

## FOUR *BACILLUS* SP. SOIL ISOLATES CAPABLE OF DEGRADING PHENOL, TOLUENE, BIPHENYL, NAPHTHALENE AND OTHER AROMATIC COMPOUNDS EXHIBIT DIFFERENT AROMATIC CATABOLIC POTENTIALS

LIDIJA ĐOKIĆ, TANJA NARANČIĆ, JASMINA NIKODINOVIĆ-RUNIĆ,  
SANJA BAJKIĆ and BRANKA VASILJEVIĆ<sup>1\*</sup>

<sup>1</sup> *Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, 11010 Belgrade, Serbia*

**Abstract** - Two novel *Bacillus* sp. were isolated from a soil sample from a bank of the Tamiš river in close proximity to a petrochemical facility. They were capable of utilizing a broad range of aromatic compounds as a sole source of carbon and energy (including phenol, benzene, toluene, biphenyl, naphthalene). The isolates were designated as *Bacillus* sp. TN41 and TN42, based on their 16S rDNA sequence. Their catabolic potential was compared to two *Bacillus* sp. strains (PS1 and PS11) isolated from the rhizosphere of the endemorelict plant *Ramonda serbica*. Specific activities of phenol hydroxylase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase were analyzed from crude cell extracts of the isolates, as well as the temperature and pH effects on enzyme activity. Although all four isolates had the ability to degrade a similar range of aromatic compounds, the specific activities of the enzymes indicative of aromatic compound catabolism of TN isolates were 2 to 90-fold lower compared to the PS isolates. Phenol hydroxylase and catechol dioxygenases exhibited broad temperature (10°C-80°C) and pH (4-9) activity ranges in all four *Bacillus* isolates. While phenol inhibited both phenol hydroxylase and catechol dioxygenases in the TN strains, it was an inducer for phenol hydroxylase in the PS strains.

**Key words:** *Bacillus*, biodegradation, phenol, toluene, phenol hydroxylase

UDC 577.21:579.852.11

### INTRODUCTION

Soils and groundwater are preferred sinks for complex contamination. As a result, different chemical, biological, and biochemical properties of soil can be profoundly altered. Their main effect is the continuous loss of soil functions in sustaining the survival of living organisms. Phenol, toluene, benzene and polyaromatic hydrocarbons (PAHs; i.e., naphthalene, phenanthrene, anthracene, etc.) are ubiquitous in the environment as complex mixtures and have been recognized as an environmental contamination problem of worldwide magnitude (Andreoni and Gianfreda, 2007). Aromatic pollutants emerge as a result of human activities, but they

occur naturally as well. Oil refineries and phenolic resin industries are major producers of phenolics. On the other hand, organic matter decomposition products contain phenol. Furthermore, higher plants under stress conditions synthesize phenolics as reactive oxygen species scavengers and release them into the soil. The majority of these substances are highly persistent in ecosystems in unaltered, less degradable chemical forms because of their low water solubility, their intrinsic chemical stability, and high recalcitrance to degradation (Andreoni and Gianfreda, 2007). Therefore, hydrocarbon-polluted sites can represent a long-term source of pollution and pose a severe risk to environmental health.

Removal of aromatic hydrocarbons from the environment employs different methods, such as physical, chemical and biological techniques (bioremediation). The bioremediation approach appears to be the most efficient with regard to commercial and environmental aspects (Andreoni and Gianfreda, 2007; Schie and Young, 2000).

The first step in the aerobic biodegradation of phenol includes its oxygenation by phenol hydroxylase (PH) to form catechol which undergoes ring fission that can take place between the two hydroxyl groups, involving the action of catechol 1,2-dioxygenase (C12O), or proximal to one of the two hydroxyl groups involving the action of catechol 2,3-dioxygenase (C23O). Most other aromatic compounds are first converted to substituted catechols and then degraded by C12O or C23O (Andreoni and Gianfreda, 2007).

The aim of this work was to isolate phenol-degrading microorganisms, preferably Gram positive *Bacillus* (as most of the known aromatic degraders are Gram negative *Pseudomonads* and closely related species), from soil collected in close proximity to a petrochemical industry (contaminated with various aromatics). We report on the isolation, characterization and identification of two novel *Bacillus* strains. In addition, we compared their aromatic degradation ability to two *Bacillus* strains isolated previously from rhizosphere soil with a naturally elevated concentration of phenolics (Djokic et al. 2011). We also examined the activity of the key enzymes involved in phenol and other aromatic compound degradation.

## MATERIALS AND METHODS

### *Reagents*

Phenol, benzene, toluene, styrene and other aromatic compounds used as a sole carbon source, as well as antibiotics and other salts, were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA). *Pfu* DNA polymerase, T4 DNA ligase, O`Gene 100 bp DNA Ladder and pUC19 plasmid were purchased from Fermentas (Burlington, Can-

ada). Tryptic soy broth, soluble starch, casein, and other media components were purchased from Becton Dickinson (Sparks, MD, USA). Oligonucleotide primers were obtained from Invitrogen (Darmstadt, Germany). The QIAprep spin plasmid mini-prep kit, QIAEX II gel purification kit, and QIAquick PCR purification kit were purchased from QIAGEN (Hilden, Germany).

