

Isolation and partial characterization of extracellular NADPH-dependent phenol hydroxylase oxidizing phenol to catechol in *Comamonas testosteroni*

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Abstract

OBJECTIVE: *Comamonas testosteroni* Pb50 is a microorganism that possesses high tolerance for phenol and shows strong phenol degrading activity. This bacterial strain is capable of utilizing phenol as the sole carbon and energy source. Although examples are known in which the *C. testosteroni* utilizes phenol for growth or metabolism, much less information are known on the nature of the phenol-oxidizing enzymes in this microorganism. Therefore, the occurrence and cellular location of phenol hydroxylase (EC 1.14.13.7), the enzyme participating in the first step of phenol degradation, catalyzing its hydroxylation to catechol in a bacterial *Comamonas testosteroni* Pb50 strain grown in the presence of phenol as a sole carbon and energy source are the aims of this study.

METHODS: Combination of fractionation with polyethylene glycol 6000 and gel permeation chromatography on columns of Sepharose 4B and Sephacryl S-300 was used for isolation of phenol hydroxylase detectable in the medium in which *C. testosteroni* was cultivated. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel chromatography on Sephacryl S-300 were used to evaluate the molecular mass of the enzyme. The enzyme activity was followed by HPLC (phenol consumption and/or catechol formation).

RESULTS: Whereas low activity of phenol hydroxylase was detected in cytosol isolated from *C. testosteroni*, more than 16-fold higher activity of this enzyme was found in the medium in which *C. testosteroni* was cultivated. The presence of phenol hydroxylase extracellular activity suggests that this microorganism may secrete the enzyme into the extracellular medium. Using the procedure consisting of fractionation with polyethylene glycol 6000 and gel permeation chromatography on columns of Sepharose 4B and Sephacryl S-300, the enzyme was isolated from the medium to homogeneity. The formation of catechol mediated by purified phenol hydroxylase is strictly dependent on the presence of NADPH, which indicates that this enzyme is the NADPH-dependent phenol hydroxylase. The enzyme is a homotetramer having a molecular mass of 240 000, consisting of

four subunits having a molecular mass of 60 000. The optimum pH of the enzyme for the phenol oxidation is pH 7.6.

CONCLUSION: The results are the first report showing isolation and partial characterization of extracellular NADPH-dependent phenol hydroxylase of a bacterial *C. testosteroni* Pb50 strain capable of oxidizing phenol to catechol. The data demonstrate the progress in resolving the enzymes responsible for the first step of phenol degradation by bacteria.

Abbreviations:

BSA	- bovine serum albumin
BSM	- base synthetic medium
DEAE-Sepharose	- diethylaminoethyl-Sepharose
HPLC	- high performance liquid chromatography
K_m	- Michaelis constant
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
PEG 6000	- polyethylene glycol 6000
r.t.	- retention time
SDS-PAGE	- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
V_{max}	- maximum velocity

INTRODUCTION

Phenol and its derivatives are found in a wide variety of wastewaters including those from the oil refining, petrochemical, coke and coal gasification industries. Removal of phenol from such wastewaters can be achieved through aerobic biodegradation in well-run activated sludge plants. Different prokaryotic and eukaryotic microorganisms such as bacteria and fungi are effective to biodegrade phenol in such plants (Bayly and Wigmore, 1973; Yang and Humphrey, 1975; Krug *et al.* 1985; Krug & Straube 1986; Watanabe *et al.* 1996; Chang *et al.* 1998; Bastos *et al.* 2000; Paca *et al.* 2002; 2010; Chen *et al.* 2003; Komarkova *et al.* 2003; Stiborova *et al.* 2003; Ahuatz-Chacon *et al.* 2004; Tsai *et al.* 2005; 2007). *Pseudomonas* is a major bacterial genus commonly found in activated sludge plants and *Pseudomonas putida* is a species capable of using phenol as a major carbon source (Bayly & Wigmore 1973; Yang & Humphrey 1975; Gurujeyalakshmi & Oriol 1989; Chen *et al.* 2003). Several other mesophilic bacteria are also able to degrade phenol, including *Alcaligenes spp.* and *Spreptomyces setonii* and also the thermophile, *Bacillus stearothermophilus* (Gurujeyalakshmi & Oriol 1989; Bastos *et al.* 2000). In addition, pure cultures of *Comamonas testosteroni* ZD4-1, R5 and Pb50 have been shown to exhibit exceptionally high efficiency to utilize and degrade phenol (Teramoto *et al.* 1999; 2001; 2002; Chen *et al.* 2003; Paca *et al.* 2010, Tobajas *et al.* 2010).

