RESEARCH LETTER

Biodegradation of *p*-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp.

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Abstract

A bacterium named LZ-1 capable of utilizing high concentrations of *p*-nitrophenol (PNP) (up to 500 mg L^{-1}) as the sole source of carbon, nitrogen and energy was isolated from an activated sludge. Based on the results of phenotypic features and phylogenetic similarity of 16S rRNA gene sequences, strain LZ-1 was identified as a *Stenotrophomonas* sp. Other *p*-substituted phenols such as 4-chlorophenol (4-CP) were also degraded by strain LZ-1, and both PNP and 4-CP were degraded via the hydroquinone pathway exclusively. Strain LZ-1 could degrade PNP and 4-CP simultaneously and the degradation of PNP was greatly accelerated due to the increased biomass supported by 4-CP. An indigenous plasmid was found to be responsible for phenols degradation. In soil samples, 100 mg kg^{-1} of PNP and 4-CP in mixtures were removed by strain LZ-1 (10^6 cells g^{-1}) within 14 and 16 days respectively, and degradation activity was maintained over a wide range of temperatures (4–35 °C). Therefore, strain LZ-1 can potentially be used in bioremediation of phenolic compounds either individually or as a mixture in the environment.

Introduction

p-Nitrophenol (PNP) is an environmental pollutant commonly used in the manufacture of dyes, pesticides, pharmaceuticals and explosives (Spain & Gibson, 1991). It is the major metabolite resulting from the degradation of parathion (Mulbry & Karns, 1989) and is a priority pollutant (as HR-3 grade) (US EPA, 1986).

Microbial bioremediation has become more attractive than traditional methods for decontamination due to convenient processes, lower cost and minimal impacts on the environment (Labana *et al.*, 2005). However, owing to the high toxicity of PNP to microorganisms, biodegradation of PNP was mostly studied at lower concentrations (Bhushan *et al.*, 2000). To date, there is a lack of information on biodegradation of higher concentrations of PNP and its related parameters such as pH, temperature or nutritional supplementation.

Pollutants usually occur in mixtures in the environment and the biodegradation of one compound is often retarded greatly by other compounds because of substrate interactions (Timmis *et al.*, 1994). Therefore, the isolation of microorganisms capable of simultaneously degrading pollutants is important. 4-Chlorophenol (4-CP), another representative *p*-substituted phenol listed as a priority pollutant (US EPA, 1986), is distributed widely in the environment as an end-product of reductive dechlorination of polychlorinated phenols (Westerberg *et al.*, 2000). 4-CP often exerts inhibition effects on the degradation of other phenols, due mainly to the incompatibility of different pathways (Saéz & Rittmann, 1993; Hao *et al.*, 2002).

In this article, we report the isolation and characterization of *Stenotrophomonas* sp. LZ-1, a strain capable of degrading unusually high concentrations of PNP and other *p*-substituted phenols via the hydroquinone (HQ) pathway exclusively. The degradation ability was retained from 4 °C to 35 °C. Particularly, strain LZ-1 could degrade PNP and 4-CP simultaneously without cross-inhibition effects. Other strains with similar properties and catabolic pathways have been reported (Bae *et al.*, 1996a; Westerberg *et al.*, 2000). The degradation activity resided on an indigenous plasmid. We also investigated the bioremediation of phenols in soils by strain LZ-1.

Materials and methods

Chemicals

PNP, 4-nitrocatechol, 4-chlorocatechol and HQ (>99%, HPLC grade) were purchased from Sigma-Aldrich (St Louis, MO). Other *p*-substituted phenols (>99%, HPLC grade) were obtained from Fluka (Bucks, Switzerland). All other chemicals used were of the highest purity available commercially.

