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Effect of temperature and additional carbon sources on phenol degradation by an indigenous soil *Pseudomonad*

Paraskevi N. Polymenakou[#] & Euripides G. Stephanou[*]
Environmental Chemical Processes Laboratory, Department of Chemistry, University of Crete, Knossos Avenue P.O. Box 1470, 714 09 Heraklion, Greece; [#]Present address: Hellenic Center for Marine Research, P.O. Box 2214, 71003 Heraklion, Crete, Greece
*(*author for correspondence: e-mail: stephanou@chemistry.uoc.gr)*

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Abstract

A new indigenous soil bacterium *Pseudomonas* sp. growing on phenol and on a mixture of phenol, toluene, *o*-cresol, naphthalene and 1,2,3-trimethylbenzene (1,2,3-TMB) was isolated and characterized. Phylogenetic analysis suggested its classification to *Pseudomonadaceae* family and showed 99.8% DNA sequence identity to *Pseudomonas pseudoalcaligenes* species. The isolate was psychrotroph, with growth temperatures ranging from ca. 0 to 40 °C. The GC–MS structural analysis of metabolic products of phenol degradation by this microorganism indicated a possible *ortho* cleavage pathway for high concentrations (over 200 mg L^{-1}) of phenol. Biodegradation rates by this species were found to be three times more effective than those previously reported by other *Pseudomonas* strains. The effect of temperature on phenol degradation was studied in batch cultures at temperatures ranging from 10 to 40 °C and different initial phenol concentrations (up to 500 mg L^{-1}). Above 300 mg L^{-1} of initial phenol concentration no considerable depletion was recorded at both 10 and 40 °C. Maximum degradation rates for phenol were recorded at 30 °C. The biodegradation rate of phenol was studied also in the presence of additional carbon sources (*o*-cresol, toluene, naphthalene, 1,2,3-TMB) at the optimum growth temperature and was found significantly lower by a factor of eight in respect to the strong competitive inhibition between the substrates and the more available sources of carbon and energy. The Haldane equation $\mu = \mu_m S/(K_S + S + S^2/K_I)$ was found to best fit the experimental data at the optimum temperature of 30 °C than the Monod equation with kinetic constants $\mu_m = 0.27\ h^{-1}$, $K_S = 56.70$ mg L^{-1}, $K_I = 249.08$ mg L^{-1}.

Abbreviations: GC–MS – gas chromatography–mass spectrometry; HPLC – high performance liquid chromatography; NAPL – non-aqueous phase liquid; RDP – ribosomal database project; TCA – tricarboxylic acid

Introduction

The extensive use of phenolic compounds in various industrial operations (e.g., polymeric resin production, oil refining and coking plants) has led to widespread environmental pollution by these chemicals (Beltrame et al. 1980; Annachhatre & Gheewala 1996; Santos & Linardi 2001). The control of these pollutants is of great importance because they are toxic and recalcitrant and hence their discharge into the environment must be regulated (Annachhatre & Gheewala 1996; Santos & Linardi 2001).

A number of both aerobic and anaerobic phenol degrading microorganisms have been isolated and characterized (Chen et al. 2004; Ryoo et al.

2000; Santos & Linardi 2001), although microorganisms capable of aerobic phenol degradation were described as early as 1908 (van Schie & Young 1998). A very important phenol-degrading microorganism that showed high biodegradability belongs to the genus of *Pseudomonas*. Numerous *Pseudomonas* strains and related gram-negative genera have been isolated from contaminated sites, and the biochemistry, genetics and growth kinetics of phenol degradation under laboratory conditions have been studied (Stringfellow & Aitken 1995; Favaro et al. 1996; Esteve-Nuvez & Ramos 1998; Poelarends et al. 1998; Potrawfke et al. 1998; Shapir et al. 1998; Bales & Antosova 1999; Filonov et al. 1999; Loh & Yu 2000; Banerjee et al. 2001; Pazarlioğlu & Telefoncu 2004).

It has been reported that the *Pseudomonas* species follows a typical *meta* cleavage pathway for metabolizing phenol at relatively low concentrations (Bales & Antosova 1999; Hall et al. 1999). However, in bacteria there are two pathways for the degradation of catechol: (a) The *meta* cleavage pathway which converts catechol to 2-hydroxymuconic semialdehyde and induced by precursors of the pathway substrates such as phenol and alkyl-substituted phenols (Shingler et al. 1989; Radnoti de Lipthay et al. 1999). (b) The *ortho* cleavage pathway which is active during growth on phenol and halosubstituted phenols and which converts catechol to *cis–cis* mucconic (Feist & Hegeman 1969).