The aerobic degradation pathways in microorganisms involve the occurrence of vicinal diols as substrates of ring-cleaving enzymes. Thus, the first step of phenol

degradation is hydroxylation of phenol to catechol. Catechol can undergo fission either by an intra-diol or an extra-diol type of cleavage (*ortho*- or *meta*-fission) (Arai *et al.* 2000; Tobajas *et al.* 2010). *Ortho*-fission, found in bacterial strain *A. faecalis* and in yeast such as *Trichosporon cutaneum*, *Candida albicans* TL3 and *Candida tropicalis*, gives rise to *cis,cis*-muconic acid, which is converted in further enzymatic steps *via* 3-oxoadipate to succinate and acetyl-coenzyme A (Ahuatz-Chacon *et al.* 2004, Tsai *et al.* 2005; Jiang *et al.* 2007; Vilimkova *et al.* 2009). Such a catechol cleavage was not found in *C. testosteroni* R5; the initial two steps of the phenol-degradation pathway in this microorganism included phenol hydroxylation to catechol and additional conversion of this metabolite to 2-hydroxybenzoic semialdehyde that is further converted to formate, acetaldehyde, and pyruvate (*meta*-fission) (Arai *et al.* 2000). Although examples are known in which the *C. testosteroni* utilizes phenol for growth or metabolism (Arai *et al.* 1999; Teramoto *et al.* 1999; Watanabe *et al.* 2002, Paca *et al.* 2010, Tobajas *et al.* 2010), much less information are known on the nature of the phenol-oxidizing enzymes in this microorganism.

Several phenol hydroxylases (EC 1.14.13.7) catalyze the initial step in the bacterial phenol-degrading pathway which produces catechol from phenol by introducing one atom of oxygen from molecular oxygen. Two major types of phenol hydroxylases, single- and multi-component phenol hydroxylases, have been identified in microorganisms degrading phenol (Pessione *et al.* 1999; Teramoto *et al.* 1999; Ballou *et al.* 2005; Viggor *et al.* 2008; Izzo *et al.* 2011). The enzymes were identified both in bacteria such as *Alcaligenes faecalis*, *Acinetobacter radioresistens* or a *Pseudomonas* genus (Pessione *et al.* 1999; Bastos *et al.* 2000; Griva *et al.* 2003; Rehfuss *et al.* 2005; Jiang *et al.* 2007; Kao *et al.* 2010; Izzo *et al.* 2011), and in soil yeasts *T. cutaneum* and *C. tropicalis* (Kälin *et al.* 1992; Bastos *et al.* 2000; Xu *et al.* 2001; Stiborova *et al.* 2003; 2004; Paca Jr *et al.* 2007). There is, however, limited information on phenol hydroxylases expressed in *C. testosteroni*. The genes of multi-component phenol hydroxylases have been identified in *C. testosteroni* R5 and TA441 (Arai *et al.* 1999; Teramoto *et al.* 1999; 2001; 2002) and the enzyme protein seems to be expressed in *C. testosteroni* R5 as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in this microorganism (Teramoto *et al.* 2002). In the case of *C. testosterone* KH122-3s, 3-hydroxybenzoate hydroxylase (EC 1.14.13.23) has also been found to be expressed and this enzyme has been characterized in detail (Hiromoto *et al.* 2006a; 2006b). On the contrary, information on the single-component phenol hydroxylases (EC 1.14.13.7) of *C. testosterone* is still scarce. This feature is however important to reach the efficient modulation of degradation processes in this bacterial strain or in a defined mixed culture of microorganisms capable of degrading phenol. In addition, despite the fact that phenol degradation has been studied, there are lim-

ited studies comparing intra- and extracellular enzyme levels responsible for phenol metabolism (Bastos *et al.* 2000; Jiang *et al.* 2007). Therefore, this study is aimed to investigate phenol hydroxylase and its location in a *C. testosteroni* Pb50 strain grown in medium containing phenol as a sole carbon and energy source.

The present paper reports for the first time the results providing evidence that NADPH-dependent phenol hydroxylase present in cytoplasm of *C. testosteroni* Pb50 is excreted into the extracellular growth medium. Furthermore, the results show the procedure suitable for isolation of this extracellular phenol hydroxylase from the cultivation medium (cell-free extract) and its partial characterization.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: NADH, NADPH, catechol, hydroquinone, catalase, Sepharose 4B and bicinchoninic acid (2,2'-biquinoline-4,4'-dicarboxylic acid) from Sigma Chemical Co., (St. Louis, MO, USA), NADH from Roche Diagnostics, (Mannheim, Germany) and Sephacryle S-300 from Pharmacia (Uppsala, Sweden), culture medium bouillon No.2 from Imuna (Slovakia). Other chemicals were supplied by Pliva-Lachema (Brno, Czech Republic). All chemicals were of reagent grade purity or better.

