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

Michal Turek 1, Lenka Vilimkova 1, Veronika Kremlackova 1, Jan Paca Jr 1, Martin Halecky 2, Jan Paca 2, Marie Stiborova 1

1 Department of Biochemistry, Faculty of Science, Charles University Prague, Czech Republic
2 Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology, Prague, Czech Republic

Correspondence to: Prof. RNDr. Marie Stiborova, DSc.
Department of Biochemistry, Faculty of Science, Charles University
Albertov 2030, 128 40 Prague 2, Czech Republic.
Tél: +420-221 951; Fax: +420-221 951 283; E-mail: stiborov@natur.cuni.cz

Submitted: 2011-05-20 Accepted: 2011-08-25 Published online: 2011-11-05

Key words: phenol; biodegradation; Comamonas testosteroni; phenol hydroxylase

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; Ahuatzi-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 & Oriel 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 & Oriel 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. 1999; 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-oxoapptide to succinate and acetyl-coenzyme A (Ahuatzi-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-hydroxymuconic semialdehyde that is further converted to fumarate, 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. 2011). 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 \textit{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 \textit{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′-bicinchoninol-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 \textit{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**

\textit{C. testosteroni} was cultivated in the base synthetic medium (BSM). This growth medium contained: 4.3 g/L K$_2$HPO$_4$, 3.4 g/L KH$_2$PO$_4$, 2 g/L (NH$_4$)$_2$SO$_4$, 0.34 g/L MgCl$_2$.6 H$_2$O containing 3.5 mg/L phenol as a sole carbon and energy source (phenol medium).

For acquisition of sufficient quantity of \textit{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 \textit{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 mM/L phenol and 1.0 mM/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 1 300 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 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 was cultivated. The phenol hydroxylation activity of these subcellular fractions and the growth medium was estimated by HPLC.
free of this metabolite even after prolonged incubation
times (not shown). The phenol metabolite was identi-
cified by co-chromatography with an authentic stan-
dard, 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 fragmenta-
tion 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 indi-
cate 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 cat-
echol 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. testos-
teroni 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. testos-
teroni 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

<table>
<thead>
<tr>
<th>Fraction activity</th>
<th>Volume (ml)</th>
<th>Proteins (mg/ml)</th>
<th>Specific activity (nmol phenol/min/mg)</th>
<th>Yield enzyme (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1300</td>
<td>0.3</td>
<td>33.9</td>
<td>100.0</td>
</tr>
<tr>
<td>PEG 6000, ppta 0-8%</td>
<td>4.5</td>
<td>2.1</td>
<td>103.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Eluate, Sepharose 4B</td>
<td>8.4</td>
<td>0.7</td>
<td>153.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Eluate, Sephacryl S-300</td>
<td>8.2</td>
<td>0.32</td>
<td>196.7</td>
<td>3.9</td>
</tr>
</tbody>
</table>

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.
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 ± 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 ± 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).
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 hydroxylase 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. tropicallis (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 microorganisms 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. testosteroni_ 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.

**ACKNOWLEDGEMENTS**

The authors would like to thank to Grant Agency of Czech Republic (grant P503/11/0163) and to the Ministry of Education of Czech Republic (grants MSM0021620808 and 1M0505).

**REFERENCES**