The above modes of catechol degradation might affect the growth of bacteria on phenol (Watanabe et al. 1998). Besides, it has been suggested (Roszak & Colwell 1987; Watanabe et al. 1998) that other factors, such as the nutrient availability (carbon sources), the presence of toxins and physical parameters can affect the bacterial growth. For example, temperature might have an equivalent or larger role, than nutrient availability, in degrading organic pollutants (Margesin & Schinner 1997). Most laboratory studies on pollutants degradation have been carried out at temperatures (mainly at 30 °C) higher than those found in nature (Hill & Robinson 1975; Poelarends et al. 1998; Potrawfke et al. 1998; van Schie & Young 1998; Loh & Yu 2000; Annadurai et al. 2002). The effect of temperature on pollutants degradation has been investigated for oil (Margesin & Schinner 1997), toluene and benzoate (Chablain et al. 1997), chlorophenols (Melin et al. 1998; Cort & Bielefeldt 2000) and nonylphenol polyethoxylate (Manzano et al. 1999). However, the effect of a wide range of temperatures on phenol degradation by free cells of bacterial strains has not been studied extensively.

In the present study, a new indigenous *Pseudomonas* strain was isolated from a petroleum-contaminated site in Denmark and characterized using the full gene 16S rDNA sequence analysis in the Ribosomal Database Project (RDP) (Cole et al. 2003). The main objectives of the present work were: (a) the determination of the biodegradability of phenol by this bacterium, (b) the identification of critical metabolic products by using gas chromatography–mass spectrometry (GC–MS) analysis and (c) the study of the effect of temperature and additional organic sources on phenol removal. Growth kinetics studies were also carried out in order to estimate the growth parameters μ_m (maximum specific growth rate) and K_S (saturation constant) according to Hill and Robinson (1975).

Materials and methods

Isolation and characterization of a psychrotrophic biodegradative strain

An enriched mixed culture from samples of petroleum-contaminated soil in Denmark (EC Pore to Core Program, European Environment) and capable of growth on phenol as a sole source of carbon and energy served as the starting material for the isolation of a pure culture. Colonies grown on plates were isolated and reinoculated into mineral salt medium. Single colonies were tested for their ability to utilize phenol. The pure culture was identified by phenol utilization and 16S rDNA sequence analysis and a stock culture of the purified strain was maintained on Luria Bertani (LB) broth medium plus glycerol at −80 °C (Sambrook et al. 1989). Purity was checked on LB plates and microscopic examination. The primary culture was prepared by transferring a loop of stock culture to 100 ml of growth medium with continuous replenishment of phenol (final concentration of 200 mg L^{-1}). The strain was tested for growth on a wide range of temperatures (0–40 °C). Consequently, four temperatures (10, 20, 30 and 40 °C) were selected for the determination of acclimatization time in order to

avoid long lag phases during biodegradation measurements. The strain was tested also on a wide range of initial phenol concentrations and was found capable of growth in 1–1200 mg L^{-1} of phenol concentration. A tertiary culture was used (exponential growth phase) for the biodegradation studies and a culture without the addition of any source of carbon and energy served as a negative control.

Media and growth conditions

The growth medium consisted of the following components: 1880 ml of sterile water, 50 ml of $20 \times$ M9 salts (140 g L^{-1} Na_2HPO_4, 60 g L^{-1} KH_2PO_4, 10 g L^{-1} NaCl, 20 g L^{-1} NH_4Cl and pH adjusted to 7.0 to 7.2 by adding 1 M NaOH), 14 ml of R salts (200 ml 10% $MgSO_4 \times 7H_2O$, 50 ml 1.0% $FeSO_4 \times 7H_2O$), 4 ml of 1 M $MgSO_4$ and 200 μl of 1 M $CaCl_2$. The phenol degrader was isolated on minimal medium agar plates. Agar plates were prepared by adding 15 g of Agar (Sigma-Aldrich Chemie, Germany) per liter of growth medium. For growth on agar plates, phenol crystals were placed in the lid of each petri dish. Purified strain was tested for its ability to utilize the non-aqueous phase liquid (NAPL) mixture by growing the microorganism in liquid minimal medium. Liquid cultures were grown in Erlenmeyer flasks fitted with Teflon-sealed screw caps on a G24 Environmental Incubator shaker (New Brunswick Scientific Co. Inc., Edison, NJ USA) at 30 °C ($\pm$ 1 °C). Phenol and other substrates were directly supplied to liquid media. The stock culture was maintained on LB Broth medium (Sambrook et al. 1989).