### *Isolation of bacteria from soil*

Soil samples were collected from the rhizosphere of *Ramonda serbica* and from river banks in close proximity to the petrochemical facility. Isolation of bacteria from the rhizosphere of *R. serbica* was previously described (Djokic et al., 2011). Similarly, bacteria were isolated from river bank soil by drying and then sieving the soil under aseptic conditions to a particle size of approximately 2-4 mm. Sterile potassium phosphate buffer (9 ml; 50 mM, pH 7.2) was added to the soil sample (1 g) and the suspension was vigorously mixed by vortexing for 5 min to homogenize the sample. Serial dilutions were performed to obtain a  $10^{-5}$  dilution of the original soil sample. The serial dilutions were spread-plated on starch casein agar (SCA) containing soluble starch ( $10 \text{ g l}^{-1}$ ), casein ( $1 \text{ g l}^{-1}$ ),  $\text{KH}_2\text{PO}_4$  ( $0.5 \text{ g l}^{-1}$ ),  $\text{MgSO}_4$  ( $0.5 \text{ g l}^{-1}$ ) and  $\text{NaCl}$  ( $3 \text{ g l}^{-1}$ ) and supplemented with antifungal cycloheximide ( $50 \mu\text{g l}^{-1}$ ) and phenol ( $2 \text{ g l}^{-1}$ ). Plates were incubated at  $30^\circ\text{C}$  for 7 days. CSA is known to promote the growth of actinomycetes (Hopwood, 1985). Various isolates were selected by visual differentiation of contrasting colony morphology. These bacteria, referred to as phenol-tolerant bacteria, were transferred to a mineral salts medium (MSM) containing  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  ( $9 \text{ g l}^{-1}$ ),  $\text{KH}_2\text{PO}_4$  ( $1.5 \text{ g l}^{-1}$ ),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $0.2 \text{ g l}^{-1}$ ),  $\text{CaCl}_2$  ( $0.002 \text{ g l}^{-1}$ ), trace element solution ( $1 \text{ ml l}^{-1}$ ) (Schlegel et al., 1961) and phenol ( $400 \text{ mg l}^{-1}$ ) as a sole source of carbon and energy. When necessary, the medium was solidified by adding 1.5% (w/v) bacteriological agar Becton Dickinson (Sparks, MD, USA).

To monitor phenol degradation ability, MSM liquid cultures (50 ml in 250 ml conical flasks, 1% inoculum) were grown with shaking at 200 rpm in

an incubator at 30°C for 7 days, supplemented with initial phenol concentrations ranging from 200–2000 mg l<sup>-1</sup> in 200 increments. After the incubation period, to determine the biomass yield as cell dry weight, aliquots of the cultures (2 ml) were centrifuged at 5000 × g for 10 min at 4°C (Eppendorf 5804 R bench top centrifuge) and the cell pellets were washed in potassium phosphate buffer (50 mM, pH 7.2) two times prior to application of the cells to the glass fiber membranes (Whatman). Cell suspensions were filtered through previously weighed membranes which were then dried at 90°C until a constant weight was achieved.

To test the ability of the isolates to use other aromatic compounds as a sole source of carbon and energy, the strains were spread onto MSM plates and the carbon source was supplemented via a vapor phase by adding in a sterile Eppendorf plastic tip (50 µl) and placing the tip in the lid of the Petri dish. In the case of naphthalene and biphenyl, a couple of crystals (approximately 5 mg) were similarly placed on the lid of the Petri dish. After 2–4 days of incubation at 30°C the plates were screened for the presence of colonies. Growth was confirmed by comparison with control plates without substrate and 20 mM glucose as a carbon source.

#### *Identification of bacteria*

Bacterial DNA was extracted by a method described previously (Nikodinovic et al., 2003). The extracted DNA was subjected to PCR amplification with the 16S rDNA bacteria-specific primers: 27f (Lane, 1991) and 1392r (Marchesi et al., 1998). PCR amplification was performed as described by Marchesi et al. (1998). PCR products were cloned in pUC19 vector and three independently isolated recombinant plasmids for each strain were sequenced on an Applied Biosystems 3130 Genetic Analyzer (Foster City, USA) using M13f/M13r primers (Yanisch-Perron et al. 1985). Homologues were identified by the BLASTN algorithm (Altschul et al., 1997).

The BLASTN program (Altschul et al., 1997) was used to search for similar sequences in the GeneBank

database services provided by the NCBI and the Seqmatch tool was used to search for similar sequences compiled by the Ribosomal Database Project-II Release 9.4 (RDP; <http://rdp.cme.msu.edu>; (Cole et al. 2009)).

#### *Preparation of crude cell extracts*

Bacteria were grown in 200 ml of tryptone soy broth (TSB; 30 g l<sup>-1</sup>) at 30°C, shaking (200 rpm). After four days the cells were harvested by centrifugation at 6,000 × g (15 min, +4°C, GSA rotor, Sorvall, USA). For the induction experiments, two hours prior to harvesting phenol was added directly to the medium at a 0.1 mM final concentration. The cell pellet was washed with 50 mM potassium phosphate buffer (20 ml; pH 7.2), followed by centrifugation under the same conditions. The cell pellet was resuspended in lysis buffer (20 ml; 50 mM potassium phosphate buffer, pH 7.2, containing 1 mg ml<sup>-1</sup> lysozyme). The suspension was incubated on ice for 30 min. The crude cell extracts were obtained after sonication (6 bursts of 10 s at 15 Hz; Soniprep 150 MSE; Beckenham, UK) and centrifugation at 12000 × g (Eppendorf 5804R bench top centrifuge) for 15 min at 4°C and used for enzymatic tests. Total protein concentration was determined by the method of Bradford using bovine serum albumin ((Sigma-Aldrich, St Louis, MO, USA) as standard (Bradford 1976).