Isolation and culture conditions

Minimal salt medium (MSM, pH 7.0) containing (g L^{-1}) K_2HPO_4, 0.75; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.09 and $FeSO_4 \cdot 7H_2O$, 0.06, and Luria broth (LB) (pH 7.0) containing (g L^{-1}) tryptone, 10.0; yeast extract, 5.0 and NaCl, 10.0 were used. Enrichment was conducted by successive subculturing of sludge samples from a wastewater treatment plant in Tianjin, China. One gram of sludge was inoculated to an Erlenmeyer flask (250 mL) containing 100 mL MSM with filter-sterilized PNP (50 mg L^{-1} final concentration) as the sole carbon and nitrogen source. Such cultures were incubated on a rotary shaker (200 r.p.m) at 30 °C for one week, and 5-mL cultures were subcultured into fresh MSM containing PNP, the concentrations of which increased gradually from 100 to 500 mg L^{-1} for five generations. The final cultures were serially diluted and streaked onto MSM agar plates containing 300 mg L^{-1} PNP. Colonies becoming visible after 3 or 4 days of incubation at 30 °C were picked randomly, restreaked several times to ensure purity and inoculated into MSM containing 100 mg L^{-1} PNP as the sole carbon and nitrogen source to test the degradation ability. The isolate turning the culture from yellow to colorless (indicative of the PNP degradation) most rapidly was selected for further investigation.

Taxonomic identification

The morphological and physiological characteristics of the strain were identified with reference to Bergeys Manual of Systematic Bacteriology (Holt *et al.*, 1994). The total genomic DNA of strain LZ-1 was prepared by phenolic extraction (Sambrook & Russell, 2001). The 16S rRNA gene was amplified by PCR and sequenced (Yang *et al.*, 2006). The determined sequence was compared with those of type strains available in the GenBank database (http://www.ncbi.nlm.nih.gov/) using the NCBI BLAST program. Multiple alignments of sequences, construction of a neighbor-joining phylogenetic tree, and a bootstrap analysis for evaluating the phylogenetic topology were accomplished by using CLUSTAL X (Thompson *et al.*, 1997) and the MEGA 3.1 program (Kumar *et al.*, 2004).

Inoculum preparation for degradation studies

The cells were grown to late exponential phase ($OD_{600\,nm} = 1.0$) in MSM supplemented with 0.2% sodium acetate and 0.02% yeast extract at 30 °C at 200 r.p.m. The culture was centrifuged, washed and quantified by the dilution plate count technique. For all experiments, 10^6 cells mL^{-1} were inoculated and samples were incubated at 30 °C at 200 r.p.m. All the experiments were performed in triplicate and repeated three times to ensure accuracy and controls were maintained throughout, unless otherwise stated.

Growth and biodegradation of PNP

Biodegradation studies were performed in MSM containing 200 mg L^{-1} PNP. The effects of different initial PNP concentrations (100–800 mg L^{-1}) were investigated. Bacterial growth, PNP degradation, nitrite release and metabolites were monitored periodically. The degradation of other *p*-substituted phenols was tested under the same conditions. The effects of extra carbon (glucose and succinate) and nitrogen [$NaNO_3$ and $(NH_4)_2SO_4$] sources, pH and temperature were also studied.

Enzyme assays

The removal of PNP, 4-CP, HQ, 4-nitrocatechol and 4-chlorocatechol was spectrophotometrically monitored in 50 mM phosphate buffer (pH 7.0) with cell-free extracts (0.1–0.3 mg) and 0.2 mM substrate in a 1-mL mixture (final volume) (Spain & Gibson, 1991; Bae *et al.*, 1996a). NADH and NADPH were added as the electron donors.

Protein concentration was determined using the method of Lowry *et al.* (1951). Enzyme activities were expressed as units (nanomoles of substrate oxidated per minute) per milligram of protein.

Analytical method

Bacterial growth was monitored ($OD_{600\,nm}$) using a Beckman DU-800 spectrophotometer. The concentration of nitrite ions and chloride ions was measured as previously described (Montgomery & Dymock, 1961; Bae *et al.*, 1996b). Phenols were quantified by HPLC and their metabolites were identified by GC-MS (Bae *et al.*, 1996a; Qiu *et al.*, 2006).

Plasmid curing and conjugation studies

Plasmid curing and conjugation studies were performed as previously described (Ditta *et al.*, 1980; Trevors, 1986). *Stenotrophomonas* sp. YC-1 (PNP^- and $4\text{-}CP^-$, without indigenous plasmids) was used as the recipient strain (Yang

et al., 2006). Plasmid isolation was conducted by the alkaline lysis method (Sambrook & Russell, 2001).