Determination of 16S rDNA sequence

Primers fD1 and rD1 were designed around the 5′ and 3′ ends of bacterial 16S ribosomal DNA (rDNA), respectively, to produce a 1.5-kb fragment of 16S rDNA by PCR amplification as described by Bresler et al. (2000). The 1.5-kb PCR product amplified from the isolate was gel purified and cloned into a pGEM-T Easy Vector Systems (Promega product No. A3600) and then subjected to sequencer using the T7/SP6 vector primers. The sequence was compared with sequences in the Ribosomal Database Project (RDP) (Cole et al. 2003) by using Sequence Match Software and Phylip Interface (for phylogenetic analysis) and with GenBank sequences by using BLAST software (Stoesser et al. 2001). The phDV1 16S-rDNA sequence has been submitted to GenBank under accession number AY435123.

Analytical methods

Cell density was monitored spectrophotometrically by measuring the absorbance at 600 nm by using a Milton Roy Spectronic 401 UV–Vis spectrophotometer. Phenol measurements were performed by reverse phase high prformance lquid chromatography (HPLC) on a Hewlett Packard 1100 Series Model equipped with a HP G.1322A degasser, a Photodiode Array Detector at 212 m and Kromasil C18 column (250×4 mm). The mobile phase was 65% sodium acetate at pH 4.5, 25% Acetonitrile and 10% Methanol at a flow rate 1.5 ml/min and at a temperature of the column of 22 °C. For the segregation of NAPL mixture the chromatographic conditions were the following: column temperature 22 °C, flow rate 1 ml/min, mobile phase 50% MeOH–50% H_2O, mobile phase program: 50–80% MeOH (18 min), 80–90% MeOH (2 min), 90% MeOH (2 min), and 90–50% MeOH (18 min).

Detection and identification of metabolites was performed by GC–MS. GC–MS analyses were carried out on a Hewlett Packard mass-selective detector with the appropriate data system in full scan mode. A Hewlett Packard GC Model 5890 equipped with a Grob-type split–splitless injector was directly coupled with the fused-silica capillary column (HP-5 MS with 0.25 μm film thickness, 30 m $\times$ 0.25 mm ID), to the ion source. Helium was used as a carrier gas with a backpressure of 55 kPa. The electron impact ionization mode conditions were the following: ion energy 70 eV; ion source temperature 195 °C; mass range 35–590 m/z. The chromatographic conditions were the following: injector temperature 270 °C; temperature program, 60 °C (1 min), 70–190 °C (3 °C/min), 190–290 °C (10 °C/min). The experimental procedure for the isolation and derivatization of metabolites was carried out according to Potrawfke et al. (1998). Briefly, culture supernatants were extracted three times with equal volumes of ice-cold ethyl acetate at a neutral pH for the recovery of non-dissociated compounds and at acid pH for dissociated compounds. The organic

phase was removed, treated with anhydrous and pre-cleaned sulfate magnesium and evaporated to near dryness under the gentle stream of nitrogen. Derivatization of metabolites was performed with freshly prepared solution of diazomethane in diethyl ether until the yellow color of the reagent remained stable. The derivatized extracts were directly analyzed by GC–MS (Potrawfke et al. 1998).

Reagents

All the chemicals used in the present study were of analytical grade. Phenol crystals (99 + % purity) were purchased from Merck. The experimental procedure for liquid phenol preparation was carried out according to Sambrook et al. (1989). *o*-Cresol (99 + % purity) was purchased from Sigma and naphthalene (> 98% purity) was purchased from Fluka. Toluene (99.9%) and 1,2,3-Trimethylbenzene (> 90%) were purchased from Merck.

Results and discussion

Strain identification and phenol biodegradation

A mixed culture degrading phenol and maintained on solid medium (unpublished data) was used as the starting material for this study. After 1 month of continuous subcultivation on solid and liquid media, a pure culture capable of growth on phenol as a sole source of carbon and energy within the range of 1–1200 mg L^{-1} was isolated. The isolated strain was gram-negative, and capable of growth also on NAPL. The NAPL mixture contains five different representative pure aromatic compounds, namely, phenol, *o*-cresol, toluene, naphthalene and 1,2,3-Ttrimethylbenzene. The first four substances are very common contaminants, whereas the fifth compound is a recalcitrant organic substance and it is used as a tracer in field studies (Vayenas et al. 2002). The isolate was psychrotroph, with growth temperatures ranging from ca. 0 to 40 °C. 16S rDNA sequence analysis of phDV1 strain showed 99.8% DNA sequence identity to *Pseudomonas pseudoalcaligenes*. On the basis of this data, this strain is referred as *Pseudomonas* sp. strain phDV1.