Microorganisms and their maintenance

A bacterial *C. testosteroni* Pb50 strain as a monoculture was used in this work. This strain was isolated from a biofilm developed on polyurethane foam particles of a packed bed reactor after 4 months of continuous phenol degradation (Paca Jr. *et al.* 2005). The strain was able to utilize phenol, toluene, xylenes, styrene, and acetone as the sole carbon and energy sources (Paca *et al.* 2010).

Growth media and cultivations

C. testosteroni was cultivated in the base synthetic medium (BSM). This growth medium contained: 4.3 g/L K_2HPO_4 , 3.4 g/L KH_2PO_4 , 2 g/L $(NH_4)_2SO_4$, 0.34 g/L $MgCl_2 \cdot 6 H_2O$ containing 3.5 mg/L phenol as a sole carbon and energy source (phenol medium).

For acquisition of sufficient quantity of *C. testosteroni* biomass, two stage cultivations were used. The first cell cultivation was carried out in shaking flasks for 68 h using batch process in the 25 g of culture medium bouillon No. 2 with 250 mg of phenol in 1 000 ml of distilled water. Cells from the first cultivation were used as an inoculum for the second cultivation. The second cultivation step, which was carried out in the growth medium (BSM, see above), was performed in a laboratory fermentor (B. Braun Biotech GmbH, Germany) at 30 °C and pH 7.0 for 48 h. The cells were separated from growth medium by centrifugation (Jouan CR3i /50 863 × g). The residual cell-free growth medium was

stored at -20 °C and used to analyze phenol hydroxylase activity. After separation, the cells were washed three times with distilled water and disintegrated using an ultrasonicator (Cole-Parmer instruments CP 130 PB-1, USA) to obtain the cell-free homogenate.

Preparation of membrane and cytosolic fractions

The isolation of the subcellular membrane and cytosolic fractions from the *C. testosteroni* cell-free homogenate was carried out by differential centrifugation (Mizerovska *et al.* 2009). The 16 000 × g sediment and the residual supernatant were taken as membrane and cytosolic fractions, respectively. Membrane and cytosolic samples were stored at -80 °C. Both fractions were used to analyze phenol hydroxylase activity. Protein concentrations in subcellular fractions and the growth medium were assessed by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) with serum albumin as a standard (Wiechelman *et al.* 1988).

Phenol hydroxylase activity assay

The phenol hydroxylase activity in cytosolic, membrane and cultivation media samples was followed by consumption of phenol and formation of catechol, measured with high performance liquid chromatography (HPLC) as described previously (Stiborova *et al.* 2003; Paca Jr *et al.* 2007; Vilimkova *et al.* 2008; 2009). The incubation mixtures contained in 0.1 ml, 40 mM sodium phosphate buffer (pH 7.6), 80 μL of membrane or cytosolic or cultivation medium samples, 1.0 mmol/L phenol and 1.0 mmol/L NADPH. Control incubations were carried out either without NADPH or with this cofactor, but with the heat-inactivated protein samples. Unless stated otherwise, incubations were performed at 37 °C for 20 min. Incubations were stopped with 20 μL of 0.6 mol/L $HClO_4$ and centrifuged. 20 μL aliquots of supernatant were injected by autosampler (ASI-100, Dionex, Germany) directly onto a HPLC column. The HPLC was performed with a Dionex HPLC pump with a spectrophotometric detector set at 275 nm. The column used was a 5 μm Nucleosil 100-5C18 (Macherey-Nagel, 4 × 250 mm) preceded by a C-18 guard column. A flow of 40% methanol in water, with a flow rate of 0.5 ml/min, was used to elute the phenol metabolites. The major reaction product formed was identified by comparison of its retention time with an authentic standard of catechol, having the retention time 7.9 min and by mass and UV/vis absorbance spectroscopy. Recoveries of phenol and catechol were around 80%. Mass spectra of the phenol metabolite (eluted at 7.9 min using HPLC) and a standard (catechol) were recorded on a FINNIGAN MAT INCOS 50 (electron impact, 70 eV, low resolution, direct inlet). UV/vis spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer.

Kinetic analyses were carried out using the non-linear least-squares method described by Cleland (1983).

Purification of NADPH-dependent phenol hydroxylase from *C. testosteroni* growth medium

All operations were carried out at 4°C. Into 1300 ml of the growth medium of *C. testosteroni* polyethylene glycol 6000 (PEG 6000) dissolved in distilled water (50% PEG 6000 solution) was gradually added into growth medium of *C. testosteroni* (300 ml) to precipitate protein fractions containing phenol hydroxylase. Three fractions precipitated with PEG 6000 were isolated (fractions obtained by precipitation of medium with 0–8%, 8–16% and 16–24% of PEG 6000). After centrifugation, the precipitates were dissolved in a minimal volume of 50 mM sodium phosphate buffer pH 7.6 and used to analyze phenol hydroxylase activity. The fraction containing the highest phenol hydroxylase activity (the protein fraction 0–8% PEG 6000) was applied to a Sepharose 4B column (1.6 × 70 cm), previously equilibrated with 50 mM sodium phosphate buffer pH 7.6.