#### *Enzyme activity analysis*

Enzyme assays for the activity of phenol hydroxylase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase were carried out as previously described. For phenol hydroxylase, the oxidation of 1 mM NADH by the cell extract in the presence of 100 nM phenol was monitored at 340 nm (Neujahr and Gaal 1975). To assay the activity of catechol 1,2- and 2,3-dioxygenase, 1 mM catechol was added to the crude cell extract. For the catechol 1,2-dioxygenase formation of *cis* molecules, *cis*-muconic acid was measured at 260 nm (Ornston and Stanier 1966); for estimating catechol 2,3-dioxygenase activity, the appearance of 2-hydroxymuconic semialdehyde was measured at 375 nm (Mars et al., 1997). Assays were carried out

in 50 mM phosphate buffer, pH 7.2 at 30°C. A unit of the enzyme was defined as the amount of enzyme catalyzing 1 mmol of substrate per 1 min. Results are shown as enzyme activity per mg of total protein in the crude cell extract.

The effect of pH and temperature on enzyme activity was determined by carrying out reactions in 50 mM sodium acetate (pH 2, 3, 4 and 5), 50 mM phosphate (pH 6 and pH 7), 50 mM Tris-HCl (pH 8 and pH 9) and 50 mM carbonate/bicarbonate buffer (pH 10 and pH 11) at 30°C. The optimum temperature was determined by assaying the enzyme activity at different temperatures, from 10°C to 80°C in 50mM phosphate buffer.

#### *PCR-based screening for phenol hydroxylase core regions*

Core regions of phenol hydroxylase were amplified using the previously described primers Pheh-3F/Pheh-3R (5'-CGKATGACSTACGGCT-GATGGGCG-3'/5'-ACGTCCTGTTCGAT-GATCTCCTTGATCCGC-3') (Sandhu et al., 2009). A 870 bp fragment was amplified under the following conditions: 1) 10 min at 95°C; 2) 30 1 min cycles at 95°C, 1 min at 60.5°C and 1 min at 72°C; 3) a final 5 min extension at 72°C and gDNA of isolates as a template. In the study, gDNA of the strain *Bacillus cereus* ATCC 14579 was also included as a negative control.

#### *Nucleotide sequence accession numbers*

The sequences for 16S rRNA genes were deposited in GenBank under accession numbers EU721601, EU721602, JN118576 and JN118577 for PS11, PS1, TN41 and TN42, respectively.

## RESULTS AND DISCUSSION

### *Isolation and characterization of phenol-degrading bacteria*

Aromatic hydrocarbon-degrading microorganisms have been isolated from various environments,

mostly polluted sites, however, they are widespread in marine, freshwater and soil habitats (Phale et al., 2007). Plant rhizospheres have proven to be a rich source of these bacteria, as higher plants usually extrude phenolics into the soil providing a suitable niche for the enrichment of phenol degraders (Toyama et al., 2009). *Ramonda serbica* is a relict plant of the ancient Tertiary flora and an endemic species of Serbia capable of surviving very long dry periods in the state of anabiosis (Quartacci et al., 2002). Due to their specific life cycle, the content of phenolic acids in this plant is at least three times higher than that of other higher plants (Beckman 2000; Sgherri et al., 2004). The rhizospheres of this plant have been proven to be a rich source of phenol-degrading bacteria, although the bacterial community associated with it had limited diversity (Djokic et al., 2010).

From the soil collected from the Tamiš River bank and from the rhizospheres of the endemorelict plant *Ramonda serbica* we isolated 58 and 13 phenol-tolerant strains, respectively. Out of these isolates, strains showing substantial growth on phenol as a sole carbon source were selected for further testing. They included two strains from the river bank sample which were identified by 16S rDNA sequencing (Table 1). As isolates TN41 and TN42 were assigned to the genus *Bacillus*, we included two *Bacillus* isolates from the rhizosphere soil sample (PS1 and PS11) for the purpose of comparative study. Isolate TN41 had 99% homology with *Bacillus megaterium* (GenBank CP001982), while the isolate TN42 had 99% homology with *B. pumilus* (GenBank JF769748) which was isolated as a halotolerant bacterium from saltern sediments. Isolate PS11 had 98% homology with *B. simplex* isolated from rape leaves (GenBank GU086427), while isolate PS1 shared only 90% homology with the known *B. cereus* strain (Table 1).

The selected strains were tested for their ability to grow on a range of additional 14 aromatic substrates as the sole source of carbon and energy on a solid MSM medium (Table 2). These substrates included benzene, toluene, styrene, biphenyl, naphthalene, etc. All of the isolates were capable of degrading 7 aromatic compounds, including benzene, toluene,

styrene, bromobenzene, guaiacol, biphenyl and naphthalene (Table 2). Under the conditions tested (30°C, medium pH7.2), the strains isolated from the river bank (TN41 and TN42) could grow on 9 and 11 compounds, respectively, while strains PS1 and PS11 could grow on 11 and 14 compounds, respectively. Strains TN41, TN42 and PS11 could grow on the rich medium (TSB) at 43°C, while none of the strains could grow at this temperature on the minimal medium (MSM) containing phenol as the sole carbon source. Strains TN42 and PS1 and PS11 were capable of growing on a rich medium at 4°C, while only PS1 and PS11 were also capable of growing on minimal medium with phenol as a sole carbon source at this temperature. All four strains were capable of growing in MSM liquid culture supplemented with phenol at different initial concentrations (200 to 2000 mg l<sup>-1</sup>). While they were all capable of growing in the presence of phenol at all concentrations, isolates exhibited growth inhibition at different concentrations. PS1 showed growth inhibition at 1,000 mg l<sup>-1</sup>, PS11 at 1,600 mg l<sup>-1</sup>, while for the strains TN41 and TN42 growth inhibition occurred at 400 mg l<sup>-1</sup> (data not shown). In comparison, wastewaters containing phenol in the range of 5-500 mg l<sup>-1</sup> are considered suitable for treatment by biological processes (Whiteley and Bailey 2000). Thus, all four *Bacillus* isolates have great bioremediation potential, with the PS strains showing growth inhibition on phenol at higher concentrations in comparison to the TN strains.