The phylogenetic analysis of 16S-rDNA sequence (Figure 1) shows that the closely related microorganisms belong to the species *Pseudomonas pseudoalcaligenes* (Moore et al. 1996; Juteau et al. 1999; Sikorski et al. 1999; Takami et al. 1999; Anzai et al. 2000). The catabolic pathway of phenol degradation was studied, after extraction of a culture containing 200 mg L^{-1} of phenol by using GC–MS analysis. HPLC analysis was used to confirm the presence of catechol (Figure 2a).

In Figure 2b is illustrated a GC–MS chromatograph of the methylated acidic extract of culture supernatants from a 2-day incubation experiment (where the color of the culture changed to yellow). This analysis confirmed the presence of one more intermediate of phenol decay, namely 2,4-hexadienedioic acid dimethyl ester (peak Nr. 1; Figure 2b). In Figure 2c is presented the corresponding mass spectra of the above compound (annotated with the same number as in Figure 2b). The mass spectrum of 2,4-hexadienedioic acid dimethyl ester contains the molecular peak at $m/z = 170$, while the ion at $m/z = 111$ shows the loss of one carbomethoxy group (Figure 2c). Both

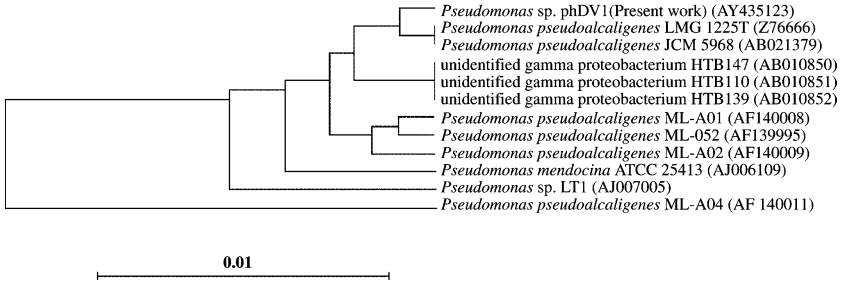

Figure 1. Constructed phylogenetic tree using Phylip Interface Software, showing the closely related microorganisms to *Pseudomonas* sp. phDV1 (accession number AY435123). The accession numbers of the related strains are also indicated (in parentheses). The scale bar indicates 1% nucleotide change per 16S rRNA position.