The enzyme was eluted using 50 mM sodium phosphate buffer pH 7.6 with flow rate of 0.5 ml/min and the fractions containing the phenol hydroxylase activity were pooled and precipitated with PEG 6000 (8% saturation) The precipitated enzyme preparation was dissolved in a minimal volume of 50 mM sodium phosphate buffer pH 7.6 and applied to a column of Sephacryl S-300 (1.6 × 60 ml). The enzyme was eluted using the same procedure as shown for chromatography on Sepharose 4B (see above). The fractions eluted from the column were pooled, frozen and stored at –80°C until used. The activity of this enzyme preparation was stable (remained unchanged) for at least one month.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the enzyme samples obtained by enzyme purification was carried out using SDS/10% polyacrylamide gels as described by (Laemmli 1970).

Determination of molecular mass of phenol hydroxylase of *C. testosteroni*

The molecular mass of the native protein was determined using gel filtration on a Sephacryl S-300 column (1.6 × 60 ml) and a series of standard proteins [catalase (250 kDa), chicken immunoglobulin IgG (170 kDa), yeast alcohol dehydrogenase (141 kDa) and cytochrome c (12.4 kDa)]. The molecular mass of the subunit of phenol hydroxylase was determined using SDS-PAGE as described by (Laemmli 1970). “Wide range” molecular mass markers (Sigma Chemical Co., St. Louis, MO, USA) were used as standards.

RESULTS

Location of phenol hydroxylase activity in *C. testosteroni* Pb50

Since a pure culture of *C. testosteroni* Pb50 has been found to be able to effectively utilize phenol as the sole carbon and energy source (Paca *et al.* 2010), we investigated the enzyme systems that can catalyze the first step in oxidation metabolism of phenol, phenol hydroxylation, and can be responsible for this phenomenon. Since NAD(P)H-dependent phenol hydroxylases are known to catalyze the hydroxylation of phenol in many microorganisms, we initially analyzed the activity of these enzymes both in the cellular compartments of this bacterium (membrane and cytosolic fractions isolated from this microorganism) and in the growth medium, in which *C. testosteroni* Pb50 was cultivated. The phenol hydroxylation activity of these subcellular fractions and the growth medium was estimated by HPLC. In the presence of NADPH, a cofactor of NADPH-dependent phenol hydroxylase, the enzymatic system of the medium used for growth of *C. testosteroni* Pb50 cells oxidized phenol to a single reaction product having the retention time of 7.9 min (see the product peak shown in Figure 1A for purified extracellular phenol hydroxylase). Control incubations carried out in parallel with heat-inactivated medium were essentially