Apart from our recent report of two *Bacillus* strains that could degrade a wide range of aromatic compounds and were successfully employed in soil model system experiments (Djokic et al., 2011), only a few other *Bacillus* strains have been reported to degrade phenol, including *B. brevis* (Arutchelvan et al., 2006), *B. cereus* (Banerjee and Ghoshal, 2010), *B. stearothermophilus* (Dong et al., 1992; Gurujeyalakshmi and Oriol, 1989) and *B. subtilis* (Tam et al., 2006). Banerjee et al. isolated two *Bacillus* strains from the oil refinery and exploration sites which could grow on phenol (1000 mg l<sup>-1</sup>) (Banerjee and Ghosal, 2010). Nevertheless, none of these strains have been evaluated for the ability to degrade a wide range of other aromatic pollutants.

#### *Phenol hydroxylase and catechol dioxygenases activities in crude cell extracts*

Having established the excellent potential of four *Bacillus* strains for bioremediation applications, we analyzed the catabolic pathways of these strains by assessing the enzymatic activity of the aromatic catabolic pathway in the crude cell extracts. Isolates were grown in rich TSB medium and TSB supplemented with phenol (0.1 mM) to assess the phenol hydroxylase and catechol dioxygenases activities in crude cell extracts (Table 3).

Phenol hydroxylase (EC 1.14.13.7) and catechol 1,2-dioxygenase (EC 1.13.11.1) activity was detected in all isolates, under both conditions tested, while catechol 2,3-dioxygenase (EC 1.13.11.2) activity was not detected in the PS1 strain (Table 3). In general, the TN isolates had substantially lower enzyme activities (for all three enzymes, under both conditions) in comparison to PS isolates. The difference ranged from 1.5-fold in the case of phenol hydroxylase (PS1 in comparison to TN42 in the absence of inducer), to 90-fold in the case of catechol 2,3-dioxygenase (PS11 in comparison to TN42 in the presence of inducer).

Phenol hydroxylase activity was highest for the PS11 isolate, and when the isolates were grown in rich TSB medium it was 1.5-, 6.3- and 2.3-fold higher in comparison to PS1, TN41 and TN42 isolates, respectively (Table 3). Similarly, the activity of catechol 1,2-dioxygenase was highest for PS11, being 1.7-fold higher in comparison to the PS1 isolate and 16.7 and 44.8 times higher in comparison to TN41 and TN42 isolates, respectively. The same trend remained for the catechol 2,3-dioxygenase with PS11 activity being 17- and 48.4-fold higher in comparison to the TN41 and TN42 isolates.

The previous report indicated that phenol may act as an inducer of phenol hydroxylase and catechol 1,2-dioxygenase (Djokic et al., 2011), which was confirmed in the case of PS isolates. However, the presence of 0.1 mM phenol in the medium for two hours prior to harvesting had a negative effect on the enzymatic activity of all three enzymes in the case of TN

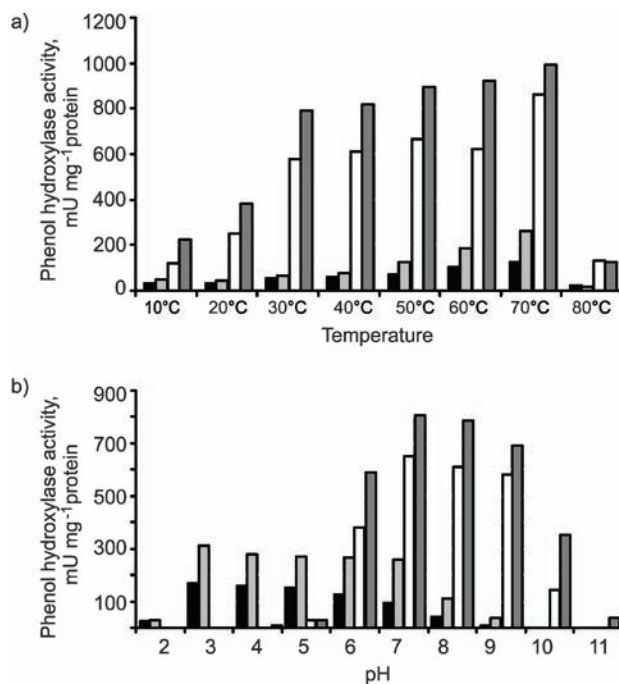
isolates (Table 3). Specific activity decreased from 1.1-fold in the case of C12O for TN42, to 6.4-fold in the case of phenol hydroxylase for the same isolate. Paller et al. found PH to be induced by its substrate (Paller et al., 1995). However, in the literature phenol hydroxylase inhibition by its substrate is more common (Neujahr and Kjellen, 1980; Viggor et al., 2008). Some other inhibitors, such as  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$  ions (Saa et al., 2010) and dithiothreitol were also reported (Cadieux et al., 2002).

Isolate TN42 had 2.8 times higher specific activity in comparison to TN41 in the TSB grown cells without addition of phenol, while the activities for catechol 1,2- and 2,3- dioxygenases were 2.8 times lower for the same isolate in comparison to TN41 (Table 3).