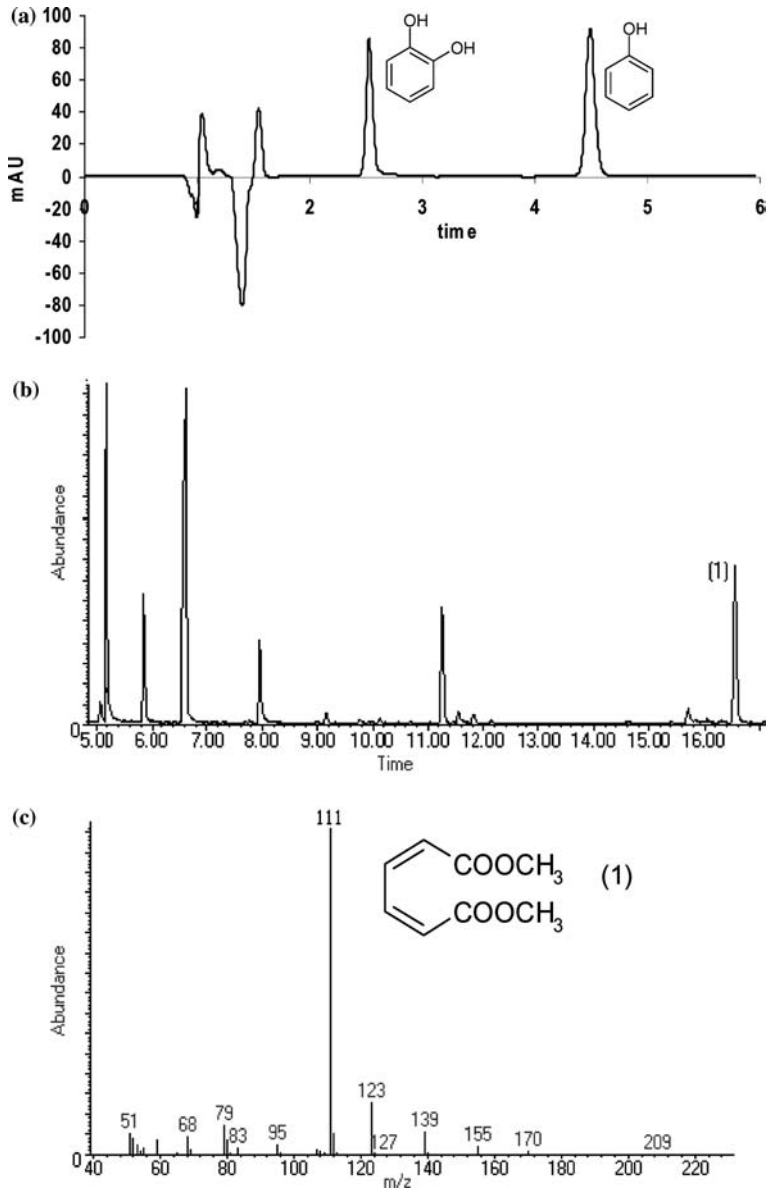

Figure 2. (a) HPLC chromatograph of phenol and its transformation product catechol. (b) GC–MS chromatograms of the methylated culture supernatant acidic extract after a 2-day incubation period. (c) Mass spectra of the determined metabolic product of phenol degradation: 2,4-hexanedioic acid dimenthyl ester (1).

GC–MS chromatograph and mass spectrum (Figures 2b, c) suggest the formation of 2,4-hexadienedioic acid (*cis–cis* mucconic acid) in high amounts (Figure 2b) during phenol decay.

The cleavage product of catechol, *cis–cis* mucconic acid, represents the critical intermediate of *ortho* decay (Feist & Hegeman 1969). The ring cleavage product of catechol can be then easily converted to tricarboxylic acid cycle (TCA cycle) intermediates, which are further broken down into CO_2 and water (Madigan et al. 2000). Our results indicated that the isolated strain can dearomatize the phenolic ring using possibly the *ortho* cleavage mechanism at high concentrations of phenol. In reverse, at low concentrations of phenol ($1\ mg\ L^{-1}$) the critical products of the *ortho* cleavage pathway were not detected indicating a possible *meta* cleavage pathway. Considerably, the

aerobic *Pseudomonas* sp. phDV1 strain disposed the capability to induce different ring fission pathways (*ortho* and *meta*) under different initial substrate concentrations. GC–MS analysis can be used as an alternative and more reliable technique (to the classical enzyme assays mostly based on changes of color medium (Whiteley et al. 2001) or UV absorbance at a specific wavelength (Briganti et al. 1997) induced by the addition of catechol) in order to characterize the cleavage mode of aromatic pollutants. GC–MS analysis offered the advantage to directly detect and confirm the presence of the critical intermediate in phenol degradation, which in this study was *cis–cis* muconic acid. However, a detailed analysis of the involved enzymes in the presence of phenol must be performed in order to thoroughly investigate the metabolic strategy of the isolated strain.

Effect of temperature on phenol degradation

Phenol degradation was carried out with free cells of *Pseudomonas* sp. under different process variables as the initial phenol concentration. Negative controls exhibited no significant change in optical density during incubation. Figure 3 shows the reduction of phenol concentration during the degradation process at the different temperatures. Firstly it can be seen that for phenol concentrations above 300 mg L^{-1} no significant decrease in the amount of phenol was observed at both 10 and 40 °C (Figure 3).

In calculating the average degradation rates of phenol we have observed that the differences in degradation rates could be erroneous when the length of the lag phase was included since this phase varied significantly between duplicate experiments. Thus, we calculated the degradation rates by dividing the net amount of phenol (when phenol accounted ca. 10 and 90% of the initial concentration) by the corresponding time as Loh and Wang (1998) have suggested. The biodegradation rates of phenol at four different temperatures were calculated and their variation was plotted as a function of phenol concentration (Figure 4). It has been commonly assumed, as in chemical reactions that biodegradation rates decrease by a factor of two for a 10 °C temperature decrease (Melin et al. 1998). However, we observed biodegradation rates almost identical at