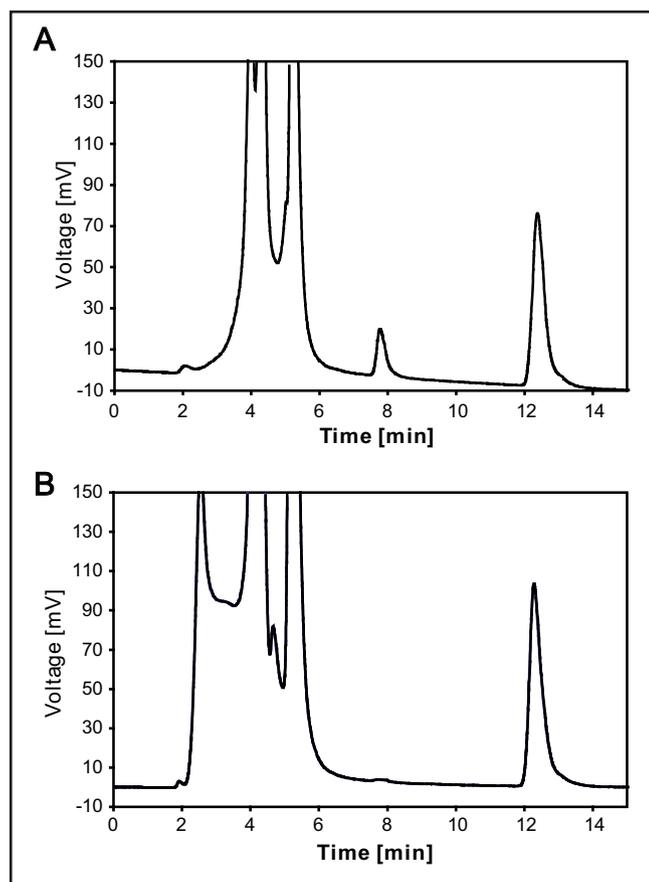


Fig. 1. Chromatograms showing the separation of phenol from its metabolite, catechol, formed by extracellular phenol hydroxylase purified from the medium used for growth of *C. testosteroni*. Chromatogram of the reaction mixture containing 0.3 mg of protein of extracellular phenol hydroxylase of *C. testosteroni*, 1 mM NADPH and 1 mM phenol incubated for 20 min at 37 °C, (A) and that of the same mixture, but containing 1 mM NADH instead of NADPH (B). Peaks eluting between 2.0–5.5 min, solvent front, NADPH (NADH) and enzyme protein; peak eluted at 7.9 min, catechol; peak eluted at 12.3 min, phenol. Experimental conditions and procedures are described in Material and Methods.

free of this metabolite even after prolonged incubation times (not shown). The phenol metabolite was identified by co-chromatography with an authentic standard, catechol, having the same retention time of 7.9 min, and by mass and UV/vis spectroscopy. The mass spectrum showed that the metabolite has a molecular weight of 110. This molecular peak and the fragmentation peaks at m/z 39, 53, 64, 81 and 92 (Figure 2) are identical with those of a standard, catechol. In addition, UV/vis absorbance spectrum of the metabolite, having an absorption maximum at 277 nm was identical with that of catechol (data not shown). All these results indicate that the phenol metabolite formed by the enzyme system of the growth medium of *C. testosteroni* Pb50 is catechol. Testing the chromatographic properties on HPLC of another possible oxidative product of phenol, hydroquinone, we excluded the possibility that this compound is formed by the enzymatic system of the growth medium of *C. testosteroni* Pb50 under the conditions used. No product having the retention time identical with that of hydroquinone (6.1 min) was found. In addition, a possible reaction product of catechol hydroxylation, 1,2,3-trihydroxybenzene (eluted at 5.8 min), was not detected.

The highest activity of phenol hydroxylase was found in the medium used for cultivation of *C. testosteroni* Pb50 cells; a value of specific phenol hydroxylase activity was 33.9 nmol of phenol/min/mg of protein. A more than 16-fold lower activity of this enzyme was detected in the cytosolic fraction of *C. testosteroni* Pb50 (2 nmol phenol/min/mg) and no activity was detectable in the membrane fraction of this microorganism. These results suggest that *C. testosteroni* Pb50 may secrete phenol hydroxylase into the growth medium.

In order to investigate hydroxylation of phenol by the enzyme found in the growth medium of *C. testosteroni* Pb50 cells further, the enzyme was isolated from the cultivation medium and partially characterized.

Isolation of NADPH-dependent phenol hydroxylase from growth medium of *C. testosteroni* Pb50 and its partial characterization

Phenol hydroxylase was purified from medium (BSM medium containing phenol, for details, see Materials and Methods) used for cultivation of *C. testosteroni* Pb50 by the procedure consisting of fractionation with PEG 6000 and gel permeation chromatography on columns of Sepharose 4B and Sephacryl S-300 (Table 1). The protein fraction precipitated by adding PEG 6000 to achieve its concentration of 8% contained most of phenol hydroxylase activity (103.2 nmol of

Tab. 1. Purification of extracellular NADPH-dependent phenol hydroxylase from *C. testosteroni*.

Fraction activity	Volume (ml)	Proteins (mg/ml)	Specific activity (nmol phenol/min/mg)	Yield enzyme (%)
Medium	1300	0.3	33.9	100.0
PEG 6000, ppt ^a 0-8%	4.5	2.1	103.2	7.4
Eluate, Sepharose 4B	8.4	0.7	153.6	6.8
Eluate, Sephacryl S-300	8.2	0.32	196.7	3.9

^appt – precipitate; Experimental conditions are described in Material and Methods.