Biodegradation of PNP and 4-CP in soils

Soil from the campus of Beijing University, Beijing, China was used in this study (pH 7.96, 2.25% organic matter content and 26.7% moisture content) and was prepared and inoculated with strain LZ-1 (10^6 cells g^{-1}, pre-grown on PNP and quantified using the dilution plate count technique) for degradation tests as previously described (Singh *et al.*, 2004). The decrease in substrate concentration was analyzed periodically by HPLC (Backman & Jansson, 2004; Labana *et al.*, 2005) and the effect of different pH was also studied.

Nucleotide sequence accession number

The nucleotide sequence of the 16S rRNA gene of strain LZ-1 has been deposited in the GenBank database under accession no. DQ784545.

Results and discussion

Isolation and identification of a PNP-degrading bacterium

After four weeks' enrichment, four different isolates utilizing PNP as the sole carbon and nitrogen source were obtained. An isolate (named LZ-1) showing the highest degradation activity was selected. Its cells were straight rod-shaped, gram-negative, strictly aerobic, motile with polar flagellum, 0.3–0.5 μm in diameter and 1.4–1.6 μm in length. Colonies of strain LZ-1 on LB agar plates were circular, smooth, glossy, convex, pale yellow and 1.2–1.9 mm in diameter after 3 days' incubation at 30 °C. Biochemical tests showed that strain LZ-1 was oxidase-positive, catalase-positive, urease-positive and nitrate-reduction-positive. Strain LZ-1 could grow aerobically from 4 °C to 37 °C, but not at 41 °C. Most of the sugars tested such as glucose, sucrose, fructose, mannose and maltose, and organic acids such as citrate, acetate, malate, succinate, lysine, histidine and leucine, could support the growth of strain LZ-1. However, alcohols did not support the growth of strain LZ-1. The physiological properties of strain LZ-1 were consistent with those of *Stenotrophomonas* sp. (Yang *et al.*, 2006).

The phylogenetic tree based on 16S rRNA gene sequences indicated that strain LZ-1 was most closely related to *Stenotrophomonas* sp. IC193 (AB196256) and *Stenotrophomonas* sp. BO (AF156709), with a sequence identity of 99.0%. All the sequence similarity values between strain LZ-1 and other *Stenotrophomonas* species were above 96.0% (Supplementary Fig. S1), indicating that strain LZ-1 was affiliated to genus *Stenotrophomonas*. Although strains of this genus have been known to be involved in the biodegradation of xenobiotic compounds (Yang *et al.*, 2006), this is the first report of the isolation of 4-halophenol- or PNP-degrading bacterium from this genus.

Cell growth and PNP biodegradation

Strain LZ-1 could utilize PNP as the sole carbon, nitrogen and energy source. PNP at a concentration of 200 mg L^{-1} was rapidly degraded in 20 h, concomitant with bacterial growth and nitrite release (Fig. 1a). No degradation was observed in uninoculated controls; however, the amounts of PNP depletion and nitrite release were not stochiometric. This might be due to the utilization of nitrite by the strain as a nitrogen source because the addition of extra nitrogen sources did not affect the patterns of PNP degradation and cell growth, but resulted in stoichiometric nitrite release (Bruhn *et al.*, 1987) (data not shown). Furthermore, in contrast to the observation of Bruhn *et al.* (1987), strain LZ-1 retained its degradation ability after prolonged