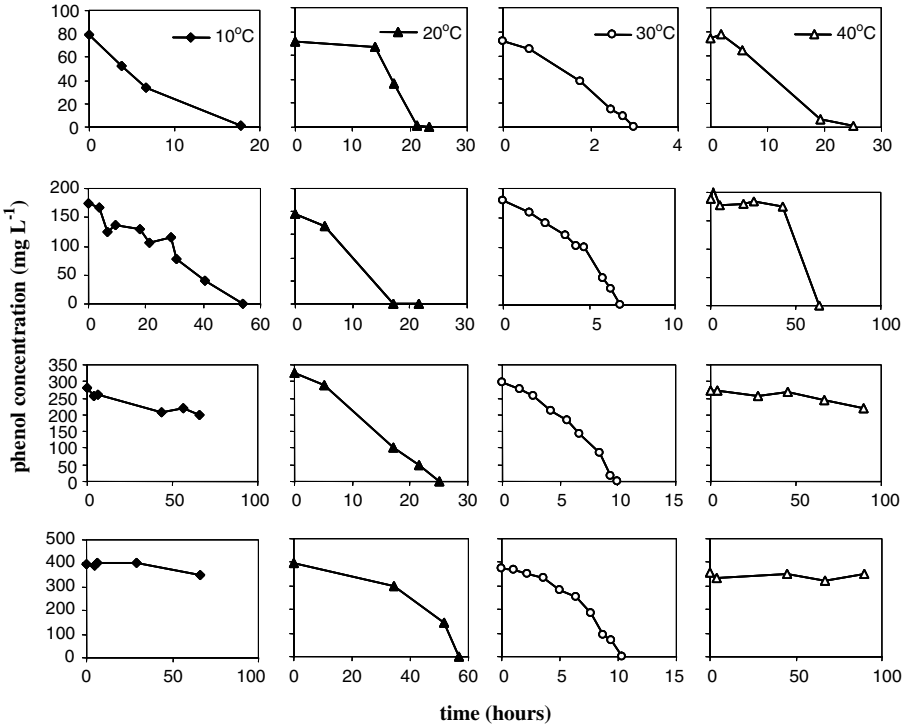

Figure 3. Phenol degradation of various concentrations at four different temperatures (10 20, 30, and 40 °C).

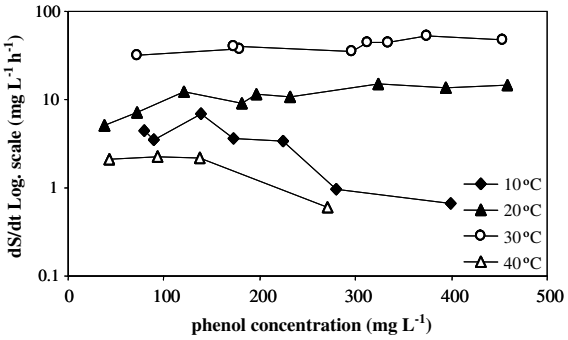

Figure 4. Biodegradation rates (dS/dt) in mg L^{-1} h^{-1} (logarithmic scale) of phenol at four temperatures.

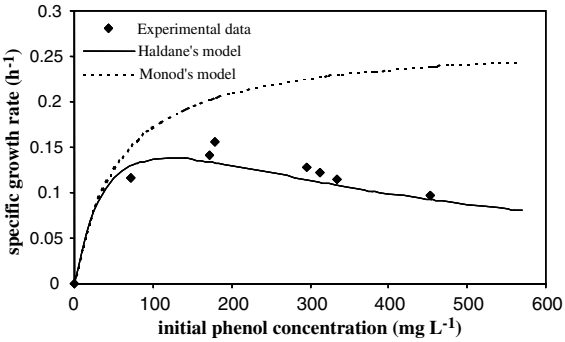

Figure 5. Experimental and predicted values of specific growth rate versus initial phenol concentration from Haldane's and Monod's model using free cells of *Pseudomonas* sp. phDV1 strain. Haldane's model best fit the experimental data.

both 10 and 40 °C, and slightly higher at 20 °C. At 30 °C we observed degradation rates significantly higher (by a factor of three) than the rates at 20 °C. At 30 °C, biodegradation rates reached values up to 52 mg L^{-1} h^{-1}(Figure 4). This differentiation from the main rule of chemical reactions might be due to the differences in penetration efficiency of phenol into the cells of *Pseudomonas* sp. strain phDV1 through the cell membrane. The biodegradation rates of phenol by utilizing this strain were higher, almost by three orders of magnitude, than the corresponding rates reported by Bandyopadhyay et al. (1998).

Kinetics of phenol degradation

Since the degradation proceeds with cell mass growth, the kinetic parameters were evaluated on the basis of Monod and Haldane growth models at the optimum growth temperature (30 °C). The implementation of these two models was used to determine the importance of the inhibition effects of phenol using the bacteria of the genus *Pseudomonas* sp. strain phDV1.