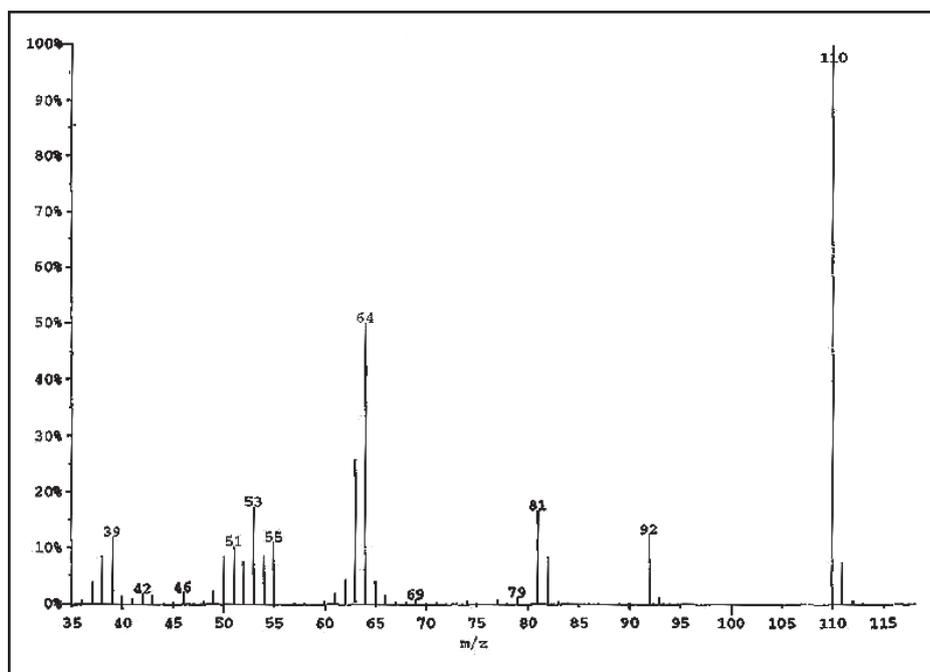


Fig. 2. Mass spectrum of a phenol metabolite formed by extracellular phenol hydroxylase of the medium used for cultivation of *C. testosteroni*.

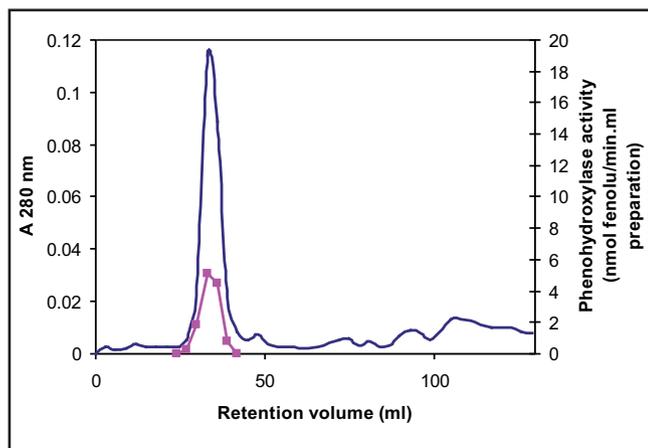


Fig. 3. Gel permeation chromatography of extracellular NADPH-dependent phenol hydroxylase of *C. testosteroni* on Sephacryl S-300. The experimental procedure is described in Material and Methods.

Fig. 4. SDS-PAGE of purified preparation of extracellular phenol hydroxylase of *C. testosteroni*. The gel shown was a 1.5-mm-thick, 10% polyacrylamide slab gel according to Laemmli (1970). It was stained with Comassie blue R. Sample was 30 µg protein per lane.

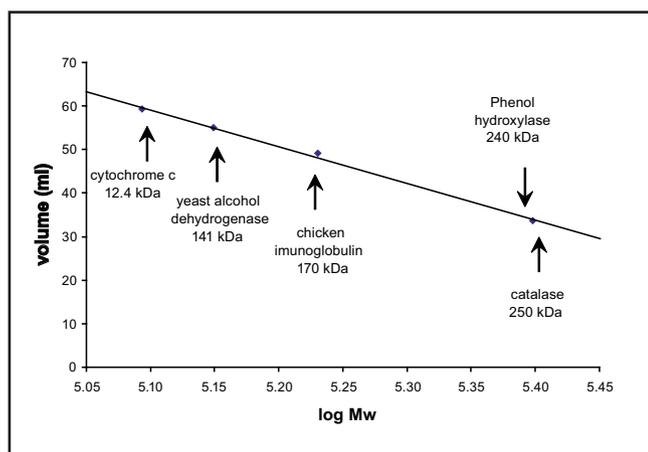
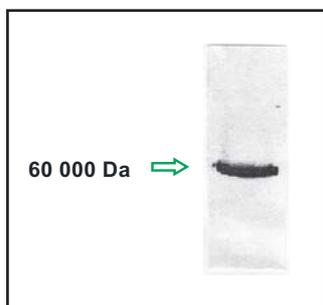


Fig. 5. Molecular mass determination of extracellular NADPH-dependent phenol hydroxylase of *C. testosteroni* by gel permeation chromatography on Sephacryl S-300. Experimental conditions are described in Material and methods.

phenol/min/mg of protein). Therefore, this protein fraction was additionally utilized for gel permeation chromatography on Sepharose 4B. Nevertheless, during this chromatography, the phenol hydroxylase preparation was still contaminated with traces of other proteins. Using a final purification step, gel-permeation chroma-

tography on a column of Sephacryl S-300 (Figure 3), a homogeneous preparation of phenol hydroxylase was obtained (Figure 4).