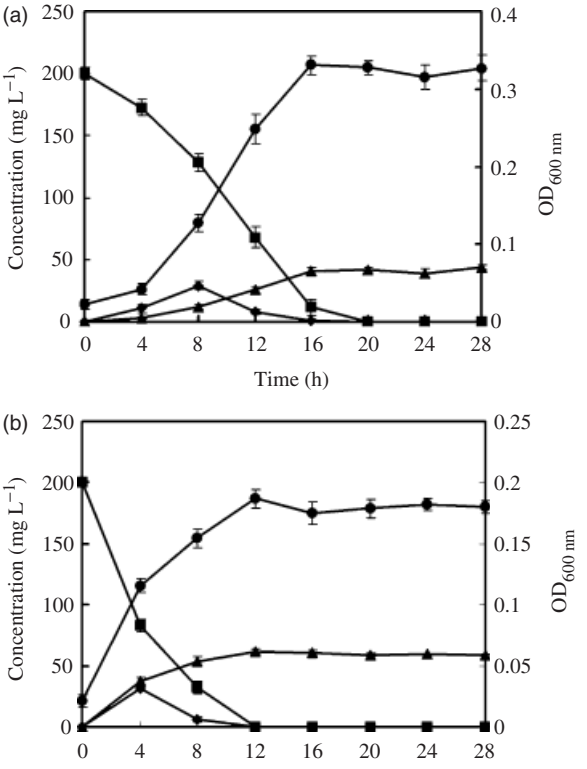

Fig. 1. Utilization of 200 mg L^{-1} PNP as a sole carbon, nitrogen and energy source by noninduced (a) and induced (b) cells of strain LZ-1. Induced cells were pre-grown in MSM with PNP (100 mg L^{-1}), harvested at mid-log phase and resuspended in MSM (10^6 cells mL^{-1}). Tested PNP (200 mg L^{-1}) was added to the cell suspensions, and 200 mg L^{-1} streptomycin was used to inhibit *de novo* protein synthesis of the resting cells efficiently (this concentration was experimentally confirmed). ■, PNP; ●, $OD_{600\,nm}$; ▲, nitrite; □, hydroquinone. Experiments were performed in triplicate and repeated three times.

cultivation in medium containing other readily utilized nitrogen sources. We also found that the addition of carbon sources improved the rate of PNP degradation through increasing biomass (Schmidt *et al.*, 1987). Therefore, strain LZ-1 could steadily degrade PNP in contaminated sites where carbon or nitrogen sources usually occur. Strain LZ-1 could degrade PNP up to 500 mg L^{-1} within 68 h and no significant inhibition was observed below 600 mg L^{-1}. However, a lag phase of about 8 h appeared when the initial concentration was 300 mg L^{-1}, and increased concomitant with the increase of PNP concentration. The existence of lag phase and inhibition effects concomitant with increasing PNP concentration could be due to the antimicrobial toxicity of PNP (Bhushan *et al.*, 2000).

Degradation was inhibited in acid conditions (pH 4.0–6.5) and was improved in alkaline conditions (7.5–10.0). The optimal pH was 9.0. The optimal temperature for PNP degradation was 32 °C, and degradation was completely inhibited above 35 °C or below 4 °C.

To our knowledge, strain LZ-1 had an uncommonly high tolerance and a rapid degradation rate among the documented PNP-degrading bacteria. These properties made it potentially useful for the biotreatment of high concentrations of PNP.

Substrate specificity

p-Substituted phenols such as 4-chloro-, 4-bromo-, 4-iodo- and 4-fluoro-phenol at a quantity of 200 mg L^{-1} were completely degraded within 18 h by strain LZ-1 (data not shown). The effects of pH and temperature were similar to the case of PNP. However, strain LZ-1 could not degrade *ortho*- or *meta*-substituted phenols. Generally, strain LZ-1 showed substrate specificity for *p*-substituted phenols such as 4-halophenols, as mentioned in previous reports (Westerberg *et al.*, 2000).

Resting-cell assay

Resting-cell assays were also conducted as described elsewhere (Rappert *et al.*, 2006). PNP-induced cells removed 200 mg L^{-1} PNP within 12 h with the stoichiometric release of nitrite (Fig. 1b). In contrast, no decrease of PNP was observed in noninduced controls. Cells pre-grown on PNP could degrade 4-halophenols, and cells pre-grown on 4-halophenols could also degrade PNP. The results revealed that the enzyme systems catalyzing their degradation were inducible and identical.

Identification of metabolites and enzyme assays

During degradation of PNP by strain LZ-1, a new chromatography peak with a retention time (RT) of 22.08 min appeared while the original peak of PNP with RT 29.09 min decreased, and a similar peak with RT 16.54 min was also detected during 4-CP degradation (Supplementary Fig. S2). The peaks were both identified as HQ based on mass spectral properties. The samples drawn overnight did not show any intermediate, indicating the complete degradation of substrates.