Typical cell growth and degradation of phenol as the substrate (the limiting nutrient) were studied by using spectrophotometer and HPLC analysis, respectively. The experimental data for cell mass (X) and phenol concentrations (S) for different batch times using feeds of various phenol concentrations were generated.

In order to evaluate the kinetic parameters of Monod and Briggs-Haldane models (μ_m, K_S, K_I), the plot of specific growth rate, versus initial phenol concentration was studied. The results are illustrated in Figure 5. As the curve shows, the maximum specific growth rate μ_m was reached at a phenol concentration of 220 mg L^{-1} and then the specific growth rate μ gradually decreased. It was obvious (Figure 5) that beyond 220 mg L^{-1}, the inhibition became prominent. Thus the parameter μ has been found to be a strong function of initial phenol concentration S_O. For each batch culture with a certain initial amount of phenol, the specific growth rate was estimated using the equation described by Hill and Robinson (1975).

The kinetic parameters for *Pseudomonas* sp. grown on phenol at 30 °C were obtained as follows: $\mu_m = 0.27$ h^{-1}, $K_S = 56.7$ mg L^{-1}, $K_I = 249.08$ mg L^{-1}. The experimental data fitted better the Haldane kinetic model than the Monod equation (Figure 5). The above values were in the range of literature values (Sokol 1987; Bandyopadhyay et al. 1998; Bales & Antosova 1999; Monteiro et al. 2000; Loh & Chua 2002).

Effect of aromatic compounds mixture on phenol degradation

In this work, we investigated the effect of supplementary carbon sources (toluene, naphthalene, *o*-cresol, 1,2,3-trimethylbenzene) on the phenol degradation at the temperature of 30 °C where the maximum biodegradability of phenol was observed. In Figure 6 is plotted the decrease of the compounds concentrations versus time, where it can be seen that phenol together with *o*-cresol and toluene were degraded at the same time where the optical density of cells remained constant and then a significant increase in cell abundance was observed.

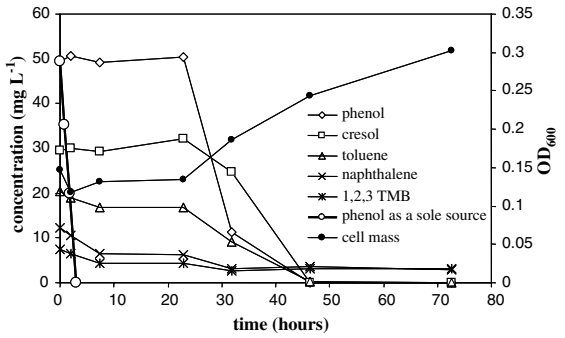

Figure 6. (a) Variation of the NAPL mixture degradation and of the phenol as the only source of carbon and energy versus time in batch cultures using free cells of *Pseudomonas* sp. strain phDV1.

Previous reports also have shown that Pseudomonads disposed the ability to degrade efficiently *o*-cresol, toluene and naphthalene (Chablain et al. 1997; Lloyd-Jones et al. 1999; Mosqueda et al. 1999; Shim & Yang 1999; Takizawa et al. 1999; Carragher et al. 2001).

When *Pseudomonas* sp. cells were transferred to the minimal medium containing phenol as a sole source, which was the same carbon source as in preculture, a lag phase was not observed. Conversely, in a medium containing the NAPL mixture, cell growth did not occur for the first 30 h (Figure 6). During the lag phase, cells might have prepared themselves for adaptation in the toluene, *o*-cresol and naphthalene. The biodegradation rate of phenol in presence of NAPL mixture was lower by a factor of eight in comparison with cultures containing phenol as a sole source, most likely due to the more available sources of carbon and energy (Figure 6).

Based on the degradation mechanism proposed by Yang and Humphrey (1975), the first step in biodegradation process of the phenol is the formation of the catechol intermediate facilitated by the enzyme of NADPH-dependent hydroxylase. Similarly, the oxidation of naphthalene, *o*-cresol and toluene are initiated also by hydroxylase enzymes to form the correspondent hydroxylated compounds (Bayly et al. 1966; Gibson et al. 1970; Barnsley 1975) where the 1,2,3-trimethylbenzene is a known non-growth substrate. Hence, these compounds expected to inhibit oxidation of each other, competitively. In the present study, competition was dominated by the oxidation of phenol, *o*-cresol and toluene. Naphthalene and 1,2,3-trimethylbenzene were rather unable to bind with the hydroxylase enzyme. Consequently, no significant depletion on the levels of both naphthalene and 1,2,3-trimethylbenzene was observed (Figure 6).