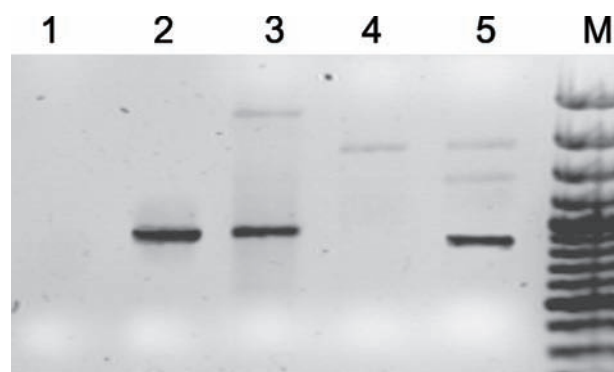
We have determined that all the strains, TN41, TN42, PS1 and PS11, degrade phenol via an *ortho* pathway, by measuring the activity of catechol 1,2-dioxygenase as the formation of *cis,cis*-muconic acid. Additionally, by measuring the formation of 2-hydroxymuconic semialdehyde which is the product of catechol 2,3-dioxygenase activity, it was shown that strains TN41, TN42 and PS11 utilize the *meta* pathway of phenol catabolism as well. In only a few instances do bacteria degrade phenol via both pathways (Jiang et al., 2006).

C12O is a biotechnologically important enzyme as its product is a precursor of the industrially important compound adipic acid and represents raw material for new resins, pharmaceuticals and agrochemicals (Di Nardo et al., 2009; Yoshikawa et al., 1993). PH is also used as a biocatalyst for chemical synthesis (Kim et al., 2005), while C23O is mostly used for the biotreatment of wastewaters and in soil decontamination (Guzik et al., 2009).

The way that enzymes respond to temperature and pH is important to many areas of biotechnology. We have also examined the effects of temperature and pH on specific enzyme activity. We have determined the conditions for the maximum specific activities (Table 4). The specific activity of PH was highest for



**Fig. 1** Effect of temperature (A) and pH (B) on the activity of phenol hydroxylase in the cell extracts of four *Bacillus* strains (; TN41, ;TN42, ;PS1 and ; PS11). Data is average of two independent experiments.



**Fig. 2** Fragments obtained using primers for amplification of the core region of large phenol hydroxylase subunit in Gram positive bacteria and gDNA of *Bacillus* strains (1, *B. cereus* ATCC14579; 2, *B. sp.* PS1; 3, *B. sp.* PS11; 4, *B. sp.* TN41; 5, *B. sp.* TN42, and M, O'Gene 100 bp DNA Ladder).

all four isolates at 70°C (Fig. 1a). This was also the case for C12O and C23O (data not shown). This may be due to the fact that many enzymes exhibit higher activity at a temperature at which they are unstable (Eisenthal et al., 2006). Under these conditions (70°C),

**Table 1.** Identification and comparison of bacterial isolates, from river bank soil and a rhizosphere associated soil capable of growth on phenol as sole carbon and energy.

The best GenBank match				
Isolate	Classification	(Accession number)	% homology	% coverage
TN41	<i>Bacillus megaterium</i>	(CP001982)	99	99
TN42	<i>Bacillus pumilus</i>	(JF769748)	99	99
PS1 <sup>a</sup>	<i>Bacillus cereus</i>	(CP001746)	90	98
PS11 <sup>a</sup>	<i>Bacillus simplex</i>	(GU086427)	98	98

**Table 2.** Aromatic substrate range used for the growth of *Bacillus* isolates TN41, TN42, PS1 and PS11.

Substrate	Result for strain			
	TN41	TN42	PS1	PS11
benzene	+	+	+ <sup>a</sup>	+
toluene	+	+	+	+
ethylbenzene	-	+	-	+
<i>o</i> -xylene	-	+	+	+
<i>m</i> -xylene	+	+	-	+
<i>p</i> -xylene	-	-	+	+
styrene	+	+	+	+
chlorobenzene	+	+	-	+
bromobenzene	+	+	+	+
nitrobenzene	-	-	+	+
nitrophenol	-	-	-	-
guaiacol	+	+	+	+
biphenyl	+	+	+	+
naphthalene	+	+	+	+

<sup>a</sup>+, confluent growth on MSM agar plate containing the aromatic substrate as the sole source of carbon and energy at 30°C

<sup>a</sup> (Djokic et al. 2011)

phenol hydroxylase activity was still highest for the PS11 isolate in comparison to all other isolates, being 1.2-, 7.9- and 3.8-fold higher in comparison to PS1, TN41 and TN42, respectively (Table 4). Phenol hydroxylase activity was comparable at 10°C and 80°C for the PS1 isolate, while it was 1.8-, 1.7- and 3-fold lower at 80°C in comparison to 10°C for PS11, TN41 and TN42 isolates, respectively (Fig. 1a). In the case of the oxygenases, specific activity remained from 5- to 10-fold higher at 80°C in comparison to 10°C for all four isolates (data not shown). Straube reported a temperature range of 10°C to 55°C, with 35°C being the optimum for *Rhodococcus* phenol hydroxylase (Straube, 1987). In contrast, the optimum temperature for phenol hydroxylase from *Bacillus thermo-*

*glucosidasius* A7 was reported to be 55°C (Kirchner et al., 2003). Di Nardo et al. reported a temperature range for the C12O from *Acinetobacter radioresistens* S13 of 10°C to 60°C (Di Nardo et al., 2009).