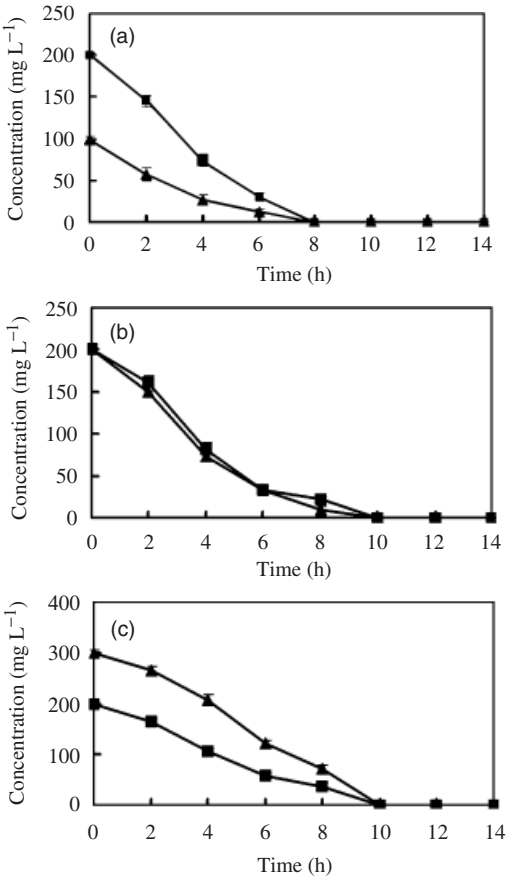

Fig. 2. Effect of different initial 4-CP concentrations on the simultaneous degradation of PNP and 4-CP by strain LZ-1. The initial cell concentration was 10^6 cells mL^{-1}. The initial PNP (■) concentration was 200 mg L^{-1}. The initial 4-CP (▲) concentrations were (a) 100 mg L^{-1}, (b) 200 mg L^{-1} and (c) 300 mg L^{-1}. The depletion of PNP or 4-CP was determined by HPLC. Experiments were performed in triplicate and repeated three times.

Potential metabolites were added separately to induced resting-cells or cell-free extracts. In spectrophotometric assays of HQ removal the original peak of HQ at 288 nm disappeared gradually and a transient peak around 320 nm appeared, indicating the formation of 4-hydroxymuconic semialdehyde (Spain & Gibson, 1991). After 30 min, the reaction mixture showed no significant absorbance in the UV region of the spectrum. No depletion of 4-nitrocatechol or 4-chlorocatechol was observed.

Under aerobic conditions, cell-free extracts of strain LZ-1 showed oxidation activities catalyzing the stoichiometric

release of nitrite ions from PNP or chloride ions from 4-CP. The enzyme activity of PNP and 4-CP was retained in the particulate fraction mainly (82% and 88% respectively). Cytochrome c, acriflavin and picric acid (50 mM), known oxygenase inhibitors, could inhibit the hydroxylation. As the electron donor, NADPH stimulated the oxidation activity more efficiently than NADH. No ring fission activity of 4-nitrocatechol or 4-chlorocatechol was detected.

GC-MS analysis and enzyme assays indicated that HQ, the product of hydroxylation of *p*-substituted phenols by a membrane-associated monooxygenase (Spain & Gibson, 1991; Bae *et al.*, 1996a), was the common metabolite rather than catechols of the degradation of *p*-substituted phenols. According to the results of intermediate identification, we proposed that these phenols were metabolized via the HQ pathway (Fig. 3) by strain LZ-1 (Spain & Gibson, 1991).

Simultaneous degradation of PNP and 4-CP

In the media containing a constant concentration of PNP (200 mg L^{-1}) and three different initial concentrations of 4-CP (100, 200 and 300 mg L^{-1}), strain LZ-1 could remove PNP and 4-CP simultaneously. The addition of 4-CP shortened the time of PNP degradation greatly [12 h for PNP alone (Fig. 1b) compared to 8 h for PNP in the mixtures of PNP and 4-CP (Fig. 2a)]. Particularly, the increase in concentration of 4-CP (100–300 mg L^{-1}) did not inhibit PNP degradation, which was different from the inhibition effects previously reported (Saéz & Rittmann, 1993; Hao *et al.*, 2002). The accelerated PNP degradation might be due to the increased biomass supported by 4-CP because, concomitant with the increase of 4-CP concentration, the biomass increased but the rate of PNP degradation was almost invariable (data not shown) (Bae *et al.*, 1996b). The simultaneous degradation was completely inhibited when the initial PNP concentration increased to 600 mg L^{-1}.