These observations indicated the equivalent contribution of the structurally analogous substrates of phenol, toluene and *o*-cresol on biomass growth of *Pseudomonas* sp. strain phDV1. The selective binding of these compounds with the hydroxylase enzyme indicated that this strain induced enzymes compatible with phenyl-ring compounds and the low biodegradation rates are likely due to the strong inhibitory effect of the non-growth substrate 1,2,3-trimethylbenzene. In a previous study, Loh and Wang (1998) reported the competitive inhibition between phenol and 4-chlorophenol, and they demonstrated that the addition of conventional carbon sources increased the toxicity tolerance of bacterial cells towards 4-chlorophenol, and enhanced biodegradation rates (Loh & Wang 1998). The addition of conventional carbon sources could be serving as a convenient approach to overcome the competitive inhibition between organic compounds.

Implications of the observations

Cometabolism is an important biological transformation process in nature (Loh & Wang 1998) and the effectiveness of bioremediation depends on the affinity between the proper microorganisms and the different organic compounds as well as on the ability of microorganisms to gain access to contaminants (Cort & Bielefeldt 2000). This ability is affected mostly by many physical, chemical and structural properties of both the contaminants and the soil matrix (Loh & Wang 1998). The ability of the isolated *Pseudomonas* sp. strain phDV1 to degrade phenol in a wide range of temperatures has an important implication for the treatment of contaminated environments. The pollution problem in the natural environments is often more serious at temperatures lower than 30 °C where the majority of the laboratory experiments were usually carried out (Kettunen et al. 1996; Margesin & Schinner 1997; Cort & Bielefeldt 2000). Hence, we demonstrate the importance of the present bacterium strain in biodegradation processes. The available sources of carbon and energy have also an equivalent role to the temperature in pollutants degradation. The degradation ability of the

isolated bacterium in the NAPL mixture was also evident despite the presence of the non-growth, and high toxic 1,2,3-trimethylbenzene, strongly contributed to the importance of the isolated strain in bioremediation processes.

Conclusions

In the present study, a new indigenous soil bacterium that can grow on a wide range of aromatic compounds was isolated and characterized by analysis of 16S rRNA gene. The metabolic products were identified by GC–MS analysis. The effect of temperature and additional organic sources on phenol degradation in batch cultures containing free cells of this bacterium was also investigated. Temperature had a strong effect on phenol biodegradation rates. A 10 °C decrease in temperature decreased the degradation rates by over a factor of three. Degradation rates at both 10 and 40 °C were low, but the optimum values were recorded at 30 °C (52 mg L^{-1} h^{-1}). The values of the kinetic constants obtained at the optimum growth temperature were in the range of literature values. The addition of supplementary organic sources (NAPL mixture) on phenol cultures, resulted in a significant decrease of phenol consumption by a factor of eight indicating the rapid adaptability of *Pseudomonas* sp. cells on the structurally analogous substrates of toluene and *o*-cresol and the possible inhibition effect of the nongrowth substrate 1,2,3-trimethylbenzene. In the present study we have shown the ability and importance of new isolated strain *Pseudomonas* sp. phDV1 in biodegradation processes.

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Appendix

Nomenclature

S	Phenol concentration (mg L^{-1})
S_O	Initial phenol concentration (mg L^{-1})
X	Initial OD_{600} of the culture supernatant
K_S	Saturation constant (mg L^{-1})
K_I	Inhibition constant (mg L^{-1})
OD_{600}	Optical density of culture supernatant in 600 nm
μ	Specific growth rate (h^{-1})
μ_m	Maximum specific growth rate (h^{-1})
dS/dt	Biodegradation rate (mg L^{-1} h^{-1})

Growth by binary fission is described by $dX/dt = \mu x$, which defined the specific growth rate. The estimation of specific growth rate versus initial phenol concentration was carried out by $\mu = (\ln X_2/X_1) / (t_2 - t_1)$.

In non-inhibited growth, commonly described by the Monod model, μ asymptotically approaches an upper limit, μ_m, as S increases. Substrate inhibition of microbial growth by this mechanism is given by Haldane equation $\mu = \mu_m S/(K_S + S + S^2/K_I)$. At sufficient high substrate concentration ($S \gg K_S$), the above equation can be linearized such that the plot of $1/\mu$ versus S gives intercept $1/\mu_m$ and slope $1/(K_I\mu_m)$. Such linearization, of course, yields no information on the value of K_S. For K_S estimation Andrews equation is usually used in which the maximum specific growth rate with inhibition, occurs at the substrate concentration where $S = (K_I\ K_S)^{0.5}$ (Hill & Robinson 1975).

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