Phenol hydroxylases of the TN strains could tolerate a broader range of pH conditions and had higher activities at lower pH values, while phenol hydroxylase activity could not be detected at pH 2 and pH3 for the PS strains (Fig. 1b). The maximum specific activity for both TN strains was observed at pH3 (Table 4). In contrast, maximum specific activity for catechol 1,2- and 2,3-dioxygenase was observed at pH values 11 and 10 for PS and TN isolates, respectively (Table 4). Even under optimal conditions for

**Table 3.** Specific activities of phenol hydroxylase (PH), catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) from cell free extracts of phenol degrading isolates grown at 30°C in rich medium (TSB) and rich medium supplemented with inducer (phenol).

Growth condition	Phenol hydroxylase mU mg <sup>-1</sup> protein				Catechol 1,2-dioxygenase mU mg <sup>-1</sup> protein				Catechol 2,3-dioxygenase mU mg <sup>-1</sup> protein			
	TN41	TN42	PS1	PS11	TN41	TN42	PS1	PS11	TN41	TN42	PS1	PS11
TSB	93±3	257±4	397±8	590±10	8.3±0.7	3.1±0.6	82±3	139±6	5.4±0.1	1.9±0.2	ND <sup>b</sup>	92±3
TSB <b>0.1 mM phenol<sup>a</sup></b>	40±2	40±7	620±7	815±7	4.8±0.2	2.8±0.5	120±6	130±4	2.6±0.2	1.5±0.1	ND	135±3

<sup>a</sup> inducer was added directly to growth medium at 0.1 mM final concentration two hours prior to cell harvesting

<sup>b</sup>ND = not detected, under conditions tested

**Table 4.** Conditions (pH and temperature) for the maximum specific activity of phenol hydroxylase and catechol dioxygenases of four *Bacillus* isolates.

Condition	Phenol hydroxylase mU mg <sup>-1</sup> protein				Catechol 1,2-dioxygenase mU mg <sup>-1</sup> protein				Catechol 2,3-dioxygenase mU mg <sup>-1</sup> protein			
	TN41	TN42	PS1	PS11	TN41	TN42	PS1	PS11	TN41	TN42	PS1	PS11
<b>70°C</b>	126±8	263±14	862±13	995±19	17±2	42±6	350±11	545±18	29±4	64±4	ND	820±17
<b>pH</b>	168±16 <sup>b</sup>	309±11 <sup>b</sup>	650±18 <sup>a</sup>	805±18 <sup>a</sup>	24±3 <sup>d</sup>	64±4 <sup>d</sup>	510±10 <sup>c</sup>	618±18 <sup>c</sup>	36±2 <sup>d</sup>	110±8 <sup>d</sup>	ND	956±9 <sup>c</sup>

<sup>a</sup> pH 7, <sup>b</sup>pH 3, <sup>c</sup>pH11, <sup>d</sup>pH10, ND = not detected, under conditions tested

the maximum value for enzymatic activities, the PS strains had higher enzymatic activities in comparison to the TN strains. In the literature, the pH optimum for these enzymes ranges from 5.5 to 8.2 (Ahuatzi-Cacon et al., 2004; Kirchner et al., 2003; Viggor et al., 2008). Catechol 2,3-dioxygenase from *Pseudomonas* sp. exhibited a pH activity range from 5.5-9.5 (Wei et al., 2009).

#### PCR-based detection of phenol hydroxylase gene

Phenol hydroxylase has been used as a molecular marker of phenol-degrading bacteria (Basile and Erijman, 2008; Sandhu et al., 2009). In addition, phenol hydroxylases can be used as biocatalysts and potential agents for organic syntheses (i.e. indigo dye production (Kim et al., 2005)), thus the detection of novel phenol hydroxylases is of high interest. We have successfully amplified the core region of a large

PH subunit in three out of four *Bacillus* soil isolates (Fig. 2).

In the PCR experiments, using the genomic DNA of four phenol-degrading isolates as a template, fragments of the expected size (870 bp) were obtained for PS1 and PS11 as previously reported (Djokic et al., 2011), while no expected fragment was detected for the TN41 isolate and the expected size fragment was obtained for the TN42 isolate (Fig. 2). The appropriate control reaction with *B. cereus* ATCC14579 gDNA yielded no detectable fragments (Fig. 2). The primers used were group-specific primers designed to amplify the core region of a large phenol hydroxylase subunit in Gram positive bacteria (Sandhu et al., 2009). The inability to amplify the PH fragment from the TN41 isolate may indicate that the sequence of this gene is sufficiently different from the primers used. The ob-



tained fragments were sequenced and compared to the available sequences in the GenBank database. The sequences of isolates PS1 and PS11 were identical, with closest similarity of 99% observed to the *Rhodococcus erythropolis* PR4 gene encoding for putative phenol hydroxylase (Djokic et al., 2011). The sequence of TN42 shows 97% sequence identity with the *Rhodococcus erythropolis* phenol degradation gene cluster.

BLAST analysis showed high similarity between the PH genes from the PS1, PS11 and TN42 strains with those from *Rhodococcus erythropolis*, with DNA identity of 98%, 99%, and 97%, respectively. High gene sequence similarity between PS1, PS11 and TN42 with *Rhodococcus*, coupled with the high promiscuity of *Bacillus* strains, could indicate that these genes underwent a horizontal gene transfer event.