The phenol hydroxylation activity of the purified enzyme was estimated by the same procedure as described above for measuring the activity determined in the growth medium, by HPLC. The formation of catechol mediated by purified phenol hydroxylase (Figure 1A), is strictly dependent on the presence of NADPH; control incubations, carried out in parallel measurements without NADPH were free of this metabolite even after prolonged incubation times (data not shown). NADH was essentially ineffective as a cofactor of the purified extracellular phenol hydroxylase of *C. testosteroni* Pb50 (Figure 1B). These findings indicate that the isolated enzyme is NADPH-dependent phenol hydroxylase.

Yields for each step of the purification process are summarized in Table 1. A 5.8-fold purification of phenol hydroxylase was achieved with yield of 3.9%. The specific activity of the purified enzyme was 196.7 nmol phenol/min/mg protein.

It should be noted that in initial experiments we also tested suitability of other methods to isolate phenol hydroxylase, namely, fractionation of proteins of the medium with ammonium sulphate and chromatography of the enzyme on a DEAE-Sepharose column. Nevertheless, these procedures were not suitable to obtain an active enzyme preparation.

A gel filtration on a Sephacryl S-300 column (Figure 3) showed that phenol hydroxylase of *C. testosteroni* Pb50 eluted with a volume corresponding to a molecular mass of about $240\,000 \pm 5\,000$ (Figures 3 and 5). As judged by disc electrophoresis (SDS-PAGE) (Figure 4), the final preparation of extracellular phenol hydroxylase of *C. testosteroni* Pb50 contains one protein component having a molecular mass of about $60\,000 \pm 5\,000$. These results indicate that the enzyme is probably a tetramer consisting of four subunits.

A time-dependent decrease in phenol followed by an increase in formation of an oxidation metabolite, catechol, was catalyzed by purified NADPH-dependent phenol hydroxylase of *C. testosteroni* Pb50 (Figure 6).

Phenol consumption was measured in the reaction medium, which contained isolated NADPH-dependent phenol hydroxylase of *C. testosteroni* Pb50, NADPH and increasing concentrations of phenol (0–0.75 mM). The reaction measured by phenol consumption followed the Michaelis-Menten kinetics (data not shown). The values of a maximal velocity (V_{max}) and an apparent Michaelis constant (K_m) for oxidation of phenol are 408.2 nmol/min/mg of protein and 0.87 mmol/L, respectively. The standard deviations of V_{max} and K_m as calculated from three different adjustments of the plot to the observed values were 10%.

Oxidation of phenol to catechol catalyzed by phenol hydroxylase of *C. testosteroni* Pb50 is influenced by pH. The optimal pH of the enzyme is pH 7.6 (Figure 7).

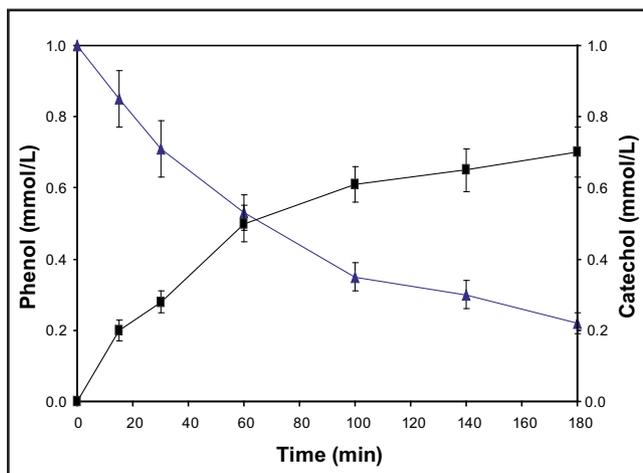


Fig. 6. Time dependence of phenol consumption (▲) and catechol formation (■) by extracellular NADPH-dependent phenol hydroxylase of *C. testosteroni*. Incubations were carried out by the procedure described in the text, except that incubation times were 0–180 min.

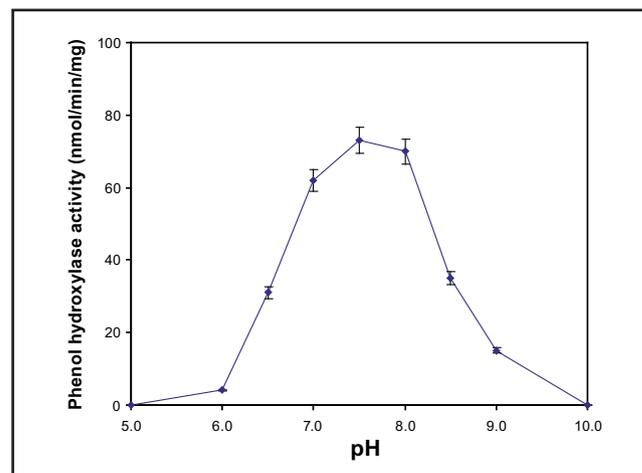


Fig. 7. The influence of pH on the activity of purified extracellular NADPH-dependent phenol hydroxylase of *C. testosteroni*. Incubations were carried out by the procedure described in the text, except that pH of the incubation mixtures ranged between values of 5.0 and 10.0.