Cells pre-grown on either PNP or 4-CP could degrade their mixtures, and the times required for their respective degradation were almost same independent of their initial concentrations. These results indicated that they shared the same metabolic pathway, and the responsible enzymes had no preference for either PNP or 4-CP. Although simultaneous degradation of different compounds is often problematical due to the incompatibility of different pathways (Timmis *et al.*, 1994), indigenous or genetically engineered microorganisms containing a sole pathway are able to degrade mixtures successfully (Rojo *et al.*, 1987; Bae *et al.*, 1996b). The simultaneous degradation by strain LZ-1 might also be due to the elimination of competition of different pathways. To date, this is the first report of simultaneous biodegradation of PNP and 4-CP in a single bacterium. Therefore, strain LZ-1 will be useful for the bioremediation of the mixtures of phenolic compounds, which generally occur in hazardous waste sites.

Location of catabolic genes for *p*-substituted phenols degradation

The involvement of catabolic plasmids in phenols degradation has been reported previously (Ledger *et al.*, 2006). In this study, an indigenous plasmid was isolated from strain LZ-1 (Supplementary Fig. 3). In plasmid-curing studies, six derivatives with PNP^- and $4\text{-}CP^-$ phenotypes were obtained, and that plasmid was not detected in them. In conjugation studies, transconjugants capable of degrading PNP or 4-CP were obtained at the frequency of 10^{-6} on selection agar, and that plasmid was detected in them. The patterns of phenols degradation of wild and transformed strains were very similar (data not shown). These results

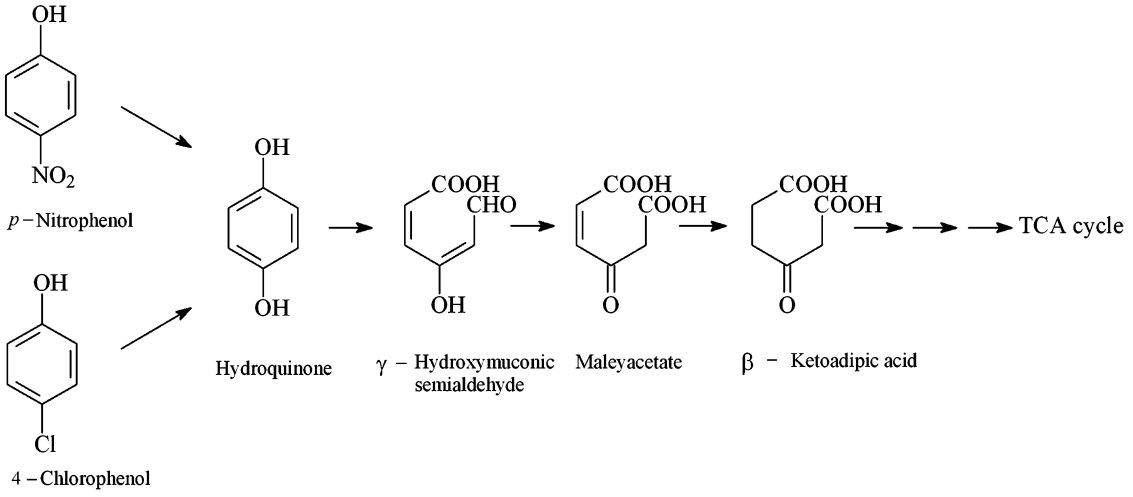

Fig. 3. Proposed pathway for the degradation of PNP and 4-CP by *Stenotrophomonas* sp. LZ-1.