Our aim was to isolate Gram positive bacteria capable of tolerating high amounts of phenol in the soil sample from the river bank in close proximity to the petrochemical facility, and to compare these to isolates from the rhizospheres of phenolic extruding *Ramonda serbica*. We aimed to isolate either *Bacillus* or *Streptomyces* strains for their advantageous spore-forming ability, due to the absence of reports on their applicability in the bioremediation efforts. Although at the beginning of this study the riverbank soil sample provided 4-fold more phenol-tolerant isolates in comparison to the rhizosphere sample, we obtained only two isolates with a good capability of utilizing phenol as the sole source of carbon and energy. These novel isolates were designated as *Bacillus* sp. TN41 and *Bacillus* sp. TN42. They were found to be less tolerant to higher initial amounts of phenol in comparison to *Bacillus* isolates from the rhizosphere. In addition, the specific enzyme activities of PH, C12O and C23O were also lower under all tested conditions. Since data on *Bacillus* wide-range aromatic degrading strains are scant, the present study provides a better understanding of phenol degradation pathways and provides the basis for the possible application of these common soil strains in bioremediation.

*Acknowledgement* - This work was supported by Ministry of Education and Science of the Republic of Serbia (Grant no. 173048).

## REFERENCES

- Ahuatzi-Cacon D., Ordorica-Morales G., Ruiz-Ordaz N., Cristiani-Urbina E., Juarez-Ramirez C., and J., Galindez-Mayer (2004). Kinetic study of phenol hydroxylase and catechol 1,2-dioxygenase biosynthesis by *Candida tropicalis* cells grown in different phenolic substrates. *World J Microbiol Biotechnol* **20**: 695-702
- Altschul S.F., Madden T.L., Schaffer A.A., Zhang J, Zhang Z, Miller W, and DJ, Lipman (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Acids Res* **25**: 3389-3402
- Andreoni V., and L., Gianfreda (2007). Bioremediation and monitoring of aromatic-polluted habitats. *Appl Microbiol Biotechnol* **76**: 287-308
- Arutchelvan V., Kanakasabai V., Elangovan R., Nagarajan S., and V., Muralikrishnan (2006). Kinetics of high strength phenol degradation using *Bacillus brevis*. *J Haz Mat* **129**: 216-222
- Banerjee A., and A.K., Ghosal (2010). Isolation and characterization of hyper phenol tolerant *Bacillus* sp. from oil refinery and exploration sites. *J Haz Mat* **176**: 85-91
- Banerjee A., and A.K., Ghoshal (2010). Phenol degradation by *Bacillus cereus*: Pathway and kinetic modeling. *Biores Technol* **101**: 5501-5507
- Basile L.A., and L., Erijman (2008). Quantitative assessment of phenol hydroxylase diversity in bioreactors using functional gene analysis. *Appl Microbiol Biotechnol* (**78**): 863-872
- Beckman C. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence response in plant? *Physiol Molec Plant Pathol* **57**: 101-110
- Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254
- Cadieux E., Vrajmasu V., Achim C., Powlowski J., and E., Muenck (2002). Biochemical, Mössbauer, and EPR studies of the diiron cluster of phenol hydroxylase from *Pseudomonas* sp. strain CF 600. *Biochemistry* **41**: 10680-10691
- Cole J.R., Wang Q., Cardenas E., Fish J., Chai B., Farris R.J., Kullam-Syed-Mohideen A.S., McGarrell D.M., Marsh T., Garrity G.M., and J.M., Tiedje (2009). The ribosomal database