DISCUSSION

The results of this study answer the question, which of the enzymes is responsible for the first and rate-determining step of phenol degradation, phenol hydroxylation to catechol, in *C. testosteroni* Pb50. This bacterial strain is, besides of *C. testosteroni* R5, very effective in utilizing phenol as the sole carbon and energy source (Paca *et al.* 2010, Tobajas *et al.* 2010). Here we show for the first time that the enzyme hydroxylating phenol to catechol in *C. testosteroni* Pb50 is NADPH-dependent phenol hydroxylase. The question whether this enzyme is a flavoprotein as other hydroxylases of this class will be examined in our further studies.

Our results also reveal that activity of this enzyme is prevalently located in the growth medium, whereas more than 16-fold lower levels of phenol hydroxylase activity were found in the cytosolic fraction of *C. testosteroni* Pb50. These findings indicate that NADPH-dependent phenol hydroxylase expressed in *C. testosteroni* Pb50 may be excreted by this microorganism into the growth medium. The capacity to secrete phenol oxidases (i.e. lignin peroxidase, Mn-peroxidase and laccase) is well known in filamentous fungi (Garzillo *et al.* 1998; 2001; Hofrichter *et al.* 1999; Duran *et al.* 2002; Zilly *et al.* 2002; Ogel *et al.* 2006; Wu *et al.* 2010; Majeau *et al.* 2010), whereas excretion of phenol hydroxylase was found only in yeast *C. tropicalis* (Bastos *et al.* 2000) and bacterial species *A. faecalis* (Bastos *et al.* 2000; Jiang *et al.* 2007). In the case of *A. faecalis*, excretion of phenol hydroxylase and another enzyme responsible for phenol degradation, catechol 1,2-dioxygenase, led to a higher phenol-degrading potential of this microorganism (Jiang *et al.* 2007). This phenol hydroxylase activity of *A. faecalis* was strictly

dependent on the presence of NADPH (Jiang *et al.* 2007). This finding indicates that the phenol oxidizing enzyme of *A. faecalis* is, similarly to extracellular phenol hydroxylase of *C. testosteroni* found in this work, the NADPH-dependent phenol hydroxylase. The phenol hydroxylase of *A. faecalis* has, however, not been isolated from the growth medium, and it has also not been characterized.

The results of this and former study (Jiang *et al.* 2007) suggest that the presence of the phenol hydroxylase, which is excreted into the medium may, at least partially, explain high phenol-degrading efficiencies of *A. faecalis* (Jiang *et al.* 2007) and *C. testosteroni* Pb50 (Paca *et al.* 2010). Based on data shown in this study and in the work carried out by Jiang and co-workers (2007), we assume that organisms rich in NADPH-dependent phenol hydroxylase similar to that found in *C. testosteroni* Pb50 might be useful for bioremediation technologies such as decontamination of contaminated soils and groundwaters, as well as removal of phenol from wastewaters.

In this work, the procedure suitable for isolation of the extracellular NADPH-dependent phenol hydroxylase from a bacterial strain, *C. testosteroni* Pb50 was developed and the enzyme was partially characterized. The NADPH-dependent phenol hydroxylase enzyme is a homotetramer having a molecular mass of about 240 000, consisting of four identical subunits. This phenol hydroxylase resembles NADPH-dependent phenol hydroxylase of *Candida* yeasts and bacteria of *Alcaligenes* spp. (Bastos *et al.* 2000; Paca Jr *et al.* 2007), rather than to multi-component phenol hydroxylases of other bacteria such as *Pseudomonas* spp. and *C. testosteroni* R5, the bacteria that are also highly effective to biodegrade phenol (Viggor *et al.* 2008; Tobajas *et al.* 2010).

Therefore, the questions whether multi-component phenol hydroxylases found to be the enzyme oxidizing phenol in *C. testosteroni* R5 are also expressed in *C. testosteroni* Pb50 remain to be answered; the occurrence of such enzymes in studied microorganism has not been investigated in this work.

In conclusions, the results presented in this work are the first report showing isolation and partial characterization of extracellular NADPH-dependent phenol hydroxylase of *C. testosterone* Pb50, the enzyme that has not been isolated from any of media employed for cultivation of microorganisms degrading phenol as yet. The results also demonstrate the progress in resolving enzymes responsible for the first step of phenol degradation by microorganisms.

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