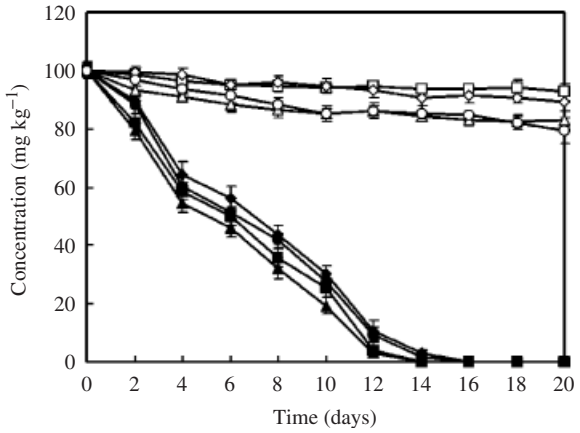

Fig. 4. Simultaneous degradation of PNP and 4-CP in soils inoculated with strain LZ-1 at the cell density of 10^6 cells g^{-1}. ■, fumigated soil containing PNP, inoculated; □, fumigated soil containing PNP, uninoculated; ▲, unfumigated soil containing PNP, inoculated; △, unfumigated soil containing PNP, uninoculated; □, fumigated soil containing 4-CP, inoculated; □, fumigated soil containing 4-CP, uninoculated; ●, unfumigated soil containing 4-CP, inoculated; ○, unfumigated soil containing 4-CP, uninoculated. Experiments were performed in triplicate and repeated three times.

clearly indicated that the genes for PNP and 4-CP degradation were located on a transmissible plasmid in strain LZ-1. Endonuclease digestion of plasmid DNA is in progress to determine its size.

Degradation of PNP and 4-CP by LZ-1 in soils

Introducing exogenous microorganisms into polluted sites is an effective approach for accelerating bioremediation (Labana *et al.*, 2005). Strain LZ-1 (10^6 cells g^{-1}) removed 100 mg kg^{-1} of PNP and 4-CP within 14 days and 16 days in soil, respectively. A much slower disappearance of substrates was observed in uninoculated controls. The decrease of substrates in unfumigated samples was slightly more rapid than those in fumigated ones (Fig. 4). Strain LZ-1 could still be isolated from the unfumigated soils, whereas phenols were removed completely, indicating that strain LZ-1 might be responsible for the removal of phenols. Therefore, the ability of strain LZ-1 to decontaminate phenolic compounds in soils was preliminarily proved.

Temperature and pH are crucial abiotic factors for the survival and activity of microorganisms (Labana *et al.*, 2005). In this study, alkaline conditions (pH 7.3–10.2) were more favorable for the removal of phenols than acidic conditions (pH 3.8–6.6), and the optimal pH was 9.0. Highly acidic (pH<4.0) or alkaline (pH>10.0) conditions did not favor the removal of phenols. Unusually, the degradation activity of strain LZ-1 was retained even at 4 °C, a low temperature unfavorable for most bacteria (Backman & Jansson, 2004), and temperature fluctuations (4 °C to 35 °C) did not influence the activity significantly. Similar strains that can degrade phenols at low temperature have been studied (Backman & Jansson, 2004). This broad adaptability to temperature made strain LZ-1 a potential tool for bioremediation in different locations, especially in cold climates.

In conclusion, due to broad substrate specificity, strong degradation ability and adaptability to temperature variation, strain LZ-1 is a promising candidate for the bioremediation of toxic phenolic compounds from the environment.

Acknowledgements

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. The phylogenetic tree of strain LZ-1 based on 16S rRNA gene sequence analysis. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points. GenBank accession numbers are shown in parentheses.

Fig. S2. GC-MS spectra and structure of intermediate of PNP (A) and 4-CP (B) degradation.

Fig. S3. Agarose gel (0.8%) electrophoresis of plasmid DNA extracted from strain LZ-1, cured derivative and transconjugant of strain LZ-1 and *Escherichia coli* DH5α (as control). Lane 1, DNA marker of λ DNA digested with HindIII; Lane 2, strain LZ-1; Lane 3, cured derivative of strain LZ-1; Lane 4, transconjugant of strain LZ-1; Lane 5, *Escherichia coli* DH5α.

Table S1. Oxygenase activities of alternative substrates by different fractions of cell-free extracts.

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