- project: improved alignments and new tools for rRNA analysis. *Nucl Acids Res* **37**: 141-145
- Di Nardo G., Roggero C., Campolongo S., Valetti F., Trotta F., and G., (Gilardi 2009). Catalytic properties of catechol 1,2-dioxygenase from *Acinetobacter radioresistens* S13 immobilized on nanosponges. *Dalt Trans* **33**: 6507-6512
- Djokic, L., Savic, M., Narancic, T., and B., Vasiljevic (2010). Meta-genomic analysis of soil microbial communities. *Arch Biol Sci*, **62**: 559-564.
- Djokic L., Narancic T., Nikodinovic-Runic J., Savic M., and B., Vasiljevic (2011). Isolation and characterization of four novel Gram positive bacteria associated with the rhizosphere of two endemorelict plants capable of degrading broad range of aromatic substrates. *Appl Microbiol Biotechnol* DOI 10.1007/s00253-011-3426-9
- Dong F.M., Wang L.L., Wang C.M., Chang J.P., He Z.Q., Sheng Z.J., and R.Q., Shen (1992). Molecular cloning and mapping of phenol degradation genes from *Bacillus stearothermophilus* FDTP-3 and their expression in *Escherichia coli*. *Appl Environ Microbiol* **58**: 2531-2535
- Eisenthal R., Peterson M.E., Daniel R.M., and M.J., Danson (2006). The thermal behaviour of enzyme activity: implications for biotechnology. *Trends Biotechnol* **24**: 289-292
- Gurujejalakshmi G., and P., Oriol (1989). Isolation of phenol-degrading *Bacillus stearothermophilus* and partial characterization of the phenol hydroxylase. *Appl Environ Microbiol* **55**: 500-502
- Guzik U., Gren I., Wojcieszynska D., and S., Labuzek (2009). Isolation and characterization of a novel strain of stenotrophomonas maltophilia possessing various dioxygenases for monocyclic hydrocarbon degradation. *Braz J Microbiol* **40**: 285-291
- Hopwood D.A., Bibb M.J., Chater K.F., Kieser T., Bruton C.J., Kieser H.M., Lydiate D.J., Smith C.P., Wards J.M., and H., Shrempf (1985). Genetic manipulation of *Streptomyces*: A laboratory manual. First edn. John Innes Foundation, Norwich
- Jiang H.L., Tiong-Lee S., Maszenan A.M., and J.H., Tay (2006). Physiology traits of bacterial strains isolated from phenol-degrading aerobic granules. *FEMS Microbiol Ecol* **57**: 182-191
- Kim J.Y., Kim J.K., Lee S.O., Kim C.K., and K., Lee (2005). Multicomponent phenol hydroxylase-catalysed formation of hydroxyindoles and dyestuffs from indole and its derivatives. *Lett Appl Microbiol* **41**: 163-168
- Kirchner U., Westphal A.H., Müller R., and W.J., van Berkel (2003). Phenol hydroxylase from *Bacillus thermoglucosidasius* A7, a two-protein component monooxygenase with a dual role for FAD. *J Biol Chem* **278**: 47545-47553
- Lane D.J. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow MM (eds) *Nucleic acid techniques in bacterial systematic*. John Wiley and Sons, Inc, Chichester, UK, pp 115-175
- Marchesi J.R., Sato T., Weightman A.J., Martin T.A., Fry J.C., Hiom S.J., and W.G., Wade (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**: 795-799
- Mars A.E., Kasberg T., Kaschabek S.R., Van Agteren M.H., Janssen D.B., and W., Reineke (1997). Microbial degradation of chloroaromatics: Use of the meta-cleavage pathway for mineralization of chlorobenzene. *J Bacteriol* **179**: 4530-4537
- Neujahr H.Y., and A., Gaal (1975). Phenol hydroxylase from yeast: Sulphydryl groups in phenol hydroxylase from *Trichisporon cutaneum*. *Eur J Biochem* **58**: 351-357
- Neujahr H.Y., and K.G., Kjellen (1980). Phenol hydroxylase from yeast: a lysyl residue essential for binding of reduced nicotinamide adenine dinucleotide phosphate. *Biochemistry* **19**: 4967-4972
- Nikodinovic J., Barrow K.D., and J.A., Chuck (2003). High yield preparation of genomic DNA from *Streptomyces*. *Biotechniques* **3** (5): 932-936
- Ornston L.N., and R.Y., Stanier (1966). The conversion of catechol and pyrocatechuate to  $\beta$  keto adipate by *Pseudomonas putida*. *J Biol Chem* **241**: 3776-3786
- Paller, Hommel R.K., and H.P., Kleber (1995). Phenol degradation by *Acinetobacter calcoaceticus* NCIB 8250. *J Bas Microbiol* **35**: 325-335
- Phale P.S., Basu A., Majhi P.D., Deveryshetty J., Vamsee-Krishna C., and R., Shrivastava (2007). Metabolic diversity in bacterial degradation of aromatic compounds. *OMICS: J Integ Biol* **11**: 252-279
- Quartacci M.F., Glisic O., Stevanovic B., and F., Navari-Izzo (2002). Plasma membrane lipids in the resurrection plant *Ramonda serbica* following dehydration and rehydration. *J Exp Bot* **53**: 2159-2166
- Saa L., Jaureguibeitia A., Largo E., Llama M.J., and J., Serra (2010). Cloning, purification and characterization of two components of phenol hydroxylase from *Rhodococcus erythropolis* UPV-1. *Appl Microbiol Biotechnol* **86**: 201-211
- Sandhu A., Halverson L.J., and G.A., Beattie (2009). Identification and genetic characterization of phenol-degrading bacteria from leaf microbial community. *Microb Ecol* **57**: 276-285
- Schie P.M., and L.Y., Young (2000). Biodegradation of phenol: mechanisms and applications. *Biorem J* **4**: 1-18

- Schlegel H.G., Kaltwasser H., and G., Gottschalk (1961). A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. *Arch Microbiol* **38**: 209-222
- Sgherri C., Stevanovic B., and F., Navari-Izzo (2004). Role of phenolics in the antioxydative status of the resurrection plant *Ramonda serbica* during dehydration and rehydration. *Physiol Plant* **122**: 478-485
- Straube G. (1987). Phenol hydroxylase from *Rhodococcus* sp. P 1. *J Bas Microbiol* **27**: 229-232
- Tam L.T., Eymann C., Albrecht D., Sietmann R., Schauer F., Hecker M., and H., Antelmann (2006). Differential gene expression in response to phenol and catechol reveals different metabolic activities for the degradation of aromatic compounds in *Bacillus subtilis*. *Environ Microbiol* **8**: 1408-1427
- Toyama T., Sei K., Yu N., Kumad H., Inoue D., Hoang H., Soda S., Chang Y-C., Kikuchi S., Fujita M., and M., Ike (2009). Enrichment of bacteria possessing catechol dioxygenase genes in the rhizosphere of *Spirodela polyrrhiza*: a mechanism of accelerated biodegradation of phenol. *Water Res* **43**: 3765-3776
- Viggor S., Heinaru E., Kunnapas A., and A., Heinaru (2008). Evaluation of different phenol hydroxylase-possessing phenol-degrading pseudomonads by kinetic parameters. *Biodegradation* **19**: 759-769
- Wei J., Zhou Y., Xu T., and B., Lu (2009). Rational design of catechol-2, 3-dioxygenase for improving the enzyme characteristics. *Appl Biochem Biotechnol* **162**: 116-126
- Whiteley A.S., and M.J., Bailey (2000). Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Appl Environ Microbiol* **66**: 2400-2407
- Yanisch-Perron C., Vieira J., and J., Messing (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103-119
- Yoshikawa N., Ohta K., Mizuno S., and H., Ohkishi (1993). Production of cis,cis-muconic acid from benzoic acid. *Bioprocess Technol* **16**: 131-147

