-3′). The BLAST program from NCBI (National Center for Biotechnology Information) was used to determine the closest available database sequences. Published sequences were obtained from the NCBI nucleotide sequence databases (GenBank).

## 3. Results and discussion

The 2,4-DCP-degrading bacteria were Gram-positive spore-forming rods with dimensions 1.9–4.0 μm. Comparative sequence analysis of the 16S rDNA (1545 bp) in the GenBank database revealed that the bacterial strain GN1 was most closely related to *Bacillus cereus* (99% similarity). Members of this genus, e.g., the thermophilic strains *Bacillus stearothermophilus BR219* (Subramanian, 1992), *Bacillus thermoglucosidasius A7* (Duffner et al., 2000), and *B. thermoleovorans strain A2* (Duffner and Müller, 1998; Feitkenhauer et al., 2001, 2003), were described to degrade phenol, cresols, nitrophenols, bromophenols. *Bacillus* strains also degrade 4-chlorobiphenyl (Sàágua et al., 1998) and polychlorinated biphenyls (Kim et al., 2004). A thermophilic phenol-degrading *Bacillus* sp. A2 was able to transform 2-chlorophenol at 60 °C at concentrations up to 1 mM, but not di-, tri- and tetrachlorophenols (Reinscheid et al., 1996). It was also observed that at 50 °C spores of the strain *Bacillus subtilis IFO3335* can produce a laccase, catalyzing oxidation of such substituted phenols as (chloro)guaiacol, (chloro)metoxyphenols (Hirose et al., 2003). The degradation potential of *B. cereus* for several pollutants, such as azo dyes (Khehra et al., 2005), polycylic aromatic hydrocarbons (Kazunga et al., 2001; Tuleva et al., 2005), chlorobenzenes (Wang et al., 2003) and polyethoxylated nonylphenols (Di Gioia et al., 2004), have been examined.

With this investigation we demonstrated the 2,4-DCP degradation capability of this species. The isolated *Bacillus* strain exhibited degradation potential for 2,4-DCP at concentrations up to 400 μM in liquid medium under aerobic conditions (Fig. 1). Transformation products, such as phenol, *o*-methylated phenol, and 2- or 4-chlorophenol, did not occur in the HPLC chromatograms, suggesting, at least, a cleavage of the aromatic ring. At concentrations of

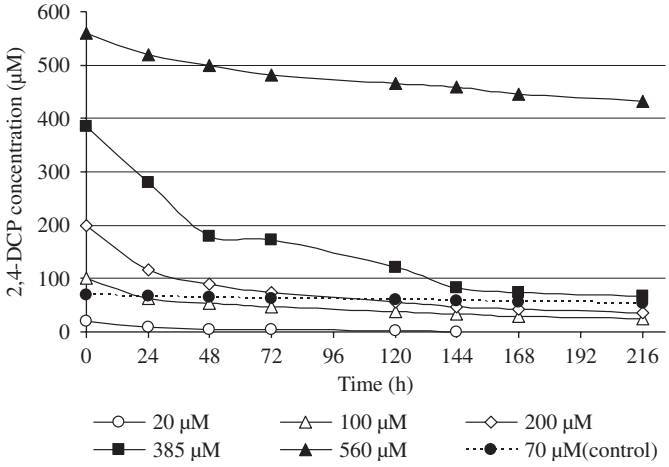


Fig. 1. Kinetics of 2,4-DCP degradation at various initial concentrations.